ASPECTS OF LEAF AND EXTRACT PRODUCTION
from
Tasmannia lanceolata

by

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Abstract

This thesis examines several aspects of the preparation, extraction and analysis of solvent soluble compounds from leaf material of *Tasmannia lanceolata* and reports a preliminary survey of extracts of some members of the natural population of the species in Tasmania.
A major constituent of these extracts, polygodial, was shown to be stored within specialised idioblastic structures scattered throughout the mesophyll, and characterised by distinctive size and shape, and a thickened wall. The contents of these cells were sampled directly, analysed and compared with the composition of extracts derived from ground, dry whole leaf. This result was supported by spectroscopic analysis of undisturbed oil cells in whole leaf tissue.

In a two year field trial, the progressive accumulation of a number of leaf extract constituents (linalool, cubebene, caryophyllene, germacrene D, bicyclogermacrene, cadina-1,4 - diene, aristolone and polygodial) during the growth flush was followed by a slow decline during the subsequent dormant season. These results were interpreted in relation to leaf dry matter accumulation, in order to propose a harvest period within which leaf material will produce consistent composition of extract.

Under four levels of irradiance in a growth cabinet experiment the plant exhibited many characteristics of a 'shade' species, in particular, a limited ability to acclimate to high light levels. Assimilation rates were highest at 150μmol $m^{-2}s^{-1}$ while elevated respiration rates and a reduced quantum yield occurred at a higher light level. Maximum assimilation rates in leaves grown at 150μmol $m^{-2}s^{-1}$ were obtained at around 250μmol $m^{-2}s^{-1}$. Optimum net assimilation rate was obtained from 18-25°C. The effect of level of irradiance on the proportion of extractable compounds in the leaf, chlorophyll levels, specific leaf area, leaf thickness and percentage dry matter in the leaf are reported and discussed in relation to a probable production system in which the new canopy is largely removed at the end of each growth cycle.
The ontogenetic patterns determining canopy architecture were observed in the field, and used, with support from the results of a trial pruning of mature trees, to discuss the likely outcome of various harvest methods. These results are combined to suggest a production strategy for maximum yield of leaf extract of consistent composition. The strategy proposes harvesting in late summer, after new leaf has achieved full maturity and may enable full canopy recovery in the subsequent growing season.

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CONTENTS

CHAPTER 1: INTRODUCTION

Interest in 'wild' populations of plant species with a view to cultivation for human consumption has enjoyed a long history. It is of particular contemporary significance, as concerns are raised about the loss of 'non-commercial' species (flora and fauna) through the careless or deliberate activities of agriculture, forestry, mining and urban development. Support for measures to preserve 'biodiversity' hinge on two premises: the altruistic assumption that human domination and extinction of fellow travellers is inherently wrong, or that useful, and (emotively) 'lifesaving' compounds, extracts, synthetic mechanisms, etc may be revealed by current and future technologies should we care to investigate.
If the current development plans for *Tasmannia lanceolata* extract evolve as planned by industry, it might become a case in point - for at least the last fifteen years, many thousands of hectares of mature trees have been cleared each year in a routine operation referred to as 'pepper rolling' in preparation for eucalypt and pine plantations by Tasmanian forestry operators. While the species is in no danger of extinction, substantial areas which might have provided commercial raw material have been lost and will require many years of managed cultivation and the expenditure of considerable capital to replace them should commercial development require it.

Tasmannia lanceolata - 'mountain pepper' - produces, upon solvent extraction, a fragrant product with unusual spicy, peppery characteristics. This extract has been received with interest by a number of flavour and fragrance industry representatives, and identified by the Tasmanian Essential Oils industry as a priority for further development.
Commercialisation of such a product demands consistency of extract quality and yield, and, as is the case with 'domestication' of any plant species, an understanding of the plant's response to management techniques with the aim of maximising productivity. The development path for most agricultural crops commenced with a long period of passive management - harvest of wild stands, and was followed, often after many hundreds of years, with efforts to improve the technology and plant material involved.

In the case of *Tasmannia lanceolata,* the technological approach has been introduced early in the development path with a coordinated research and market development program of which this study is a part.
This study, therefore, begins a long process of interaction between the population of the species found growing naturally, and the interests of those who would develop it as a horticultural crop. The ultimate object of the interaction is the cultivation of selected plant material, under carefully managed growing conditions and coupled to harvest and

product preparation technology. All of this must result in a product which fulfills the strict requirements of the food and flavour extract industry.

The study attempts firstly to resolve several fundamental issues relating to the extract itself, - its preparation and composition, where certain of the components reside in the plant tissue and how the process of accumulation of these components relates to the normal annual growth cycle.

Secondly, and following the previous point, it investigates the basic ontogenetic patterns in the plant, and relates these to the problem of harvest of those plant parts containing the extract, on an annual basis.

Lastly, the impact of such a harvest on the photosynthetic capacity of the canopy is investigated, providing basic information on the potential productivity of the species, and a guide to the suitability of local light environments for maximum productivity.

Combining these findings, a suitable harvest strategy is proposed, providing for consistent extract composition at optimum yield, and with adequate provision for regeneration of vegetative growth.

CHAPTER 2: LITERATURE REVIEW

2.0 Introduction

This review commences with a general introduction to *Tasmannia lanceolata* , and the family to which it belongs, considering the morphological and developmental literature relating to the species, then referring to published work dealing with oils and extracts obtained from members of the family. Particular attention is devoted to polygodial, an unusual and potent extractive, and the subject of a number of continuing inquiries in its own right.

Secondly, five topics are discussed as they relate to the experimental approach in this thesis- the study of oil cells as a morphological phenomenon, seasonal changes in extract composition and their significance in biological studies, literature relating to canopy structure and manipulation in tree species, light acclimation in understorey species (of which *Tasmannia lanceolata* is one) and lastly a general review of harvest strategies employed in the production of essential oils and extracts.

2.1.1 Distribution of the Winteraceae

The family Winteraceae is confined to the southern hemisphere reflecting its origins on the Gondwanan supercontinent. Against a background of some dispute regarding the systematics of the family and its genera, Cronquist (1981) is followed here in considering the family together with the Magnoliaceae, Annonaceae and seven other families as belonging in the order Magnoliales. Smith (1945) placed six genera in the family, distributed as follows -

Exospermum (2 spp.) and *Zygogynum* (6 spp.)- New Caledonia
Pseudowintera (2 spp.)- New Zealand
Bubbia (30spp.) New Guinea, New Caledonia, Lord Howe Is., Queensland
Belliolum (8spp.) New Caledonia and the Solomon Islands
Drimys southern Mexico to Cape Horn and the Juan Fernandez group, Australia, Tasmania, New Guinea , Celebes, Borneo and the Phillipines.

The inclusion of *Tasmannia* as a section within the genus *Drimys* has since been displaced by a recognition of two separate genera (Smith 1969), principally as a consequence of morphological studies by Bailey (1944), Bailey and Nast (1943a,b:1945a,b) and chromosome studies by Ehrendorfer *et al.*, (1968). The genus *Drimys* is confined to South America while *Tasmannia* is limited to Australia, Tasmania, New Guinea and one wide ranging species in the Phillipines, Borneo and the Celebes.

A further addition to the family has been that of the genus *Takhtajania* recorded from one collection in Madagascar (Leroy 1980 cited in Vink 1988), bringing the widely held number of genera to eight.
Vink (1988) argued that recognition of *Tasmannia* as a separate genus was not appropriate and detailed an evolutionary relationship to support this as well as combining *Bubbia, Zygogynum, Exospermum* and *Belliolum* , renamed *Zygogynum* so that he only recognised four genera. Vink's system appears not to have prevailed in subsequent literature and the genus *Tasmannia* appears generally recognised.

About seven species of *Tasmannia* are distinguished in Australia (Sampson *et al.* 1988), with distributions as follows -

glaucifolia; local, disjunct confined to Barrington Tops, NSW
stipitata; common,SE Queensland to Hastings R NSW
xerophila; high altitudes, SE NSW, ACT and Vic.
insipida; SE Qld, east NSW
purpurascens; Barrington Tops, Gloucester Tops NSW
membranea; NE Qld, south of Cape York above 500m.
lanceolata; wet sclerophyll forest to alpine heath, SE NSW, Vic and Tasmania

Tasmannia lanceolata (Poir.) AC Smith, referred to previously as *Tasmannia aromatica,*, *Winterania lanceolata* and *Drimys aromatica* , (Smith 1943) is described as a much branched shrub up to about 5 metres high with dark green glabrous leaves and distinctive crimson young stems. The dioecious plant bears black fruit, the size of a pea, containing numerous small seeds. The plant inhabits cool wet habitats from sea level to about 1200m in Tasmania, preferring disturbed sites in which it is an early coloniser, preceding wet eucalypt forest and *Nothofagus* rainforest (Read and Hill 1983) and is found in similar situations in Victoria and at high altitudes in NSW as far north as the Hastings River.

2.1.2 Medicinal and Culinary Uses of the Winteraceae

Several Winteraceous species have been associated with medicinal use amongst indigenous peoples in the regions in which they occur. New Guineans (presumably Solomon Islanders) are reported to have used the pounded leaves of a *Belliolum* species for treatment of 'diseased spots' on the skin of pigs and decoctions of *Tasmannia* species were taken as an abortifacient (Kloppenburgh-Versteegh cited in Perry 1980). *Drimys wintera* Forst., (locally known as 'canelo', 'foique' or 'casca d'anta'), distributed between the Straits of Magellan and central Chile (to about 32°S) forms a large tree to 30 metres and is reported to be used in Brazil as a treatment for cholic, cattle itch and as a 'stomachal tonic' (Retamar 1986).

Decoctions of *Pseudowintera axillaris*, a New Zealand shrub were used by Maori people as a stimulant, for skin diseases, venereal diseases and stomach ache. The leaves were chewed to relieve toothache (Salmon 1980).
European use of the family began in 1597 when Captain Winter, Commander of the Elizabeth, under Drake, used the bark of *D. wintera* to relieve scurvy amongst his crew. The species subsequently enjoyed some European use as a herbal remedy until it became hard to obtain and was partly replaced by *T. lanceolata*, *D. chilensis* and False Winter's Bark - *Cinnamomum corticosum* from Jamaica and the West Indies. Winter's Bark either True or False appears to have become increasingly scarce, and fell from favour as a herbal remedy during the twentieth century (LeStrange 1977). Bark of *Pseudowintera axillaris*, was used by pioneering New Zealanders as a quinine substitute while the sap was used for treating skin diseases (Salmon 1980).
During the nineteenth century and the first half of this century, *Tasmannia* species have been intermittently referred to as presenting economic possibilities. *T. lanceolata* was mentioned by Maiden (1889) for its potential as a pepper or allspice substitute, and for its resemblance to Winters Bark. *T. aromatica* (syn. *T. lanceolata*) was suggested by Maiden (1899) as a possible source of a succulent, though insipid (!) fruit.
Dr. Beth Gott (Dept. of Ecology, Monash University - pers. comm.) has found no direct evidence of Aboriginal use of *Tasmannia* species for any purpose, but quotes two aboriginal names ascribed to the species.

Historical reference suggests use of *Tasmannia* has been mainly medicinal or herbal in nature, although reference to preparation of materials and doses is invariably vague. Australian 'bush tucker' writers refer to the use of *Tasmannia lanceolata* and *T. insipida* as flavouring herbs in preparation of meat and savoury dishes (Cribb and Cribb 1975, Low 1988, Cherikoff 1989), and one current estimate of Australian consumption for this purpose is approximately 2-3000 kg dry weight annually (I R Farquhar: pers. comm).

2.2 Morphological and anatomical studies of *Tasmannia*

Scientific interest in the genera *Drimys* and *Tasmannia* has centred on the significance of a number of morphological characters and developmental patterns for an understanding of the development and evolution of angiosperms in general.
The vesselless xylem in the wood of the species, first observed in the mid nineteenth century, was studied in some detail in the early part of this century (Jeffrey and Cole 1916, Bailey and Thompson 1918). Debate at the time focussed on whether the nature of vessel-like structures found in the wood of members of the genus was that of a simplified vessel structure developed from an earlier, more organised transport system as found in other members of the Magnoliaceae or rather (as is now generally accepted), an undeveloped primitive characteristic indicating a gymnospermous

ancestry. The long, scalariformly pitted tracheids found in the wood of *Drimys* and *Tasmannia* species as well as nine other genera of woody angiosperms from five different families are considered to represent the precursors of the more advanced xylem element in which the membranes of bordered pit pairs towards the ends of the cell have disappeared, and which have become increasingly broader and shorter. Pitting of the thick lateral walls of tracheids found in the older wood of *Drimys* is usually circular, with occasional scalariform pitting towards the ends of the cells (Foster and Gifford 1974). The development and specialisation of the vessel members in dicotyledonous angiosperms is considered (Cheadle, 1953) to have arisen successively in the secondary xylem, the metaxylem and the protoxylem.

Carlquist (1989) related a number of anatomical characteristics of the wood of members of *Tasmannia* to regional climatic factors - most importantly temperature. Growth rings, arising from the distinction between 'earlywood' and 'latewood' in secondary thickening, were more prominent in populations found in colder climates, the proportion of 'latewood' being greater in collections from high altitudes and more southerly distribution. Latewood is characterised by narrower tracheids, more resistant to embolism should groundwater remain frozen on a sunny day when transpiration could lead to collapse of vessel walls. Similarly, the degree of complex, prominent vesturing (wartiness) on the internal walls of the tracheids paralleled the above trend, being most obvious on populations from cold areas. These trends are observed where frosts occur in spring and autumn - higher altitudes and latitudes. Carlquist (1989) details these morphological trends for *Tasmannia* and summarises aspects of wood anatomy and morphology in Winteraceae, having addressed the subject in a series of papers on the family cited therein.

Relevant to this was Bongers' (1973) survey of epidermal leaf characters in the Winteraceae, examining the range of epidermal characters in the family. Within *Tasmannia* a range of cuticular thickness, stomatal densities and the presence of plugs of alveolar material occluding the stomata in a few species were observed. The significance of the stomatal occlusion in association with the vesseless nature of the wood, and the adaptive significance for water transport in the genus was discussed. Bongers suggests that since genera of the *Winteraceae* occur entirely within mesic environments, the apparent failure of the family to develop a more efficient (broad, unobstructed, more or less continuous) vessel system may reflect a lack of evolutionary advantage of such developments in these situations, contrary to Carlquist's (above) interpretation of the trend as conferring some advantage with respect to frost and freezing resistance.

The vesseless character was of interest to Ritman and Milburn (1991) in their observations of the attenuation of audible and ultrasonic emissions in *Tasmannia stipitata* and *Thuja occidentalis* in which the detection of such emissions was used to detect sites of cavitation in whole plants.

Bailey and Nast (1945c) summarised an extensive survey of the comparative morphology of the family, examining pollen, stamens, carpels, leaf vascularisation and epidermis.

This study directed its attention to characteristics of systematic value and the findings were later invoked in a revision of the taxonomic status of the genus (Smith 1969).

The seasonal development of meristematic tissue at the shoot apex of *Tasmannia lanceolata, T. wintera* and *Pseudowintera axillaris* (together with representatives of four other ranalian families exhibiting primitive characters) is described in detail by Gifford (1950).

Tucker and Gifford (1964, 1966a,1966b) devoted their attention to details of the floral ontogeny of *Tasmannia lanceolata* . The species is dioecious, and the development of vascular bundles in the carpellate flower, the development of the carpel, and floral development in the carpellate inflorescence itself drew further attention to the distinction between the two sections (Tasmannia and Wintera) of the genus *Drimys*. Carpel development, is described as conduplicate - the carpel enfolds a series of ovules attached to the adaxial inner surface, and the margins, although closely appressed, remain separate as a distinct cleft in the mature fruit. Pollen tubes arising from pollen adhering to the crests pass amongst a mat of hairs between the appressed ventral surfaces of the carpel, but do not penetrate the carpel tissue. This is regarded (Fahn, 1982) as a manifestation of a primitively developed carpel from which gradual fusion of the margins, and development of a stigma from the paired crests by progressive closure of the margins towards the upper part of the carpel could have occurred.

Stamen venation was the subject of a study by Sampson (1987) who examined all genera in the Winteraceae and reported the presence of a single vascular bundle branched slightly at its tip in *Tasmannia lanceolata.* The vasculature of the carpel after ovule initiation was the subject of a paper by Tucker (1975) in which the contribution of the dorsal and ventral vascular bundles to ovular supply and the phylogenetic significance of the predominantly ventral supply observed in *D. wintera* and *T. lanceolata* was discussed.

Vink's (1970) assessment of the morphology of the Winteraceae provides extensive detail of leaf, flower, fruit and seed morphology, a summary of flower ontogeny in 'Sect. Tasmannia' and a discussion of the consequences for taxonomy of the genus. *'Drimys lanceolata'* is then typified and its cytology, distribution and ecology briefly discussed.

Vink (1970) describes the flush of growth which occurs together with or immediately after anthesis as a 'periodic shoot increment - p.s.i.', along which the spirally inserted leaves may be evenly distributed or may be crowded below the apex. The mean lengths of leaves along the twigs increased distally and leaves on lateral twigs had lower mean leaf lengths. Fine white crystals found on twigs of herbarium specimens were considered to arise from preparation techniques, recrystallising from 'substances'

originating in the twig. On leaf specimens, however, the occlusion of stomata by waxy plugs is considered a characteristic of 'sect. Tasmannia', and their absence in some herbarium specimens was considered to be due to the melting or dissolving of wax crystals during processing.
Vink's description of leaf morphology mentions the wide variation in general outline and xeromorphy, particularly cuticle thickness and the angle of leaf venation to the midrib. Vink also included 'sharp peppery taste' as a distinguishing characteristic for entities within *Tasmannia piperita* collected in the New Guinea highlands.

Petals of some genera in the family have been shown to have cells on their inner surfaces which are densely packed with polysaccharide granules (Thien *et al.* 1990). Hydrolysis of the granules results in the rapid uptake of water and the opening or closing of the petals, while chewing activity observed upon these floral parts suggests that the granules may serve also as a pollinator reward for visiting insects.

Casey (1991) used *Tasmannia lanceolata* from several Tasmanian localities, together with specimens collected on Flinders Is., in Victoria and towards the northern end of the distribution in NSW, in a study of the effects of light climate and altitude on leaf morphological and physiological characteristics. He examined variation in leaf weight and specific leaf area, leaf and cuticle thickness, palisade cell length and number of layers and stomatal densities, and undertook a comparison of frost resistance and photosynthetic efficiency in leaves of plants collected from these localities and grown under controlled conditions. In general, genetic influences were considered responsible for leaf size, cuticle character, frost resistance and photosynthetic activity amongst the types, and lowland types tended to demonstrate a more plastic response to environmental influence on leaf size than those originating at the higher altitudes.

Despite the attention to the morphology and anatomy of *Tasmannia* reviewed above, there appears to be no specific consideration of the distinctive oil-bearing characteristics of the species, or even the genus. Bongers (1973) and Vink (1970), despite detailed study of leaf morphology and epidermal characters in Winteraceae scarcely mention oil cells as a feature. West (1969) examined a range of genera in several related families, including Winteraceae, (but not *Tasmannia)* for the presence of oil cells and gave a general account of their structure and occurrence. Carlquist (1989) mentions that oil cells occur in rays of *Tasmannia* wood.
Oil cells are considered to offer systematic utility in some families, particularly Annonaceae (Bakker and Gerritsen 1992) and have been described in detail for the others in which they occur (Maron and Fahn 1979, Platt-Aloia *et al.* 1983, Bakker and Gerritsen 1990), but their presence in the Winteraceae has not been examined in detail in light of the extractable compounds reported for leaf and wood of many members of the family.

The distribution of *Tasmannia lanceolata* and indeed most members of the Winteraceae, in moist environments, often associated with rainforest or wet sclerophyll vegetation types might lead to a general assumption that ideal cultivation practices would provide for moist, shaded growing conditions.

T. lanceolata appears to tolerate a wide range of temperature environments- from southern high altitude sites to warm temperate conditions on the mid coast of New South Wales, provided moisture is neither limiting nor inundating. The tracheal transport system appears to provide at least one physical explanation for its confinement to such situations.

The significance of light level, a critical factor at rainforest margins and in canopy breaks, in the performance of the species both in establishment and growth is less clear. It would appear that while the species does not flourish at high light levels found on completely open or north facing environments, it does respond to the temporary increases in light associated with canopy breaks, and colonises open habitats in high rainfall (cloudy) situations but does not persist under dark, closed canopies. Again, this suggests a fairly narrow range of preferred light environments and may constrain cultivation practice with regards site selection, plant density etc.

The success of the species during the early stages of colonisation might be due to the pungent properties of the leaf and stem, providing some protection from browsing.

2.3.0 Extracts of the Winteraceae

The self evident presence of aromatic and volatile compounds within the foliage, fruit and bark of members of the *Winteraceae* has prompted several investigations of their extracts and volatile oils. With modern analytical techniques many interesting constituents have been identified, but until the 1970's the plant species themselves were regarded as little more than curiosities and no serious attempt at cultivation or even use of products of wild plants other than *D. winteri* appears to have occurred.

Petrie (1912) reported positive reaction to a test for cyanogenetic glucosides in a water infusion (40°C, 24hrs) of chopped leaves of *Drimys aromatica* (*T. lanceolata*) and *Drimys dipetala* (*Tasmannia insipida)* in a survey of Australian species for hydrocyanic acid. More recently, Collins *et al.* (1990) included the plant in their survey of plants of the Australasian region, and using Mayer's and 'silicotungstic' tests for the presence of alkaloids in two Victorian samples of plant material found 0.005 - 0.01% by dry weight in bark and none in leaf and stem samples.

Atkinson and Brice (1955) tested the essential oil of *T. lanceolata* for its activity in suppressing growth in broth and agar dilutions of *Staphylococcus aureus* , *S. typhae* and *Mycobacteria phlei.* , finding it effective in suppressing growth of *S aureus* and *M phlei* at 'reasonable' titres.

Stevens (1955) investigated the essential oil of *T. lanceolata* , distilling relatively large quantities of leaf and stem material from a number of sites around Tasmania,

determined some physical and chemical constants for the oil, and attempted to determine the structure of a crystalline compound guaiol, which occurred in large concentrations in oils from several of the sites.

Loder (1962) first reported the occurrence of a sesquiterpene dialdehyde 'polygodial' in petroleum extracts of *T. lanceolata* , (the compound first described from solvent extracts of *Polygonum hydropiper* by Barnes and Loder (1962)), but reported no evidence of alkaloids in the extract.

Drimys winterii, and its compatriots *Drimys brasiliensis* and *Drimys confertifolia* have been investigated since the late fifties at which time Appel *et al.* (1963) showed the presence of a number of drimane type sesquiterpenes in extracts of dried bark of a number of samples of *D. winteri* from different regions of Chile. Subsequently, as part of a survey of Chilean flora for anti-tumor compounds, hexane and acetone leaf extracts of *D. winteri* were shown to be active against mouse leukaemia lymphocytes and a number of compounds were recovered from the extracts, including cryptomeridiol, cirsimaritin, quercetin, taxifolin, astilbin and quercitrin, amongst which taxifolin was found to be active in antitumor tests (Cruz *et al.* 1973). Sierra *et al.* (1986) reported the isolation and structural determination of 3ß- acetoxydrimenin and the isolation of safrol, drimenol and polygodial from the leaves of *D. winteri* . Dried leaves yielded approximately 10% w/w of petroleum (60-80°C) extract from which 0.9g (0.09%) of polygodial was recovered by TLC methods.

Vink (1970) describes an earlier report in which the flavonols quercetin, kaemferol and hydroquercetin were found throughout the Drimys (both sections) and Pseudowintera. The flavones luteolin and apigenin were often found within the 'sect. Tasmannia'.

Drimys brasiliensis was shown (Vichnewski *et al.* 1986) to contain two drimane derivatives, 1ß-p-coumaroyloxypolygodial and 1β-p-coumaroyloxyvaldiviolide as well as confertifolin, previously isolated from *D. confertifolia* (Appel *et al.* 1958)

Some thirty constituents, sixteen of them identified, of the volatile oil of *Pseudowintera axillaris* were reported by Corbett and Grant (1958). A more comprehensive summary of the composition of the essential oils for both *Pseudowintera axillaris* and *P. colorata* was published by Cambie (1976), in which 29 compounds common to both species were listed, and the identification of two compounds, (-) - cyclorotenone and (+)-α- santalene in the bark of *P. colorata* was reported. McCallion *et al.* (1982) report the isolation of polygodial from the leaves of *P. colorata* and the results of tests of the activity of the compound against the yeast *Candida albicans*. Keller *et al.*(1992), as part of a survey of New Zealand plants for enzyme inhibitory activity found methanolic extracts of *P. colorata* showed 20-40% inhibition of α-chymotrypsin and α- glucosidase.

Dragar (1984) obtained essential oil and petroleum ether extracts of *T. lanceolata* and confirmed some physical constants and the chemical composition of the oils.

Southwell and Brophy (1992) included *T. lanceolata* in a comparison of the Australian species of the genus by the composition of their essential oils. Both ethanol extracts and

the hydrodistilled oils were analysed by liquid chromatography and GC-MS to identify the major constituents and some conclusions were drawn regarding the chemotaxonomy of the Australian species as a result, specifically that the oils could serve as an adjunct to classical morphological characters in distinguishing *Tasmannia* species. The material used for the steam distilled oils was taken from three specimens originating in the vicinity of Clyde Mountain NSW, three from the ACT, and one from 'the southern highlands' of NSW. All but one of the solvent extracted samples were taken from plants originating in the Tasmanian highlands, but were provided by the Australian National Botanic Gardens in Canberra, presumably from specimens grown in the gardens. The authors noted that the peppery taste present in the leaf of most species was not evident in the volatile oils.

Reports of the chemical constituents of *Drimys*, *Pseudowintera* and *Tasmannia* species are summarised in Table 2.1.

Table 2.1: Reported constituents of extracts and oils of members of the Winteraceae; see Key to references and extraction method at end of table. Continued.....

COMPOUND	Species (Reference:for extraction method see key at end of Table)						
	Pseudowintera colorata (1)	Pseudowintera colorata (2)	T. lanceolata(3)	T. lanceolata(4)	Drimys spp (5,6,7,8)	Tasmannia (exc. lanceolata) (9)	Tasmannia lanceolata (10)
α-pinene	√	√		√		√,√	√,√
α-thujene	√	√				√,√	-,-
ß-pinene	√	√		√		√,√	√,√
myrcene	√	√		√		√,√	√,√
(+)- limonene	√	√				√,√	√,√
γ- terpinene	√	√				√,-	√,-
α- terpinene		√		√			
terpinene - 4-ol						√,√	√,√
terpinolene	√	√		√			
α-terpineol						√,√	√,√
γ- terpineol				√			
hexenyl-n valerate	√	√					
eugenol	√	√		√		√,√	√,√
bicyclic sesquiterpene	√						
(+)- aromadendrene	√	√					
humulene	√	√					
guaiol			√	√			
valvidiolide					√(5)		
fuegin					√(5)		
winterin					√(5)		
futronolide					√(5)		
camphene				√	√(5)		
sabinene				√		√,√	√,√
α-phellandrene						√,√	√,√
ß- phellandrene		√		√		√,√	√,√
p- cymene		√		√		√,√	√,-
1,8-cineole				√		√,√	√,√
linalool				√		√,√	√,√
piperitone				√			
safrole				√	√(8)	√,√	-,-
sabinyle acetate				√			
eugenol						√,√	√,√
methyl eugenol				√			
caryophyllene				√		√,√	√,√
myristicin				√		√,√	-,-
∂- cadinene				√		√,-	√,-
confertifolin					√(6)		
1ß-p-coumaroyl-oxypolygodial					√(6)		
1ß-p-coumaroyloxyvalvidiolide					√(6)		
cryptomeridiol					√(7)		
quercetin					√(7)		
quercitrin					√(7)		
taxifolin					√(7)		
astilbin					√(7)		
cirsimaritin					√(7)		
(-)- 3ß acetoxydrimenin					√(8)		
drimenol					√(8)		
polygodial					√(8)		
car-3-ene						√,√	-,-
α-cubebene						√,√	√,-

Table 2.1: Reported constituents of extracts and oils of members of the Winteraceae; (Continued).

COMPOUND	Species (Reference:for extraction method see key at end of Table)						
	Pseudowintera colorata (1)	Pseudowintera colorata (2)	T. lanceolata(3)	T. lanceolata(4)	Drimys spp (5,6,7,8)	Tasmannia (exc. lanceolata) (9)	Tasmannia lanceolata (10)
α-copaene				√		√,√	√,-
α-gurjunene		√				√,√	√,√
α-amorphene						√,-	-,-
germacrene-D						√,√	√,√
viridiflorene						√,-	√,-
viridiflorol						√,-	√,√
bicyclogermacrene						√,√	√,√
cadina-1,4-diene						√,√	√,-
calamenene		√				√,√	√,-
elemol						√,-	-,-
globulol						√,√	-,-
croweacin						√,-	-,-
spathulenol						√,-	√,-
caryophyllene oxide						√,-	-,-
bulnesol						√,-	-,-
α-eudesmol						√,-	-,-
ß-eudesmol						√,√	-,-
γ-eudesmol						√,√	-,-
(-)-cyclocolorenone		√					
α-santalene		√					
(-)-ß-elemene		√					
dipentene		√					
tricosane		√					
pentacosane		√					
farnesol						-,√	√,-

References and extraction method

1 Corbett and Grant (1958) : Steam distilled
2 Cambie (1976) : Steam distilled
3 Stevens (1955) : Steam distilled
4 Dragar (1984) : Steam distilled
5 Appel *et al.* (1958) : Petroleum ether extract
6 Vichnewski *et al.* (1986) : Hexane / ethyl acetate extract
7 Cruz *et al*(1973) : Ethyl acetate extract
8 Sierra *et al.* (1986) : Petroleum ether extract
9 Southwell and Brophy (1992) : Steam distilled (first check), ethanol extract (second check)
10 Southwell and Brophy (1992) : Steam distilled (first check), ethanol extract (second check)

2.3.1 Extraction Methods

a) Aqueous Extracts

Use of bark, leaves and fruit of Winteraceae species by indigenous peoples, presumably over a very long period, has usually depended upon the preparation of aqueous decoctions. This has been the case with *Drimys winteri* in Brazil and Chile, with *Pseudowintera* in New Zealand and with the *Belliolum* species in South East Asia. Early European use of these and other aromatic species often took the form of 'tonics' prepared as infusions of ground or chopped fresh and dried plant material.

b) Steam Distillation

Stevens (1955), using fresh, whole leaf and twig material recovered between 0.28 and 0.53% of volatile oil in an apparatus capable of distilling up to 400kg at a time, cohobating the aqueous distillate after separation of the oil in two glass jars, one containing a layer of ether to serve as an additional trap for oil. In these trials it was found that guaiol crystallised from the dried oil, within the condenser and inside the lid of the distillation vessel. The date of collection of material is not mentioned.
Corbett and Grant (1958) used whole fresh leaves and terminal branchlets of *Pseudowintera colorata* in 110kg batches recovering 0.417% v/w of a green oil from material collected in autumn.
The work of Southwell and Brophy (1992) was based upon the hydrodistillation to exhaustion (about 4-6 hrs) of individual plant specimens.

c) Solvent Extracts

Barnes and Loder's (1962) recovery of polygodial from *T. lanceolata* commenced with the preparation of a petroleum extract of the dried, milled leaves of the plant, yielding about 4.5% of oil. The purification of polygodial was carried out by partitioning the oil between petroleum-70% methanol-water, the methanolic layer diluted with an equal volume of NaCl solution. An ether extract of the aqueous portion of this, combined with the oily portion was concentrated and redissolved in petroleum (b.p. 40-60) before being chromatographed on a silica gel column. Elution of the column with 1:1 petroleum:benzene and with benzene and removal of the solvents gave fractions which crystallised on standing, yielding colourless crystals of polygodial.
Appel et al. (1958) employed fractional distillation, together with chromatographic techniques on their crude petroleum(70-80°) extract of powdered *D. winteri* to separate a number of sesqiterpenes (see Table 2.1). Sierra *et al.* (1986) using similar plant material recovered about 10%w/w of crude petroleum extract which they chromatographed on silica gel to separate safrole (0.19% in leaf) with 19:1 v petrol: EtOAc, drimenol (0.07%)with 9:1 Petrol:EtOAc, an unidentified crystalline compound (0.07%) from hexane and polygodial (0.1%in leaf), from pentane.

McCallion *et al.* (1982) used crushed, freeze dried leaves, extracted for 48 hrs with acetone. The extract was then concentrated, taken up in petroleum and the petroleum fraction used for their chromatographic procedures. Using a 10% deactivated alumina column (25x80mm) two fractions were separated. The first, eluted with petroleum ether consisted of plant carotenoids while the second, biologically active fraction, eluted with acetone, was 'decolourised' with activated charcoal, concentrated and rerun on a smaller deactivated alumina column. Bioassays (soaked paper discs on seeded nutrient agar and monitored for development of zones of inhibition) were used to identify the active band resulting from the second chromatography, and this was further purified using preparative thin layer chromatography to separate the α - and β- epimers of the active compound, polygodial. Percentage yields of the compounds were not given.

Southwell and Brophy (1992) used 30 hours ethanol extraction of 'plant material' followed by liquid column purification of components in their comparison of Australian *Tasmannia* species.

Other reports examining crude solvent extracts for new terpenoid or phenolic compounds of Winteraceous species have used hexane (Cruz *et al.* 1973) and hexane-EtOAc (Vichnewski *et al.* 1986).

2.3.2 Polygodial -a bioactive sesquiterpene dialdehyde

Polygodial (a.k.a. tadeonal by Asakawa *et al.*, 1978), Fig 2.1 has attracted interest as a biologically active component in extracts of a diverse array of organisms. Since the identification of the sesquiterpene by Barnes and Loder (1962), it has been isolated from a nudibranch mollusc (*Dendrodoris limbata*), (Cimino *et al.* 1982), three *Porella* species of liverwort (Asakawa *et al.*, 1978), and from members of three higher plant families:

-the Polygonaceae (*Polygonum hydropiper)* - (now *Persicaria hydropiper*)a cosmopolitan species used as a colourful and spicy relish in Japanese sushimi and from which the original identification was obtained (Barnes and Loder 1962),

- the Winteraceae, from members of the genera *Drimys*, *Tasmannia*, and *Pseudowintera* (Sierra *et al.*1986, Loder 1962, McCallion *et al.* 1982),

- the Canellaceae - the East African *Warburgia ugandensis* and *W. stuhlmannii* which have traditional use as sources of spice and medicine (Kubo *et al.* 1976, Kubo and Ganjian 1981, Taniguchi and Kubo 1993).

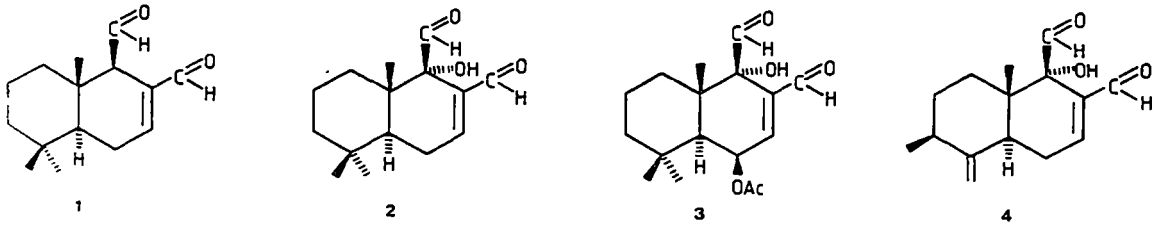


Figure 2.1: Naturally occurring drimane sesquiterpene dialdehydes with antibiotic and hot tasting properties. 1- polygodial; 2-warburganal; 3-cinnamodial; 4-muzigadial.

a) Biological Activity

A number of interesting properties have been demonstrated for the compound, some of which relate to its biological utility in the organisms concerned. Among these are piscicidal, antibiotic, hot taste to humans and insect antifeedant properties. Also attributed to polygodial in extracts of *P. hydropiper* were plant growth inhibition and anti-inflammatory properties by Furuta *et al.*(1986) in a study of a related drimane type sesquiterpenoid polygonolide.

i) Piscicidal activity

The nudibranch mollusc was shown (Cimino *et al.* 1982,1983) to synthesise polygodial and to store it in its mantle where it serves as an effective defence against predation- in fish feeding trials, the freshwater fish *Carassius carassius*m and the marine species *Chromis chromis* immediately rejected food particles treated with as little as 30μgcm^{-2} of surface area. Cimino *et al.* (1985) attempted to establish a product-precursor relationship between sesqui-terpenoid esters found in the digestive gland of the animal and polygodial, found only in the skin, concluding that there is no such relationship, and that the two products were synthesised by separate pathways, and to differing degrees depending upon some seasonal factor related to a defence requirement of the animal.

The use of fresh water extracts of shoots and leaves of *P. hydropiper* to kill predatory fish in commercial fish farms prior to stocking has been investigated (Kulakkattolickal 1989a and 1989b) and appears to have some potential due to its very low LC50 levels for the predatory species concerned and the rapid loss of toxicity to fish (about 5hrs).

ii) Antibiotic activity

Purified extracts of *P. colorata* containing the compound were examined by McCallion *et al.* (1982) for activity against a range of bacteria, fungi and yeast isolates using paper disc bioassays, for zones of inhibition surrounding 6mm discs containing polygodial. With *Staphylococcus aureus* and *Candida albicans* , at 20μg per disc, a large zone of inhibition (> 8.8 mm) was maintained around the discs for at least 13 days. The authors noted that microscopic examination of affected cells showed them to be smaller, and deformed in comparison with control cells and that their material of *P. axillaris* and *P. traversii* did not contain polygodial and did not exhibit antibiotic activity.

Kubo and Taniguchi (1988) used polygodial, warburganal and muzigadial, isolated from *Warburgia* species to examine antibiotic activity on fifteen microorganisms. The three compounds proved to have similar antimicrobial spectra and were particularly effective against *Saccharomyces cerevisiae, Candida utilis* and *Sclerotinea libertiana.* Polygodial was 2-8 times more potent than the other two compounds against all species of yeasts and filamentous fungi and slightly more effective than the commercially available amphotericin B. Actinomycin B, when used together with polygodial demonstrated a sixteenfold increase in potency against *S. cerevisiae* and powerful antifungal activity against *C. utilis* . Microscopic examination of cells after treatment led the authors to conclude that the plasma membrane was the primary site of action of the polygodial, and that by 'punching' holes in the membrane the polygodial allowed ingress of the antibiotic, greatly enhancing its activity. They proposed that its use in combination with less effective antibiotics, might offer opportunities for new treatment of persistent mycoses or for reduced doses of current treatments.

Similar synergistic effects of polygodial on a range of yeasts, filamentous fungi, and bacteria species with a number of antibiotics and other naturally occurring compounds have been reported- Taniguchi *et al* (1988a,b), Kubo (1988), Kubo and Himejima (1991), Himejima and Kubo (1992, 1993), Kang *et al.* (1992)

Interestingly, Kang *et al.* (1992) suggested that the use of (polygodial containing) *Polygonum hydropiper* and *Perilla frutescens* , (shown to contain perrillaldehyde, an effective antibiotic in its own right) together as spicy relishes ('tade' and 'shiso' or 'aoziso', respectively) in the consumption of raw fish in the Japanese Sushimi tradition may indicate an underlying wisdom with respect to control of *Salmonella* poisoning. Anke and Sterner (1991) include polygodial in a comparison of antimicrobial and cytotoxic properties of a number of similar compounds, finding antibiotic but no mutagenic capability.

iii) Hot taste and insect antifeeding

In an effort to determine the mechanisms involved, Kubo and Ganjian (1981) compared insect antifeedant activity and the hot taste experienced by humans for a number of similar sesquiterpenes (polygodial, warburganal, muzigadial and ugandensidial - Fig 2.1) and for four related, but tasteless compounds (including the 9α- epimer of polygodial). They found a systematic relationship between the taste, antifeeding activity and the spatial geometry of the compounds and suggested that the crucial criterion was the distance between the double bond end of the -enal moiety and the nucleophilic dipole of the aldehyde. D'Ischia *et al.* (1982) also explored the mechanism of activity, comparing the reactivity of the compound with thiols and primary amines and showed that the mechanism for activity was most likely to be a reaction with $-NH_2$ groups, explaining the relative unreactivity of the 9α- isomer. Fritz *et al.* (1989) reviewed Kubo and Gangian's proposals, described two other contributions by Sodano *et al.* (1982, 1987) and in a series of experiments using army worm larvae *Spodoptera frugiperda* for their bioassay, examined a number of substituted analogues of

polygodial and warburganal finally proposing a scheme involving both the D'Ishia *et al.*(1982) and Kubo and Gandjian (1981) mechanisms in reactions at the chemoreceptor.

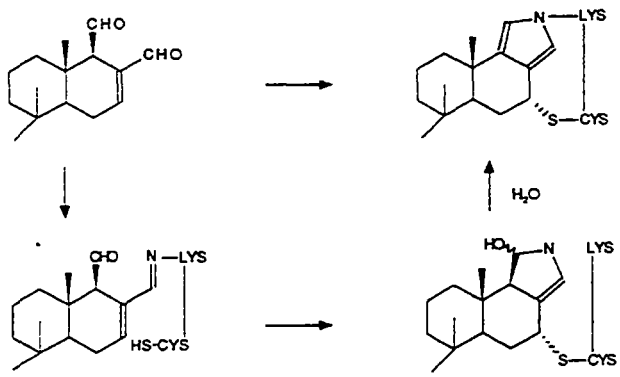


Figure 2.2: Proposed pathway for reaction of polygodial with L-cysteine in vivo. (from Fritz *et al.* 1989)

This reaction (Fig. 2.2) involves the formation with the -NH_2 of L-lysine of an aromatic pyrrole and a reaction between the sulfhydryl of L-cysteine and the enal at C7. This accounts for several of the observed characteristics of the antifeedant reactions - the behavior of the epimer of polygodial, the importance of the aldehyde groups, the weak activity of large analogues and the more potent activity of warburganal, with its additional α-C9 hydroxyl group.

Caprioli *et al.*(1987) discussed the stereochemistry of several isomeric bicyclic dialdehydes of slightly different configuration to natural polygodial, and concluded that a reaction *in vivo* with a primary amino group would depend upon specific spatial arrangement of the CHO groups and upon distances between them in the formation of intermediate compounds.

Schoonhoven and Fu Shun (1989) showed, using larvae of *Pieris brassicae* that stimulation of deterrent receptors and a reduced sensitivity of most other receptors explained in large part the antifeeding activity of polygodial, warburganal and muzigadial. After termination of treatment, receptor cells regained their sensitivity and in most cases showed some hypersensitivity after recovery.

Several groups have investigated the possible applications of the antifeeding response in agriculture. Polygodial was shown to decrease acquisition of some aphid borne plant viruses (Gibson *et al.* 1982) by altering probing and settling behaviour in the aphid concerned (*Myzus persicae*). However, Hardie *et al.* (1992) reported that the behaviour of starved aphids was not affected by treatment of the substrates with polygodial, despite the earlier findings of Dawson *et al.* (1986) with beet yellow virus, and Gibson (1982)-barley yellow dwarf virus, that such treatment altered acquisition and transmission. The discrepancy was resolved by Powell *et al.* (1995) using choice/no-

choice assays, finding that the insects, when given choice, concentrated their settling, searching and feeding behaviour on untreated leaf areas, whereas, in a no choice situation, aphids on treated leaves did not behave significantly differently to those on untreated leaves. The authors speculate on the significance of this finding for pest control strategies in the field situation.
van Beek and de Groot (1986) review the significance and variety of terpenoid antifeedants of natural origin.

b) Synthetic preparation of polygodial.
Methods for producing the compound have been reported by several workers: Pickett (1985) reported the synthesis and resolution of polygodial resulting in a racemic mixture which, if it contained trace quantities of the (+)- isomer, proved highly phytotoxic and the use of natural polygodial from plant sources was recommended. Ley (1990) discussed synthetic production of polygodial and warburganal as a 'case study' for development of new synthetic insect control systems and callus and suspension cultures of *Polygonum hydropiper* were used by Banthorpe *et al.* (1989, 1992) to accumulate polygodial and its parent drimenol. The latter paper presents work conducted on purification of the enzyme system, farnesyl pyrophosphate:drimenol cyclase from cell cultures of *Polygonum hydropiper* in levels comparable to those in extracts of the parent plant material. These papers discuss the difficulty of using the dialdehyde directly as a plant protectant owing to its volatility and instability, and raise the possibility of gene transfer from *Polygonum hydropiper* to endow commercially important crops with the capacity to synthesise the antifeedant themselves.
de Groot and van Beek (1987) review previous efforts to synthesise members of the group of drimane sesquiterpenes including polygodial, warburganal, and cinnamodial, noted for their activity, and describe a variety of suitable synthetic approaches for each. Subsequently contributions by Urones *et al.* (1994, 1995), Barrero *et al.* (1994), employ zamoranic acid and sclareol, respectively as starting compounds for multiple step syntheses. Jansen *et al.* (1989) used dihydrocarvone to prepare chiral intermediates for polygodial synthesis, and Kutney *et al.* (1990) proposed a synthetic route to polygodial employing derivatives of thujone.
Separation of enantiomers and analysis of the compound were reported by Brooks *et al.* (1985, 1988) and Hariguchi *et al.* (1993) and van Beek *et al.* (1994) report HPLC methods for determination of polygodial.

2.3.3 Concluding Comments

The forgoing discussion highlights the relative rarity of polygodial in nature, (although it appears often among members of the family Winteraceae), and invites speculation upon the biological utility of the compound where it occurs. Similarly it raises questions about the suitability of plant material or extracts containing the compound for human consumption.

In this respect, a parallel with capsaicin might be drawn. Present as the hot principle in a variety of capsicum fruits, capsaicin is injested in substantial quantities around the world. The neurotoxic effects of the compound are well documented, but information on carcinogenicity and mutagenicity remains limited and contradictory and there is even some suggestion that the compound may induce chemoprevention in some instances (Zhang *et al.* 1993). Surh and Lee (1995) review the conflicting literature surrounding the toxicology of capsaicin, and conclude that minute ingestion of the compound (present in dried fruit at approximately 0.5%) results in little or no toxic effect, while heavy injestion may result in saturation of the relevant defensive mechanisms, with noxious effect.

Traditional consumption of at least one of the species containing polygodial as a flavouring relish (*Polygonum hydropiper)* and more recently, over twenty years of use in Australia of *Tasmannia spp.* as novel foodstuffs (Cribb and Cribb 1975, Low 1988, Cherikoff 1989) would seem to offer anecdotal evidence for the safety of small quantities of the compound for human consumption.

In particular, some indication of the 'typical' concentration of the compound in natural leaf and in the appropriate extracts of *Tasmannia lanceolata* is required. These should be compared with those concentrations typically found in *Polygonum hydropiper* - (0.06% w/w in dried plant material and 12% in the petroleum ether extract - (Barnes and Loder 1962)).

The presence of polygodial in *T. lanceolata* together with its demonstrated antifeedant properties suggests that the apparent success of the species in colonising open areas in wet situationsmight be due in some part to the discouraging effect of polygodial on browsing vertebrates and invertebrates.

Lastly, the large variety of secondary compounds reported for *T. lanceolata* suggests that other commercially useful compounds might emerge from an exhaustive identification of extract constituents across a range of plant material, habitats and geographic locations.

2.4 Oil cells as storage sites for secondary metabolites

Amongst the higher plants a diversity of storage and secretory systems associated with the production, transport and sequestration of the products of the secondary metabolism are encountered. Such structures include glandular hairs and trichomes, multicellular cavities, secretory idioblasts, resin ducts and areas of epidermal cells (Fahn 1988) and are borne both on, (and sometimes articulated from the surface of) the plant, and within the plant body.

Superficially the simplest of these is the oil cell or oil idioblast found embedded in parenchymatous tissue, often in association with similar structures containing mucilaginous deposits, or with tannin containing cells.

Oil cells occur in a taxonomically diverse range of angiosperm species, but are most common in, and have been described as characteristic of, the primitive woody Ranalean plant families by West (1969), who surveyed their development in nine of these families, including Magnoliaceae, Lauraceae, Annonaceae and Winteraceae. West describes a 'normal oil cell ontogeny' typical of most of the 46 species (27 genera) studied and details those secondary structural developments which deviated significantly from this. West's study included *Drimys winteri* (a specimen collected in Brisbane, Australia) and *Pseudowintera axillaris* as representatives of Winteraceae and confirmed only the presence of mature oil cells in both species and the normal pattern of development in the latter.

These systematic aspects of oil cell occurrence and distribution patterns have been examined in *Cinnamomum spp.* by Bakker *et al.* (1992), in *Annona* by Bakker and Gerritsen (1992) and in the dicotyledons generally by Baas and Gregory (1985). Oil cells have not been used for systematic studies in Winteraceae, and are mentioned by Bailey and Nast (1945b) only as a typical characteristic of the family. Vink's (1970) examination of Winteraceae did not mention oil cells as having potential as a systematic tool, despite the use of numerous other anatomical and morphological characters to determine the taxonomic status of the 'Old World' genera - *Pseudowintera, Drimys* sect *Tasmannia* (sic), and the other tropical and sub tropical members of the family. Whole extracts of leaf material were used by Southwell and Brophy (1992) to support certain taxonomic distinctions within the genus *Tasmannia.*

While the single cell oil idioblasts typically found in the woody Ranalean plant families have undergone considerable scrutiny from the point of view of ultrastructural development, the content of the cells and the development and accumulation of those contents has not been well examined.

2.4.1 Oil Cell Development and Structure

Several oil cell-containing species are described in the literature as exemplifying the process of oil cell development. Maron and Fahn (1979) examined oil cells in the leaves of *Laurus nobilis* L and described four 'stages' in cell development:

1 - the presence of only the outer cellulose wall, when cytoplasm contains many ribosomes grouped together, a few , short profiles of endoplasmic reticulum, many mitochondria and dictyosomes and variously shaped plastids containing electron translucent vesicles,

2 - presence of a lamellated suberin layer inside the cell wall, wall protuberances with plasmodesmata, a vacuole which increases in volume as suberin deposition proceeds, numerous polysomes (groups of ribosomes), stacks of ER in parallel arrays, decreasing numbers of dictyosomes, and occasionally plasmalemma invaginations containing vesicles and disorganised membrane structures,

3 - an inner cellulosic wall appears, commencing near where the suberin layer is thinnest, at which location cupule formation commences with a thickening of the inner layer. Ground cytoplasm becomes more electron opaque containing only a few small vacuoles and a 'large space develops which contains the oil drop', and which is lined with the plasmalemma which adheres to the inner surface of the cell wall, particularly at the site of the cupule, a peglike projection of the oil body,

4 - the oil drop occupies most of the volume of the cell, cytoplasm becomes very opaque and organelles can no longer be distinguished.

Oil cells were first encountered at the third leaf primordium from the apex, although stage of development was not correlated with ontogenetic leaf stage.

Bakker ands Gerritsen (1990) proposed a slightly different scheme in their study of *Annona muricata* , adding that the young cell may have arisen from an unequal cell division and would be found with a smaller 'sister' cell, and preferring to subdivide a 'stage 3' (in which the inner cell wall was present) into three parts, based on the size of the oil globule, the amount of smooth tubular and vesiculating endoplasmic reticulum and the degeneration of other organelles in the fully mature cell. (See Fig 2.3)

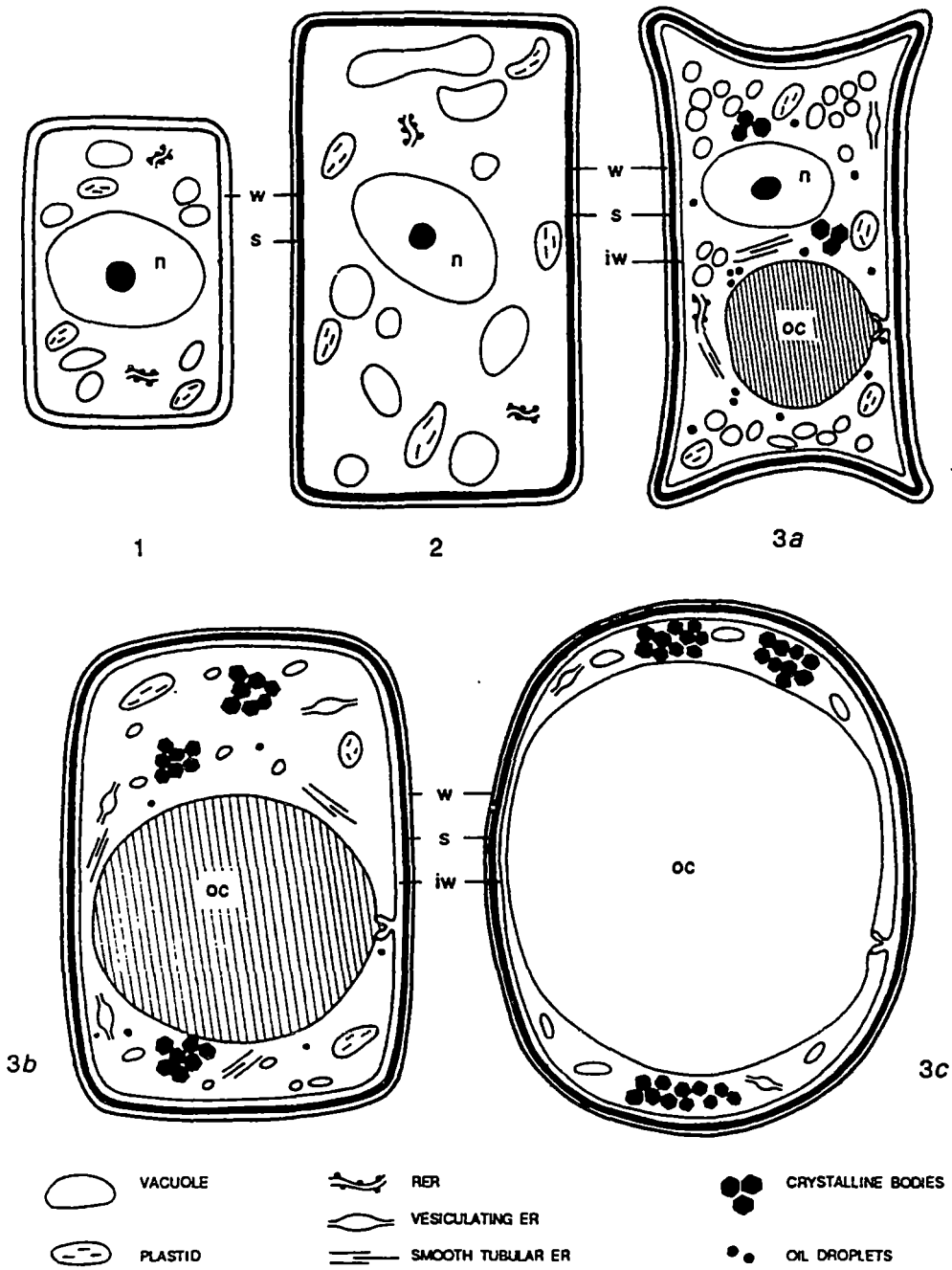


Figure 2.3: Schematic representation of developmental stages in oil cell differentiation in *Annona muricata*. 1: Young cells with abberent plastids and an electron translucent cytoplasm. 2: Suberised layer deposited against the primary wall. 3a: inner wall deposition initiated, oil cavity develops, tubular ER and crystalline bodies appear in cytoplasm. 3b: Enlarged and degenerating organelles. w= initial cell wall,n=nucleus, s= suberized layer, iw=inner wall, oc=oil cavity.(Adapted from Bakker and Gerritsen 1990)

A number of other studies of oil cell development and structure in *Magnolia grandiflora* (Postek and Tucker 1983), *Persea americana* (Platt-Aloia *et al.* 1983, Platt and Thompson 1992), *Cinnamomum spp* (Bakker and Gerritsen 1989, Bakker *et al* 1992), *Liriodendron tulipifera* (Mariani *et al.* 1989), *Annona muricata* (Bakker and Gerritsen 1990) have contributed to the model for secretory cell development.
The occurrence of the cupule, involvement of plastids in oil production and the tripartite wall structure appear generally accepted although Postek and Tucker's paper questioned the concept of the cupule, suggesting it might be an artefact, and found no more than two wall layers in their survey of 16 families. There are no reports of entry of oil droplets into a differentiated oil cell, nor any details of the mechanism of filling of the drop although Fahn (1988) describes the probable movement of oil drops from points of synthesis (plastids) into the cytoplasm and then fusion with the growing droplet, probably by reverse pinocytosis through the surrounding membrane.
The function of suberised layers or walls is proposed by Bakker and Baas (1993) to serve to compartmentalise potentially toxic substances, by preventing apoplastic transport of the cell contents. It is not clear from this paper whether plasmodesmata are occluded by the suberin layer in oil secreting cells, although such a situation is noted by Platt-Aloia *et al.* (1983) in avocado mesocarp, in which case all transport pathways are presumably blocked, and no further apoplastic or symplastic exchange is possible.
In general, idioblast differentiation and development are detected in ground meristem associated with very young tissue and are distinguished by size, a less electron dense cytoplasm, fewer starch granules, the presence of osmiophilic material associated with several different organelles and the eventual development of the suberised layer generally regarded as typical of the structure (Platt-Aloia *et al.* 1983, Bakker and Gerritsen 1990, Fahn 1988).
Tucker (1964) reported the occurrence of secretory oil cells in close association with terminal veinlets in at least 138 Magnoliaceous species, describing the adjoining wall of the tracheal element as sometimes appearing pitted, although this association has not been mentioned in the many subsequent publications on the subject.

2.4.2 Contents of Oil Cells in Plant Tissue

In considering the process of development of oil cells and the accumulation of secondary products in plant tissue generally, it is important to distinguish between the contents of the specialised structures, and the composition of oils and extracts obtained from the whole tissue system.

Essential oil/ extract composition and yield serve to provide an indication of overall metabolic activity at sites of synthesis. They may be correlated with structural changes such as gland filling (in hops for maturity - Menary and Doe 1983) or resin canal

frequency in pine trees (to assess genetic variation - White and Nilsson 1984); with ontogenetic and seasonal patterns in plant growth (Leach and Whiffin 1989; Zavarin *et al.* 1971); or with geographic variation within or between species (von Rudloff 1972, 1975; Zygaldo *et al.* 1990)

Solvent extracts (or steam distillates) of whole leaf tissue will contain not only the contents of oil bearing structures associated with the leaf tissue, but also lipid soluble (or steam volatile) compounds present elsewhere in the leaf, for example, as inclusions in synthetic organelles in epidermal, mesophyll cells, or in intercellular spaces in the leaf and as elements of cuticular and alveolar deposits. A study of changes in the composition of 'total extract' during plant growth will not distinguish between extractable compounds associated with maturation (development of cuticle, cell walls and tracheary elements, increase in cell size, production of secondary products in epidermal and mesophyll cells) and progressive development and filling of the oil bearing structures themselves. Furthermore, many plant tissues contain more than one type of secretory organ which may independently accumulate quite different suites of secondary compounds (Fahn 1988). Lastly, the high temperatures and often acid environment of the plant material during steam distillation might result in the formation of artefacts particular to the oil and plant species. This is examined in detail with respect to α-terpinene and terpinolene present in steam distilled tea tree oil by Southwell and Stiff (1989) who also cite a number of examples of other terpenes similarly implicated in artefact formation.

The problem of distinguishing between the contents of the structures and the 'whole' oil or extract was raised by Russin *et al.* (1988) in considering previous examinations of the steam distilled oil of *Tagetes erecta.* None of those studies cited had distinguished between the contents of the various structures, nor addressed the possibility that some oil constituents might be artefacts produced by the high temperature environment during distillation, A similar situation applies to most studies of spatial or temporal variation in oil composition in essential oil species. Bicchi *et al.* (1985) did show the difference in composition between the oil contained within secretory cavities and that obtained by steam distillation, and Russin and his colleagues developed a procedure for isolating secretory cavities and examining their contents (Russin *et al.* 1988, 1992) -see 2.4.3 below.

Once again this issue was set aside by Zygaldo *et al.* (1990) for the related *Tagetes minuta,* where a generalised oil composition was reported in geographically separate populations and proposed as a suitable indicator for chemosystematic comparisons. Kobiler *et al.* (1993) separated oil cells from avocado mesocarp in order to show that the structural integrity of these cells prevented antifungal compounds held within them from acting to control infection, while the same compound, present in the pericarp but not sequestered in oil cells, was able to prevent infection in this tissue. This was practical recognition of the importance of identifying oil cell constituents *per se,*

distinct from 'total extract', in studies of the mechanisms of such biological defence systems.

More generally, detailed qualitative investigation of biochemical processes at sites of terpene synthesis and storage (eg isolation and investigation of secretion products and enzyme systems, incorporation studies, examination of synthetic and secretion processes *in situ* and study of plant chemical defence systems), require the same discriminating analysis of essential oils obtained from the plant structure in question. Therefore methods are required for separation of the secretory structure from the rest of the plant tissue (ideally intact and vital), removing the contents of the storage sites, or by some other means determining the nature of their chemical content. Some of these methods are reviewed below.

A) Isolation of oil bearing organs/cells

The liberation of intact mesophyll cells and protoplasts from leaf tissue is well established for use in cell culture, photosynthetic and biochemical studies. Grinding techniques (Colman *et al.* 1979, Oliver *et al.* 1979) or enzyme digestion, followed by sieving and centrifugation using density gradients (Franceschi *et al.* 1984) have been shown to produce large numbers of mesophyll cells or protoplasts, usually assessed for purity and viability by means of staining and microscopic examination or gas exchange measurements.
Due to the variety in morphology of the oil bearing structures, methods for their isolation have tended to vary according to the size and structural integrity of the system.

i) Superficial glands
In species in which oil bearing structures are borne on the leaf surface, a number of mechanical techniques are reported for obtaining preparations of the cells or their contents, ranging from the homogenisation of leaf tissue and separation of the gland heads on a Percoll density gradient (Slone and Kelsey 1985), abrasion or scraping of leaf surfaces (Croteau and Winters 1982; Gershenzon *et al.* 1987; Keene and Wagner 1988), preparation of epidermal peels (Croteau 1977)and use of adhesive tape (Keene and Wagner (1985), although none of these methods appeared to generate preparations of sufficient purity or vitality to permit purification of biosynthetic enzymes. For this type of secretory material, the method of Gershenzon *et al.* (1992) appears to be the most useful, in which a gentle abrasion of the leaf surface with glass beads in buffer at 4°C is followed by filtration through a series of nylon meshes, washing with buffer and suspension of the resulting cell clusters in buffer for biosynthetic studies and preparation of cell free extracts. This method was further improved by Hashikodo and Urashima (1995) shaking leaflets of *Rosa rugosa* with a mixture of quartz and sea sand, to produce intact trichomes without inducing browning and enzyme denaturing in

the process. Yerger *et al.* (1992) report the use of powdered dry ice, added to leaf material and 'vortexed' to produce large quantities of pure trichomes from a variety of plant materials -geranium (*Pelargonium sp.*), tomato (*Lycopersicum*), squash (*Cucurbita sp.*), and velvetleaf (*Abutilon sp.*).

ii) Oil Cavities

Multicelled secretory cavities embedded within the leaf lamina of African marigold (*Tagetes erecta)* were examined by Russin *et al.* (1988) after manual dissection from the surrounding mesophyll. The structures were then extracted in petroleum ether and the composition of the extract determined by HPLC. Russin *et al.* (1992) took the isolation approach further, and used a digestion and filtering technique to obtain secretory cavities entirely free from surrounding mesophyll cells, which they then examined microscopically for structural integrity and with vital stains for viability.

iii) Single oil cells

Platt and Thompson (1992) report a method for isolating oil cells from the mesocarp of soft ripe avocado fruit by homogenisation and filtration and from immature or mature unripe fruit by digestion of small pieces in a cellulytic medium followed by homogenisation and filtration. Cells appear intact and relatively free from cellular debris and were tested using histochemical techniques for the presence of alkaloids and terpene compounds with the result that both classes of compound were indicated, to different degrees in the variously mature fruit tissues examined.

B) Direct sampling of the contents of secretory structures

There are a number of reports of direct sampling of the contents of secretory structures for analysis. Lanyon *et al.* (1981) examined one of three types of secretory structure found on *Cannabis sativa* - the secretory sac of the capitate stalked gland, - using a micropipette to remove the contents of the sac without damage to the underlying disc cells and showed that the cannabinoid profile of this oil did not differ significantly from that reported for whole gland extracts, suggesting that secretory product is almost entirely removed to the secretory sac upon production. Turner *et al.* (1978) using similar techniques compared drug and fibre strains of the species, and glands taken from vein and nonvein areas of the leaf and bract surfaces. Capitate-sessile glands on the leaves contained low levels, and in some strains none, of the principal cannabinoid associated with that strain, while high levels of the compound were detected in capitate-stalked glands on bracts of the same clone.

Venkatachalam *et al.* (1984) also considered stalked and sessile glands, but on *Salvia officinalis* , while Bicchi *et al.* (1985) concentrated on a comparison of direct sampled and microdistilled oils from *Tagetes* spp and *Humulus lupulus* finding marked differences in each case- that distilled oils contained a predominance of monoterpene and oxygenated monoterpene compounds, and lacked the less volatile higher carbon

number terpenoid compounds of the extracts, the difference being most marked in the case of hops. A similar approach was used by Menary *et al.* (1986) to compare oils from lupulin glands and glandular trichomes and to investigate the consequence for storage stability of alpha acids of the natural partitioning of some of the components of these oils.

Microsampling techniques were used by Spring (1991) to analyse the sesquiterpene lactones of the *Asteraceae* in a consideration of the chemosystematics of the family. The isolation and analytical techniques appropriate for these relatively non- volatile compounds include GC (with derivatisation) and HPLC methods. These trichomes are quite large, and collection of trichome contents using an insect pin or needle, or breaking the basal cell away on dried plant material appeared to be quite straightforward.

C) Other methods of determination of 'oil cell' contents

Rather than separating the cells to determine the exact nature of their contents, some techniques are available for analysing the contents *in situ* . The histochemical methods employed for oil cells in avocado fruit by Platt and Thompson (1992), included two for compounds of a terpenoid nature -sulphuric acid (red reaction with sesquiterpene hydroperoxides) and Nadi reagent (pink for resiniferous acids, blue -essential oils and violet a mixture of these). The results for this technique, (performed in tandem with cell isolation, extraction and TLC techniques) were not completely satisfying, indicating differences in oil cell content associated with fruit maturity which were not detected in chromatography of the extracts, and only providing a general reaction for the large classes of compounds likely to be present.

Zarate and Yeoman (1994), similarly examined oil cells *in situ* in sections of ginger root tissue.They used UV spectral results to confirm visual evidence of colour change in oil cell contents upon the addition of 10% sodium carbonate, due it is suggested, to the presence of curcumin derivatives or flavonoid-like compounds, rather than to the main pungent principle gingerol. A ferric chloride/ potassium ferricyanide mixture was used to detect phenolics, and Nile red for the detection of lipidic material. An attempt to correlate oil cell number with gingerol yield and cryo-fractured rhizome material visualised under SEM are also presented. The authors suggest that the collective evidence of staining reaction and the freeze fracture results confirm the presence of lipid material within the cells, and that the probable location of gingerol in (non-polar) lipid solution together with the correlation of cell numbers to gingerol yield points strongly to the likelihood that the compound is accumulated and stored within the 'cells with yellow contents'.

Karwazki (1993) detected the enzyme chalcone synthase in tannin containing idioblasts and oil cells in *Kalanchloe daigremontiana* and *Acorus calamus* and leaves of *K tubifera* by means of indirect immunoflourescence, and found that the distribution of the enzyme did not change during the period of organ development.

Hagendoorn *et al.* (1994) employed FTIR spectroscopic methods to examine whole oil cells of *Polygonum hydropiper,* specifically for the presence of polygodial. This report suggests that the compound was localised in these cells and not present in neighbouring tissue, nor in similar cell structures in a related species which did not yield this compound upon extraction. The spectra presented appear to suffer from the presence of substantial quantities of water, and include several departures from spectra of the pure compound presented in the same paper. Nonetheless, sufficient agreement is in evidence to confirm that polygodial is localised in the cells themselves and that oil cells in this genus do not invariably accumulate this compound. No discussion of what compound(s) might be characteristic of the cells in related species is offered.

2.4.3 Concluding remarks

The above discussion identifies and describes the oil cell system found in the Winteraceae, and underlines the fact that in the case of *Tasmannia lanceolata* , the presence of these cells has not been considered in either morphological or chemical studies. Clearly the oil cell system should be considered with respect to the complex of secondary compounds obtained by extraction of leaf material, and in particular, some effort made to distinguish between extract and oil cell contents, for the purposes of experimental comparisons. Of the methods described above, direct removal of oil from the cells, separation of oil cells by digestion and filtration and use of FTIR spectroscopy appear to offer the most promise.

2.5 Seasonal Changes in Oil Composition

Seasonal changes in oil composition, together with the developmental and morphological changes associated with the annual growth cycle may be employed definitively in several areas of research, in particular, commercial production, chemosystematic studies and ecological studies of species interaction where chemical influences are implicated.

2.5.1 Commercial production of essential oils

Since for most essential oil crops the compositional requirement for oils and extracts is quite specific, selection of suitable cultivars from an unselected population depends upon a critical comparison of chemotypes available, and careful management of harvest with respect to timing and the nature of the material harvested. With established commercial essential oil species, the importance of ontogenetic changes in determining the composition of oils and extracts is clear, as is selection of the plant parts to be harvested.

Hay (1993) notes the significance of phenology and ontogeny in commercial production of volatile oils from field crops, observing that selection of species and cultivars depends upon matching phenology to the available growing season, and management and profitability depend upon interrelationships amongst three aspects of ontogeny -

1) time course of biomass (DM) production,
2) time course of oil content per unit dry matter, and
3) time course of production of oil constituents.

These parameters are reported for many traditional essential oil crops, and characterisation of the seasonal change in relation to dry matter and oil yield continues, even for widely established species such as peppermint (Court *et al.* (1993), Bouverat-Bernier (1992)).

Studies of this kind for a range of common essential oil crops are frequent in the literature-

Apiaceae eg *Petroselenum* -Porter (1989a) , *Anethum* -Clark and Menary (1984);
Lamiaceae eg *Majorana, Melissa, Ocimum, Origanum, Salvia* Basker and Putievsky (1978), *Thymus* - McGimpsey *et al.* (1994), *Lavandula* - Lammerinck *et al.* (1989), *Mentha* -Clark and Menary (1979) ;
Asteraceae - *Tagetes* - Thappa *et al.* (1993), *Artemesia* Putievsky *et al.* (1992);
Myrtaceae - *Melaleuca alternifolia* - Southwell and Stiff (1989).

Such studies provide a basis, biochemically and agronomically, for selecting an optimum harvest date.

For example Porter's (1989a) study of two cultivars of parsley- *Petroselinum crispum* , monitored twelve components in the steam volatile oil at six stages of development for each cultivar, corresponding to developmental stages between the rosette and mature, brown secondary umbels. Oil yield was expressed as % of fresh weight and the levels of each of the compounds as a percentage in the oil. The plants were also separated into portions- stem, leaf, primary secondary and tertiary umbels to provide an indication of the contribution of each to the whole oil yield. Composition and yield varied continuously during the development cycle, reflecting the large changes in proportion of the plant parts and changes in oil composition within them. In general, as oil accumulation proceeds from 'herb' to 'seed', oxygenated compounds (eg myristicin) begin to dominate the oil, and yields per plant increase. Harvesting of 'herb' oils, characterised by β- phellandrene and *p* -mentha 1,3,8 triene, required some compromise in yield, as it was necessary to cut short the development of the oil rich inflorescence. Porter notes that because of the continuum of compositional changes during the developmental cycle, each crop 'offers a wide range of oil compositions' enabling achievement of target oil qualities demanded by the market.

Where crop response to local growing conditions is not well understood, there is a requirement for study of the phenological and ontogenetic responses to prevailing conditions. In this respect the quality of the oil at the time of harvest will be of paramount importance. This situation applies to any introduction of a conventional crop to a new region, for example Kallio and Jünger-Mannermaa(1989) - juniper berry production in Finland, Hay *et al.* (1988) - Summer Savoury for Scottish conditions and Clark and Menary (1984) -dill for Tasmanian conditions. In these examples, the phenology of the crop is 'fitted' to the prevailing temperature and daylength regime. On the other hand, in consideration of a wild population for commercial use, the natural variety of habitat conditions, ecotypes and genetic diversity at the individual level complicates examination of phenology and ontogenetic change in yield 'quotient'. Examples of this are found with Holm *et al* (1988) - Dragonhead oil in Finland, Hegerhorst *et al.* (1988) - commercialisation of Rubber Rabbitbush, Dragar and Menary (1992) - essential oil from a Tasmanian *Asteraceae*, and the series of studies of *Satureja douglasii* -by Lincoln and Langenheim (1976, 1978,1979) and Rhoades *et al.* (1976).

The last example represents a case in point. *Satureja douglassii* , a mint naturally distributed on the west coast of the USA north to British Columbia, found in a diversity of habitats and offering a wide range of ecotypes, was examined for systematic chemical groupings. Five chemotypes were discerned from among 51 separate populations, the leaves of all types containing substantial proportions of bicyclic monoterpenes, but differing in their remaining constituents, named 'carvone', 'pulegone', 'isomenthone', 'menthone' and 'bicyclic' (compounds- camphene, camphor, borneol) for the high relative proportion of each in the so-called chemotypes. These studies considered developmental changes only cursorily, and as a result, in the 1978

report of the effects of irradiance and temperature on oil composition, showed that the net influence of these factors was complicated by ontogenetic trends, particularly leaf position on the stem, and changes in yield/leaf dry matter *and* yield of leaf dry matter. In the Rubber Rabbitbush study (Hegerhorst *et al.* 1988), seasonal trends *were* considered for several major terpenoid compounds and rubber and resin content in green tissue, one year old tissue and tissue several years old were determined over an eleven month period. This showed that rubber and resin were strongly negatively correlated and changed steadily over the sampling period and provided an indication of the metabolic interrelation between the two products, as well as suggesting an optimum harvest date. Holm' *et al*'s (1988) interest in Dragonhead (*Dracocephalum moldavica*) arose from the possibility of producing a 'Lemon Balm' -like oil in the short cold growing season at high latitudes. The highest oil yield (the optimum for harvest) was found to coincide with flowering, although the desirable geranial continued to increase thereafter, offering, as in the case of parsley, the possibility of trading yield against quality requirement.
Russin *et al.* (1988) were more specific in their examination of oil development in *Tagetes minuta* , studying the changes in the contents of isolated secretory cells, rather than those in the essential oil obtained from the whole leaf. The significance of this approach lies in the difference between the elements of foliar secretion. The authors observe that in this species, three types of secretory structure are found, and presumably three (or more) different sequences of oil accumulation contribute to the whole.

Clearly, the processes of synthesis and accumulation of oil components together with the extent of volatile emissions, or catabolism of stored compounds underly the development of the final oil composition. Since the relevant economic parameters are harvestable yield of oil /dry matter, and oil composition at harvest, it is a fundamental requirement that the pattern of oil metabolism and dry matter accumulation be correlated with phenological events and that these events 'fit' the available environmental circumstances.

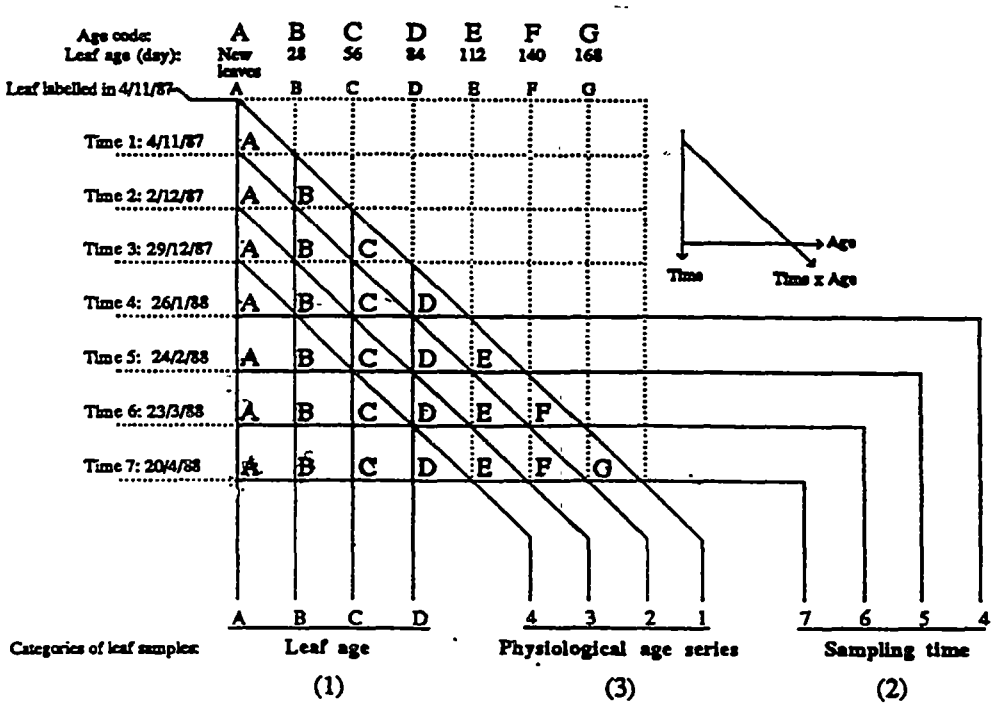
2.5.2 Chemosystematic studies

The importance of seasonal changes in terpene and other secondary compounds should be considered in chemosystematic and genetic studies. There is a great deal of literature in which volatile or extractable plant metabolites are used to compare plant entities, from comparisons within species and between individuals, regional groups or ecotypes to studies of familial and higher order relationships. Such studies rely on similarities or differences in levels of such metabolites to justify changes to accepted taxonomic groupings, and ideally should clearly separate any effect of time of sampling or type of plant material sampled, from the genetic component of variation. Many, however fail

to specify the time of year, physiological age or even the type of plant material used in the comparisons.

Menadue and Crowden (1983) included ethanol leaf extracts in a study of two *Richea* species from 24 populations (4 of one, 20 of the other) from which they concluded that the smaller group should be submerged in the larger and that no discontinuity in the chemical or morphological characters used justified retaining the separation. The report describes the material sampled as terminal portions of single branches but does not mention the time of year of sampling nor the age of leaves used in the analysis. Southwell and Brophy (1992) suggested that essential oil chemistry of the Australian *Tasmannia* species might be a useful adjunct to morphological studies in confirming certain slightly contentious aspects of the taxonomy of the genus. However, their preliminary investigation and conclusions regarding affinities and separation of some members of the genus depended upon oils and extracts obtained from samples of a few plants from only three wild locations or a few Tasmanian collections grown in an institutional gardens, for which the time of harvest or the physiological age of the material in each case is not recorded. Berry *et al.* (1985) concentrated on cluster analysis of sesquiterpene compounds found in Rimu (*Dacrydium cupressinum*) foliage to impute biosynthetic linkages between them. Stating that 'no effort was made while sampling Rimu foliage to distinguish between old and new growth, and samples taken in Spring 1981 may well have included some new growth', these authors rely on the assumption that 'sesquiterpene levels are largely unaffected by environmental factors' to conclude that the levels are genetically controlled. Adams *et al.* (1970), in establishing the taxonomic status of a new species of *Juniperinus*, compared the oil obtained from type samples taken on a specific (and carefully reported) date, with oils obtained from other recognised taxa some five years previously on an unspecified date. The above examples show that systematic studies may be less than conclusive when the nature of the plant material or growing conditions are not specified.
On the other hand, many chemosystematic studies do attempt to address the problem of seasonal or ontogenetic changes.
Zygaldo *et al.* (1990) comparing populations of *Tagetes minor* used fully open flowers as a sampling unit to avoid the problem, while Butcher *et al.* (1994) confined their sampling of eleven populations of *Melaleuca alternifolia* to a two week period in early autumn, using only the previous season's growth. The latter approach requires care, particularly when applied to populations in widely different environments wherein the stages of ontogeny may not be synchronous. The former approach, on the other hand, ignores the difference in time of season or even the kind of season which has occurred during the development of the flowers. Li (1993) incorporated three 'dimensions' of influence in his study of volatile oils of provenances of *Eucalyptus nitens* , *E. delegatensis* and *E. globulus*. growing in similar environments. These he described as ontogenetic, physiological and seasonal. The sampling system provided

an opportunity to separate the effects of a) time of initiation/emergence, b)physiological age at time of sampling and c) any trend in composition over the season (after consideration of a and b). The scheme in Fig 2.4 shows the approach taken, with each successive sampling date providing leaves of an age series which can be compared with those of the matrix developed over previous sample dates for composition changes. The scheme relies on a regular sequence of newly emerging leaves for development of the 'leaf age' series - appropriate for the Eucalypt species and the growth season of the experiment described but not for short, or changeable growing seasons where it would not be possible to compare a current date 'D' leaf with the previous 'C' leaf etc., nor for species which undergo determinate growth cycles for a short part of the season eg spring, or which exhibit uneven patterns of leaf emergence during the season.



Categories of leaf samples and sources of variation for each category of leaf sample:

(1) Leaf age classes: leaves initiated at different time, same physiological age sampled at different times throughout the study period. Variation between leaf samples within each age class are affected by sampling time factor and could compound environment/seasonal effects with ontogenetic effect.

(2) Time series: leaves initiated at different times but sampled at same time. Variation between leaf samples within each time series is affected by age factor and could compound seasonal/time effects on initiation of leaves, ontogenetic and physiological aging effects.

(3) Physiological age series: leaves initiated at same time but sampled at different time. Variation between leaves within each series are mainly affected by physiological age differences, but could compound differences due to environment at time of sampling.

Figure 2.4: Sampling matrix for comparing the effects of sampling date, physiological age and time of initiation of *Eucalypt* leaves on the composition of their volatile oil content (from Li 1993).

The importance of addressing the prospect of changes during growth and maturation in systematic studies is increasingly recognised. Zavarin *et al.* (1971) noted the implications for chemotaxonomy and many publications since have dealt specifically with the problem in order to determine the appropriate sampling regimen for the species' concerned (von Rudloff 1972, 1975; Bernard-Dagen *et al.* 1979; Pitarevic*et al.* 1984; Scora *et al.* 1984; Simmons and Parsons 1987; Holm*et al.* 1988; Southwell and Stiff 1989; Whiffin and Hyland 1989).

Whiffin and Hyland (1989) for example, incorporated seasonal variation in the level of several terpene compounds into a systematic study of Australian rainforest trees, using monthly samples of leaf to establish patterns in composition of steam distilled oil for ten trees of *Syzigium canicortex* an Australian tropical rainforest species. The study concluded that for this species, the variation in oil composition during the year was less than that between individuals and that systematic studies might safely be conducted at any convenient time of the year. For another species *Litsea leefeana*, principle coordinate analysis was used to account for the variation amongst three trees during the year, and showed that they remained clearly distinct at all times. Lastly, the study showed that there was no consistent pattern of oil composition over the year for single trees of six different genera in the Lauraceae, nor was the extent of divergence among them noticeably greater at any particular month. This result may be unexceptional for tropical species experiencing less distinct seasonal cycles, but is clearly not the case in many temperate species.

For example Nerg *et al.* (1994) compared extractable compounds in leaves of Scots pine seedlings grown from seed of nine different geographical origins and held at three latitudes (approx. 58°, 62° and 66°N) in Finland and Estonia and found that seed origin was insignificant in comparison with the changes during the season and between the location of growth.

Simmons and Parsons (1987), showed the confounding of genetic and seasonal effects in a study of seasonal variation of the steam volatile oil of *Eucalyptus ovata* and *E. camphora* . They concluded that patterns of variation were most likely to be due to leaf ageing, and that the genetic characteristics of particular trees would determine response to ageing effects, leading to the likelihood of large differences within and between species and between individuals in a population. Canonical variate analysis demonstrated a clear grouping of different trees despite considerable variation over the sampling period.

Importantly, the authors concluded that for chemotaxonomic work with Eucalypt spp., winter and autumn sampling, together with a suitable sampling method should enable elimination of leaf ageing and seasonal effects from chemosystematic studies. Scora *et al.* (1984) distinguished between terminal, juvenile, 'adolescent' and mature foliage in *Artemisia douglasiana* and noted that although oil yield was lowest for the latter group, the homogeneity of the samples indicated it was the most suited for chemtaxonomic purposes.

White (1983) discussed patterns of terpene mixtures in wild stands of lodgepole pine and other pine species in a study of correlations between terpenes by family and in the wild stand, but other than specifying sample date and type of leaf material did not incorporate any seasonal aspect in the work. Subsequently, White and Nilsson (1984), examining heritability of resin canal frequency and levels of mono- and sesquiterpene compounds, concluded that terpene production and storage was compartmentalised in the canals, that their frequency determined total terpene yield, and that this was only moderately under genetic control. Again there was no consideration of the ontogeny of the storage organ or the progressive accumulation of oil constituents.

A series of studies of North American *Pinus* species - *P. mariana* (von Rudloff 1975a); *P. contorta*, (von Rudloff and Nyland 1979); *P. banksiana*, (Lapp and von Rudloff 1982) and *P. pinaster* (Zimmerman-Fillon and Bernard Dagan 1977) depended upon an earlier determination of the nature of the seasonal development and change in terpene components in the foliage of white spruce (*P. glauca)* (von Rudloff 1972). In that investigation, von Rudloff sought to determine the source and site of seasonal variation, comparing buds, new shoots and older plant parts during a full year of growth. Comparison of oils sampled during autumn over five years from a single tree showed that type and relative amount of terpene present was under strict genetic control.

The author did not employ a weight basis for expression of terpene compositions, preferring to avoid errors associated with complete evaporation of the ether used to collect the distilled oil. Similarly, he pointed to seasonal changes in non-volatile components in the leaf as the reason for not using leaf dry weight as the basis for presentation of results, preferring 'relative percentages' of components within the oil against a summation of areas of all recorded peaks.

The most significant change occurred in new shoots after flushing of the buds in spring, although the pattern of change was different for each of the major terpenes, and older leaves showed only slight variations in early summer. Pinenes decreased from high relative percentages (in the oil) in the bud prior to bud flush, to a minimum about three weeks later, after which they rose to approximately the level detected in leaves on older shoots. Limonene and myrcene showed the opposite effect, while the remaining four terpene compounds examined each rose from trace amounts in the buds to an early maximum in May. After this, relative amounts dropped gradually to the typical values found in autumn and winter foliage.

Hence von Rudloff (1972) distinguished between three phases of terpene synthetic activity in the white spruce foliage - in new buds during autumn and early winter, high activity during the early summer flush, and low activity in older leaves during the same period. Sesquiterpene alcohols and hydrocarbon were at a maximum in the oil (about 10% of each) in early summer when total yield of volatile oil was about 0.05% by FW of foliage, but by midsummer, when total oil yield had risen to 0.35%, the percentage

of these combined was less than 1%. On a dry weight of foliage basis, this represented a fall from 0.01to 0.004% of actual amounts of the sesquiterpenes and it was suggested that further metabolism of the compounds must be occurring, and that some resinification may take place in the warmer summer weather.

Bernard-Dagen *et al.* (1979) integrated ultrastructural and extraction studies to examine needles of maritime pine (*Pinus pinaster)* and were able to distinguish three 'zones' in the needles, and to show that epithelial cells lining the resin ducts were functional mainly in the basal parts of the needle, where they were responsible for the elaboration and secretion of monoterpene compounds into the resin duct. These cells deteriorate during the growth of the needle so that in the mid- and tip regions of the needle, they no longer perform a secretory function. Monoterpene levels remained steady during the season in all portions of the needle - highest at the base, and lowest at the tip, supporting the proposition that they are substantially synthesised early in the growth period and are subsequently metabolised or diluted as the resin canal ages and its surrounding epithelium deteriorates . Sesquiterpene synthesis, however, continued in all parts of the needle, progressively and continuously enriching the resin as the season proceeded. The paper is not specific in suggesting where these compounds are synthesised other than to refer to the 'permanent secretory activity typical of these unspecialised tissues'. This work was taken further to compare eight phenotypes of the species in a study which also examined wood and cortical tissue for terpene composition (Zimmerman-Fillon and Bernard-Dagen 1977).

Taking the question of suitability of sample technique one step further, a number of studies have examined diurnal variation in leaf oils, for example Leach and Whiffin (1989) who showed that in *Angophora costata,* diurnal variation was small, and significant only for two compounds (of the 86 numbered peaks resolved in this study) which tended to decrease during the night and rise to maxima during the day. Scora *et al.* (1984) expected, but failed to find, a diurnal pattern in composition of the essential oil of *Artemesia douglasiana* .

2.5.3 Ecological and other studies

The apparent use by many plant species of secondary compounds as defences against herbivory and infection has interested ecologists because of the implications of investment in irretrievable compounds by species for which resources may be difficult to obtain. Such investigations usually assume that a reduction in predation occurs as a result of changes in levels or nature of the compounds in question.

Similarly, turnover (recycling of energy, carbon etc on a continuous basis) in these compounds and their retrieval upon senescence of the plant part has been incorporated into several theories and models on evolution, cost, and availability of plant defence strategies (Coley *et al.* 1985, Fagerstrom 1989). The notion of 'mobility' of the particular compounds and ultimate loss to, or recovery by the plant is invoked in

comparisons between long lived plants in resource-poor environments for which the investment in immobile defence mechanisms is a 'least cost' strategy, and short-lived, fast growing plants for which recovery of the resources may be preferred. Recently part of the basis for some of these theories has been called into question with the paper of Mihaliak *et al.* (1991) and Gershenzon *et al.* (1993) in which the earlier work demonstrating rapid loss of labelled monoterpenes from apical peppermint cuttings was shown to be artifactual and not applicable in intact plants of a variety of oil bearing species. Ultimate loss of terpene compounds from senescing leaves (Croteau 1988) may not be exhaustive, and such compounds may be evident in leaves after senescence.

Such consideration of animal and insect interactions with plant species are complicated by the concept of coevolution, whereby natural predators will develop strategies to deal with an evolving defence system and within the populations of protagonists there will be more or less successful individuals on both sides. Eliminating the ontogenetic or diurnal elements of phytochemical change allows a more powerful determination of such interactions.

Halls *et al.* (1994) examined Rubber Rabbitbush (*Chrysothamnus nauseosus*) which contains a variety of active antimicrobial, antifungal and insecticidal properties, and showed that attention from browsing animals could be correlated with reduced winter levels of the volatile secondary chemicals. This correlation was not supported by clear evidence of active 'antiherbivory', since an earlier study (Ward 1971) showed that winter foliage of the plant was of comparable digestibility for the browsing species.

Cedarleaf *et al.* (1983) followed annual changes in monoterpene content in Big Sagebrush (*Artemesia tridentata*) proposing that mule deer browsing patterns might be determined by preference for seasonally low terpene content. The study showed substantial seasonal variation in 'monoterpenoid' content of current season's growth, cycling between 1 and 5% on a dry matter basis but did not examine the browsing pattern in detail, or include choice or digestibility assays. The authors concluded that on the basis of earlier reports of browsing patterns, there was no support for the suggestion that browsing and terpene changes were related. A similar conclusion was drawn by Lincoln and Langenheim (1979) in relation to twice yearly yield determinations of monoterpenes and herbivory in *Satureja douglasii*..

On the other hand in a study of six 'chemovars' of *Thymus vulgaris* , each distinguished by a single dominant monoterpene, Linhart and Thompson (1995) showed consistent patterns of feeding preference amongst adult and immature individuals of the snail *Helix aspersa* . This study did demonstrate the interaction of specific monoterpenes with choice and digestibility of the leaf material but did not consider changes in the levels of the active compounds during leaf development and maturity. Wagner *et al.* (1990) noted seasonal changes in secondary metabolite levels, including terpenes, in three hosts of the western spruce budworm and found consistencies with feeding patterns in the insect.

Studies of the type described above may not conclusively distinguish between endogenous seasonal cycles in the relevant 'defensive' compounds, and injury-induced reactions such as those detected by Barnola *et al.* (1994) in which defoliated *Pinus carribaea* appeared to increase the needle content of several monoterpenes. Neither, in many cases, do they establish causal connections between herbivory and secondary compounds present. However, they do provide basic data upon which more definitive feeding and browsing assays might be based.

Extract or oil composition has been employed effectively for each of the areas of research outlined above - commercial production, chemosystematics and ecological investigation. As shown, in many cases the seasonal cycle of synthetic, accumulative and degradative processes is the single most significant aspect of the system under study. For this reason, a basic and rigorous analysis of this cycle must underly the research, or mistaken assumptions can easily detract from the conclusions.
The basic study must incorporate a sampling method which will resolve the difference between ontogenetic processes and environmental changes, it must use an extraction and analysis technique which identifies the key compounds and an appropriate means for the expression of results must be employed.

In *Tasmannia lanceolata*, from the review of the available literature, it appears that ecological studies of the type represented above (herbivory and successional status) might arise from recognition of the properties of polygodial. Certainly this compound, and the whole extract are of interest from the commercial point of view, and any attempt to establish a production system based on the species will require information regarding ontogeny and oil accumulation and composition. Lastly extract analysis has already been proposed in the area of chemotaxonomy for the genus (Southwell and Brophy 1992).
It would appear necessary, therefore, that a basic study of the type described, identifying the annual pattern of extract composition, and expressing it in terms of the annual growth increment, is essential to inform any such research in the future.

2.6 Canopy architecture and harvest strategies

Porter (1989b) described a principle objective of pruning models as the removal of as many individual 'units' (leaves, buds, limbs) from the plant body as would allow for their complete replacement in one growth period. Harvest of vegetative material from mature plants on a regular, (usually annual) basis requires an understanding of the normal patterns of growth determining canopy form in the undisturbed plant, and a knowledge of the response of the plant to harvesting.
This is the issue with vegetative harvesting systems (such as applies to tea, and would be relevant to *Tasmannia*), for which the particular unit of concern is the annual shoot growth - usually on the top or outside of the remaining canopy. Productivity in this context refers to annual dry matter production of leaf and shoots, is usually closely related to light interception (Palmer 1989), and was calculated by Linder (1985) for a range of forest stands, to approximate 1.7 g MJ^{-1} photosynthetically active radiation.
From a physiological point of view, there are two aspects to consider.
The first of these is the effect of removal of storage resource and photosynthetic organs on the capacity of the plant to recover its energy investment. This will reflect the ecological habit of the plant, its usual level of tolerance to browsing, wind damage etc. This in turn will depend on the productivity of the remaining photosynthetic organs, the distribution of resources in the whole plant and the speed with which replacement leaves and twigs become net contributors of carbohydrate.
Intervention in the normal progression from growth, through maturity to senescence of leaf and stem structures will disturb source/sink relationships, allocation of resource to reproductive *vs* vegetative activity and will alter the significance (in energy terms) of independent factors such as frost damage, herbivory and disease agents.
Secondly, the phenological mechanisms which operate to respond to natural predation, injury or senescence of growth points, will determine the optimum position, timing and extent of such harvests.
In addition, of course, there is the question of tissue injury and possible infection associated with any 'harvest' technique.

2.6.1 Growth patterns in *Tasmannia lanceolata*

Gifford (1950) describes the activity of the shoot apex during the cycle of growth and dormancy, with particular attention to the shape and cytology of the apex itself, and refers to 'future papers' devoted to leaf development and comparative studies of inflorescence and floral apices in the Winteraceae.
One of these, Tucker and Gifford (1966b) provides a growth chronology in a discussion of floral ontogeny in *T. lanceolata*, referring to the typical cycle in San Francisco. Shoots expand actively from spring till late summer (earlier, and for longer than the

typical Tasmanian situation), followed by a period in late summer-autumn when extension growth ceases or is greatly reduced, but buds undergo a development phase. By late autumn/winter the terminal bud contains an apical meristem, about 8 spirally arranged leaf primordia, each subtending a leafless vegetative meristem, then 6-23 floral bracts also spirally arranged and each subtending a single flower, with no bracteoles. The apex is essentially dormant during winter, and open flowers are first observed in late September.

Floral initiation and development occurs between December and April (southern hemisphere), initiation continuing until late summer - February. By late January, the older flowers have carpels and young flowers of all ages are present.
Vegetative axillary buds remain undeveloped throughout the dormant season, and in spring each produces two sub-opposite prophylls and a succession of vegetative leaves. The floral axillary bud appears convex, when compared to the vegetative bud at a comparable, pre-appendage formation stage. These buds produce two sepals, also sub-opposite.
Vink (1970) describes a typical cycle of growth in *Drimys* spp. which can be paraphrased as follows:
The twig bears leaves in clockwise or anticlockwise direction, in a 2/5 spiral terminated by a bud, whose scales continue the spiral of the foliage leaves. The bud is either vegetative (producing a new shoot flush) or mixed (producing a shoot and flowers in axils of bud scales) and the shoot may extend simultaneous with, or subsequent to anthesis. Leaves on one shoot increment (bud scale scars to terminal bud) are usually evenly spaced or crowded below the apex - pseudoverticillate.
A lateral shoot usually produces one or more vegetative terminal buds (one per cycle) after which mixed buds are formed until shoot apex terminates its activity, whereupon a new lateral shoot takes over as leader.
Mean of leaf lengths per shoot increment increases distally along the twig, and lateral shoots have lower values for these than leading shoots. Increase of leaf length ceases with termination of activity of meristem at which time a lateral takes over as leader.
Flowers are inserted in axils of scales of mixed terminal bud and occasionally in the axil of the first leaf above the scales. The inflorescence is terminated by a vegetative apex. Bud scales of the mixed bud are referred to as 'bracts' since they can subtend florescences.
For Vink's '*sect. Tasmannia*', he notes that within the terminal bud, bracts increase in size acropetally, that initiation and anthesis of flowers tends to be acropetalar, but that in the lower 1-4 florescences the trend may be reversed, and flowers may be replaced by vegetative buds. Similarly, vegetative buds may occasionally be found in the axils of the highest bracts.

2.6.2 Classical Canopy Models

Canopy morphogenesis and the structural consequences of differing mechanisms are considered by Hallé *et al.* (1978) and a number of 'architectural' models are described in detail. The essence of this approach is that the model implies change and proposes the plan of growth rather than shape or physiognomy of the resulting mature plant. These authors use a *definition* approach (the provision of precise boundaries for inclusion of an example) to designating models rather than *typification* (using a taxonomic point of reference as representative) and distinguish 23 models for canopy growth. An illustrated key is provided and examples of species (usually tropical or sub tropical tree species) typical of each are listed.

Criteria of importance in this analysis are the life span of meristems (monopody *vs* sympody), the differentiation of vegetative meristems into sexual (determinate) or vegetative (indeterminate) structures, plagiotropic or orthotropic habit, rhythmic or continuous growth cycles and the chronology of branch development.

Applying this approach to *Tasmannia lanceolata* classifies the typical habit as conforming to Rauh's Model, specifically: monopodial trunk, rhythmic growth, branches morphogenetically identical to the trunk, flowers lateral.

For the 'ideotype', branches usually develop by prolepsis in temperate species, or syllepsis in tropical species, thc inflorescence is always lateral, and may be on first part of a renewal shoot subtended by leaves or scale leaves (eg avocado), or may occur at distal end of an extended shoot. Periodicity reflects latitude and environment, less tropical examples tending towards more periodic growth. The 'strategy' of the model is its simplicity and ability to regenerate. Usually damage to the trunk meristem results in the ready substitution of uppermost lateral meristem and little disturbance to growth, while rhythmic growth allows suspension of meristematic activity during periods of (seasonal) stress, most usually cold or drought.

Commercially important examples conforming to this model include *Hevea* (Rubber), several timber species (*Sweitenia, Khata, Triplochiton*) and the subtropical fruit species *Artocarpus (*breadfruit), *Persea* (avocado) and *Mammea* .

For these species, the most comprehensive literature on canopy management applies to the avocado, (family Lauraceae)which, although directed towards reducing vegetative growth and maximising reproductive productivity, does examine in detail some of the elements of canopy formation, and presents a number of empirical approaches to the problem of stimulating and supressing vegetative growth at specific axes. It also includes useful discussion and data to show how such intervention in the normal growth pattern can alter the balance between reproductive and vegetative growth.

The general problem of management for vegetative productivity in perennials - the removal of much of the photosynthetic tissue and growth axes by harvest, in direct

conflict with the requirement to retain carbohydrate production and perenniating growth, has been addressed most thoroughly in the development of manual and mechanical pruning and harvest strategies for production of tea (*Camelia sinensis*).

Although both these species are tropical/ subtropical in distribution, the general relevance of the principles is explained below. There is no published literature on the response of *Tasmannia lanceolata* to physical 'damage', - even the frequent insect injury of buds which is observed in the Tasmanian field situation (P.McQuillan, University of Tasmania - pers. comm).

2.6.3 Pruning and canopy management - evergreen tree crops

Whiley *et al.* (1988) summarise the problems for avocado orchard management associated with canopy development and tree vigour. The productivity of such orchards is low compared with that for other sub tropical fruits largely due to the energy cost of producing the large oil-storing fruit with a large seed. Trees tend to vegetative growth due to a high turnover of short lived leaves typical of trees evolved to inhabit understory niches. Root systems are shallow and relatively inefficient ('litter feeders').

Management techniques are directed towards reducing fruit drop at times when competition between vegetative growth and reproductive activity or fruit development is most intense, and usually involve water management, fertiliser applications, *Phytopthora* control, rootstock choice, strategic pruning, etc.

With a detailed analysis of canopy architecture, bud phenology and shoot growth in avocado Thorp and Sedgley (1993b) and Thorpe *et al.* (1994) showed the significance for productivity of modular growth (rhythmic extensions of growth twice or thrice per annual cycle), apical dominance (influence of the primary axis over subordinate axillary buds on the growing shoot) and acrotonous growth (growth of proleptic shoots resulting in development of many major limbs). Node numbers were higher in reproductive shoot modules, but were not influenced by cultivar, rootstock, location, climate etc, but were regulated endogenously.

The architectural analysis approach was also used by Cutting *et al.* (1994) who devised an experiment in which pruning cuts applied at two morphologically and physiologically different locations and times were shown to influence shoot number and complexity and to enable control of shoot vigour. In avocado, lateral buds abscise when less than one year old, while those in the bud ring may last for many years. Cuts were applied through the bud ring and midway along the previous growth flush in late summer and late autumn. The experiment showed that pruning to increase complexity is possible but that the position of the cut is critical. The vigour of the many shoots generated (as flush shoot diameter) was less than that of unpruned shoots, resulting in a smaller canopy diameter and increased shoot number.

Thorp and Sedgley (1993a) aimed to contain vegetative growth in established avocado trees, by removal of apical or axillary buds and removal of the whole summer (second) flush and compared these treatments with the normal (unpruned) growth arising from buds on primary and secondary axes for vigour, number and position. In the same trial, and in that of Wolstenholme *et al* (1990), growth regulators were used to reduce spring flush and divert resources into fruit set.

Scholefield *et al.* (1985) examined carbohydrate levels during the annual cycle and found them to be lowest after the summer flush, and just prior to floral initiation. Levels increased over winter, reaching a maximum in early spring before decreasing sharply during flowering, shoot growth and fruit development. This paper describes methods used for recording shoot flushing, bud examination and carbohydrate sampling of trees in southeastern Australia. Fruit drop seemed to be related to competition for falling carbohydrate reserves by the post-anthesis flushing of the vegetative terminal buds in the inflorescence. The authors suggest that these cycles of flush and associated carbohydrate cycling are similar to those found in citrus. A similar competitive situation might also apply to the mixed bud found frequently on shoots of *Tasmannia lanceolata*.

In the production of tea, (*Camelia sinensis),* vegetative productivity is the primary objective. There is an extensive, though specific, literature on the subject, typically discussing growth response to plucking and pruning (eg Mwakha 1989; Mwakha and Ankuya 1990; and Smith *et al.* 1990), seasonal and clonal differences in growth rates (Stephens and Carr 1990; Mwakha 1991) or light as a factor in productivity (Barua 1969).

The usual pattern of canopy management is to gather the tender shoots (two leaves and the terminal bud) from the plants at regular intervals depending on growth rate, and at longer intervals, to reduce the height of the canopy by pruning back to some predetermined level. The extent and timing of the pruning operation is a major factor in bush recovery, and subsequent yield, (Mwakha 1989, 1990) and local practices tend to vary. Of particular interest is the use of 'lung pruning' techniques, in which a varying proportion of maintenance foliage is retained after pruning, shown (Mwaka and Anyuka 1990) to increase subsequent yields, but adding to the difficulty of hand harvesting, and supressing underlying shoots if left beyond the time of first tipping. The growth equations developed by Smith *et al.* (1990) are particular to the clonal material and location studied (Malawi), although some adaptation for the general situation may be possible.

Barua's (1969) discussion of the effect of light on plantation productivity includes some commentary on the effect on productivity of retaining maintenance foliage in the lower parts of the canopy, and the difference amongst clones in foliage habit between the horizontal 'Assam type' and the semi-erect 'China type' of canopy. Agronomic factors resulting in poor canopy development and incomplete ground cover are shown to have

been the reason for favourable responses to shading treatments reported for many experiments carried out on Assam type bushes in NE India. Redirection of assimilates toward economic yield rather than increases in total net production resulted from reductions in irradiance to about 60% of full sunshine over these poorly developed bushes. Barua also notes that some endogenous control of this partitioning is implicated, to some extent independent of total radiation and net productivity.

With respect to the subject of this study it appears that the key aspects for consideration are firstly the nature of the response to pruning or harvesting 'events' by the plant, including a determination of the availability and behaviour of growing points on the mature canopy. While normally responsible for directing canopy development, in a pruned plant this response will control development of the annual harvest 'quotient'. Secondly, physiological factors determining productivity in the canopy, most importantly, the response to changes in light levels brought about by such manipulation of the plant canopy should be examined.

2.7 Light acclimation in understorey species.

Björkman (1981) points out that distribution of photosynthate between photosynthetic and other tissue within the leaf, (and the allocation to leaf vs other plant parts) is substantially affected by the light environment experienced by the plant,- in broad terms 'sun' vs 'shade'. This influence is likely, therefore, to extend to the proportions of secondary compounds synthesised and stored within leaves during growth and development.
Changes in light environment brought about in the natural situation (either suddenly, by treefall or herbivore damage, or gradually by overtopping of a plant canopy by neighbouring species) require a capacity for adjustment of canopy structure, leaf orientation, photosynthetic apparatus and the balance between storage and expenditure in the plant as a whole.
For leaves on a particular plant, the photon flux density (PFD) conditions during leaf development and maturation, and the acclimation capability of the species may constrain its response to instantaneous extremes of light.
Within the whole canopy, a wide range of light levels will be experienced by each leaf depending upon aspect, position in the canopy, orientation and changes in ambient light conditions with cloud cover, time of day and season.
As explained in Section 2.6 and discussed by Barua (1970) in relation to tea (a perennial crop in which fresh vegetative growth is harvested), pruning and harvesting of a perennial species imply drastic changes to the light environment of leaves during a typical seasonal cycle.

2.7.1 Photosynthesis in *Tasmannia lanceolata* and *Drimys spp.*

This section will consider the literature relevant to photosynthetic capacity in *Tasmannia lanceolata* , discuss two areas of research activity in which acclimation of species to changing light regime is of interest, and summarise the expected outcomes for photosynthetic and leaf morphological parameters of large changes in light climate.

For *Tasmannia lanceolata*, Read and Hill (1989) concluded that the species occupied the niche of early invader at disturbed sites likely to support closed canopy rainforest, and determined that in comparison with dominant species and other more persistent understory species *Tasmannia lanceolata* had a higher light compensation point (approx 35 μmol $m^{-2}sec^{-1}$) and lower rate of photosynthesis (4.3 mg CO_2 $dm^{-2}hr^{-1}$) at light saturation (around 600 μmol $m^{-2}sec^{-1}$) and would not grow quickly enough to secure a position in the upper canopy. Casey (1991) found with collections from altitudes ranging from 1160m to about 600m that leaf morphology, frost tolerance and

photosynthetic performance tended to reflect site of origin. Seedling plants were collected from the field and grown under glasshouse conditions before the trials commenced. Light saturation for photosynthesis occurred at about 500 $\mu E\ m^{-2}\ sec^{-1}$. Temperature optima (standardised as % of maximum rate) for photosynthesis amongst the sampled populations decreased with increasing elevation, from 18-20°C for low altitudes to about 14-15°C . Predictably, higher elevation sites showed broader response curve, 70% of P_{max} at 5°C, while lowland sites only 50% P_{max} at 5°C. The temperature optimum for photosynthesis appeared to correlate well with mean annual temperatures at site of origin.
Armesto and Fuentes (1988) and Rebertus and Veblen (1993) noted a similar niche habit with *Drimys wintera* which was uncommon in the rainforest canopy but which regenerated in gaps produced by treefalls to the exclusion of other canopy species. *Drimys wintera* was gradually overtopped by large trees growing around the gap edge, particulary where it grew on unsuitable substrates.

2.7.2 Ecological significance of acclimation

The question of successional adaptation was considered by Turnbull (1991). In a study of early, mid and late successional subtropical rainforest species, he used seedlings grown under four levels of neutrally shaded sunlight and filtered shade to analyse interactions between photon flux density and reduced red/far red ratio on selected photosynthetic characteristics of each species. The generalised response expected was for pioneer species to exhibit higher rates of A_{max} than late successional species. Growth irradiance significantly influenced light saturated photosynthesis (A_{max}) in 5 of the species - maximum assimilation rate decreasing with increasing shading. In a second paper Turnbull *et al.* (1993) report on the dynamics of acclimation to these changes in light levels and ratios. Acclimation was significant for all the photosynthetic parameters examined for transfer of plants between high and low light regimes and vice versa. Morphological/structural characteristics were not included in this study and the authors concede that acclimation will probably be ultimately limited by structural parameters. Osunkoya *e*and Ash(1991) used only growth rates and biomass allocation parameters to compare acclimation in seedlings of rainforest tree species and showed that relative growth rate as a measure of carbon economy in the plant could be used to compare acclimation ability in the species to reduced irradiance.

The question of whether ecotypes from differing light environments will retain their 'sun' or 'shade' photosynthetic capacity or will acclimate fully, has not been satisfactorily resolved. As cited in Boardman (1977), previous work by a number of researchers had shown that ecological races of several species retained aspects of their photosynthetic response. For example, *Solidago virgaurea* clones from shaded habitats did not fully adjust to high light intensity, and those from sunny habitats

consistently showed higher light saturated photosynthetic rates (Björkman 1968, Holmgren 1968). Gauhl (1976) found a similar result for *Solanum dulcamara*, while Teramura and Strain (1979) reported signficant differences in phototosynthetic and diffusion resistance response between shaded, flecked and open-grown adjacent populations of *Plantago lanceolata* , when cloned and grown in standard conditions and concluded that this indicated genetic differentiation between them.
The *Solanum dulcamara* example was further examined in Gauhl (1976), in which he found that the depressing effect of mild water stress on photosynthetic capacity persisted long after changed light conditions might have induced light acclimation. Clough *et al.* (1983), on the same species, agreed that persistent changes in leaf water potential, correlated with light levels may be the more important element of differences within and between so-called sun and shade ecotypes, and that real differences in survival and reproduction (the prerequisites for recognition of distinct ecotypes) may be more difficult to attribute to photosynthetic performance or acclimation ability. Clough *et al.* (1983) then questioned whether the widely reported persistence of limits to photosynthetic capacity in shade and sun 'ecotypes' might in fact often be attributable to this phenomenon.

2.7.3 Light level and the accumulation of secondary compounds

The allocation of photosynthetic resource to primary or secondary metabolic processes reflects the functional significance of the compounds in question and the availability of the resource.

Where defensive properties are attributed to compounds, consideration of the interaction between light and secondary product metabolism focusses on the ecological implications. Givnish (1988) included a consideration of defence compounds in discussing the 'economics' of allocation of resources in sun and shade plants, referring to the papers of Coley (1983) and Coley *et al.* (1985) in which she predicts that shade adapted species will allocate more resource to their defensive mechanisms and will be more specific in their herbivore targets than sun species. These arguments are not extended to the intraspecies level, although one apparent example of a visual defence adjusting to open and closed canopies is cited (Smith 1986).
Lincoln and Langenheim (1979) examined the effects of irradiance on monoterpenoid yields in *Satureja douglasii* correlating these with the degree of herbivory observed in an open meadow adjoining a *Sequoia* dominated forest. High yielding types were associated with low light and high herbivory, and although genotypic factors were implicated in the yield variation, it was not eliminated in the comparison between light levels. Neither was the covariance of herbivory with monoterpene yield and light level

confirmed as causal (seedling selection or stimulation of defence reaction) or consequential (selective browsing, or preference of the herbivore for shaded habitat).

Lincoln and Lagenheim (1978), using clonal material of *Satureja douglasii* compared the effects of light and day temperature on composition and yield per leaf of pentane extracted oils. In leaf pairs on the same species they showed that yield per unit leaf dry weight decreased during development, and was markedly reduced at high light levels, irrespective of day temperature. This was attributed to an increase in total leaf dry weight under high light levels.
On the other hand there was a negligible affect of light level on yield per leaf pair. These findings are discussed in relation to possible direct influences of light on the compounds concerned such as photochemical reactions or volatilisation, and previously reported reductive conversions of several monoterpene constituents resulting in alterations to oil composition in *Mentha piperita* (Burbott and Loomis 1967).
This result - falling terpene yield per unit leaf dry weight - was not observed in a number of other studies, even in other members of the family -Lamiaceae - Burbott and Loomis' (1967) study in peppermint, Yamaura *et al.* (1989) for *Thymus vulgarus*, and Firmage (1981) in *Hedeoma drumondii* .
In summary, monoterpene (and more generally, secondary metabolite) production seems to be readily influenced by light regime, and indeed by any experimental condition which influences the photosynthetic performance of the leaf (Gershenzon and Croteau 1990).
The cumulative nature of oil synthesis results in sensitivity of oil quality to the balance of photosynthetic and respiratory activity during leaf development. This was shown in *Mentha piperita* by Clark and Menary (1980a), in a dissection of 'apparent photosynthesis' into its component photosynthesis, photorespiration and dark respiration to estimate true photosynthesis. The result was incorporated into an oil accumulation model to show the influence of environmental factors, most importantly night and day temperatures, on the oil composition at any time.

2.7.4 Effects of light level on photosynthetic and morphological characteristics

a) General

The review of Björkman (1981) details factors determining photosynthetic performance under strong and weak photon flux densities and the pattern of acclimation of plant species representative of shade and sun environments to each, respectively, and discusses the phenomenon of photoinhibition.
At low light levels, a strategy of high efficiency (photosynthesis *vs* respiration) and minimal direction of resources away from photosynthetic tissue tends to occur.

Chlorophyll content in obligate shade plants is at least as high as that found in sun plants, and tends to be enriched in chlorophyll b (chl b) relative to chlorophyll a (chl a). This reflects the increased proportion of total leaf chlorophyll associated with the light harvesting chlorophyll ab- protein complex, considered to be primarily associated with the PSII photosystem (see Fig 2.5 below).

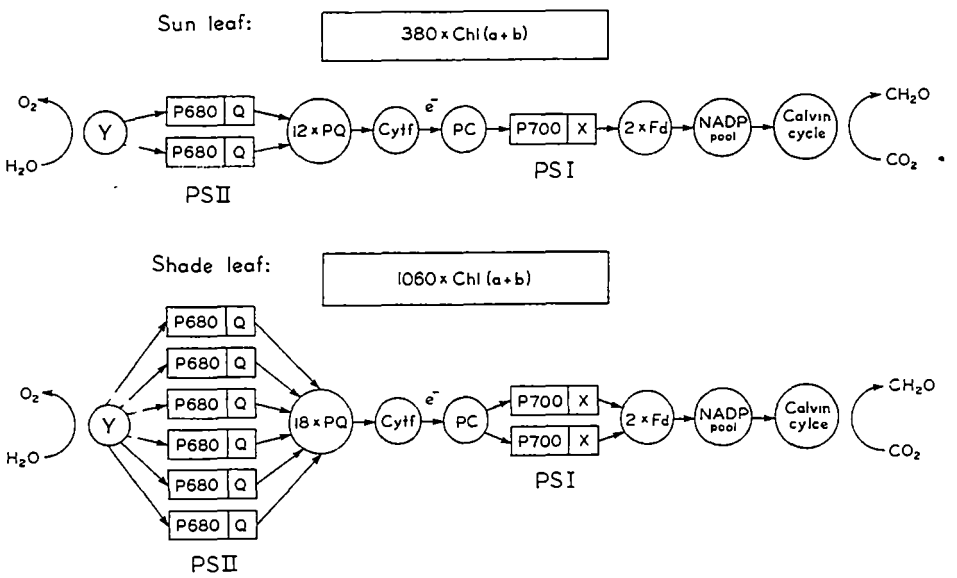


Figure 2.5: (from Bjorkman 1981): Schematic proposal for organisation of photyosynthetic system in sun and shade leaves. The important feature is that the electron transport chains (via cytochrome f) are served by larger numbers of PSI and PSII units in the shade leaf, and that the ratio of these is higher in the sun leaf. Consequently the total amount of chlorophyll per chain is much higher in the shade leaf and more of the chlorophyll is associated with the light harvesting chlorophyll complex, including all the chlorophyll b.

Quantum yield is not found to differ markedly between sun and shade grown leaves of the same species and appears to be essentially the same in normal leaves of higher C3 plants regardless of the species and light regime, provided that for shade plants the light regime is not extreme and temperature and leaf water potential are within a normal range.

Distribution of dry matter within the plant (and, by extension, allocation to defensive and structural tissues), are strongly and consistently influenced by QFD. Leaf weight ratio (W_{Leaf}/W_{Plant}) and specific leaf area (A_{Leaf}/W_{Leaf}) are both increased under low light regimes, while increases in leaf allocation may be at the expense of root growth.

Björkman (1981) presents a model of sun and shade leaves which attempts to account for the experimental experience, at the heart of which is the schematic model for photosystem organisation shown in Fig 2.5 in which the basic difference is shown as the PSI:PSII ratios - 2:1 for sun leaves, 3:1 for shade leaves.

Other differences discussed by Björkman (1981) include those of leaf structure - ratios of mesophyll surface area to that of the leaf, mesophyll morphology and volume to surface area ratio, and stomatal conductance.

Photoinhibition - the over-illumination of shade adapted leaves or plants - is observed as a reduction in A_{max} and sometimes in the quantum yield at high light intensities. The phenomenon may be partly or fully reversible, and is usually detected only in low light-grown leaves. Obligate shade plants exhibit a limited capacity to adapt to high light regimes, while leaves on sun plants grown at low light levels appear capable of quickly adjusting to higher light levels.

b) Recent studies

Wild and Wolf (1980) compared the effects of different light levels on morphological and physiological aspects of leaf development in *Sinapis alba*, in particular frequency and size of stomata and mesophyll and guard cell number, size, and chlorophyll content.

Pons and Pearcey (1994) found that leaf mass and respiration per unit area declined sharply after shading, and shaded leaves lost N/unit area progressively before senescing. This process occurred more quickly in shaded leaves on unshaded plants. A_{max} also declined with shading, more rapidly than leaf N suggesting that N exported from the leaf mostly came from N involved in photon absorption, (PSII core chlorophyll), rather than from compounds involved in the provision of photosynthetic capacity, (light harvesting complex II), which underwent a relative increase upon shading (indicated by a decrease in chlorophyll a/b ratio).

Sims and Pearcey (1991) distinguished two functions of respiration in non-growing leaves on plants moved from low to high light. Firstly the requirement for maintenance of the photosyntyhetic apparatus, and secondly the (larger) component arising from other effects of high light eg higher rates of protein turnover with repair of photoinhibition, transport and processing costs for increased amounts of photosynthate or increases in other energy-requiring metabolism (nitrate reduction). Comparing costs and benefits of acclimation to high light they concluded that it involved larger leaf construction commitment than simple maintenance of the shade leaf, and as a consequence, for light conditions at which either sun or shade leaves achieve maximum assimilation rate, shade leaves obtain higher 'return on investment' in carbon.

Givnish (1988) also reviewed the literature on the subject of adaptation to sun and shade and re-evaluated the classical paper of Björkman *et al*(1981) (in which leaves were shown to perform best, photosynthetically at the light level to which they have been grown) in terms of 'whole plant energetics'.

Brooks *et al.* (1994) examined the progress of acclimation in mature (that is, structurally 'determined') foliage of *Abies amabilis* using paired branches on mature trees and subjecting foliage to shade or no shade as well as removal of buds to prevent new growth and to identify the extent to which acclimation was dependent on the ageing process and source-sink factors.

With respect to *Tasmannia lanceolata*, the key issues here would be a more detailed assessment of basic light response parameters, and a consideration of the acclimation capacity of the species. The second point is relevant in the context of a pruning or harvesting system which would remove substantial portions of the photosynthetic canopy after the completion of the seasonal growth cycle.

A general consideration of the response of leaf morphology and production of secondary compounds to different light levels might also be useful in developing a production strategy, where opportunity exists to select sites and plantation system (for example monospecific plantations *vs* use of the species as an understory crop).

2.8 Harvest Strategies for Yield and Composition of Secondary Compounds

'Harvest strategy' may be considered to embrace the following aspects of crop management:

- physiological status of the plant and its oil product and the scope for manipulation of this by agronomic means,
- disease and pest management imperatives,
- practical limitations of harvesting equipment and processing facilities and
- product durability and storage methods

Much of the relevant literature underlying agronomic management strategies for essential oil crop harvesting has been reviewed in Section 2.5.1, since the primary requirement is an understanding of ontogenetic changes in oil composition and biomass production, as summarised by Hay (1993), and exemplified by Porter's (1989a) study of parsley .

Agronomically there may be some scope for alteration of the phenological process and consequently the timing of harvest by means of manipulation of nitrogen and irrigation levels, planting date or planting density (Clark and Menary 1980b, Hay *et al.* 1988, Svoboda *et al.* 1990), and these aspects of production might be incorporated in a harvest strategy. Many environmental factors on the other hand are shown experimentally to alter oil production and phenology, for example temperature, irradiance level and photoperiod (Skubris and Markakis 1976, Clark and Menary 1979, Yamaura *et al.* 1989) but are only under the control of the grower in field conditions to the extent that choice of site and geographic location may determine the limits for these parameters.

An example of the type of strategy which might be applied to control the quality and yield of essential oil, is the proposal of Clark and Menary (1979). Harvest date is determined on the basis of regular field samples for composition, while the broad strategy is to take two harvests and blend the resulting oils, and to control vegetative development of each crop using high levels of nitrogen and irrigation, while retaining flexibility to cut prematurely should a disease outbreak occur.

In practice, disease or herbivory on canopy structure and loss of oil bearing organs may, in the field situation, dictate a harvest timetable quite independent of oil quality, requiring the blending of crops between seasons, locations and producers to obtain the desired result.

Removal of only certain plant parts, on the basis of known variations in extract composition within the plant (eg. parsley seed*vs* herb oil- Porter 1989a), or in order to retain the canopy framework in a perennial species, offer both opportunities and constraints for development of harvest strategies.

In addition to the purely physiological framework, many practical constraints may operate during commercial harvesting and should be incorporated into harvest

strategies in light of the above. Most obviously, limitations in handling, transport, distillation and extraction capacity, together with the durability of the harvested plant material, fresh, frozen or dried may dictate compromise on the 'ideal' composition or yield requirement. These issues are usually specific to the crop, location and market concerned and would not be usefully reviewed here.

2.9 Conclusion

The requirement to develop a generalised production and harvesting strategy for *Tasmannia lanceolata* provides a unifying context for the issues discussed in the forgoing review:

- there is a general technical requirement for a *methodology of extraction, preparation and analysis* which will generate extracts of a reliable quality at maximum yield from the plant material used,
- preparation of the leaf extract requires detailed knowledge of the *storage system within the species*, the structure(s) involved and their location and morphology, and the role of the structures in accumulation of components in the extract,
- the timing and method of harvest will specifically depend upon matching market requirements of the extract to *the pattern of seasonal change*,
- site selection, plantation system and pruning strategies will depend upon the *requirement of the plant for light* and space,
- a *harvest strategy* must be devised to retain the productive integrity of the plant - bud and leaf production and the canopy framework must be preserved, - while dry matter (and extract) yields are maximised.

CHAPTER 3 GENERAL METHODS, DEVELOPMENT OF EXTRACTION SYSTEM AND PRELIMINARY STUDIES

3.1 Extractions

a) Solvent

Unless otherwise specified, the extraction solvent used was redistilled petroleum ether (bp 40-60°). Solvent composition was checked for irregularities by inclusion of solvent blanks in all analytical runs.

b) Leaf Preparation

Except where otherwise mentioned, fresh leaf was dried in a thermostatically controlled drying cabinet incorporating a circulation fan and set to 35°C. Leaves were separated from stems and placed in open kraft paper bags on racks, and dried for 48hrs.

With the exception of the drying experiment (Sect 4.2), all yields and percentage composition are expressed in terms of 'dry weight' (%w/w of DM)- that is weight of leaf dried at 35°C for 48hrs. For the drying experiment results are expressed in terms of leaf dried to constant weight at 70°C.

Leaf samples were broken up and combined in a small mortar and pestle, and ground to a fine, uniform powder which was weighed into 20ml glass vials with close fitting plastic caps.

5ml of solvent containing 1mg of C18 standard octadecane was added and the vials shaken (Janke and Kunkel shaker table KS500) for two hours at 150cpm, and allowed to settle before a 1ml aliquot was transferred to a GC vial, and the vial capped and analysed as described below. All extractions were conducted in the laboratory where room temperature is controlled to approximately 20°C.

c) Preparation of whole extracts

Where complete extraction and recovery of a sample of extract was required (*cf* analytical determination of yield) the standard procedure employed was to grind the plant material to a very fine powder in a mortar, immerse, sonicate briefly and then shake in solvent (2 hrs, 150cpm, 20°C), and filter the solution through a sintered glass funnel, washing the leaf residue and the funnel at least twice in fresh solvent. The combined solution was then evaporated to dryness in a small rotary vacuum evaporator (Büchi, Rotavapor R) using a warm water bath at 60°C, and applying a maximum vacuum of 680mm Hg for approximately two minutes. The condenser was chilled using recirculated refrigerant at 2°C. In the text of this thesis, such an evaporation is referred to as 'removal of solvent from the extract on the RVE'.

The duration of maximum vacuum is critical, here, particularly with very small (< 500mg) quantities of extract. Prolonged application of these conditions continued to

reduce final mass, probably by loss of some lower boiling point fractions of the extract, while inadequate removal of solvent could easily result in innacuracies. In any event, 2 minutes of maximum vacuum was chosen as a compromise 'standard condition'. The effect of extended evacuation of a single 250 mg sample of extract is shown below- Figure 3.1

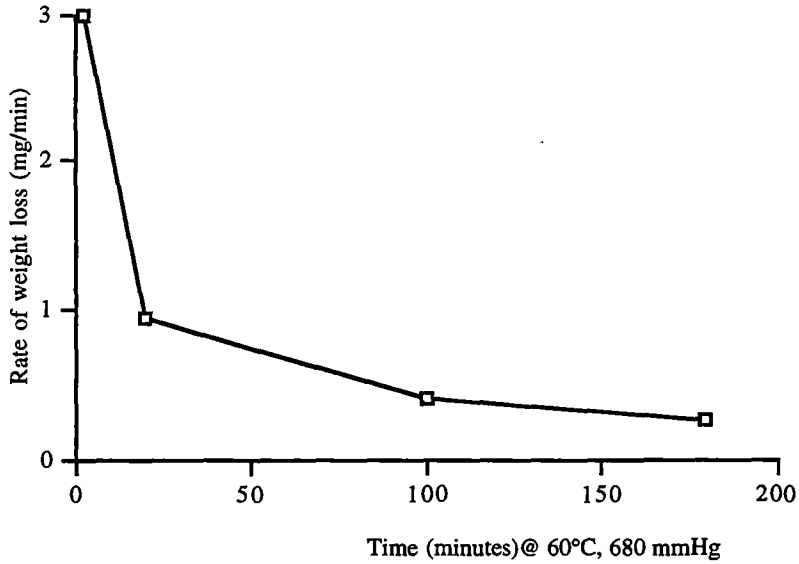


Figure 3.1: Result of prolonged heating and evacuation on *Tasmannia lanceolata* extract - (each point represents the mean weight loss/ minute since the previous measurement).

3.2 Extract Analysis

a) General

With the exception of certain analyses conducted at the University's Central Science Laboratory (CSL), which are described in the text, all GC analysis was conducted on an HP 5890 gas chromatograph fitted with an HP7673A automatic injector and FID detector, operation and data analysis by HP/Chemstation 3365 software.

Column was a 15m HP1 column (i.d. 0.22mm, phase thickness 0.33μm) operating with head pressure of 8 psi, the carrier being high purity nitrogen with column flow of 2 ml min^{-1}. Injector mode was arranged for split flow, the ratio being 25:1, injector temperature 250°C, detector temperature 280°C and oven temperature was programmed: 50°C (1 min) - (20° min^{-1}) -150° - (5° min^{-1}) - 260° (5 mins). Sample size was 1μl.

After a number of trials using the solvent sampling method described elsewhere (Sect. 4.7) it was decided to establish the integration parameters to reject peaks with areas less than 400, to commence integrating with the peak corresponding to 1,8 cineole (sometimes a mixture with limonene) with retention ratio approximately 0.32 (*cf* C18 std) and to cease with that of kaurene (retention ratio approx. 1.25). Total peak area

calculated by this means (excluding the standard) was described as representing '% volatiles' and is usually included in the data presentation.

FID response was determined only for polygodial (see below), for which a pure sample, prepared by solvent partitioning, thin layer chromatograpy and recrystallisation, and authenticated using GCMS, was used (RIRDC Report 1995). Since, for the purposes of most experimental extractions the relative proportions of compounds in the extract was of interest, all compositional data is presented with reference to the internal C18 standard used. That is, all percentage yields of extract constituents, including polygodial, are calculated from peak areas on the FID output, by the following formula:

% of Compound Z in extract sample =

$$\frac{\text{wt (g) } C_{18}\text{ Std}}{\text{Wt (g) Sample}} \times \frac{\text{Area Z}}{\text{Area}_{Std}} \times \frac{100}{1} \quad \text{.....(3.1)}$$

b) FID response to polygodial

Method

A standard curve for the FID response factor to polygodial in the analytical GC procedure above was determined as follows:

A series of solutions of the pure polygodial sample were prepared from a stock solution containing 4.796mg ml^{-1} redistilled hexane together with a standard solution containing 1.2560g octadecane (C_{18}) in 25mls redistilled hexane.

Results and conclusions

The response factor for polygodial is included in the equation 3.2 and is determined from the inverse of the slope of the curve: area ratio for polygodial and C_{18} *vs* concentration ratio of polygodial and C_{18}.

$$\% \text{ polygodial} = \frac{\text{Area polygodial}}{\text{Area } C_{18}} \times \frac{\text{Mass } C_{18}}{\text{Mass sample}} \times \text{Response Factor} \times 100 \text{.....Eqn. 3.2}$$

The mean for duplicate analysis of each of the solution mixtures is summarised in Table 3.1. The relationship is shown in Figure 3.2 and displays a slope of 0.663. The response factor for use in determining % polygodial by weight. is therefore 1.51.

Table 3.1: Solution concentrations and FID response ratios (mean of two analyses) for determination of response factor for % polygodial.

polygodial conc'n (mg ml^{-1})	C_{18} concentration (mg ml^{-1})	Area ratio: polygodial/std	Conc'n ratio polygodial/std
4.796	1.256	2.484	3.818
3.837	1.256	2.027	3.055
2.878	1.256	1.471	2.291
1.918	1.256	0.960	1.527
0.996	1.256	0.449	0.793

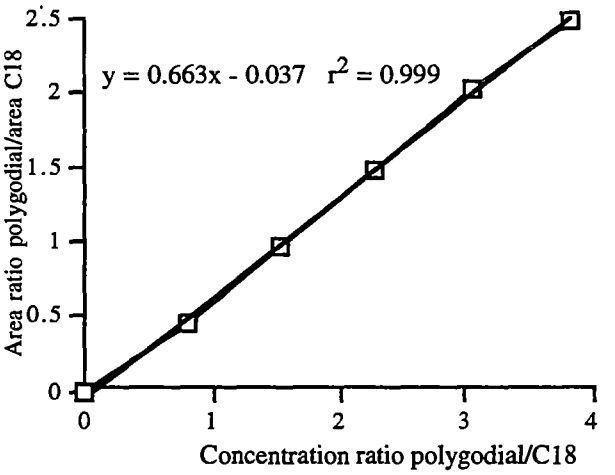


Figure 3.2: Standard curve to determine FID response for polygodial*cf* C_{18} hydrocarbon standard. Response factor = inverse of slope = 1.508

3.3 Plant materials, growing conditions

Much of the physiological work in Sections 5.0, 7.1, 7.2 of this project was carried out using clonal plant material designated W3 which was found to propagate readily and to yield an extract of roughly median composition (see Figure 3.4). The material was produced from several cuttings taken from bushland on Mt Wellington, Tasmania, and propagated on a mist bed at 20°C. A voucher specimen has been lodged with the Tasmanian Herbarium - HO 312698. This material was multiplied several times to produce approximately fifty plants of each type which were maintained in 150mm pots in a shade house at ambient temperature and 75% of ambient light until required. Plants were watered daily and received a weekly application of normal 'Hoaglands' nutrient solution. The soil medium used was a 3:1 mixture of coarse washed river sand and spagnum peat moss (TEP Medium, Tartu Estipeat BV), containing 100g/20l of Osmocote (17-1.6-8.7; 8-9 month release). Where specific growing conditions were part of an experimental regimen this is detailed in the text.
Mature plants used in the seasonal development work are described in Sect. 6.0.

3.4 Microscopy

a) Light microscopy

Micrographs used to compare leaf morphology (Sect. 7.1) were prepared from discs of leaf tissue fixed in 3% glutaraldehyde in cacodylate buffer and hand sectioned and mounted in glycerol. Bud sections were fixed and infiltrated with resin as in (b) below, then stained with toluidine blue. All photographs were taken through an Arrow 80B blue filter and recorded on Kodacolor 100ASA film.

b) Transmission Electron Microscopy

Samples used in sect. 5.2 were prepared by first fixing the plant material in 3% glutaraldehyde in sodium cacodylate buffer (pH 7.2) for 12 hrs at room temperature, followed by 5 rinses in buffer and dehydration in ethanol series. Samples were then, post-fixed with osmium and infiltrated with Spurr's medium, then sectioned for examination under the transmission electron microscope. Sections were stained with lead citrate and uranyl acetate, mounted on grids and examined under a Phillips CM100 (Phillips Scientific and Industrial, Australia) electron microscope at 80kV. Micrographs were recorded on Kodak EM 4489 film.

c) Scanning Electron Microscopy

Samples used in Section 5.2 were prepared by placing fresh, whole leaves on filter paper soaked in osmium tetroxide in aqueous buffer (pH 7.2) in a glass petri dish for 24 hours, then slicing the leaf at an oblique angle to obtain a broad exposure of the mesophyll. Small pieces of this tissue were then mounted on microscope stubs and freeze dried for 24 hrs before being sputter coated with gold and then examined under the scanning electron microscope (Phillips 505 SEM) at approximately 15kV. Micrographs were recorded on a Rolex 120, using Ilford FP4 film.

3.5 Preliminary investigations

At the commencement of the investigation, several general investigations were undertaken, to establish the scope of variability in the Tasmanian population at large, to determine whether the observed trend in leaf morphology from low to high altitudes was accompanied by a trend in extract composition, and to compare the oil obtained by steam distillation with solvent extracts.

3.5.1 Plant Collections

Extracts obtained from previous collections of *Tasmannia lanceolata* leaf material have shown widely different yields and composition (C Dragar- pers. comm., Stevens 1955) between locations, while at least one author (Southwell and Brophy 1992) has suggested the utility of extract chemistry in differentiating amongst members of *Tasmannia* on the basis of fresh material collected from a relatively small number of locations.

In order to obtain a general indication of variability in extract yield and composition within and between sites in Tasmania, a 'preliminary' collection was conducted as described below.

Method

During March 1993, leaf material of *Tasmannia lanceolata* was collected from twelve locations around Tasmania (Site details Table 3.2), mature leaf from 2 - 6 plants per location was dried, ground and shaken in petroleum ether. The extract was obtained by

filtration followed by evaporation of the solvent in a rotary vacuum evaporator (see 3.1). The extract was weighed, and a sample analysed for composition.

TABLE 3.2: Site data for samples collected in 'preliminary' survey.

ID	Location	Altitude	Tasmap Ref.	No Plants
W	Mt Wellington	600	EN201492	6
B	South Bruny Is. Allonah	<100	EM959199	6
M	Middleton, Cox's Rd	<100	EN119191	6
P	Tasman Peninsular, Macgregor Pk	500	EN411760	6
N	Winnaleah	200	EQ703535	6
S	Smithton, Salmon River Rd	<100		5
G	Talbot Lagoon, Guildford	600		6
Y	Takone Rd, Yolla	350		2
H	Hellyer Gorge/Parrawe	500	CQ816277	4
Q	Murchison Hwy, Queenstown	300		6
D	Derwent Bridge	800		2
L	Lost Falls	400		3

Results

Yield of extract and specific components was expressed as % weight of dry leaf material, and these results, together with some observations for the plants concerned and the resulting extracts are included at Appendix 2.1. Note that peak assignment is tentative, and that the temperature programme for this data was - initial temperature 50°C, 20°/minute to 150°C, 5°/minute to 260°, final temperature held for 7 minutes; other GC parameters as for the standard analysis (sect. 3.2).

Analysis of the results with respect to variation between and within sites for the small number of samples was limited to a comparison of polygodial levels and the result of this for the twelve sites is shown in Fig 3.3, the error bars showing standard error for % polygodial w/w of dry leaf for the relevant number of trees at each site.

The proportions of polygodial, 'volatiles' and non volatile components in the total extract can be seen in Fig 3.4, showing each of these elements of the extract as percentages of dry leaf extracted. Yields vary between 0.88% and 13.3%of DM, and polygodial from 0.11% to 2.9% of DM, and the collections are arranged in order of increasing *concrete* yield.

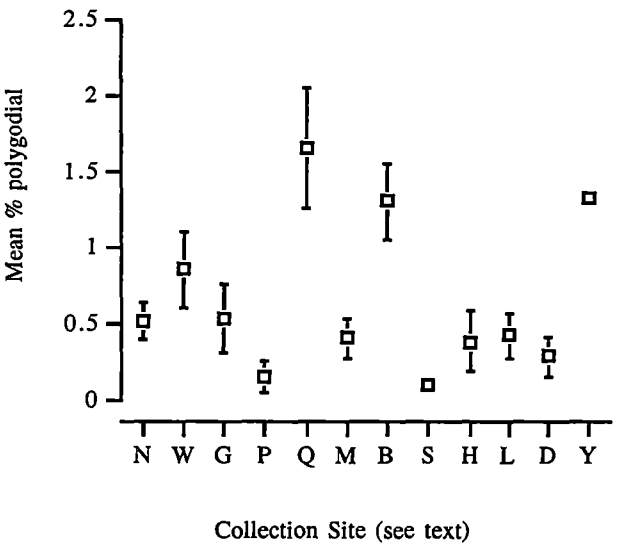


Figure 3.3: Polygodial levels- mean and ± se of mean for each collection site in 'preliminary collections'.

Discussion

While the limited site selection presented here could not be regarded as representing the range of extract compositions to be encountered in the whole Tasmanian population of the species, (in particular there were no representatives from the South West coast, where the species is particularly abundant and vigorous), it does provide a graphic indication of the spread of this particular characteristic, and of the scope for selection of plants on the basis of polygodial level or extract yield, without singling out any specific commercial requirement. The survey also shows clearly that this variability must be considered in comparative experimental work undertaken in any study of the species.

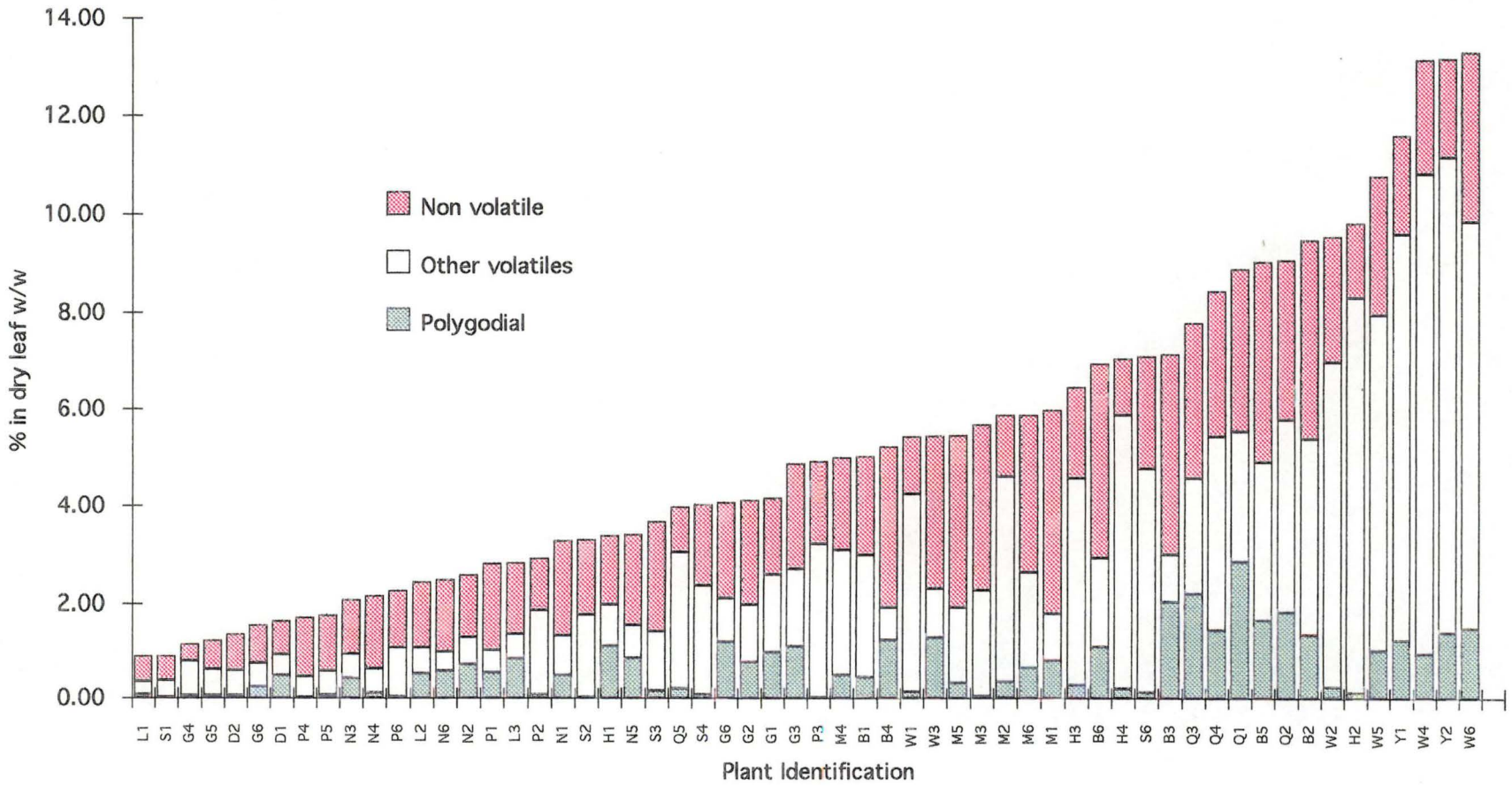


Figure 3.4: Extract composition characteristics for mature leaf from 58 plantscollected from sites around Tasmania in March 1993. Arranged by increasing yield of extract by dry weight, irrespective of site.

3.5.2 The effect of altitude on extract composition

Amongst natural populations of *Tasmannia lanceolata* variation in leaf thickness and size with altitude is typical of that found with other species found across wide altitudinal range (Korner *et al.* 1989, Casey 1991). As a preliminary trial, material collected from plants spanning the middle to higher elevations found in close proximity on Mt Wellington, near Hobart, were compared for extract characteristics below.

Method

Leaf and twig material collected from plants from small local populations along an altitude transect on Mt Wellington was dried and analysed. The populations were not discrete, and all collections were within 1km of the next nearest neighbouring collection. Material from only one plant per altitude was split into two, and one analysis conducted on each portion.

Results

Results are shown in Table 3.3, with the same restrictions regarding identity of components as applied to Sect. 3.5.1, above. Percentage dry matter of sub samples of leaf (to constant weight at 70°C), and leaf area to dry weight ratios (Specific Leaf Area) were determined for as many leaves as possible per altitude sample (Table 3.4).

Table 3.3: Leaf extract composition, means for two analyses of leaves taken from plants along an altitude transect on Mt Wellington, Hobart

Component	Ret.Time	400	600	700	800	900	1000	1100	1200
1,8 cineole°	5.42	0.22	0.24	0.19	0.79	0.31	0.37	0.08	0.14
linalool	5.99	0.13	0.25				0.20		
cubebene	8.83	0.20	0.45	0.29			0.39		
caryophyllene	9.84	0.22	0.14	0.09	0.07	0.08		0.07	0.07
a-cadinene	10.68	0.11	0.27	0.16			0.23		
Unknown	10.91	0.25	0.18	0.11	0.40	0.31	0.21	0.17	0.14
calamenene°	11.15	0.21	0.66	0.36			0.43		
cadina-1,4 diene	11.43	0.78	1.95	1.17			1.70	0.02	
guaiol (?)	12.37	0.23	0.90	0.41			0.58	0.15	0.10
polygodial		2.17	2.44	0.65	4.75	0.05	1.90	0.02	
% Volatiles*		**5.98**	**9.45**	**4.16**	**8.22**	**1.48**	**7.84**	**1.14**	**0.85**

° Peak area may include other compounds

* Total area integration between peaks at 5.47 mins and 20.92 mins (ex. C18 std)

Table 3.4: Leaf area and dry matter determinations of leaves from the Mt. Wellington transect.

Elevation	No leaves	Leaf area (mm^2)	%DM	SLA m^2kg^{-1}
400	57	709	34	7.1
600	61	532	33	7.1
700	43	567	33	7.1
800	32	680	37	5.0
900	23	411	41	5.6
1000	75	126	38	5.3
1100	53	138	42	3.7
1200	79	97	43	3.7

Collections from the wider distribution of *Tasmannia lanceolata* have shown (3.5.1, above) that variation between plants at a site is such that the data presented here must . be considered exploratory in nature only. However, three tendancies are suggested by the data in the Tables above: a general fall in the levels of terpenoid volatile constituents in the leaf, an increasing proportion of dry matter, (resulting in decreasing specific leaf areas), and a marked reduction in leaf size at the higher altitudes. The latter two trends are widely reported for species occurring along altitudinal gradients, (Korner *et al.* 1989), and although in comparison with the range of altitudes discussed in that report, 400-1200m represents only a small span, the higher elevation does equate to the maximum altitude at which the species is found in Tasmania. Expression of 'percentage volatiles' on the basis of area, (using the data for SLA) does not remove the obvious irregularities in this data, and while the very low levels found at higher altitudes were at the low extreme for the survey reported in 3.5.1 above, it is not possible, without further sampling, to predict any trend in this characteristic with altitude.

3.5.3 Steam distillation of *Tasmannia lanceolata*

For comparison with the solvent extract, a quantity of steam distilled essential oil of *Tasmannia lanceolata* was prepared.
Stevens (1955) distilled several batches of leaf and stem material collected from six sites around Tasmania, and found that yield of oil varied between 0.28 and 2%, depending on the type of material - the higher yield obtained from separated leaf alone. These distillations were allowed to continue for several days until no oil was detected in the distillate. Stevens found very high proportions of guaiol in the steam volatile oil, in one case resulting in crystallisation of the compound from the oil and Dragar (1984) reported almost 3% of this compound and identified 22 other terpenoid compounds in a small quantity of steam distilled oil obtained from plants collected on Mt Wellington, Tasmania. Use of laboratory scale microdistillation apparatus produced only minute quantities of oil, so a large distillation using a small commercial still was undertaken.

Experimental method

A large batch of mixed leaf and twig gathered from the site at Parrawe was packed into a small commercial distillation vessel. Steam was passed through the plant material and condensed in a simple water cooled vapour-in-tube condenser. The distillate was allowed to settle in a separation tank whereupon the oil accumulated on the surface of the water. The distillation was continued for two hours, by which time no oil could be detected on the surface of distillate at the condenser outflow.

Samples of leaf taken from the vessel after distillation were dried and extracted to determine whether the leaf still contained components found in extracts of fresh or dried leaf.

Results and Discussion

The complete distillation of 680kg of mixed leaf and twig material yielded 2.2 litres (0.32% v/w) of a dark yellow oil. A sample of leaf material taken from the vessel at the completion of the distillation was dried, ground and extracted in the usual way. The oil, and the extract solution were analysed by GCMS, the results of which are summarised in Table 3.5, together with the average percentages of components obtained from a selection of *T. lanceolata* leaf extracts.

Table 3.5: Composition of steam distilled leaf oil (as % of peak area) compared with 'typical' extract of *Tasmannia lanceolata* leaf, and extract obtained from distilled leaf taken from still after two hours of distillation (the 'marc')

Peak identity	Constituent as a percentage of total peak area		
	'average' extract	distilled oil	extract of marc#
α-pinene	1.64	12.7	-
β-pinene	0.38	3.74	-
α-phellandrene		1.69	-
1,8 cineole +*	1.00	6.35	-
linalool	2.38	10.14	-
δ-terpineol	0.10	1.07	-
piperitone	0.58	0.87	-
safrole	-	0.11	-
eugenol	1.38	2.25	0.50
γ-allemene	-	0.47	-
α-cubebene	1.51	1.09	4.77
methyl eugenol	-	1.39	-
α- copaene	0.57	-	-
caryophyllene	1.37	1.48	1.40
bicyclogermacrene+ *	1.18	4.59	-
calamenene	1.01	5.15	6.67
δ-cadinene	3.79	2.99	-
palustrol +*	1.85	2.62	-
cadina- 1,4 -diene	3.80	-	14.38
guaiol	6.32	6.33	1.60
drimenol	-	0.72	0.93
polygodial	19.7	0.75	4.77

* peak may be mixed with other compounds

Significant discrepancy between the distilled oil and a typical leaf extract may be observed in the results above. Steam distilled oil, in which the monoterpene compounds tend to predominate, contained very little polygodial or cadina- 1,4 -diene. This might be the consequence of degradation during distillation in the case of the former, or simply due to high boiling point and the resulting low partial pressure in the vapour phase during distillation. Unfortunately the results allow only a general comparison between extraction and distillation since no pre-distillation sample for extraction was taken. Southwell and Stiff (1989) note that artefact formation during distillation has been implicated for several terpene compounds, and report their own observations in relation to steam distilled tea tree oil.

3.6 Asexual Propagation of *Tasmannia lanceolata*

Introduction

For the purposes of this project, it was necessary to obtain cutting material from mature plants and to establish this in the glasshouse as quickly as possible. Seedling production was not practicable both because of the slow germination of the species (T. Walduck, Tasmanian Forest Seeds, pers. comm.), and the uncertainty of using open pollinated seed for preparing uniform experimental material.

Secondly, in any subsequent commercial cultivation of clonal selections of*Tasmannia lanceolata* it will be necessary to undertake vegetative multiplication programme, having the following objectives:

1) acquisition and cultivation of vegetative material from identified wild plants of varying age/vigour/environmental circumstances, etc., and

2) multiplication of favourable selections as quickly and economically as possible.

The following general observations were made in preliminary trials with cutting propagation of *Tasmannia lanceolata* :

- Cuttings taken in autumn, stripped of their lower leaves, inserted in an unpasteurised medium containing 30% peat and 70% coarse sand and maintained under leaf controlled mist with bottom heating (20 - 23°C) will produce functional roots in approximately 90% of cases, within 8 weeks. However the rate and vigour of root formation varies widely, some cuttings producing a mass of vigorous roots within a few weeks, while others may require 6-7 weeks before producing two or three fragile roots. As a consequence the resulting plants are very variable, and the management of large quantities of cutting stock would be difficult. Similarly, the rate of shoot growth subsequent to rooting and potting on is extremely variable.
- Where cuttings have been taken in winter, spring or early summer a less reliable strike appeared to occur, root development was variable, and cuttings were more inclined to collapse prior to forming roots.
- In the natural situation the plant exhibits a vesicular- arbuscular mycorrhyzal association.
- Cuttings used in these trials were taken of recent shoots from plants of widely varying maturity (in many situations the plant grows slowly and some shrubs may be many decades old) without regard for the age of the plant.
- Opportunities exist for obtaining many hundreds of cuttings from single plants in their natural situation.

This investigation aimed to determine the effect of bottom heat and a rooting hormone (indole butyric acid - IBA) treatment on root initiation and development in clonal cutting material taken from the parent plant at different stages of seasonal development. No

attempt was made to determine the effect of juvenility in the stock plant, nor to investigate use of wood older than one growth cycle.

Experimental Methods

1) Plant material

Uniform age, clonal plant material (initially generated from cuttings of a 'wild' plant, and allowed to grow unpruned after potting) was maintained in 6" pots in a shade tunnel for more than 14 months. At the commencement of the experiment, plants bore the well-developed buds characteristic of winter conditions -swollen and awaiting lengthening days to commence growth.

A comparison of 'spring', 'summer' and 'autumn' growth for suitability as cutting material would normally need to be conducted at different times of the year, complicating the control of factors such as air temperature and daylength. The three growth types used in this trial were obtained by treating the plants as described below and taking the cutting 'types' simultaneously.

48 large plants were repotted (16/6/95), and treated as follows:

16 plants -Group C, were placed in a temperate glasshouse (21° Days, ca 14° nights, under lights to provide 14hour days, in order to stimulate a vigorous growth flush .

The remaining 32 plants were held in a growth tunnel under 21° C, 8 hr days, 2° C night temperatures.

When the first group had completed shoot extension, (effectively at the 'summer solistice'), it was then moved to 'autumn' conditions (shorter, cooler days) in an outdoor shade tunnel (16/8) and held at these conditions for one month.

At the same time, half the second group of plants (Group B- 'summer') were moved into long day conditions, while the remaining plants -Group A, 'spring', were held under winter conditions until one week before the commencement of the experiment, when they were moved to the temperate glasshouse.

At the time of setting, 'spring' cuttings could have been described as hardwood, the stems being firm, though still reddish, and leaves mature and toughened, while 'autumn' cuttings were still semi-soft, with green-red stems and fully expanded but soft leaves, and a slightly swollen apical bud. 'Summer' cuttings were 'soft', the more distal leaves incompletely expanded and the stems green and soft and the apex remained small, though no longer extending.

2) Treatment of cuttings

IBA (Sigma Chemicals) at 3 concentrations and a control:0, 500, 2000, 8000 ppm IBA in 50% ethanol. Bundles of cuttings were dipped in 1cm depth of solution for six seconds, allowed to dry briefly and then inserted into the rooting medium.

3) Rooting Environment

Cuttings were placed in a peat:sand mixture(1:3) under leaf controlled mist and ambient air temperature and light levels. Two temperatures in rooting bed - heated (20-22°C) and unheated (12-15°C), see Fig 3.5

The temperatures of the sandbed, high and low temperature cutting trays, and air temperature within and outside the mist enclosure were monitored continuously using a Foxboro Multichannel recording thermograph. Shade mesh was spread over the igloo as soon as air temperatures above the bed began consistently to exceed 20°C.

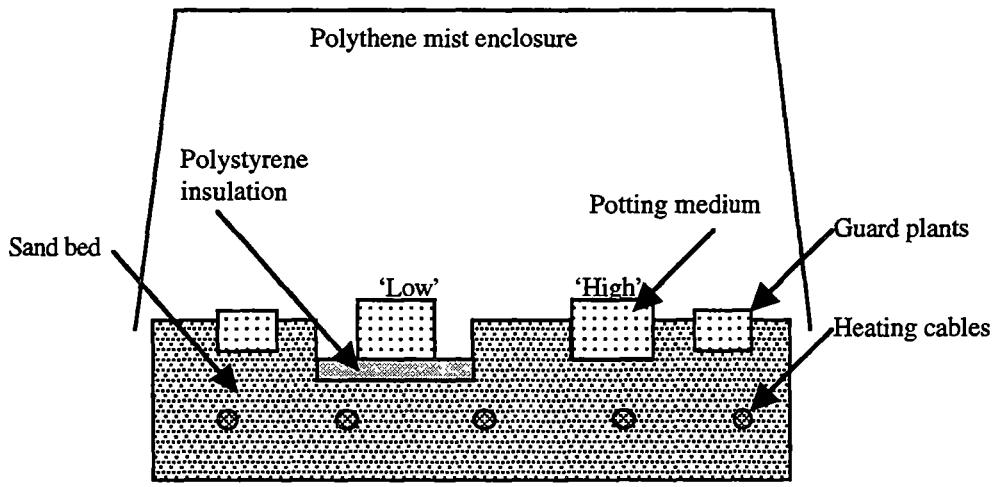


Fig 3.5: Mistbed propagation system used to establish high and low rooting medium temperatures

4) Experimental Design

The experiment was conducted as a randomised complete block design, with two replicates of the 24 treatments, each plot (a small plastic seedling tray) contained 4 cuttings; cuttings taken from the 16 plants in each season group were mixed and allocated randomly amongst treatments; blocks were situated adjacent to one another in the mistbed; temperature treatments were arranged on a plot by plot basis using insulated trays; the blocks were surrounded by a double row of 'guard cuttings' to ensure a uniform misting atmosphere among the plots;

Time of Cutting	Hormone Treatment	.
'Spring'-A (yellow tag)	Nil - '0'	Low (= air temp) - L
'Summer'-B (yellow tag)	500ppm - '1'	High (20-22°C) - H
'Autumn'-C(white tag)	2000ppm - '2'	
	8000ppm - '3'	

5) Measurements

After ten weeks under misting conditions, cuttings were removed from trays, washed free of soil, and assessed as follows:

• Score for root develoment, 0-4 as follows.The total score for four cuttings in each plot was used in the analysis:

0 - cutting completely dead or at least necrotic below the soil surface
1 - no callus development but cutting fresh and healthy
2 - callus development but no visible root development
3 - roots present, less than ten of more than 2mm length
4 - roots present -more than ten greater than 2mm long

• Mean length of longest roots, in millimetres (mean for four cuttings in each plot was used in the analysis).
• Dry wt of roots (not including callus), taken after drying to constant weight at 50°C (mean for each plot used in the analysis).
Analysis of results was conducted using the SAS anova procedure(SAS Institute-Software vers. 6.07) to determine means and least significant differences for the dependent variables 'score', length and dry weight, (summarised in Appendix 2.2)
Temperature data recorded in the propagation unit are summarised below, Table 3.6.

Table 3.6. Temperature data recorded over the duration of the trial.

Temperature range:	Maximum °C	Minimum°C
Outside enclosure	20	<5
Inside enclosure	11.4	5.8
Low treatment	23.5	10
High treatment	26	18.5
Sand bed	28	21.9

Temperarure Comparison	Differential°C
'Low' vs mistbed air temperature	2.0-4.0
'Low' vs bottom heat	3.5-10.5
'High' vs mistbed air temperature	9.5-12.5
'High' vs bottom heat	2.0-3.0
Low vs High	6.5-8.0

Results

Treatment means and LSD's are summarised in Table 3.7 below. There were no significant interactive effects amongst the three treatment dimensions. For 'Score', the number of deaths (or zero scores), associated with each treatment total (two blocks) is given in brackets.

Table 3.7: Treatment means for each parameter and LSD (p<.05) values for season, IBA treatment and heat/ no heat in rooting zone. 'Score' indicates root development (max. 16); 'Length' is mean of longest root for 4 cuttings per plot in mm.; 'Dry wt '- all roots per plot determined after drying @ 70°C

	'SEASON'	Mean	IBA(ppm)	Mean	TEMPERATURE.	Mean
SCORE	Spring	11.81 (1)	0	10.92 (3)	High	11.38 (9)
	Summer	8.63 (17)	500	11.00 (3)	Low	8.63 (14)
	Autumn	9.56 (5)	2000	11.67 (2)		
			8000	6.42 (15)		
	LSD(P>.05)	*1.83*		*2.11*		*1.49*
LENGTH	Spring	15.2	0	12.18	High	15.69
	Summer	8.54	500	13.43	Low	5.7
	Autumn	8.35	2000	11.3		
			8000	5.88		
	LSD (P>.05)	*4.4*		*5.08*		*3.59*
DRY WT	Spring	10.82	0	7.93	High	11.76
	Summer	5.76	500	10.08	Low	2.63
	Autumn	5.01	2000	6.1		
			8000	4.68		
	LSD (P>.05)	*4.89*		*5.64*		*3.99*

Conclusions

The use of bottom heat in rooting cuttings of *Tasmannia lanceolata* would appear to improve root initiation and root vigour and in this experiment resulted in fewer losses of cuttings. IBA dipping of cuttings did not improve rooting performance for these parameters and at the highest level of 8000ppm resulted in significantly reduced development, length and dry weight. This effect was particularly pronounced in softwood cuttings where no bottom heat was applied (data not shown), and no cuttings survived the ten week propagation period.

Most importantly, use of well hardened 'spring' type cutting material appears to give the most vigorous result with substantial benefit to root length and dry weight of roots generated with this type of cutting material. Good survival rates in 'autumn' -semihard wood - suggests that this material would ultimately generate adequate roots for propagation purposes, although for a uniform and rapid result, it appears that cutting material taken from mature plants late in winter would be more satisfactory.

Irrespective of this, the experiment shows that vigorous cutting stock can be produced by controlling the growing conditions of the parent plant prior to taking cuttings - simulating the conditions during the dormant and spring growth periods. In the development, at the appropriate time, of a strategy for multiplication of selected clonal material it may be assumed that two to three generations of cuttings might be produced during a calendar year, with careful management of light and temperature for rooting and maintenance of stock plants.

3.7 Other

The growth cabinets used in Section 7.1 were large insulated chambers, with electronic temperature control of separate heating and cooling circuits. The chambers were illuminated by banks of timeswitch-controlled fluorescent tubes supplemented by incandescent lights. Air circulation in the cabinets was provided by axial fans positioned beneath a perforated baffle on the floor of the chamber. Temperature within the cabinets was recorded continuously during the experiments.

Field radiation measurements presented in Chapter 7 were obtained using a Delta T Devices Tube Solarimeter and a Li-Cor inc. Pyranometer coupled to Delta T Devices Type MVI millivolt integrators. Methods used to obtain readings are described in the relevant methods section.

Assimilation measurements in Sect. 7.1 were conducted using an open circuit, infra red gas analysis system, using an ADC LCA3 gas analyser coupled to a perspex PLC3 leaf chamber. The apparatus was installed in an air conditioned room in which the temperature was maintained at 18-20°C during all measurements.

Illumination was by means of a GTE Sylvania 400 Metalarc lamp mounted directly above the leaf chamber and providing a maximum of 1500μmol $m^{-2}sec^{-1}$ photon flux, and separated from the leaf chamber by a cool water bath approximately 25 mm deep. The required reduction in photon flux was obtained by placing screens of Sarlon® shade cloth over the chamber. Quantum flux (PFD) at the level of the leaf surface was determined using the quantum flux sensor (Lambda Instruments LJ185 recording in the range 4-700nm) for each light level.

Leaf chamber temperature was controlled by fitting the chamber into a copper block, through which the contents of a water bath were recirculated. Temperature was measured continuously using the thermocouple installed in the leaf chamber. Gas flow to the IRGA and leaf chamber was supplied from gas bottles via short lengths of low density polyethylene tubing and was passed through copper tube immersed in an insulated waterbath maintained at 10°C by the addition of crushed ice at intervals during measurement periods. Gas was then bubbled through three simple humidifying flasks containing distilled water at room temperature, then passed through an F&P Co precision bore flowmeter calibrated from 0-1000ml min^{-1} air at 20°C, and into the inlet port of the instrument, from where it was directed automatically either directly, or via the leaf chamber, to the gas analyser for comparison.

Leaf area was determined by comparing manual measurement with the output of an electronic planimeter for two sets of forty leaves, for which the mean difference between the two methods was approximately 4%. Areas used in calculating the following results were obtained by multiplying the width of the chamber (25mm) by a manual measurement of the width of the region of lamina spanning it. The humidity of air passing into, and leaving, the leaf chamber was measured continuously on the

instrument, and these figures recorded simultaneously with the CO_2 differential and leaf temperature.

A calibrated source of reference air was used to set analysis parameters on the instrument, and to determine CO_2 concentration in bottled, compressed air containing 330-340ppm CO_2 which was used thereafter for the experimental work.

CHAPTER 4: PREPARATION, EXTRACTION AND ANALYSIS OF *TASMANNIA LANCEOLATA* LEAF

4. 0 Introduction

An account of the history of development of techniques for recovery of volatile and non-volatile lipid soluble compounds from plant parts is given in Geunther (1948), together with a brief, if slightly dated outline of 20th century practices for extraction of natural flower oils. The salient points here are the low temperature of the process and 'trueness' of the resulting extract, its dark colour resulting from the presence of plant pigments, and the relative costliness of the process rendering it suitable only for the more valuable of plant products.

Choice of solvent, the preparation of the plant material, parameters for the extraction process, and final product preparation are usually tailored to the specific requirements of the consumer of the product, subject to constraints of costs of production and processing.

Of the available solvents, purified petroleum ether- (its constituents mostly saturated paraffins - chemically relatively inert, completely volatile at low temperatures and, thus far, regarded as safe for use in preparation of food and flavour products), is the usual solvent of choice. Ethanol offers similar utility and a slightly different extraction performance, as do purified diethyl ether, ethyl acetate, hexanes and pentanes. On the other hand, while chlorinated solvents such as dichloroethane and dichloromethane, or aromatic solvents, for example benzene may offer technical possibilities, their hazardous properties render them unsuitable for commercial use.

Commercial methods, usually subject to confidentiality or patent, are not well reported in the literature in their entirety.

Georgiev and Gantchev (1983) compared five solvent types - petroleum ether, ethanol, methanol, dichloroethane and a mixture of petroleum ether and dichloroethane for the yield and quality of extract of *Pinus sylvestris* , and found that while ethanol and methanol gave high yields, the nature of the resulting extract was unacceptable. Only petroleum ether and the mixed solvent yielded suitable products, the latter a slightly higher yield and improved delivery of absolute of subsequent treatment. Georgiev and Gantchev(1983) also compared fresh and dried plant material and found that yield increased with reduced moisture content for both petroleum ether and mixed solvent, particularly with petroleum ether alone, while mixed solvent performed slightly better than petroleum ether in fresher material, as a result of the slightly more polar nature of the solvent.

Commercial extraction methods are developed to generate a reproducible extract of the desired quality at maximum yield, at a cost commensurate with the value of the commodity, though these objectives often involve compromise between yield and quality. In commercialisation of a plant extract, efficiency of extraction, composition

and yield are of fundamental importance, while for experimental comparison of plant materials and in studies of synthesis and accumulation, a fast and precise means of thorough extraction of the particular suite of secondary chemicals is more important.

The following study addresses two questions in relation to this:

a) commercial requirement for high yield and predictable composition, consistent with economical use of solvent, ease of handling and durability of both raw plant material and the extract product (sect. 4.2 -4.6).

b) requirement for a fast and precise experimental extraction technique using relatively small quantities of plant material, and providing extracts representative of a commercial extraction process (sect. 4.7-4.8).

Leaf material of *Tasmannia lanceolata* represents the dry matter bulk of annual incremental growth, and will contain the greater part of the extractable yield of secondary compounds of commercial interest. The leaf also represents the probable site of synthesis and storage of many of these compounds.
Freezing or drying of harvested material would appear to offer alternatives for extending the time frame for extraction, (assuming a limited harvest 'window'), particularly since experimental methods for preparing and extracting green leaf were less succcssful than those for dry leaf (see sect. 4.7 below). Practical considerations such as the separation of leaf from twigs and branches, the difficulty of breaking up fresh material, and the relative cost of frozen storage, packing and transport for fresh rather than dry leaf point to the benefits of preparing a dry, bulk product.
In the following series of experiments, it is assumed that dry leaf will be used in commercial extractions, and several aspects relating to the preparation of a material suitable for this extraction are investigated.

4.1 The effects of time and temperature during drying

Tasmannia lanceolata leaf epidermal characters, as described by Bailey and Nast (1945b) and Bongers (1973), - the shiny glabrous adaxial surface, the presence of cuticular deposits, sometimes occluding the stomata on the abaxial leaf surface and the cuticle varying from thin to quite thick would seem to mitigate against the rapid and uniform desiccation of leaves at normal temperatures. Variation in rate of drying and equilibrium dry matter content between leaves in preliminary trials when simple air drying at room temperatures was undertaken indicates the need for some acceleration of the process and a more thorough circulation of air around the leaf.
On the other hand, high temperatures might accelerate degradative processes in secondary metabolites, and the loss of lower boiling point compounds which might prove important from a flavour or fragrance point of view.

Experimental methods

Whole, fresh leaves taken from twenty pot grown plants maintained in shadehouse conditions were combined, and samples were taken randomly from these. Only undamaged leaves were used and those significantly larger or smaller than usual were discarded.

Six leaves per sample were weighed and placed in Kraft paper bags on wire shelves in the drying oven (see 3.1b). At the appropriate time, the samples, (referred to in this experiment as 'cured samples'), were reweighed, and three leaves per sample were weighed and transferred to a 70°C drying oven for dry matter determination (36 hrs, dried to constant weight). The remaining leaves were ground and extracted in the usual manner (see sect. 4.7) and the extract was analysed for yield and composition of a selection of components including a monoterpene tentatively identified as terpineol (N.Davies pers. comm.) and an unidentified oxygenated sesquiterpene eluting at 12.16 minutes. Each treatment was replicated three times.

Dry matter determinations for three samples of fresh leaves were also conducted.

Treatments

a) Drying temperature -

Temperatures of 25, 35, 55 and 70°C were applied for 48hrs each. In the case of the 25°C treatment an additional 12 hrs of drying was necessary to achieve dryness equivalent to that of the other treatments.

b) Drying time-

Leaf samples were maintained at a temperature of 35°C for 24hrs, 36 hrs, 48 hrs 72hrs, 96hrs and 120hrs.

Statistical Analysis

Component levels (as % peak area relative to C18 standard) were calculated for each sample, and least squares means calculated using the SAS (SAS Institute Software vers. 6.07) 'proc glm, lsmeans' procedure. A summary of the output from this procedure is given in Appendix 2.2.

Results and Conclusion

Yield of each of the selected compounds expressed as a percentage of the dry leaf sample (dry matter at 70°C) is presented for the two experiments in Appendix 2.3a and 2.3b and presented graphically in Figures 4.1.1 and 4.1.2 respectively.

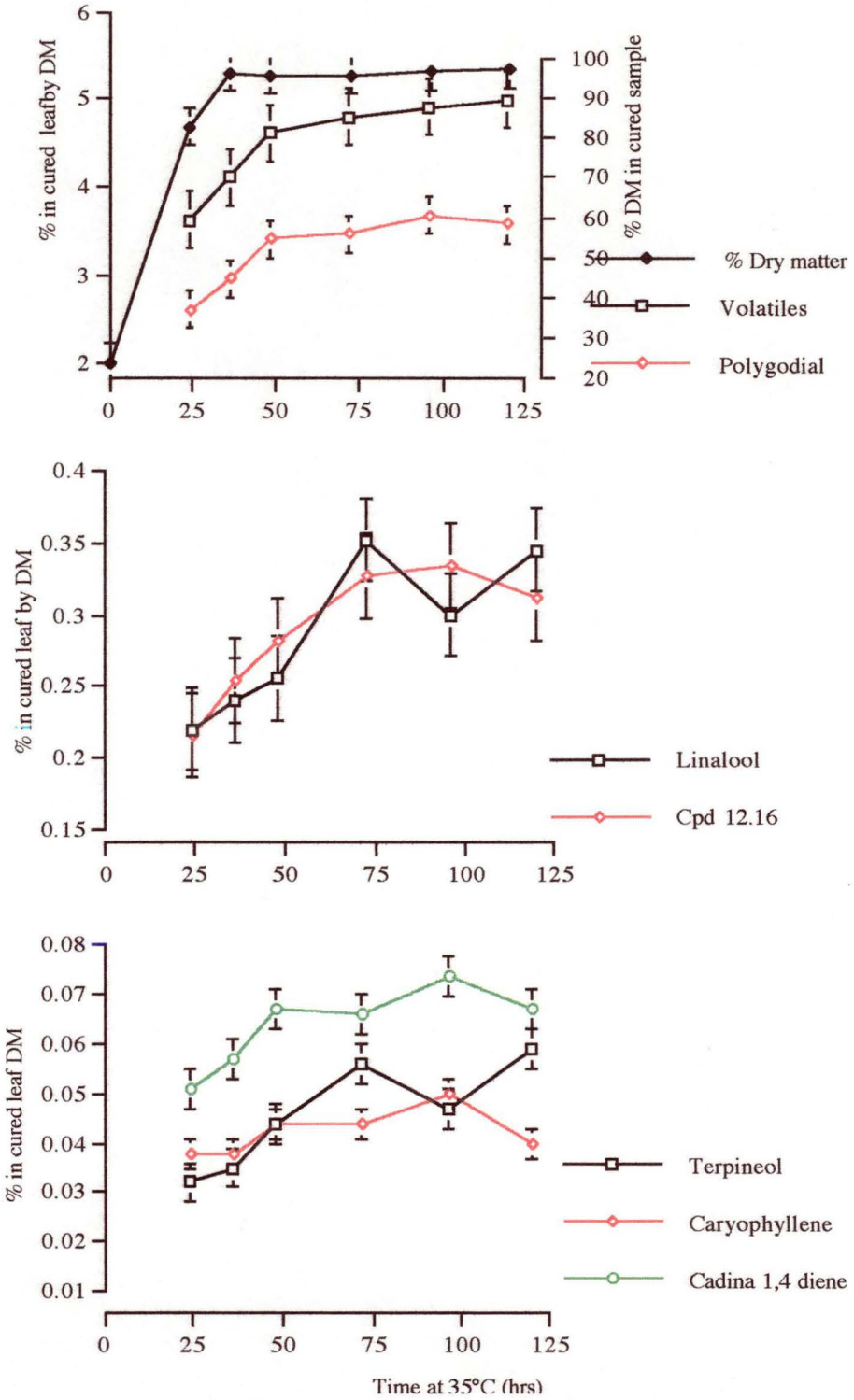


Figure 4.1.1 Effect of drying time on composition of leaf extract -percentage of each component in leaf dried at 35C (error bars show standard error of means of three replicates).

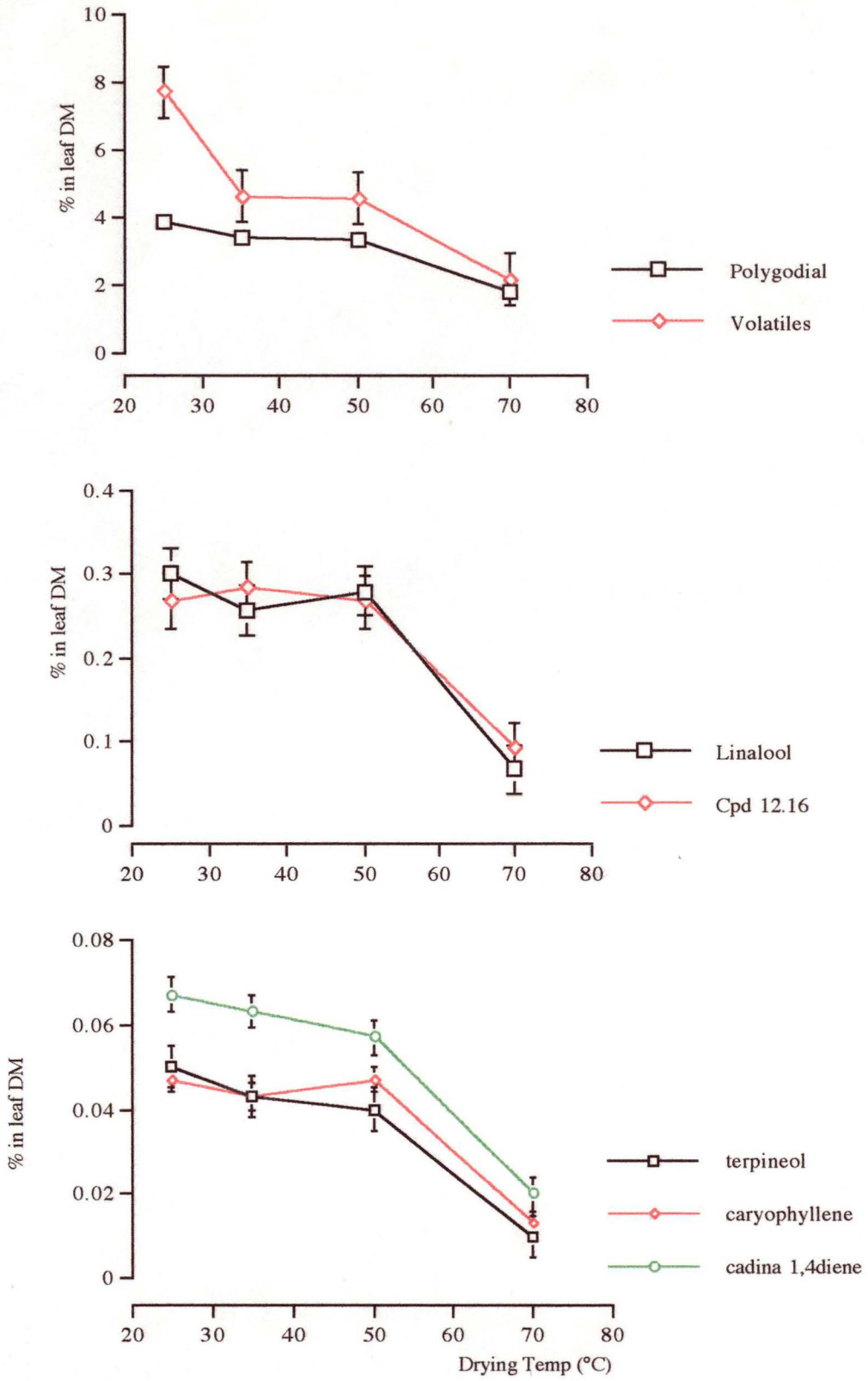


Figure 4.1.2: Effect of drying temperature on extract composition -percentage of each component after 48 hrs at each temperature (error bars show standard error of means of three replicates).

The result shown in Fig 4.1.1 suggests that a temperature of approximately 35°C for at least 50 hours should enable retention of most components of interest. Longer periods of drying up to 125hrs as might result in a less 'ideal' situation appear not to jeopardise recovery of the compounds monitored here, nor the collective 'volatiles' parameter. Figure 4.1.2 clearly shows the loss of all of the components recorded when drying was conducted at 70°C, and while the result for most of these compounds is not significantly reduced at temperatures between 25 and 50°C, the fall in 'volatiles' observed at the warmer temperatures indicates a significant loss of some lower boiling compounds even at 35°C. The sesquiterpene cadina- 1,4 -diene - (a significant yield component of many of *T. lanceolata* leaf samples but not as it happens, in the plant W3 used in this trial) appears to suffer some reduction during drying at 50°C.

Yields of all compounds reported increased with time of drying, the initial improvement being attributable to improvement in solvent penetration of fully dried ground leaf material, particularly since comminution of leaf seems to improve as dryness increases.
None of the compounds exhibited significant change after 75 hrs of drying, but for most (polygodial, linalool '12.16', terpineol and cadina- 1,4 -diene), there is evidence that levels continue to increase with time after the leaf is effectively fully dry. A possible explanation of this may be that it is dry matter (non extractable) which is changing -for example loss of non-structural proteins (sugars and fructans) as was detected by Trevino *et al.* (1995) in field dried oats. Such an explanation requires that some respiration (either plant or microbial in nature) continues in the *Tasmannia* leaf even at DM contents over 90% at 35°C, which seems unlikely.

4.2 Effect of comminution of leaf on rate of extraction

For commercial extraction, the intact leaf, with its tough cuticle and epidermis, and oil cells embedded well within the mesophyll presents a problem for rapid exposure of oil bearing structures to the solvent. Immersion of whole leaves in solvent during preliminary investigations resulted in imperceptibly slow extraction of secondary compounds even when sonication and gentle heating were employed. Destructive crushing, grinding or milling will increase the exposure of leaf tissue and ensure maintenance of the maximum concentration gradient across remaining membranes or cell walls and it was found that a large hammer mill, fitted with a fine screen, produced a powdery, friable dry leaf material which extracted quickly. To confirm this result, a mixed, milled leaf sample was used in the following trial comparing rate of extraction with leaf particle size.

Experimental methods

Hammer milled leaf material was prepared using a commercial mill designed for use with animal feed preparation (Ferguson, Coventry) fitted with a screen made from perforated steel sheet with 1.2mm holes. The resulting powder was then screened using a set of ten Endicott's US Standard soil sieves to determine the particle size distribution . then recombined into three size groups -

- d > 0.841 mm screen (13% of total)
- 0.841> d > 0.177 (75%)
- d < 0.177mm screen (13%).

Duplicate 5g samples of material from each size range and triplicate samples of unseived bulk material were extracted in firmly capped glass bottles (petroleum ether, shaken at 160cpm) using 20 ml solvent containing the C18 standard, for a range of times as detailed in Table 4.2.1. A 10ml sample was withdrawn from the bottle after a few minutes of settling, and the weight of extract determined by evaporating the sample to dryness in the RVE (see Sect. 3.1) and a percentage yield calculated on the basis of dry leaf material extracted.

A 'baseline', or 'total' extraction was performed on a separate larger sample by pounding the leaf in a mortar and pestle to an extremely fine consistency, adding solvent, sonicating for 15 minutes, followed by shaking for 24hrs.

GC analysis was conducted on extracts of the middle size range and polygodial (as % of C18 std.) is given for the mean of two analyses.

Results and conclusions

Figure 4.2.1 shows the distribution of particle sizes produced when the dry leaf material is milled using the commercial mill. The two larger, three smaller and remaining five fractions were recombined to produce the three size categories used in the experiment.

Mean percentage yield of extract (w/w of dry leaf) at each time and for each size category is given in Table 4.2.1. Also shown is the mean polygodial percentage of the total extract at each time (for the middle size range only). 'Baseline' result represents a 'theoretical' maximum extract for the leaf concerned after the extremely stringent preparation and extraction regime used. These results are presented graphically in Figure 4.2.2 below.

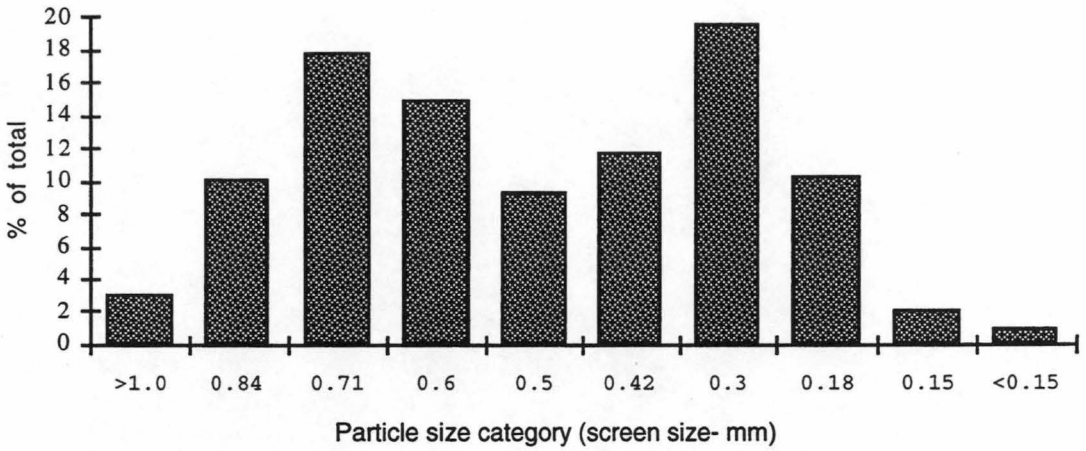


Figure 4.2.1 Particle size distribution in crude hammer milled dry leaf material- partitioned using standard soil seives.

Table 4.2.1: Yield of extract expressed as a percentage of the dry leaf sample (w/w) for each size category and time of extraction. Standard errors are given for the mean of duplicate samples. Polygodial percentages quoted for the middle particle size are as mean % of total extract (w/w) for two analyses.

Particle size (mm)	Extraction time (hrs)	Yield	*Std Err*	%polygodial
> 0.844	0.5	2.62	*0.03*	
	2	3.37	*0.132*	
	8	3.58	*0.004*	
	21.5	4.78	*0.018*	
	48	7.42	*0.094*	
0.84>d>0.18	0.5	2.06	*0.258*	13.6
	1	3.34	*0.108*	14.2
	2	3.85	*0.14*	14.4
	21	4.87	*0.106*	12.4
d< 0.18	0.3	4.68	-	
	0.5	6.54	*0.172*	
	0.75	7.63	*0.158*	
	4	7.96	*0.4*	
Bulk	0.5	3.44	*0.066*	
	1	3.64	*0.232*	
	2	4.28	*0.02*	
	16	4.89	*0.204*	
	24	5.32	*0.064*	
'Baseline'	-	9.16	-	

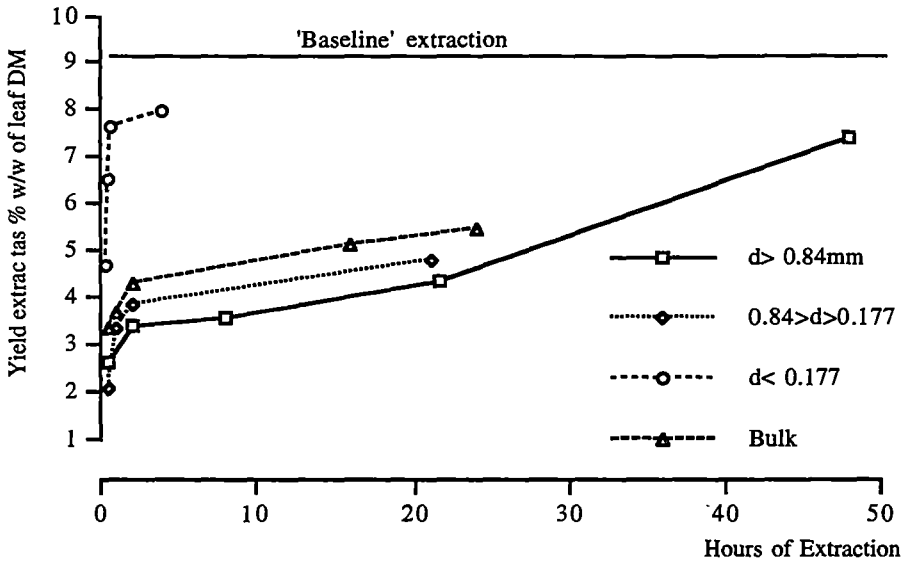


Figure 4.2.2: Time course of total extract solution for fine, medium and coarse fractions of hammer milled dry leaf material of *Tasmannia lanceolata* 'Baseline' represents yield obtained under 'ideal' conditions of preparation and extraction - see experimental methods.

As expected, the more finely divided material extracted very quickly, after 4 hours producing a solution containing 2% w/v of extract, equivalent to about 8% w/w extract from the dry leaf powder. This approached, but did not equal the yield obtained when an intensive grinding, sonication and shaking extraction was applied to the 'baseline' sample, suggesting that even with this degree of fineness, some extract still proved difficult to dissolve.

There was no significant trend in either % polygodial or 'total volatiles' (data not shown) over the range of extraction times used with the middle size range, (75% w/w of the crude milled material).

The results suggest that a slightly higher yield of extract was ultimately obtained from the finely divided fraction compared with the coarse fraction even after 48hrs of shaking, perhaps due to the latter containing a higher proportion of tougher vascular tissue and cuticle and less of the more friable (oil cell rich), mesophyll tissue.

In summary, the rate of solution of the extract is dependent on the degree of comminution and (for the concentration range employed here - up to 2% w/v) is not limited to any extent by saturation of the solution. With the hammer milling system employed in this experiment, it would appear that a substantial portion of the available extract will remain within the leaf matrix, even under extended periods of immersion in unsaturated solvent.

4.3 Extraction of pelletised leaf powder

The difficulty of recovering extract-rich solvent from powdered preparations of leaf due to the friable and absorbant nature of the material, and industry experience with solvent extraction of pelleted plant products (hops, pyrethrum flowers) suggests a comparison of rate and extent of recovery of extract compounds between loose milled leaf and compacted, cylindrical pellets approx 5mm in diameter and 10-15mm long, from which extract solution may be readily drained leaving very little solvent retained within the pellets themselves.

Experimental method

Freshly ground leaf powder (G) and freshly prepared pellets (P) of the same bulk leaf sample were produced using a Lister feed-pelletizing plant. Three samples of each were then weighed, immersed in 100ml petroleum ether in conical flasks, stoppered and shaken gently (150cpm). A further three samples of each material were weighed and dried at 70°C to determine %DM. At 1, 2, 4, 7 and 67hrs, 5 ml samples of each solvent were withdrawn and dried in a weighed RVE flask, and the resulting extract weight determined. Note was taken of the level of solvent in each conical flask at the outset and at each subsequent sampling in case of evaporative loss.

Two of the flasks, G2 and P2 proved not to be adequately stoppered and lost 10ml solvent by the first sample time. The problem was rectified for G2 but not entirely for P2, which lost a further 5ml by 2hrs and 10ml more by the 4hr sample, whereupon replacement of the stopper prevented further evaporative loss. Adjustment for these losses is made in the calculations.

All other sample weights are adjusted for the proportion of solvent sampled at each successive sampling.

After the 67 hour sampling, the combined extract was sampled and analysed using the GC method described in Chapter 3 and the percentage of selected components was determined.

Results and Conclusions

Pellets and powder comprised 86.9 and 86.7% dry weight respectively.

Table 4.3.1 below gives the mean yield of extract (expressed as w/w of pellets or ground leaf) for each set of three determinations per time of extraction. The graph of these results (Figure 4.3.1) shows the similar initial rates of extract solution and the ultimately lower yield of extract from powdered material.

At the completion of the trial, an attempt was made to decant as much solvent as possible from each of the flasks, then to weigh, dry and reweigh the leaf material to obtain an indication of the ease of recovery of solvent from spent material. The results of this varied widely amongst the three replicates, but pellets appeared to retain less

than 15% of their weight in solvent, and powder retained more than 100% of its weight in solvent after draining for 30 minutes.

Table 4.3.2 shows the mean (for three analyses) for each of seven compounds commonly detected in bulk collections of leaf, expressed a) as a percentage in the extract, and b) as a percentage of the leaf used in the extraction.

Table 4.3.1 : Mean percentage (w/w) of extract in dried leaf (s.e. in brackets).

	% of Extract w/w	
Extraction Time	**Pellets**	**Powder**
1hr	3.885 *(0.154)*	2.616 *(0.240)*
2hr	7.711 *(0.235)*	5.629 *(0.028)*
4hr	11.23 *(0.140)*	8.857 *(0.323)*
7hr	13.47 *(0.364)*	10.49 *(0.394)*
67hr	14.87 *(0.209)*	11.42 *(0.553)*

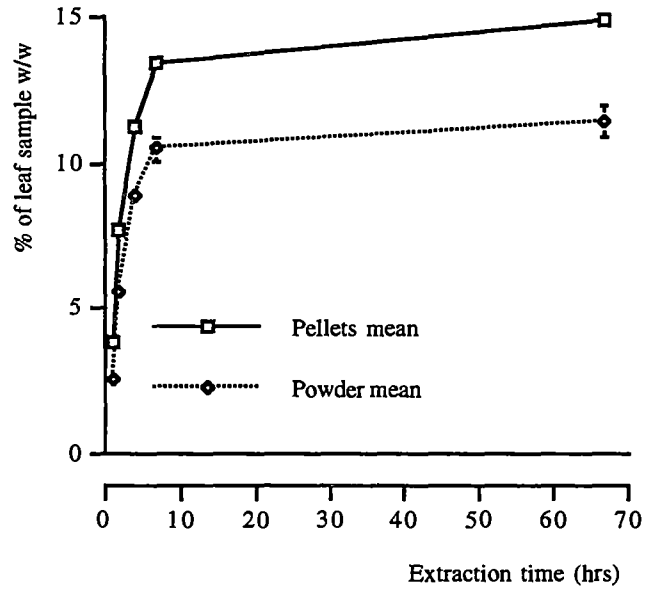


Figure 4.3.1: Rate of extraction of pelletised and powdered leaf material (error bars show standard errors of mean of three replicates).

Table 4.3.2: Composition and yield of major components extracted from ground leaf as loose powder and pellets.

	Percentage w/w in whole extract				Percentage w/w in leaf Dry matter			
	Ground leaf		Pelletised leaf		Ground leaf		Pelletised leaf	
Component	Mean	Std Err	Mean	Std Err	Mean	Std Err	Mean	Std Err
'Volatiles'	**50.84**	*(0.569)*	**49.12**	*(0.437)*	5.80	*(0.268)*	7.30	*(0.142)*
linalool	**0.89**	*(0.028)*	**1.03**	*(0.009)*	0.10	*(0.003)*	0.15	*(0.003)*
cubebene	**1.53**	*(0.027)*	**1.63**	*(0.001)*	0.17	*(0.006)*	0.24	*(0.003)*
caryophyllene	**0.84**	*(0.009)*	**0.88**	*(0.002)*	0.10	*(0.004)*	0.13	*(0.002)*
Germacrene D	**1.58**	*(0.025)*	**1.51**	*(0.015)*	0.18	*(0.006)*	0.23	*(0.001)*
cadinal,4diene	**5.05**	*(0.056)*	**4.81**	*(0.020)*	0.58	*(0.027)*	0.71	*(0.010)*
aristolone	**0.61**	*(0.041)*	**0.65**	*(0.009)*	0.07	*(0.006)*	0.10	*(0.003)*
polygodial	**16.34**	*(0.088)*	**13.70**	*(0.110)*	1.87	*(0.087)*	2.04	*(0.043)*

Clearly, use of pellets in the extraction process offered many benefits, with all component percentage yields were improved with respect to the dry leaf material for

the pellet sample, and a greatly improved rate of extraction and recovery of solvent from the spent plant material.
However, it should be noted that this approach does alter the composition of the extract, reducing the proportions of of some components (germacrene D, cadina- 1,4 -diene and polygodial), and increasing that of others (linalool, cubebene, caryophyllene and aristolone). The components measured in this comparison were selected on the basis that they represented significant proportions of each of the groups of terpenes in the extract, and not in relation to their impact on organoleptic or physical characteristics of the oil. Use of pelletised leaf material must be considered conditional on further commercial information regarding preference for physical and organoleptic and composition properties .

4.4 Ethanol Degradation of Extract Components

Since, as mentioned above, ethanol represents a safe and commonly used extraction medium, and further refinement of crude extracts into absolutes for use in the flavour and fragrance industry usually involves separating waxy extractives on the basis of solubility in cold ethanol, an experiment was devised to determine the effect of alcohol on the stability of a key extract component, the sesquiterpene dialdehyde polygodial.

Experimental Method
Approximately 25mg of each of three extracts was dissolved in 1ml HPLC grade ethanol containing 1.1726 mg C18 standard in a GC analysis vial. The solutions, maintained at room temperature (20°C), were immediately analysed by the standard method (Sect. 3.2) and re-analysed at 2, 5 and 95 hours.

Results and Conclusions
GC FID peak area ratios for the two polygodial peaks and the C18 standard, and a response factor of 1.5 (determined for polygodial in extract in a separate experiment and reported elsewhere (RIRDC Report 1995)) were used to estimate percentage polygodial for each extract and time. The mean percentage polygodial and standard error of the mean for each analysis period are presented in Table 4.4.1 below. Analysis of variance revealed no significant difference between the percentage of polygodial detected in ethanol solution even after 95 hours at room temperature.
This suggests that, should a commercial requirement for ethanol soluble 'absolute' arise, there would be no loss of polygodial during the period in which the extract was dissolved in cold ethanol- usually 24 -48 hours. Since the object of such a procedure is the removal of a proportion of the extract (insoluble plant waxes), and results in a reduction in yield, the decision would involve economic considerations as well as concerns for the stability of key constituents.

Table 4.4.1: Mean percentage of polygodial (area ratio with C18 standard) remaining in ethanol solution over time

Hours in Solution	Mean % polygodial	Standard Error
0	21.24	1.75
2	21.07	1.78
5	21.46	1.68
95	20.51	2.07

4.7 Effect of Storage conditions on extract quality

In order to assess the stability of the key extract components during storage, a trial was established using a representative bulk extract in a variety of storage regimes and the effect on composition with respect to seven components was monitored for over ten months. Storage conditions most amenable to control and likely to affect extract stability were considered to be light, temperature, headspace atmosphere and time.

Experimental Method

Samples of extract were subjected to the following six storage methods. Each treatment was applied to three replicate samples.

Table 4.5.1 describes the storage conditions applied to each of the treatments. Dark storage was provided in amber glass bottles wrapped in aluminium foil and the ambient temperature treatment was simply placement of the bottles in an air conditioned laboratory where temperatures ranged between approximately 12-22°C over the experimental period. The headspace of containers stored using nitrogen (1,2,4) was flushed with industrial grade nitrogen at the outset and after each sampling.

Table 4.5.1: Storage treatments for leaf extract, three replicates per treatment.

Treatment No	Temperature	Light	Headspace
1	-18°C	Dark	Nitrogen
2	2°C	Dark	Nitrogen
3	2°C	Dark	Air
4	Ambient	Dark	Nitrogen
5	Ambient	Dark	Air
6	Ambient	Light	Air

Samples were taken from the storage containers for analysis at fortnightly, then at increasingly longer intervals as the trial progressed. Components monitored during the trial were: α-pinene, linalool, calamenene, cadina- 1,4 -diene, polygodial and kaurene. GC FID analysis was conducted on the samples using a slightly different method from that described previously (sect. 3.2):

Sample: 20-30mg in 1ml hexane, plus 20-25µl 0.1g ml^{-1} C_{18} standard

Injection mode: 1ml solution, 50ml min^{-1} split vent flow

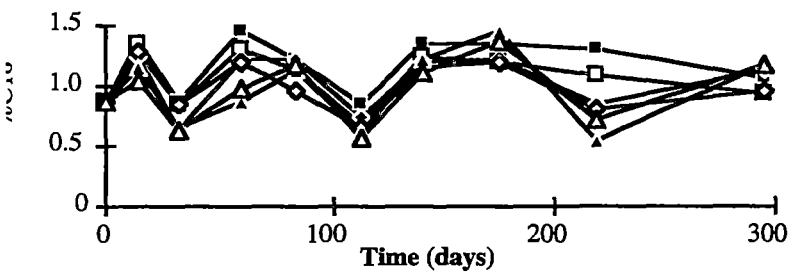
Column: SGE 15m BP1, 0.22mmi.d., 0.25µm phase thickness

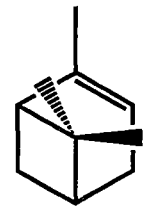
Gas flow: 12psi head pressure, 2ml min^{-1} flow

Results and Discussion

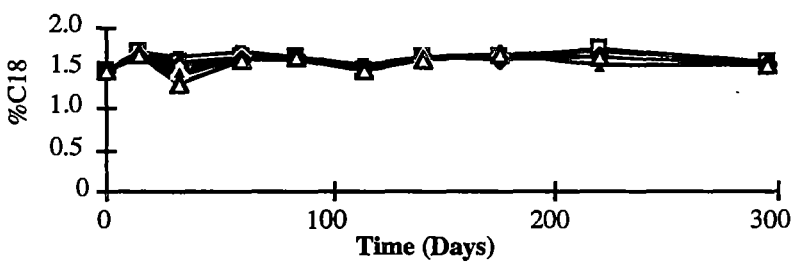
Mean percentage compositions (% C18) for this experiment are given in Appendix 2.4, together with standard deviations for each treatment and time. These results are presented below in Figure 4.5.1. Note that 'total polygodial' is the result of summation of percentages for polygodial and its injection breakdown product (see Sect. 4.9).

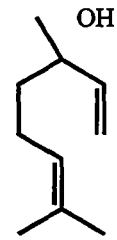
a) a-Pinene (0.28)



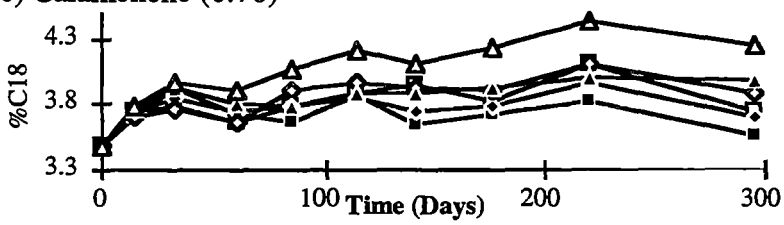


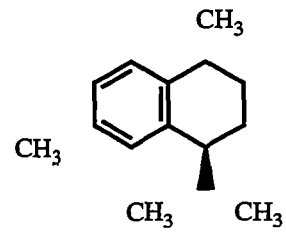
b) Linalool (0.55)



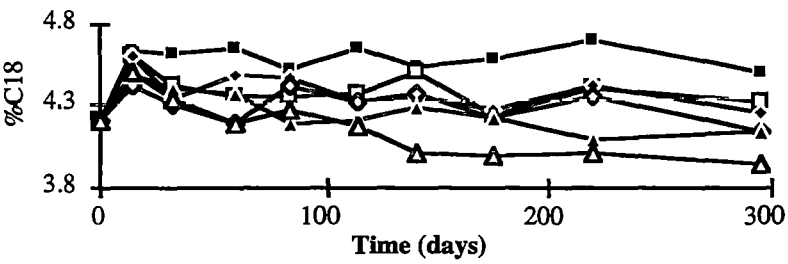


c) Calamenene (0.70)





d) Cadina-1,4-diene (0.71)



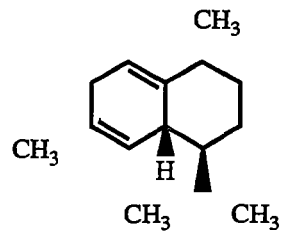
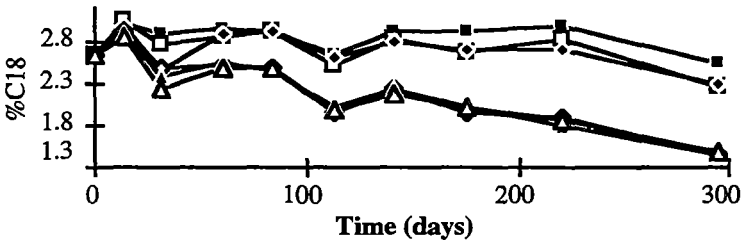


Figure 4.5.1a: Major constituents of *Tasmannia lanceolata* extract monitored over 299 days. Figures in brackets are retention ratios with the C_{18} std.

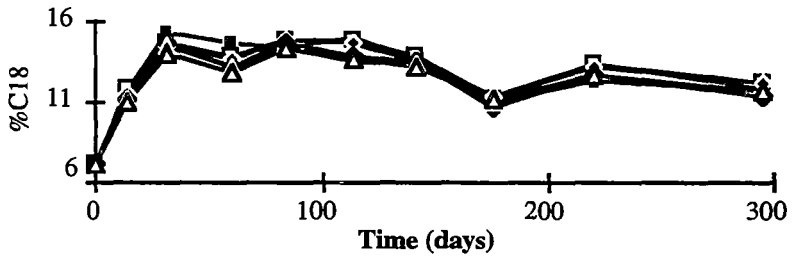
Key: Filled squares- control (dark, -18°C, Nitrogen)
Open squares-dark, 2°C, nitrogen
Filled diamonds- dark, 2°C, air
Open diamonds- dark, ambient temp., nitrogen
Filled triangles- dark, ambient temp., air
Open triangles- light, ambient temp., air

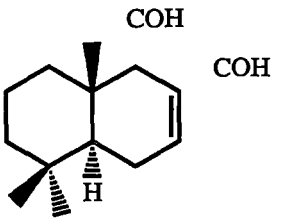
e) Unknown 3 (0.77)



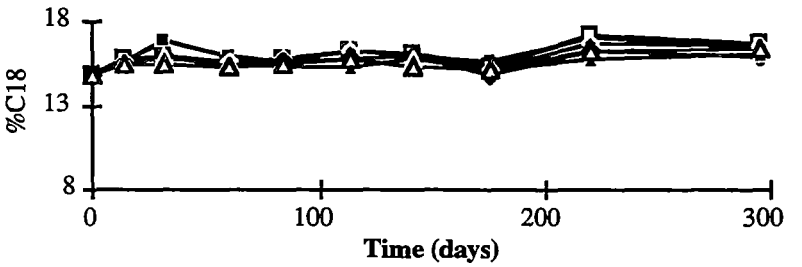
Structure Unknown

f) Polygodial (1.19)





g) Total polygodial (1.05 + 1.19)



h) Kaur-16-ene (1.27)

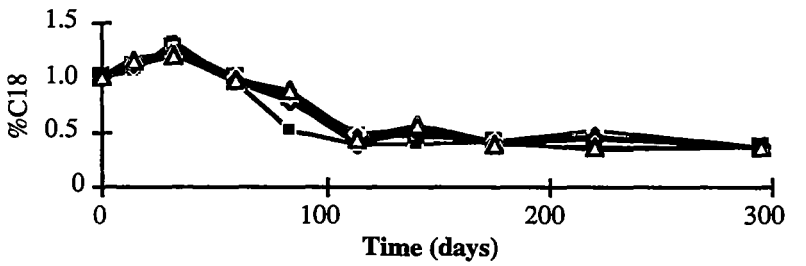


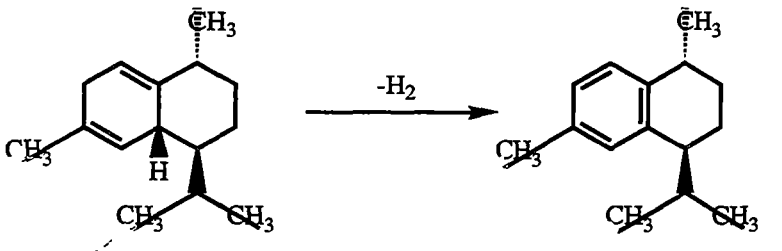
Figure 4.5.1b: Major constituents of *Tasmannia lanceolata* extract monitored over 299 days. Figures in brackets are retention ratios with the C18 std.

Key: Filled squares- control (dark, -18°C, Nitrogen)
Open squares-dark, 2°C, nitrogen
Filled diamonds- dark, 2°C, air
Open diamonds- dark, ambient temp., nitrogen
Filled triangles- dark, ambient temp., air
Open triangles- light, ambient temp., air

Fluctuation between sampling dates was attributed to errors introduced by use of solvents at changing ambient temperatures, altering concentration of C18 and extract in the analysed solutions. This problem could have been overcome by use of a standard temperature during pipetting.

Some general trends were, nonetheless apparent, both with time and between treatments for certain of the compounds examined. Alpha-pinene, linalool and

polygodial remained unchanged over the duration of the experiment in all storage regimes, while the proportion of kaurene appeared to diminish as the experiment progressed, although there was no difference between storage methods in the extent of this change. Unknown III content also fell over time, but this trend was more marked in the warmer conditions, with a slight fall evident in storage at 2°C. Steady changes were detected for calamenene (increase) and cadina1,4 diene (decrease), in both cases most obvious with the warmer storage conditions as might be expected if the dehydrogenation conversion of one to the other (Figure 4.5.2) is involved. These trends appeared unaffected by use of nitrogen vs air in the storage headspace.



Cadina-1,4-diene Calamenene

Figure 4.5.2: Dehydrogenation of cadina-1,4-diene to calamenene

In conclusion, for the compounds monitored here, in particular polygodial, the major component of commercial interest, no benefit was gained by using nitrogen in the headspace, and for storage at 2°C or less in lightproof containers, at least ten months storage without detriment to might be reasonably expected.

4.6 Quantitative analysis of leaf samples

A quick and precise method for determining extract composition using small samples of plant material is required. The resulting extract should equate as closely as possible (for the purposes of this study) to that obtained from a commercially practical method of leaf preparation and extraction. This set of experiments examines firstly a suitable means of determining the composition of the extract, and secondly, the possibility of using fresh leaf samples in such assays.

4.7.1 Sampling technique

Initially, in determining yield and composition of leaf extracts, (eg in 'Collections' and 'altitude', sect. 3.5), a technique of grinding, filtration and washing of the leaf powder, followed by evaporation of solvent under vacuum at 60°C was employed in which dry extract of each leaf sample was obtained. This was required for determination of organoleptic or physical properties, or an estimation of percentages of volatile compounds in the extract.

The method gave much opportunity for yield under-estimation by incomplete recovery of solvent from the leaf powder, over-estimation by accidental inclusion of very fine leaf material, and the possible loss of volatile components under evaporation. This, and the slowness of the technique suggested that unless 'whole' extract was required, use of an internal standard and sampling of the extract supernatant directly over the ground leaf after settling might be more appropriate. This method is referred to here as 'direct sampling' method.

A simple solvent sampling method is developed and compared with the 'whole extract' recovery method for yield of each of eleven components distinguished as peaks in the normal GC analysis and expressed as a percentage (of C18 std) of the dry leaf used in the extraction.

Experimental Method

Air dry leaf samples (W4) of approximately 1 gram were chopped, frozen in liquid nitrogen and ground in a mortar and pestle. Ten mls petroleum ether containing 7.5mg octadecane (Sigma Chemicals) as an internal standard was added and the mixture ground briefly and allowed to stand for a few minutes to settle before a 1ml aliquot was carefully transferred to a GC vial (direct sampling method).

The remaining solvent was filtered through a sintered glass funnel (Duran Jena Glass #3), the ground leaf washed twice in fresh solvent and the combined filtrates evaporated to dryness on a small rotary vacuum evaporator (at 60°C and 720mm Hg for 5 mins). A small sample of the resulting extract was transferred to a GC vial (total extract method).

This procedure was repeated for five samples.

Results and discussion

Analysis, and calculation using the ratio:

$$\% \text{ x in leaf} = \frac{\text{mg } (C_{18})}{\text{Area}(C_{18})} \times \frac{\text{Area Peak}}{\text{Wt. leaf (mg)}} \times 100,$$

provided estimates of yield (by dry weight of leaf) for each of eleven components appearing as peaks in the FID output and 'total volatiles' -total peak area between 1,8 cineole (eluting at 5.3 mins) and polygodial (*ca* 18.9 mins) (Table 4.7.1a). These peak identities were not verified for this experiment and include a number of 'unknown' compounds. Leaf dry weight was established by drying two subsamples at 70°C to constant weight, and adjusting sample weight accordingly. Analysis of variance for the two methods for each of the eleven components and the percentage of volatiles in dry leaf is given in Appendix 2.5.

Total yield of extract, that is, 1.11 times that finally recovered from evaporating the solvent from the five leaf washes, is given in Table 4.7.1b below.

Table 4.7.1a: Mean yield of eleven components and 'total volatiles' for two methods of analysis as percentage in leaf DM_{70}

		% in leaf DM (w/w)		
Component	**Retention Time**	**Direct sample**	**Total extract**	**LSD (0.05)**
1,8-cineole	5.3	0.16	0.08	0.05
cubebene	8.66	0.49	0.52	n.s.
caryophyllene	9.63	0.11	0.12	n.s.
Unknown I	10	0.11	0.11	n.s.
germacrene D	10.45	0.21	0.23	n.s.
calamenene	10.94	1.02	1.16	n.s.
cadina1,4 diene	11.21	1.55	1.71	n.s.
Unknown II	11.84	0.23	0.25	n.s.
Unknown III	12.11	0.68	0.79	0.12
aristolone	14.78	0.08	0.10	0.02
polygodial	16.61,18.88	0.91	1.12	0.14
total volatiles		6.40	7.18	1.12

Table 4.7.1b: Total extract yield from evaporated solvent corrected for volume expressed as a percentage of dry leaf material.

Sample	Dry wt (g) (@70°C	% yield of extract
1	0.5436	14.95
2	0.5663	14.68
3	0.5493	12.07
4	0.5555	11.36
5	0.5240	11.79

Note that the addition of the internal standard prior to grinding was employed in both cases to determine yields by leaf dry matter. If the results shown in Table 4.7.1b are typical, then taking a GC sample after evaporation, adding a standard, analysing, then calculating yield on the basis of total extract weight and GC sample composition would result in a wide variation of % yield estimates, since total yield itself was very variable. This variation was attributed to problems with filtration of extremely fine leaf particles and losses of some volatile components (note the significantly lower level of 1,8 - cineole for example). While the two methods resulted in no significant difference for most of the components considered, 1,8-cineole levels were reduced by the complete recovery, probably by evaporative losses during drying down of the solvent.
On the other hand, polygodial was detected in significantly reduced levels by the solvent sampling method. One explanation for this might be that polygodial is adsorbed to the particulate solids in the mixed phase system, while the preparation of complete extract, by filtration and rinsing of the leaf residue ensures more complete removal of the compound from the solids (but does not similarly affect the other compounds examined). Alternatively, undetected GC artefacts (see sect. 4.9) may have been produced in greater proportion in samples prepared from the solution than from those prepared by redissolving dried extract in petroleum ether for analysis.

4.6.2 Extraction of fresh leaf material

The method of direct solvent sampling outlined above was next employed to compare the efficacy of extraction by petroleum ether of fresh and dry leaf material and the use of a more polar solvent mixture: 50:50 hexane/acetone in fresh leaf extraction.
Fresh plant material proves difficult to extract thoroughly with non polar solvents such as hexane, pentane etc. which penetrate wet tissues poorly, even after thorough comminution. Solvent mixtures containing hydrophilic organic solvents, such as ethanol or acetone might be expected to penetrate intact wet cellular material more readily, and probably to dissolve additional compounds such as chlorophyll and other water soluble pigments and tannins in the process.

Experimental Method

To compare the performance of polar and non polar solvent mixtures on *Tasmannia lanceolata* leaf material, samples of fresh and dry leaf as used in the above experiment were extracted using petroleum ether and a 50:50 hexane: acetone solvent mixture. As with the previous experiment, leaf (fresh or dry) was frozen in liquid nitrogen, ground to a fine powder and 10mls of the solvent containing 7.5 mg of the C18 standard was added. A 1ml aliquot was withdrawn after further grinding and a few moments' settling and a GC analysis performed on the sample.

Results

Five samples were extracted using each method, and the resulting yield of each component expressed as a percentage of dry leaf (70°C) is given below (Table 4.6.2)

Table 4.6.2: Mean levels of eleven components and 'total volatiles' extracted from fresh or dry leaf with petroleum ether or fresh leaf with a hexane:acetone solvent mixture.

Component	Retention Time	% in leaf DM w/w Pet. Ether Dry leaf	Pet. Ether Fresh leaf	Hexane/acetone Fresh leaf	LSD (P<0.05)
1,8-cineole	5.3	0.16	0.12	0.11	0.03
cubebene	8.66	0.49	0.43	0.37	0.06
caryophyllene	9.63	0.11	0.10	0.09	0.02
Unknown I	10	0.11	0.11	0.08	n.s.
germacrene D	10.45	0.21	0.19	0.16	0.04
calamenene	10.94	1.02	0.90	0.58	0.25
cadina1,4-diene	11.21	1.55	1.35	1.17	n.s.
Unknown II	11.84	0.23	0.20	0.11	0.05
Unknown III	12.11	0.68	0.60	0.29	0.15
Aristolone	14.78	0.08	0.06	0.02	0.02
polygodial	16.61,18.88	0.91	0.82	0.24	0.24
% vols in leaf		6.40	5.35	3.61	1.01

Conclusions

As noted above, use of the 'direct sample' method generates an analytical result which appears to underestimate polygodial percentage slightly in dry leaf but in most respects is similar to that obtained by the complete preparation of solid extract, a slow and messy procedure in which slight underestimation of some compounds may result. In most cases these differences will not justify full extraction, and where a comparative result is sought the direct sampling method is indicated by these results.

Further simplification of comparative analyses by use of fresh leaf tissue was not supported by the results of 4.6.2 above- in the case of every extract component examined, petroleum ether extraction recovered more compound as detected by direct sampling, and in no case was the recovery from fresh leaf greater than that from the dry material. The use of a mixed, more polar solvent did not appear to be helpful in this regard.

The method to be used for the comparative analyses in this thesis (Chapters 5, 6, 7) is the direct sampling of solvent over ground dried (35°C, 24hrs) leaf.

4.7 Identification of components in *Tasmannia lanceolata* extract

Although typical extracts of leaf of *Tasmannia lanceolata* may be resolved chromatographically into more than fifty components, for the purpose of the physiological work, and comparisons of extraction techniques in this thesis, ten peaks were selected, representing clearly unambiguous (ie, never mixed with neighbouring compounds) entities across the range of elution times for terpenoid compounds commonly found in the extract. Peaks chosen were identified as the monoterpene linalool, sesquiterpenes cubebene, caryophyllene, germacrene, bicyclogermacrene and

cadina- 1,4 -diene and oxygenated sesquiterpenes aristolone, drimenol, and polygodial (two peaks). Use of the trivial name 'aristolone' here indicates only that the spectrum closely resembles the reference spectrum for aristolone included at Appendix 2.6. While the compound in the extract producing the peak and spectrum will normally be referred to as aristolone this was not an unequivocal assignment.

A number of other common terpene compounds were readily recognised in the typical analysis such as 1,8 cineole, terpineol, eugenol, α-copaene, calamenene, guaiol and kaurene but were often confounded with neighbouring peaks or were occasionally either absent or present in insignificantly small proportions. The ten peaks chosen are identified by elution time and comparison of mass spectroscopic data.

It should be noted that both retention indices and mass spectra for α-copaene and α-cubebene are not well distinguished in the literature. Separate analysis of essential oils of Cubeb and Copaiba provided clarification of retention times and MS for each of the compounds for our conditions (data not shown) and by comparison with the result for *Tasmannia extract* used here it was confirmed that the larger peak was α-cubebene.

Experimental

A sample of 17.8mg of *Tasmannia lanceolata* bulk leaf extract (W3) and small samples (a few milligrams each) of hydrocarbon standards (C_nH_{2n+2}) ranging from C_{13} to C_{22} were dissolved in 1ml acetone (analytical grade) in a GC vial. The solution was analysed by GC MS using the following parameters:

Injection Mode : manual on-column injection. Detection Temp : 290°C

Column : 20m HP1, 0.22mm id, 0.52 μm thickness.

Program temp : 40°C (1min) - 6°C/min - 290°C(7mins). Total time of 50 min.

Results

Table 4.7.1 lists the retention times for the $C_n H_{n+2}$ standards; Figure 4.7.2 the regression of carbon number with retention time; and Table 4.7.2 the Kovat's indices for the relevant compounds, calculated using the formula:

$$I^{a}{}_{b} = 100N + 100n \frac{t'R(A) - t'R(N)}{t'R(N+n) - t'R(N)} \quad \text{.....Eqn 4.1}$$

Where I is the retention index on phase a at temperature b and $t'_{R(N)}$ and $t'_{R(N+n)}$ are the adjusted retention times of n-paraffin hydrocarbons of carbon numbers N and $(N+n)$ that are respectively smaller and larger than the adjusted retention times of the unknown, $t'_{R(A)}$ (Jennings and Shibamoto 1980).

No index was calculated for linalool, (the retention time for this peak falls below that for the smallest standard used). No literature values for 'aristolone', drimenol or polygodial were obtained.

The gas chromatogram, annotated with peak identities and the associated retention time data are given in the Appendix 2.6, together with mass spectral data and the respective reference spectra for comparison. For polygodial, the spectrum obtained from a pure sample is provided.

Table 4.7.1. Retention times for hydrocarbon standards taken from the chromatogram at Appendix 2.6 and regression of hydrocarbon number n, with retention time data.

Standard Peak	Ret. Time (mins)
C13	9.71
C14	11.97
C15	14.33
C16	16.65
C17	18.91
C18	21.10
C20	25.22
C21	27.11
C22	28.97

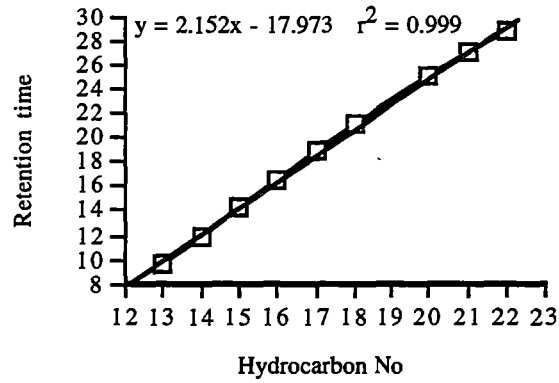


Table 4.7.2. Calculated Kovat Indices, common names and literature values for selected peaks in GC FID chromatogram shown in Appendix 2.6

Peak No.	Retention Time (mins)	Common Name	Kovat Index	Literature Value*
1	5.62	linalool	-	1092
2	10.74	a- cubebene	1345	1381
3	12.271	caryophyllene	1413	1428
4	13.59	germacrene D	1469	1468
5	13.93	bicyclogermacrene	1483	1490
6	14.79	cadina 1,4 -diene	1520	1518
7	19.38	aristolone (?)	1721	
8	19.62	drimenol	1732	
9	20.43	polygodial isomer	1769	
10	24.12	polygodial	1873	

* literature values are drawn from Davies (1990) and Ramaswami *et al* (1986)

For GC analysis used in the remainder of this work, direct comparison of GC FID output with these confirmed peak patterns by retention time ratios with the C18 standard was used to identify the relevant peaks.

A typical GC FID chromatogram from which this data might be obtained is shown in Figure 4.7.1, upon which some peak identities determined by comparison with GCMS data are indicated by reference to the key in Table 4.7.3. Many of these identities are unconfirmed and are the subject of continued investigation.

Table 4.7.3: Suggested peak identities for Figure 4.7.1 determined by comparison with GCMS data.

Peak Number (Fig. 4.7.1)	Peak Identity
1	1,8 cineole
2	cis-ocimene
3	linalool
4	α-terpineol
5	piperitone
6	eugenol
7	α-cubebene
8	gurgenene
9	caryophyllene
10	'Unknown I'
11	germacrene D
12	calamenene (poss. mixture)
13	cadina- 1,4 -diene
14	'Unknown II'
15	'Unknown III'
16	guaiol
17	δ- cadinol
18	aristolone
19	drimenol
20	polygodial
21	polygodial
22	kaurene

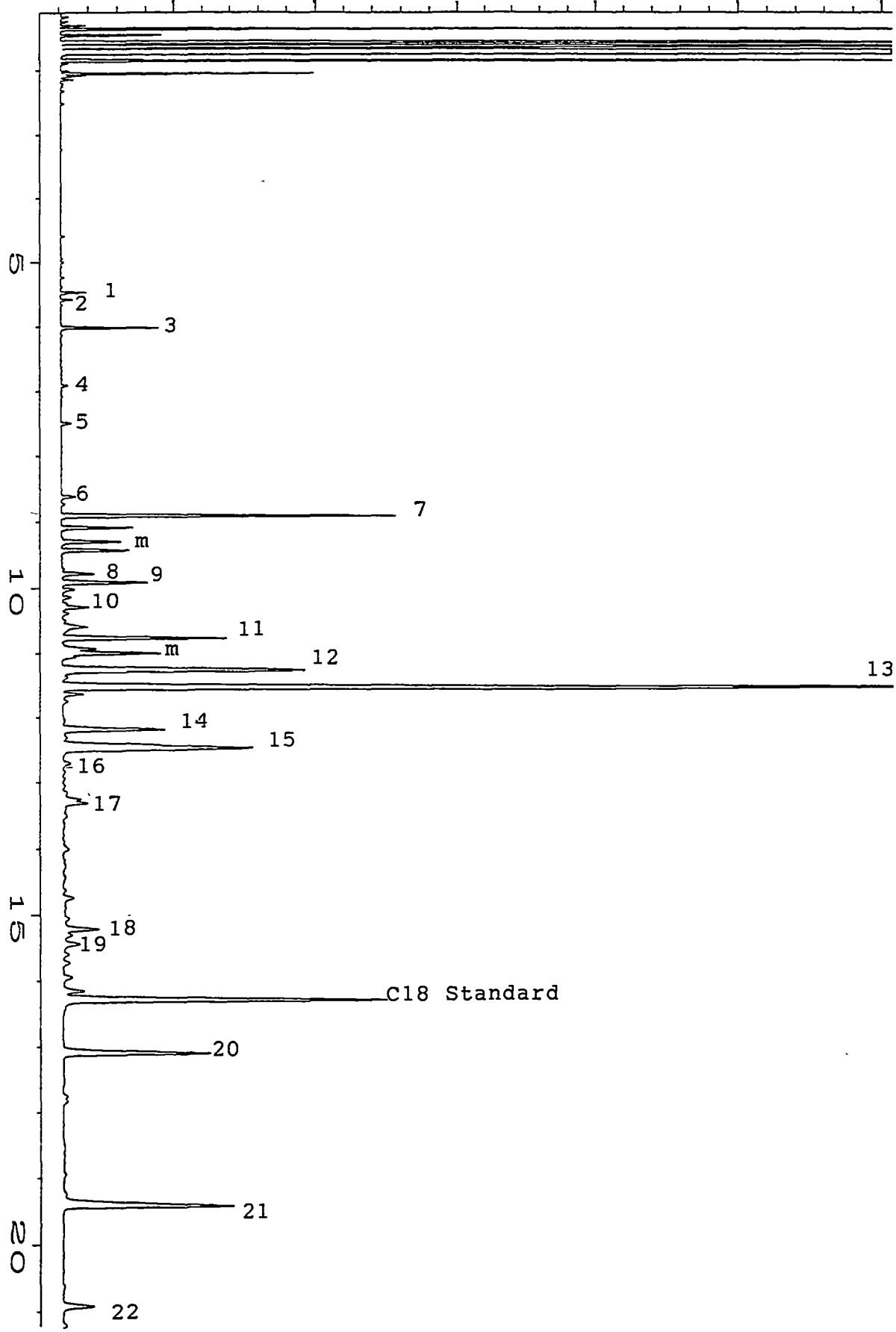


Figure 4.7.1 Typical GC FID chromatogram used to determine percentage composition in extracts of *Tasmannia lanceolata* showing suggested identities (see key - Table 4.8.3) for many of the peaks determined by comparison with GCMS analyses. 'm' denotes mixture.

4.9 Injection artefacts of polygodial

Most GC analysis conducted in this thesis employed an automatic sampling and injection system (see Sect. 3.2), and the normal mode for injection usually resulted in the appearance of a peak confirmed as a probable isomer of polygodial by GC-MS analysis. In fact, in a separate study, not reported here, levels of five 'breakdown products' detected in a pure sample during injection and analysis were determined and the total of the two major isomers of polygodial was shown to account for 92% of the compound injected, while a further three substantial peaks representing other degradation products accounted for a further 7.5-8.0 % of the total (RIRDC Report 1995).

A simple and direct comparison of automatic and manual injection of pure polygodial is shown in Figure 4.9.1 - the TIC output of the GC analysis of a pure polygodial sample by automatic injection into the injection chamber, with two large peaks, together with that of the same sample eluted under the same conditions but with direct manual injection onto the GC column.

The extent of conversion of the naturally occurring isomer to this artefact appeared not to be related to temperature conditions in the injector but was reduced slightly in analyses of extracts *cf* pure sample and appeared to decrease slightly over the course of a few hours during repeated analyses (S. Garland pers. comm).

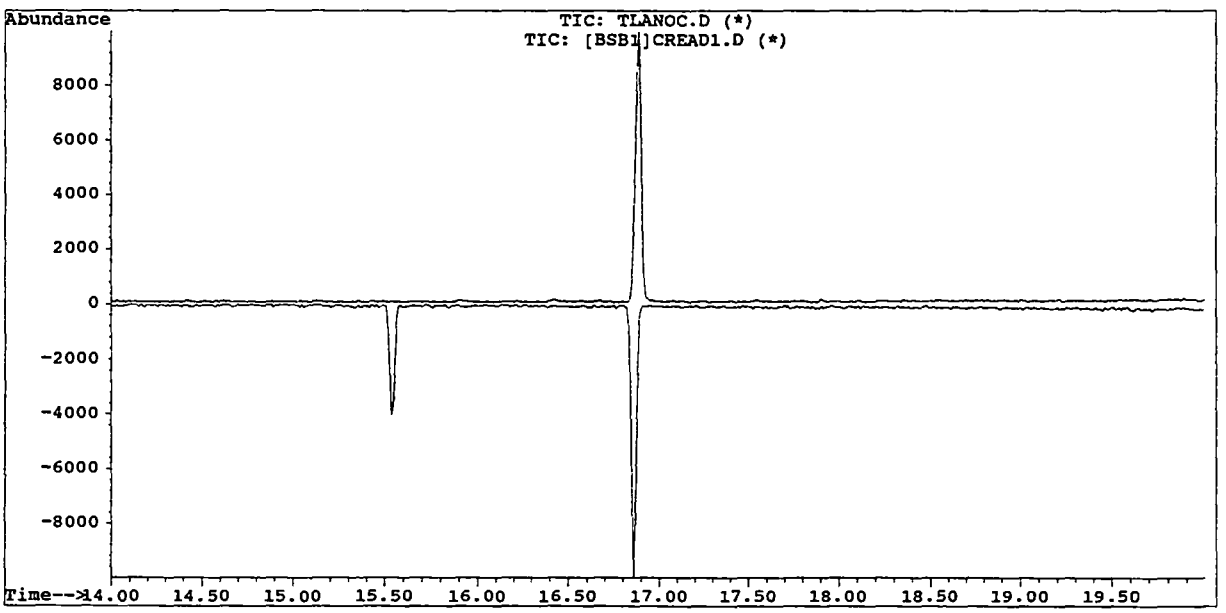


Figure 4.8.1: Pure polygodial sample analysed by GC TIC by direct (on-column) injection - top, and automatic injection- bottom, showing the presence of the dominant injection artefact.

4.10 Summary and Conclusions

The foregoing series of experiments indicates a straightforward extraction and analysis protocol which satisfies the requirements for commercial use and for screening of the *Tasmannia lanceolata* population for extract yield and composition .

The use of dry leaf material which is easily separated from the twigs upon which it is harvested and dried in the current semicommercial context and the application of a modified commercial hammermill and pelleting plant provides a good approximation of the pelletised, finely divided material used here which extracted quickly and thoroughly and with minimum loss of solvent in the residue.

Drying at low temperatures and for approximately 48hrs gave a consistent result, while the commonly used solvent mixture 'petroleum ether' (bp.40-60°) resulted in a clean product containing a wide range of terpenoid compounds characteristic of the leaf material in each case.

The extract was shown to be stable under normal cool storage conditions, over periods up to twelve months.

The confirmation of identities of a limited suite of constituents in the extract included those represented most commonly in proportions of greater than 0.02% of the GC volatile fraction, and provided an adequate set of reference peaks for the physiological investigations carried out in this project. Identification of the full range of compounds present was beyond the scope of the project and is the subject of further investigation.

The fast and reproducible solution sampling method described requires less than 200mg of leaf material and small quantities of solvent and carbon standard.

CHAPTER 5: OIL CELLS IN *TASMANNIA LANCEOLATA*: - CONTENTS AND STRUCTURE

5.0 Introduction

While the presence of oil cells in leaf tissue of Winteraceous species is well recognised (West 1969) there has been no reported effort to determine the exact composition of the contents of the storage body within these cells.
As shown in Section 2.4, the composition of distilled oils or extracts need not represent that of the contents of such structures and detailed interpretation of yield and composition data is improved by specific information regarding partitioning of oil components in the tissue system.
Leaf extracts of *Tasmannia lanceolata* contain a high proportion of polygodial, a compound found in very few other species, and which exhibits unusual, (perhaps phytotoxic - Furuta *et al.* (1986)) bioactivity, and which might require sequestration within isolating structures in the tissues in which it is found. The levels of the compound are observed to change rapidly within developing leaf tissue (Section 6.0) and tend to remain stable after leaf expansion is complete, suggesting very active sites of synthesis, and a durable storage system.
The purpose of this study was firstly to determine if possible, the composition of the contents of the oil cells in leaf material of *Tasmannia lanceolata* and secondly to examine some aspects of oil cell structure which have not been previously reported for the species.

5.1 Contents of Oil Cells

Oil cells in leaf of *Tasmannia lanceolata* are large, distributed throughout the mesophyll of the leaf, and in fresh tissue are invariably quite spherical and turgid. This study aims to determine the composition of oil in mature cells, using direct sampling and spectrophotometric methods.

Experimental Methods

a) Direct sampling of oil from cells *in vitro.*
Microsyringes with tip diameters of 20-25 μm were prepared and connected to a vacuum tweezer unit. Oblique slices of fresh leaf (W3) were placed on the stage of a dissecting microscope and allowed to dry slightly, revealing the glistening, spherical oil cells amongst the shrivelling mesophyll cells. By pressing the tip of the syringe against these cells and maintaining a steady low pressure within the tube, the cells could be punctured to release their contents which were immediately drawn into the syringe. The contents of approximately 20 cells could be collected in a few minutes whereupon the syringe was dipped briefly into HPLC grade hexane to allow a small quantity of solvent

to mix with the oil in the syringe, and the solution discharged into a glass GC vial partly filled with hexane. This process was repeated eight or ten times for each sample.
Analysis was conducted on an HP 5890 gas chromatograph (25m x0.32mm i.d. HP1 column, phase thickness 0.52nm, helium carrier at 15 psi head pressure, injector 250°C, detector 290°C, programmed from 60°C at 10° min^{-1} to 290°C, 1µl splitless injection), coupled to an HP 5970B Mass Selective Detector operating from m/z: 40-500, and performing 1.4 scans sec^{-1}. The sample was also injected manually directly onto the column, under the same conditions, for comparison.

b) Fourier Transform Infrared Spectroscopy
Pieces of fresh leaf were stripped of epidermis and allowed to dry slowly at room temperature between glass slides, reducing the water content while keeping the sample flat.
Infrared absorption measurements were carried out using a Bruker IFS 66 FTIR spectroscope coupled to a microscope fitted with a mercury/cadmium/telluride IR detector. The dried sample was placed on a ZnSe plate and positioned under the microscope so that the still-turgid oil gland occupied the field.
A sample of pure polygodial was dissolved in hexane and spread over the ZnSe plate in order to obtain a spectrum for comparison.
Data collection was conducted at a resolution of 4 wave numbers, processing by Opus software and each sample obtained by averaging 32 scans.

Results

a) Direct Sampling of oil cells

Samples of oil were analysed (typical gas chromatogram and mass spectra presented in Fig. 5.1a,b) together with the chromatogram obtained for a sample of pure polygodial, (presented elsewhere Section 4.9) and the resulting area percentages summarised below to provide an indication of relative proportions of the compounds detected.
The injection of the oil cell sample was repeated by direct, on-column injection to identify possible injection artefacts and the spectrum for this is presented 'back to back' with that for the normal injection mode (Fig. 5.1a -lower trace) and shows that several of the peaks represent only artefacts of the injection process.
Peak identity for polygodial (peaks 'e' and 'f') was confirmed by comparison of mass spectra (Fig 5.1b) with those of the the pure sample and by retention time, the former representing an injection artefact as demonstrated by the fact that this peak and peaks 'a' and 'c' do not occur in the lower chromatograph generated by direct injection of the same sample. The identity of guaiol (Peak 'b') was confirmed by comparison with library mass spectra, but no attempt was made to identify the artefacts 'a' and 'c'.
The small peak at 'd' which was not reduced by use of on-column injection technique was reported to be an impurity characteristic of the column at the time of this experiment (Noel Davies pers. comm.)

Peak	Common name	Retention Time*	Approximate relative percentages Sample 1	Sample 2
a*		11.99	1.6	2.6
b	guaiol	12.63	7.4	9.0
c		12.78	4.9	4.2
d	column artefact	13.60	1.9	1.3
e*	polygodial*	15.42	24.6	37.2
f	polygodial	16.87	59.7	45.7
		Total for e & f	**84.3**	**82.9**

* Injection artefacts- see above

Table 5.1: Area percentages (TIC) for components in oil obtained from oil cell in vivo.

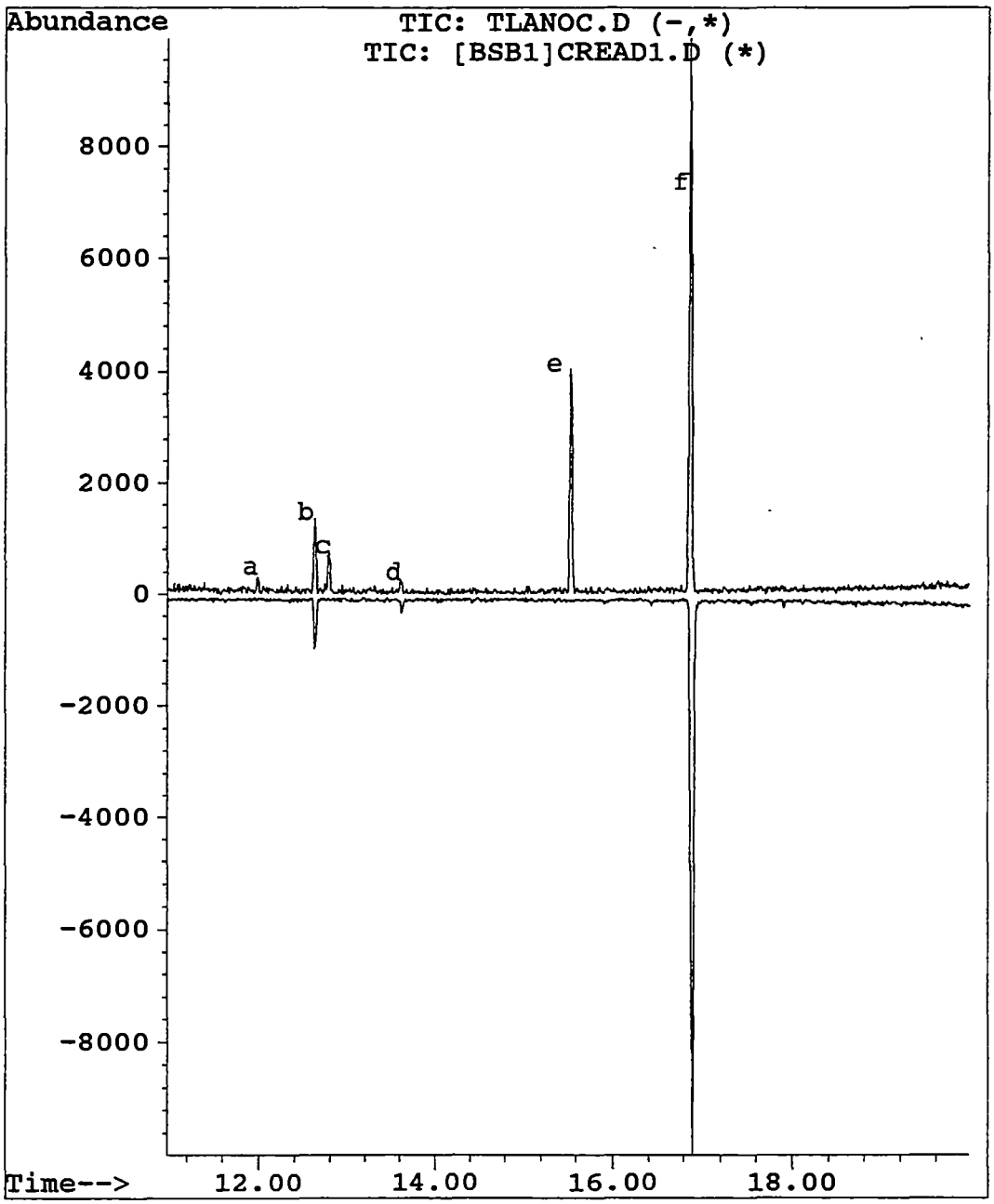


Figure 5.1a: Chromatograms of content of oil cells. Automatic injection-upper trace, direct injection - lower trace. See Table 5.1 for peak identities.

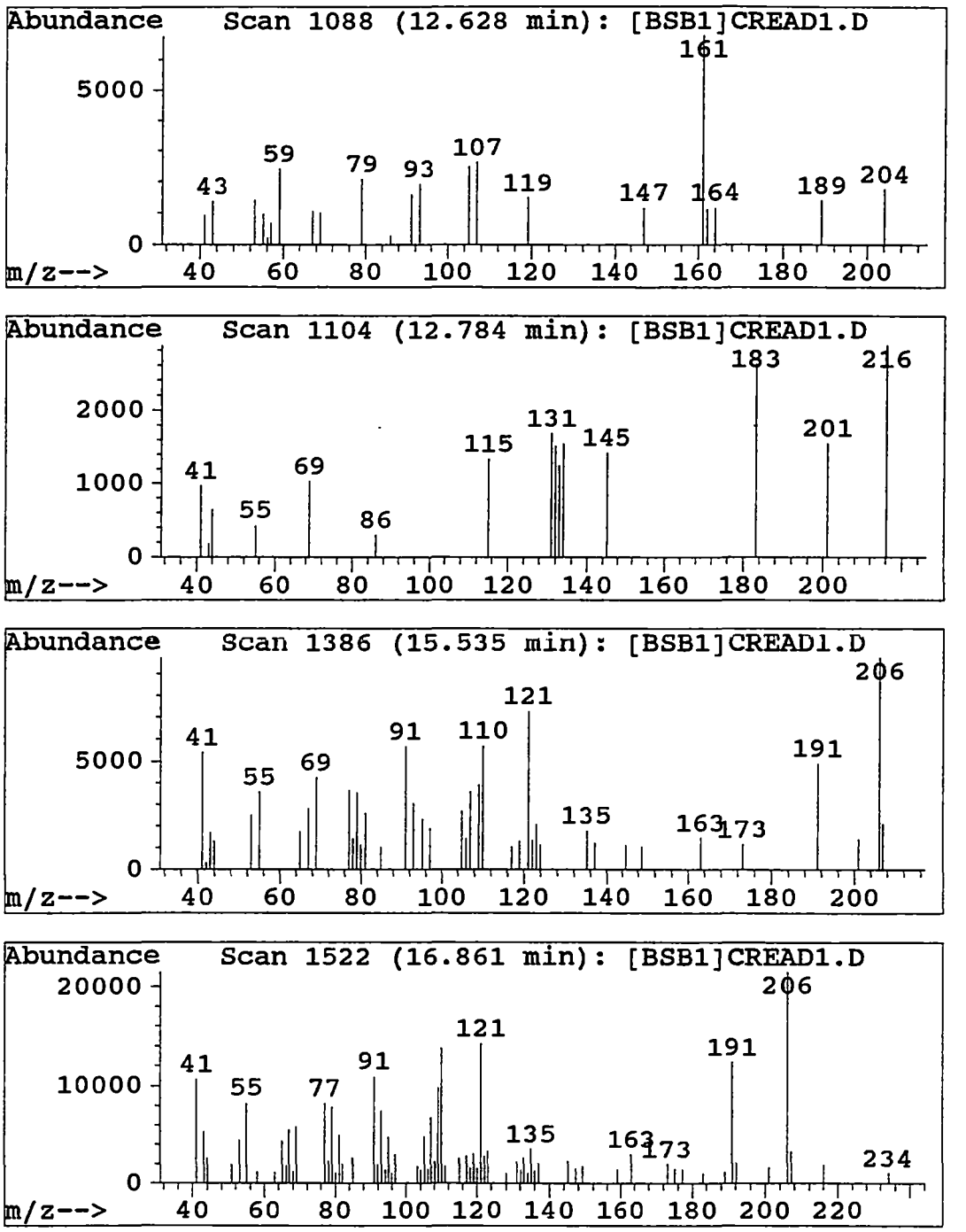


Figure 5.1b: Mass spectral data for the larger peaks from Fig. 5.1a - oil cell contents. From top: peak b (guaiol), peak c (injection artefact), peak e (polygodial isomer injection artefact), peaf f (polygodial).

b) FTIR Spectroscopy

A spectrum recognised as that for cellulose (G. Rowbottom, pers. comm.) was obtained from a part of the leaf tissue devoid of oil cells, and subtracted from that of the whole oil cell to produce a 'corrected' spectrum for the oil cell. These spectra, together with that for pure polygodial are shown in Fig 5.2.

As may be seen, there was good correspondence between the two spectra for the oil cell and pure compound, particularly in the two major areas of absorption. The distinct peak at about wavenumber 1690 was the major departure from this agreement and was not explained by concealment beneath either of the adjacent peaks.

The FTIR technique as used here can only be considered to provide support for the more sensitive determination of oil composition by GCMS. The absence of major differences in the 'fingerprint' region of the spectrum supports the contention that the predominant compound present *is* polygodial, and that the enantiomeric arrangement *in vivo* is similar to that in the pure sample.

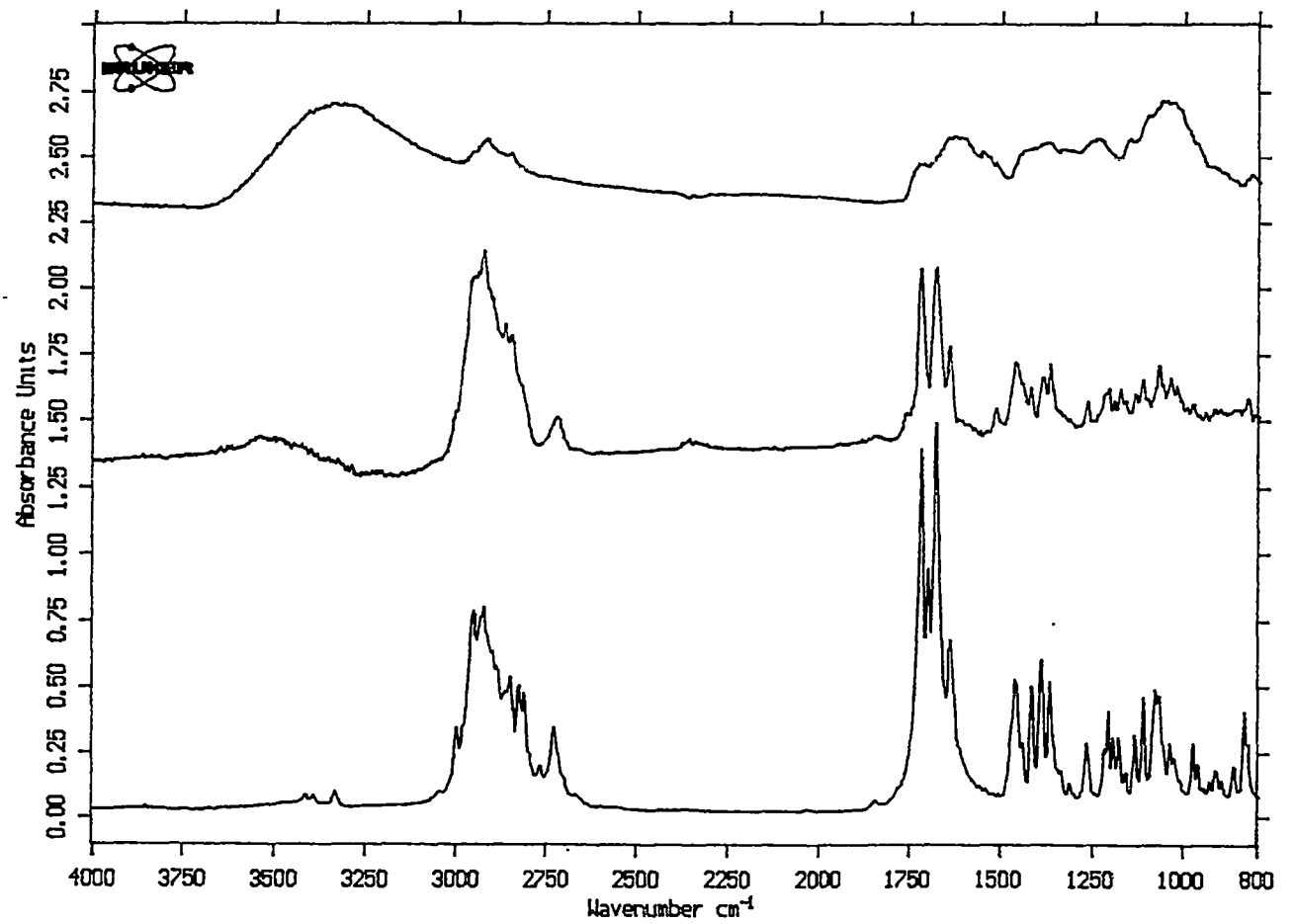


Figure 5.2: FTIR Spectra of contents of *Tasmannia lanceolata* leaf oil cell (adjusted for cellulose) and reference polygodial sample. Typical cellulose spectrum at top.

Discussion

Direct sampling of oil cells has not been reported to my knowledge. The same simple technique has been applied to the larger secretory cavities and more accesible trichome structures in an effort to distinguish between the secretory product and the essential oil or extract obtained from the whole organ (Turner *et al.* 1978, Lanyon *et al.* 1981, Venkatchelam *et al.* 1984, Bicchi *et al.* 1985, Menary *et al.* 1986).

Hagendoorn *et al.* (1994) examined the distribution of polygodial in various plant organs of *Polygonum hydropiper* and showed FTIR spectra obtained from oil gland and non-oil gland regions of the polygodial rich 'tepals' (*sic.*) and pure polygodial as evidence that the compound predominated in these structures, and claimed 'remarkable resemblance' between the spectra for oil cells and pure compound, despite a number of apparent differences in detail and the evident presence of water indicated in the spectra obtained from the oil cell.

By comparison the spectra presented here do not show the absorbances in the region 3000-3100cm^{-1} associated with water, suggesting that drying of the tissue was adequate and helpful. The absence of any significant additional peaks in the oil cell spectrum which might be attributable to other compounds, (for example the guaiol found in small amounts by direct sampling) confirms only that polygodial is the *predominant* constituent of mature oil cells in the leaf, since the FTIR spectroscopic method is much less sensitive to the presence of minor compounds than GCMS used on the sampled contents of oil cells, and alone, cannot be considered proof of the absence of compounds other than polygodial.

In the case of *Tasmannia lanceolata* the compounds detected in the oil cell itself represent only a small part by weight of the normal leaf extract. Polygodial normally represents 25 - 35% by weight of the GC volatile analysis of leaf extract, while guaiol, not identified in the analyses presented elsewhere in this thesis, is present at levels normally well below 2-3% (data not presented). Stevens (1955), in his examination of steam distilled oil of the species, reported very high proportions of guaiol in the volatile oil obtained from leaf gathered at certain sites.

Dilution of the contents of the oil cell with other solvent soluble compounds derived from non-oil cell origins is occurring to a significant degree during extract preparation, while in obtaining the essential oil from fresh leaf material, in which polygodial is not detected, different compounds may predominate.

These selective recovery or dilution effects must be considered in any comparison of plant material, and underline the importance of using an approriate method to express component yields. Unless qualitative description of the 'total extract/ oil' is required (such as in the commercial situation) characteristic compounds such as cannabinoids in *Cannabis sativa*, lupulins in hops,or in this case polygodial, should be expressed not as percentages in the 'oil' or 'extract' but in relation to the organ or plant material from which they are derived, eg as % of dry weight of leaf, or weight per gland.

5.2 Oil Cell Ultrastructure in *Tasmannia lanceolata*

West's (1969) study of nine families broadly described as 'Ranalean' included *Winteraceae* but relied on a very small subset of the genera and species within this family, using only one example of each of two species -*Drimys winteri* and *Pseudowintera axillaris.* There have been no other reports of examination of oil cells in Winteraceous species despite the interest in several unusual secondary compounds obtained in relatively large quantity from them. Lauraceae oil cells, on the other hand, have been examined in detail in most of the contributions to the subject of oil cell structure and function, particularly those in the avocado (*Persea*) fruit (eg Platt- Aloia *et al.* 1983) and *Cinnamomum sp* (Bakker and Gerritsen 1989).
The second part of this study aims to confirm aspects of oil cell ultrastructure reported for other 'Ranalean' species, for *Tasmannia lanceolata*, to visualise the gross structure and distribution of the cells in leaf tissue and to relate these to stages of leaf ontogeny.

Experimental Methods

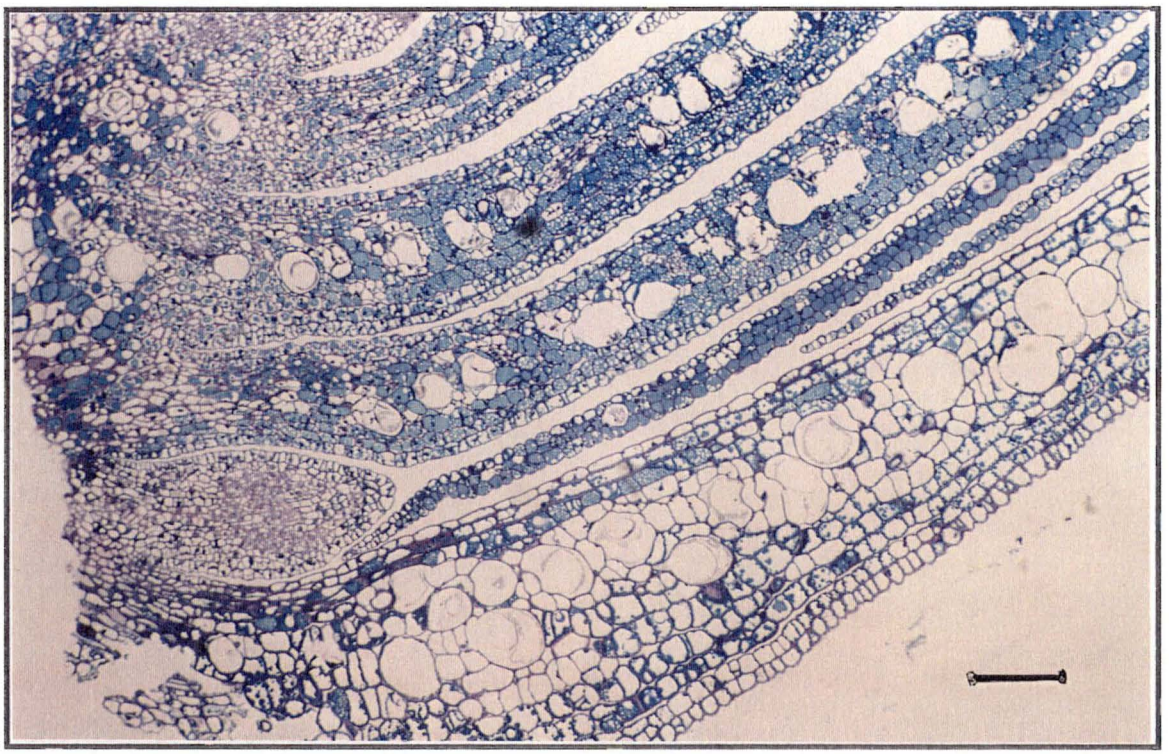
i) Scanning Electron Microscope (SEM): Mature leaf material of several different selections of *Tasmannia lanceolata* was prepared for examination by the method described in Section 3.4 (fixed in OsO_4 and freeze dried).
ii) Transmission Electron Microscope (TEM): Similarly, buds and very young leaf (<3mm long) collected from plants held in the shade house were fixed, dehydrated, embedded and sectioned for TEM examination (see Sect. 3.4). At the time of this experiment the buds were more than two months into the dormant phase, and exhibited advanced development of new leaf primordia within the bud. The bud was stripped back in each case to approximately 1.5 mm diameter, by removal of all but the last 7-8 leaf primordia.

Results

The dormant apical bud typical of the late winter condition is shown in Plate 5.1 and again, in longitudinal section in Plate 5.2, in which well developed oil cells occupy a substantial part of the leaf mesophyll, and are surrounded by undeveloped mesophyll cells (lower right). The techniques commonly used in preparation and fixing of fresh tissue tend to result in stripping of lipid soluble compounds from large storage bodies and these oil cells are clearly empty, while smaller, less well developed, (though still empty) oil cells may be discerned in the younger primordia closer to the apex (top left). Oil cells are already quite spherical and distinct from the rest of the mesophyll in even the very young leaf primordia nearest the apex, although the difference in size is less pronounced. Most cells in the mesophyll rapidly increase in size, roughly doubling in diameter from the first to the fourth primordia in this case, but throughout, the oil cells remain noticeably larger than their non-oil cell counterparts.

Plate 5.1 (above): View of typical dormant bud prior to the onset of spring growth; bar = 5mm.

Plate 5.2 (below):Longitudinal section of dormant bud, shoot apex to the upper left of the frame, showing the basal portion of four leaf primordia containing oil cells; bar = 100μm



The two SEM micrographs in Fig. 5.3 typify the appearance of the leaf mesophyll observed in several samples from each of six plants. The areoles, (the area delineated by schlerenchymatous transport tissue), (Fig. 5.3a) each contain very many large (*ca* 50μm dia.), nearly spherical cells embedded in the matrix of the mesophyll. Fig 5.3b shows one such cell surrounded by a 'supporting' system of radiating mesophyll cells, smaller, more elongated and still containing intact chloroplasts adhering to their walls. The continuity of the open intercellular spaces within the mesophyll and the virtual complete exposure of the surface of the mesophyll cells to this space may be seen. On the other hand, much of the surface of the oil cell itself is covered by the adjoining mesophyll. There are no chloroplasts visible in the interior of the oil cell.

Figure 5.4 shows a portion of a leaf primordium very close the the bud apex, most cells showing many signs of normal metabolic activity (intact organelles, abundant endoplasmic reticulum), while the distinctive cell on the left of the field contains one large space, surrounded by a dense cytoplasm, containing several plastids. Cells having this appearance were scattered through the tissue, usually with obviously empty central spaces, occasionally, as in this case, with some homogeneous, electron translucent matrix in the central space. Although these cells were not distinctly larger than other developing mesophyll cells, their frequency, and the compression of the cytoplasm leads to the conclusion that they represent an early stage of oil cell development.

Detail of oil cell cytoplasm is shown in Fig 5.5 in which dictyosomes and plastids are still intact, and in which very many endocytotic events are occurring in the membrane surrounding the oil body (arrows). This oil cell was found in a leaf primordium 4-5 nodes from the bud apex*, and was typical of the greatly enlarged oil cells in primordia at this stage, in which cytoplasmic integrity accompanied the engorged oil space and movement of oil into this space continued.

Fig. 5.6 shows an oil cell in a leaf primordium approximately 8 primordia from the apex in which the traces of cytoplasm which remain appear disorganised and fragmented. The fragmentation may be a result of the sectioning process, but in oil cells in leaf primordia at this stage, no distinct organelles could be distinguished, the cytoplasm appearing as a uniform, (or sometimes alveolar) and granulated dark mass usually adhering to the inside of the cell wall, and lacking any distinct membrane adjacent to the oil body. On the other hand, in oil cells of this maturity, the presence of the typical wall structure was evident, in this example as a lamellate region adjacent and interior to the middle lamella (arrow), shown in more detail in Plate 5.7.

Dark, somewhat diffuse bodies present in adjacent cells resemble, in appearance and location, those described by Bakker and Gerritsen (1990) as possible oil globules.

*The exact number of nodes between the apex and a sectioned leaf primordium could not be determined in a two dimensional specimen - the implication of 'age' of the leaf must be considered as relative only.

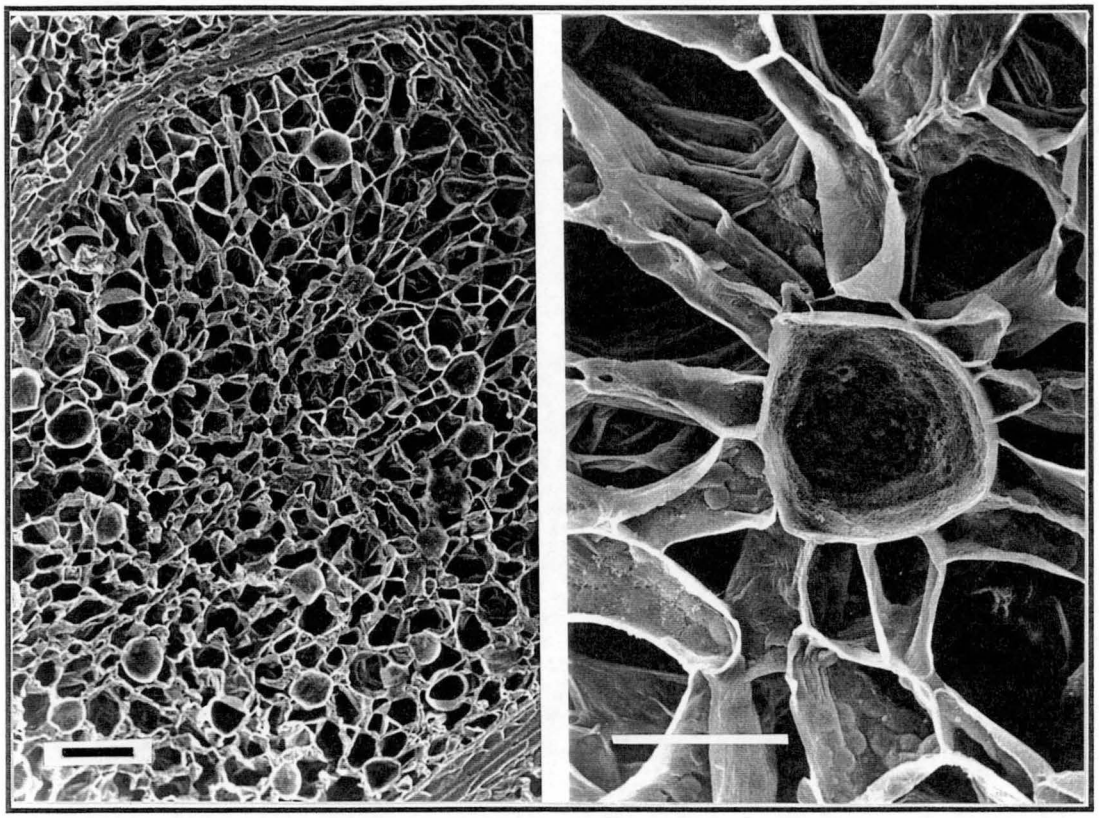
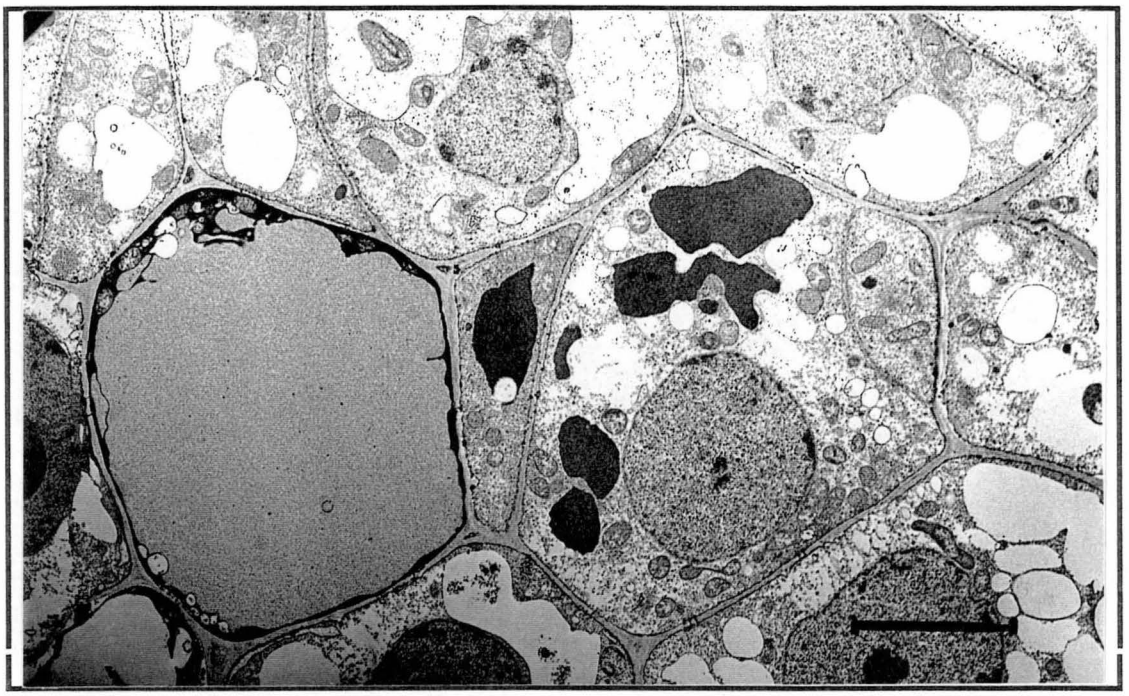


Plate 5.3 (above): (a) SEM micrograph of portion of leaf sectioned to reveal oil cells embedded in the mesophyll; bar= 100μm (b) Single oil cell within an array of unspecialised mesophyll cells. Note absence of chloroplasts in, and relative size of oil cell; bar = 45μm.

Plate 5.4 (below): TEM micrograph of leaf primordia close to bud apex. Note large, homogeneous body within the cell on the left side of the field; bar = 5μm.



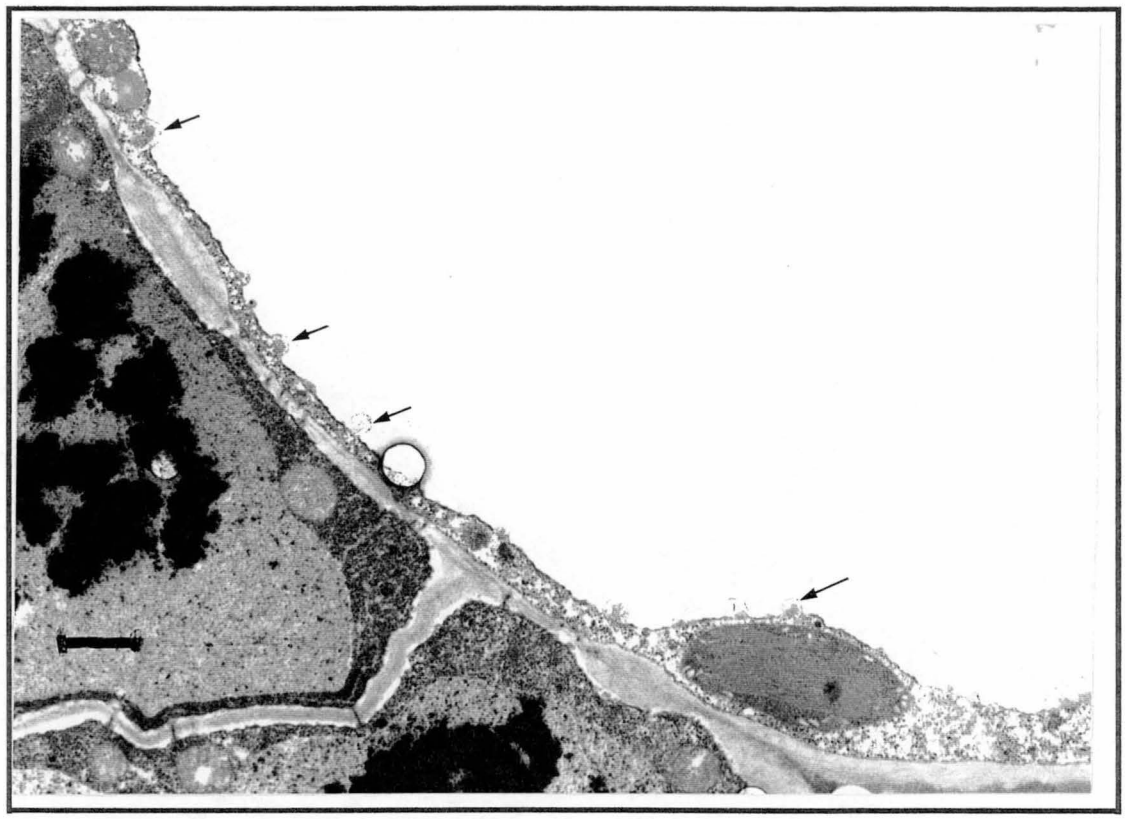
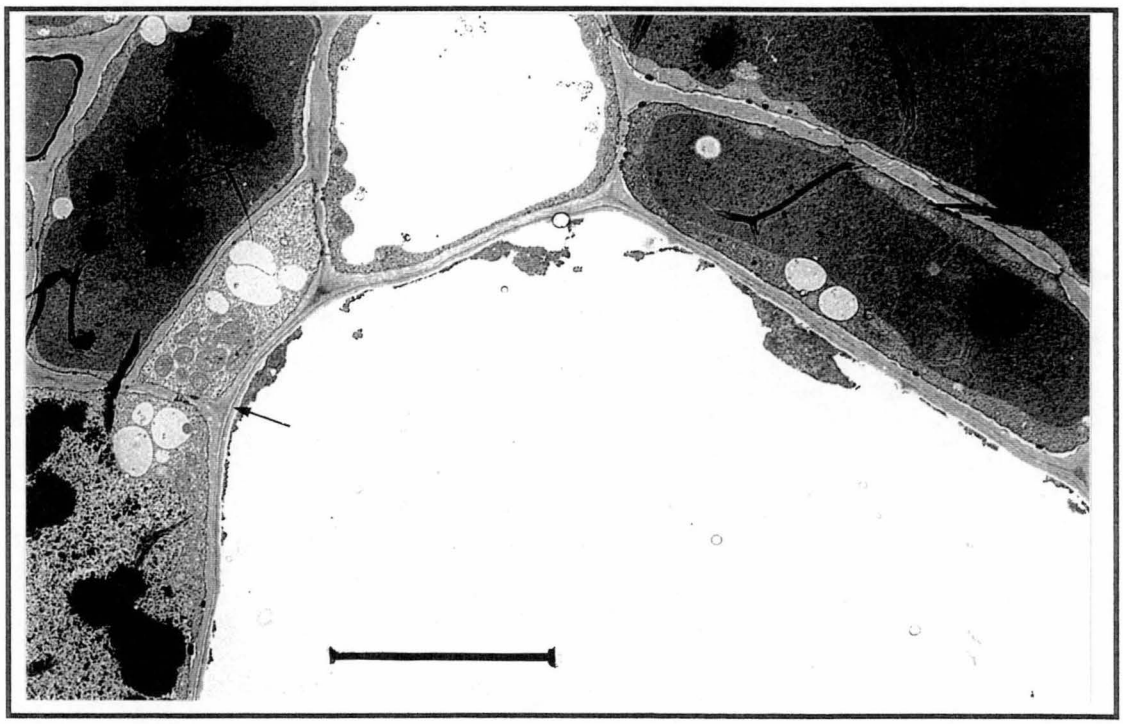


Plate 5.5: Oil cell showing cytoplasm, intact plasmalemma and organelles appressed to wall. Ribosomal activity, several apparent cytotic events, localised thickening of cell wall may be seen; bar = 1μm.

Plate 5.6: Mature oil cell, 7-8 primordia below the apex. Note aditional wall layer, not present in non-oil cell walls; bar = 1μm.



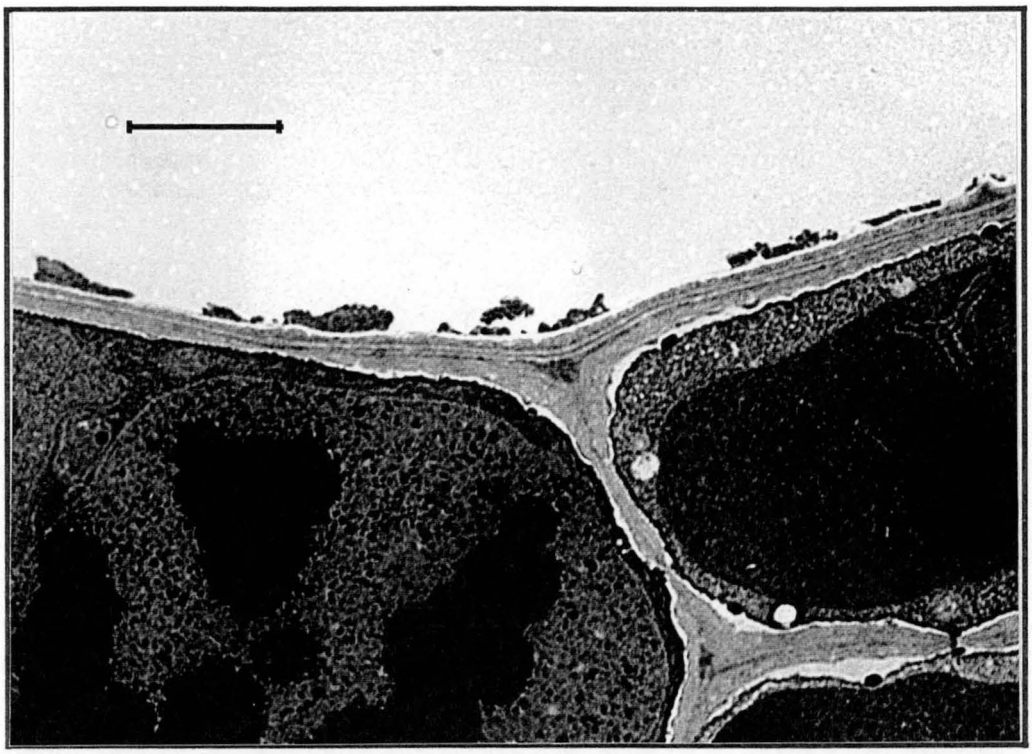


Plate 5.7: Detail of mature oil cell wall, showing additional layers associated with the oil cell, *cf* junction between other mesophyll cells. Bar = 2μm

Discussion

The observations recorded here appear to agree with the generalised models for oil cell development in Magnoliaceous species reported by Platt Aloia *et al.* (1983), Mariani *et al.* (1989) and Bakker and Gerritsen (1990):

The lamellate wall structure of the mature oil cell was seen, although the composition of the parts of the wall (suberin, polysaccharide or cellulose) was not confirmed, and three distinct layers could not be discerned in all mature cells. The specialised wall structure was not observed in oil cells until cytoplasm degeneration was well advanced, although Bakker and Gerritsen (1990) report that in *Annona muricata* the complex wall develops quite early in oil cell development while cytoplasmic organisation is still complete. Bakker and Gerritsen (1990) also found that in most sections it was possible to find a variety of stages of oil cell development, until the later primordia in which most cells were fully 'mature', but avoided correlating oil cell development stage with leaf ontogeny, as did Mariani *et al.* (1989), Maron and Fahn (1979) and Bakker and Baas (1993).

Oil cells could be distinguished in primordia 2-3 nodes below the apex, and thereafter development was very rapid, largely complete to the stage of cytoplasmic breakdown only a few primordia further from the apex.

No cupule was detected in any of the sections examined, although, as observed by Mariani *et al.* (1989), observation of this feature in thin TEM sections is dependent on locating the appropriate section, and some writers have either failed to detect (Platt-Aloia *et al.* 1983) or questioned the authenticity of the feature (Postek and Tucker 1983). Convincing evidence for the structure has been presented by Maron and Fahn (1979) and Bakker and Gerritsen (1990), and it must be concluded here that the small number of samples examined simply failed to include an appropriate section.

Lysigenous development of the oil space, reported for representatives of Magnoliaceae (West 1969) has not been supported since, for any members of the species studied, and was not observed here.

Fahn (1988) observes that different cells and cell compartments appear to contain osmiophilic droplets, and suggests that different oil components may be synthesised in different compartments. In the case of the results of this investigation, several observations might be resolved by this proposal. The presence of dark, electron dense material in a number of the normal mesophyll cells - see Plate 5.5, 5.6 , together with the apparently unassociated movement of droplets from the oil cell cytoplasm into the cavity suggests that at least two oil 'systems' may be present. This would be supported by the results of Sect. 5.1 in which only part of the solvent soluble mixture obtained from dry leaf was found in the oil cell structures themselves.

An interesting variation on this is inherent in the conclusion of Kobiler *et al.* (1993) that biologically active compounds may be 'at large' and active in certain tissues but not in others- their example was the antifungal compounds found sequestered in impervious

structures in avocado mesocarp but not in the pericarp where, they propose, protective antifungal action may occur. A similar example was the spatial separation of reactive compounds demonstrated by Menary *et al.* (1986) in hop secretory cells and secretory spaces.

Evidence that secretory structures such as trichomes, and secretory cavities contain specific portions of the essential oil or extract commonly associated with the whole organ or plant is common in the literature eg Turner *et al.* (1978), Russin *et al.* (1988, 1992), Gershenzon *et al.* (1992).

CHAPTER 6: SEASONAL CHANGES IN LEAF EXTRACT COMPOSITION

6.0 Introduction

Commercial exploitation of an essential oil or extract - producing species requires a detailed knowledge of the changes associated with development, maturation and senescence of the oil - containing organs in question. This information, together with estimates of dry matter production during the growing season enable determination of a harvest date which will result in the maximum yield of oil of an acceptable composition.

At a fundamental level this implies knowledge of the regulation and kinetics of the relevant synthetic processes, the physiology of storage and transport of the products of this synthesis and a parallel model for growth and development of the balance of the plant body.

In fact there are few examples of essential oil crop species for which a comprehensive model for all these dynamic processes is even partly complete.

Substantial elements of the model have been developed for members of the diverse and commercially important family *Lamiaceae.* For example, details of the monoterpene synthesising enzymes present in the spearmint trichome were discussed by Gershenzon *et al.* (1989), structure, distribution of, and ontogenetic trends in density for glandular trichomes in peppermint by Maffei *et al* (1989), and the relationship between yield and biomass in *Salvia spp.* by Pitarevic *et al.* (1984). Similarly in *Apiaceae* another significant essential oil bearing family, the ontogenetic changes in overall oil composition in parsley were examined by Porter (1989a), and Clark and Menary (1984) reported the changes in yield and composition of dill oil during crop development in New Zealand and Tasmania respectively.

Acquisition of such a model begins with an empirical determination of the general pattern of oil production in the organ, on the plant, and under characteristic environmental conditions. This reveals the normal pattern of accumulation (and perhaps loss) of oil components and enables correlation of this with the phenology of vegetative and reproductive development.

More broadly, it would require consideration of dry matter yield, (the annual vegetative increment for a perennial species), providing a general context for determination of commercial viability.

Examination of biochemical interrelationships amongst oil components and storage system structure and function, will enable the interpretation of the process of oil synthesis and accumulation. This interpretation is a necessary first step towards developing the production models mentioned at the beginning of this section.

6.1 Changes in extract composition during the annual growth flush

To undertake an unambiguous determination of ontogenetic changes in overall extract composition in leaf tissue on a perennial species, a number of aspects of seasonal change must be accommodated.

For shoots in which extension and leaf initiation occur together and over an extended period (for example where no pulsed growth occurs, or in species for which the growing season is very long), there are three temporal aspects of leaf development to be considered - the time of initiation of the leaf, the physiological age of the leaf and the prevailing seasonal conditions at the time of sampling.

ie. a) Regular collection of marked leaves (expanded at some point early in a growth cycle) does not enable identification of the impact of 'time of initiation' on changes in extract composition.

b) comprehensive sampling at a specific date, and sorting leaves by age ignores the implications of time of year (season) on oil composition.

c) collecting leaf samples of a particular maturity through the season, will not allow consideration of the effect of physiological age on oil composition.

The identification of sets of leaves initiated over a series of dates and sampled at a number of points in their development should enable prediction of oil composition typical of leaves of known initiation date sampled at any time during an annual growth cycle.

The three sample series in the scheme in Fig 6.1 (after Li1993) would provide the following information:

1) Composition of leaf initiated at time i_n - combined leaf age x time of sampling.
Difference between series 1, 1' 1" 1'" reflects importance of initiation date

2) Composition at leaf age A, B, C etc- combined time of sampling x time of initiation.
Difference between series 2, 2',2", 2'" reflects importance of leaf age.

3) Composition of leaf at sample date s_n - combined leaf age x time of initiation.
Difference between 3, 3', 3", 3'" reflects the importance of sample date

With respect to extract composition the latter two will probably be the more important - metabolic activity will reflect the prevailing conditions, and the cumulative result of production and storage of secondary metabolites will depend on the age of the leaf.

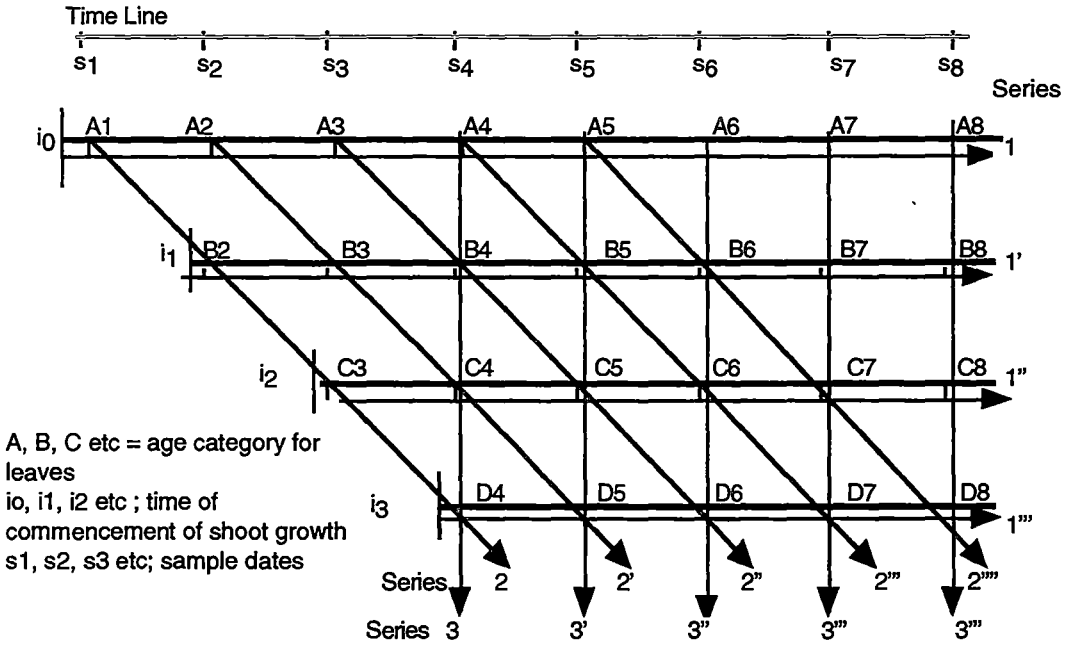


Figure 6.1: Schematic representation of a sampling matrix designed to allow comparisons of initiation date, season and physiological age, (after Li 1993)

The success of this scheme, particularly the 'series 2' comparisons - following a particular leaf cohort as it matures, (with the possibility of removing season and position effects), depends upon regular leaf emergence and fitting of sample dates to ensure that C5 is equivalent to B4 and A3 etc.

In *Tasmannia lanceolata* mature vegetative buds contain a full complement of leaf primordia which emerge over a short period following the opening of the bracts in spring (Gifford 1950). A preliminary investigation showed that detectable levels of several of the components of interest were present in very young leaves and that these levels changed rapidly as the leaves unfurl and expand. Although only a few day's development separate emergence of the first and last leaves on the new shoot, it was decided to adopt a simplified version of the above, to separate three leaf 'types' by position on the new shoot and to compare extract quality among them at each date, as well as comparing leaves at each position between dates. Leaf weight, percentage dry matter and a representative suite of mono- and sesquiterpenes including polygodial were to be monitored in the leaves as they emerged, matured and ultimately were overtopped by a succeeding generation in the subsequent year.

Plate 6.1: General view (above) from southeast corner of the Parrawe site at which the trees used for the seasonal variation trial were located, and (below) trees typical of those used in the trial ((approx. 3m high)

6.1.1 Experimental Procedures

a) Field experiment

The majority of the study was undertaken on an old field site at Parrawe, an abandoned township situated some 40km inland in Northwest Tasmania at an elevation of 600m and in a rainfall belt of 1750-2000 mm. Mean sunshine hours for Savage River, 30km to the west of the site and monthly rainfall and daily maxima and minima for the nearby recording station at Waratah are presented in the Appendix 1.2. The site, on tertiary basalt, was cleared of *Nothofagus cunninghammii* and *Atherospermum moschatum* rainforest between 1915 and 1930, sown with pasture species and grazed for some years before being abandoned some time during the 1950's. *Tasmannia lanceolata* has colonised the area (about 50ha) almost completely, forming clumps and isolated individuals between a few years and upwards of thirty years of age over the whole site, tending to be most successful in the vicinity of old logs and tree stumps (Read and Hill (1983) (see Plate 6.1).

Individuals for the experiment were solitary trees, of similar aspect, age/size, position in relation to shelter, drainage lines and frost hollows. Ten such trees were chosen, including five each of staminate and carpellate individuals on the basis of dissections of flower buds at the site. Trees were tagged with surveyors tape. A choice was first made in early October and revised in late November, as it became clear that the proportion of vegetative buds (less globose and smaller than the rapidly swelling flower buds) was too small on some trees to allow for sufficient sample material. Monitoring of bud development on three occasions during this time confirmed closely similar progress amongst the chosen plants. The scheme in Fig 6.2 shows the chronology and comparisons planned for this experiment.

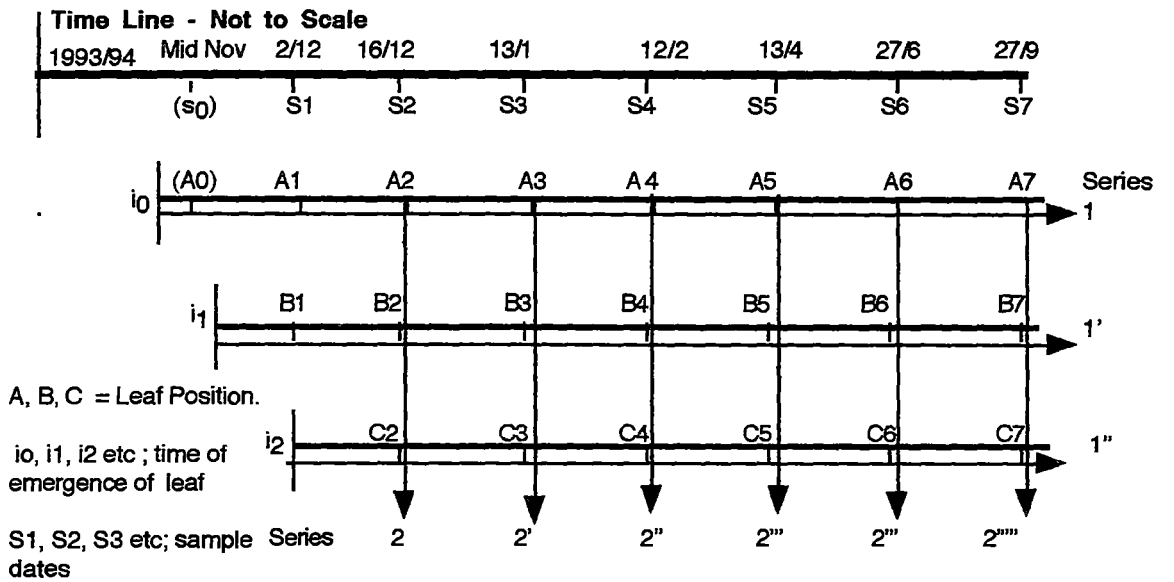


Figure 6. 2: Sampling matrix for field trial at Parrawe -see text for explanation. Budburst occurs in mid-November and shoot growth is complete by mid January. Sample dates (S_1-S_7) equate to 20, 40, 70, 100, 160, 240 and 320 days after budburst.

Comparisons of interest are those between A, B and C at each sample date - series 2 etc - comparing the effect of leaf position, and comparisons between A1, A2, A3 etc - the progress of maturation in leaves at each position.

At the first harvest date, a few days after budburst (DAB), when shoots had begun to extend, 10 'typical' shoots on each tree were tagged as markers to enable the selection of approximately equivalent shoots at each subsequent harvest. At each harvest, shoots were selected on the basis of their similarity in length, leaf number and maturity to the tagged shoots. In this way a degree of 'equivalence' between leaves of consecutive harvests was obtained.

For ten shoots gathered from each tree (twenty each for harvests at 20 and 40 DAB), leaves corresponding to positions 'A' -the oldest, 'B' -a leaf in approximately the mid position (numerically) on the shoot and 'C' -the fully formed leaf closest to the apical bud, were removed from the shoot, weighed and bulked together for each tree and each leaf position,. These samples were then dried at 35°C for 48 hrs. A sample of extract was prepared by grinding and extraction in petroleum ether containing C18 internal standard, and the extract analysed using the GC method ('Chris Mth' -see Sect. 3.2).

A sample of 10 'B- type' leaves was used at each harvest to establish a mean %DM of the 'dry' leaf material used in the analysis for all trees at that date. The number of leaves on the shoot was averaged for the ten shoots from each tree, and a mean fresh weight determined for the ten leaves of each type collected per tree.

A number of leaf samples providing a longitudinal comparison of leaves from each of three seasons was obtained as shown in the scheme below (Figure 6.2a). The leaf cohort entitled '1993 leaf' was that examined in the main part of this study, above, and for this group, the '93 Mature' result was obtained from the mean for the harvests 4-7, while the other results presented were of the means for ten trees, one sample each as indicated by the asterisk.

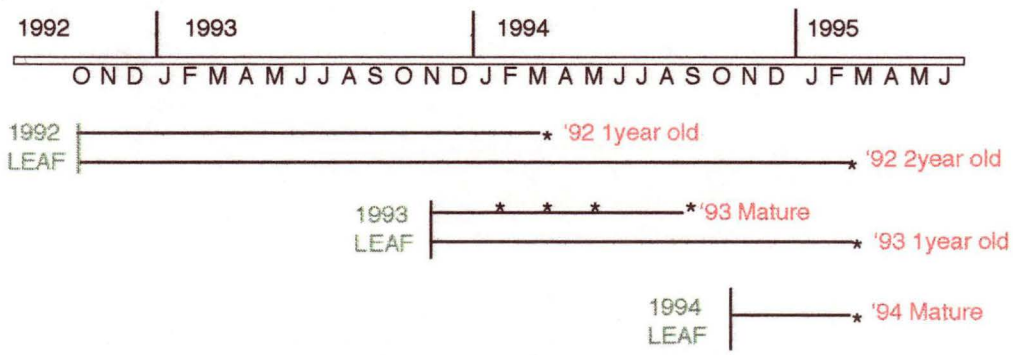


Figure 6.2a: Longitudinal comparison of leaf samples at the Parrawe site.

For the last two harvests before budburst the developing apical buds including bud scales were removed, weighed, dried and analysed as for the leaf samples.

Extract analysis was carried out for the following parameters

- the total for compounds eluting between the '1,8 cineole' peak at a relative retention time to the C18 standard of 0.34, and that of kaurene (relative retention time 1.27) inclusive, termed 'volatiles',
- compounds identified as linalool (relative retention time 0.37), cubebene (0.55), caryophyllene (0.61), germacrene D (0.66), bicyclogermacrene (0.67) cadina 1,4 - diene (0.71), aristolone (0.93) and polygodial (1.05 +1.19) which between them accounted for most of the volatile constituents identified in the analysis.

b) Changes prior to, and at budburst

The results obtained in the field trial described above did not provide useful detail of the changes occurring around the beginning of budburst and during the emergence of the first leaf. The first data point obtained in that experiment was approximately 20 days after the first sign of budburst, and it would have been impractical to collect samples during the initial period due to the remoteness of the site, and limited number of suitable growth points per tree. To focus on this very early period, a second experiment was conducted using clonal plant material (W3) held in the glasshouse. The first leaf of emerging shoots on as many plants as possible was sampled during the first 8-10 days of bud break, commencing with furled outer leaves inside the elongated, (but still closed) bud, continuing through the emergence of the furled bud from the bracts, as the bud unfolded, and as the shoot began to lengthen until the outer leaves were about 15 mm long. This point roughly corresponded to 'S3' in the field trial. Two further samples were collected to confirm the pattern discerned in plants in the field. Dissected leaves were weighed fresh and dried, and ground as described previously, before being sealed in a glass vial and stored at 2°C. At the completion of sampling, 3ml of solvent plus 0.306 mg C_{18} standard were added to each tube, the tubes shaken for two hours and a GC sample analysed. Sample sizes of approximately 20 -100mg (dry) were obtained, two samples at each date and one analysis conducted per sample. Six stages of development were arbritarily identified as follows:

0: elongated, unopened buds
1: shoot tip just protruding from sepals, less than half leaf visible above bracts
2: rosette not unfurled but at least half leaf visible above bracts
3: rosette unfurled prior to extension of shoot
4: shoot about 1.5 cm long, outer leaves approx. 15mm long (approx date 3)
5: shoot almost fully extended, lower and middle leaves about 25mm long
6: leaves fully expanded (40-50mm), shoot fully extended.

Extract parameters reported for this experiment include:

- the total for compounds eluting between the '1,8 cineole' peak at a retention time relative to that of the C18 standard of 0.34, and that of kaurene (relative retention time 1.27), termed 'volatiles',

• a compound tentatively identified (N. Davies, pers. comm.) as cis-ocimene (relative retention time 0.342), linalool (0.37), two unknowns (0.80 and 1.05), aristolone (0.93) and polygodial (1.05 +1.19) and kaurene (1.27), which between them accounted for most of the constituents identified in the analysis.

6.1.2 Results

a) Field trial

Statistical analysis

i) Comparison between trees - the SAS 'proc glm' procedure (SAS Institute -Software vers. 6.07) was applied to the 'percentage in leaf' data- Table 6.1, using only the results for harvests 4, 5, 6 & 7 to obtain an estimate of mean 'mature '93 leaf' levels of the nine parameters for each tree (Appendix 2.9).

ii) Comparisons between 'positions', - analysis for 'position' using the 'proc glm' procedure indicated no significant differences between the three positions for any of the parameters at the first three harvest dates (see Appendix 2.10). In subsequent harvests the three positions became still more similar and no further comparisons for 'position' were conducted. Thereafter the three positions were treated as replicates for each tree.

iii) Comparison between harvest dates - compositional data (nine parameters x ten trees x three samples (positions) x seven harvests) was expressed firstly as percentage of each component in dry leaf weight, and then transformed, using the data for percentage dry matter (mean for each harvest), mean number of leaves per shoot (for each tree) and average fresh weight of leaf on the shoot (mean of three leaf types for each tree) (Table 6.3) to estimate weight of each compound per shoot. The two datasets were then subjected to statistical analysis using the 'proc glm' procedure to generate Type III analyses of variance, means and standard errors for each compound by harvest date, tree number and date x tree (Appendices 2.11 & 2.12). Where a compound was undetected a zero value was used in the analysis, as an approximation assuming that the compound *was* present.

iv) Composition data for buds collected at harvests 6 and 7 are presented as means for ten trees (one sample each) for each harvest.

Data

Composition results (%w/w in leaf dry matter) for sample dates 1-7 are summarised in Table 6.1.and depicted in Figure 6.3. Standard errors reported refer to harvests 2-7 (40-320 DAB) - the initial harvest (2 positions *cf* 3) produced slightly larger standard errors in each case (not reported). For each harvest, fresh weight of leaves, leaf number and % DM data are presented in Table 6.2 and the change in dry matter of leaf per shoot at each harvest is shown as a blue curve on the top graph in Figure 6.3.

Table 6.1: Leaf extract composition - expressed as % of leaf dry weight LS means for ten trees, three samples per tree.

Component	Harvest Date (DAB)							
(% per leaf DM)	20	40	70	100	160	240	320	*Std err*
volatiles	1.65	2.16	3.97	4.71	5.31	5.41	4.62	*0.043*
linalool	0.032	0.06	0.08	0.09	0.14	0.14	0.14	*0.005*
cubebene	0.08	0.11	0.17	0.24	0.26	0.26	0.22	*0.003*
caryophyllene	0.01	0.03	0.07	0.09	0.11	0.11	0.09	*0.002*
germacrene D	0.03	0.06	0.10	0.14	0.15	0.15	0.11	*0.002*
bicyclogermacrene	0.04	0.10	0.09	0.09	0.04	0.05	0.06	*0.004*
cadina- 1,4 -diene	0.36	0.42	0.77	0.93	1.03	0.99	0.77	*0.018*
aristolone	0.13	0.11	0.10	0.06	0.06	0.06	0.05	*0.002*
polygodial	0.52	0.75	1.70	1.49	1.71	1.69	1.40	*0.018*

Table 6.2: Fresh weight per leaf (on each tree and at each harvest), % DM average for harvest and number of leaves on new shoots- average for ten shoots and seven harvests. Right hand columns give mean dry weight of leaf per twig (mg) at each harvest derived from the foregoing (tree by tree) and standard error for mean of these ten results.

Tree No		1	2	3	4	5	6	7	8	9	10	Mean	
Mean leaf no.	Mean	5.7	8.4	5.8	7.8	5.2	6.4	6.9	5.7	6.1	6.5	Dwt/	*Std*
	% DM	Mean fwt/leaf (mg)										twig	*Err*
20 DAB	32.0	17	18	21	14	14	14	10	16	15	22	33	*2.8*
40 DAB	35.3	40	36	38	34	28	22	18	14	26	33	66	*7.7*
70 DAB	36.8	108	134	132	92	93	86	90	73	110	123	249	*23.1*
100 DAB	38.8	152	181	162	155	176	146	144	146	190	178	408	*25.7*
160 DAB	43.1	164	214	131	140	158	195	183	161	194	214	492	*42.3*
240 DAB	42.0	163	189	189	180	156	172	124	151	227	194	474	*35.9*
320 DAB	42.0	177	197	182	156	179	159	163	175	224	227	498	*32.0*

As can be seen from Figure 6.3 a number of substantial changes occur in leaf extract composition, simultaneous with the rapid accumulation of dry matter early in the season. Total dry leaf weight per shoot doubled from 20 to 40 DAB and then increased fourfold by 70 DAB. This period of rapid growth was complete by 150DAB with the shoot bearing approximately 475mg of dry leaf. With respect to the concentration of components in the leaf:

• Most of the compounds monitored follow a roughly similar pattern of increasing percentage in the leaf. Germacrene D, linalool, caryophyllene, cubebene, cadina - 1,4 - diene and polygodial all accumulate rapidly during this initial growth period, reaching a plateau, usually around the time dry matter accumulation has peaked, and then fall slightly during late winter as the subsequent generation of buds begin to swell. Exceptions to this amongst these compounds were polygodial, which reaches its maximum concentration in the leaf by 70DAB, when leaf weight has reached slightly more than half its final figure, and linalool, in which the late winter decline was not observed.

• Aristolone and bicyclogermacrene followed a distinctly different trend during the growth cycle. Aristolone, present at its maximum concentration at 20 DAB declined rapidly to a stable level of less than half the original concentration by 150 DAB and remained steady for the remainder of the sampling period. Bicyclogermacrene, detected at low levels at the first sampling appeared to increase rapidly, then decline to a low at 150 DAB after which a steady increase appeared to occur for the rest of the season. With respect to this compound, the concentration detected in the same leaf cohort late in the following growing season approached that detected at the maximum (40 DAB). Close inspection of the 20DAB result for this compound revealed that the mean derived from a possible 20 loci (position x tree) in the statistical analysis, including only 8 detections in the extract samples, the mean of which was 0.08%, and this result must be regarded with some caution in light of the high 'one year old' result for the same leaves. The possibility that this compound follows roughly the same pattern as detected for aristolone - high at budbursts, then declining steadily, cannot be ruled out.

• The trend for 'volatiles' reflects the pattern described for the compounds above which account for a substantial proportion of the FID integration. Three additional peaks, two not identified in this work, the third a mixture of two compounds and including calamenene (N. Davies, pers. comm.) also contributed to this trend.

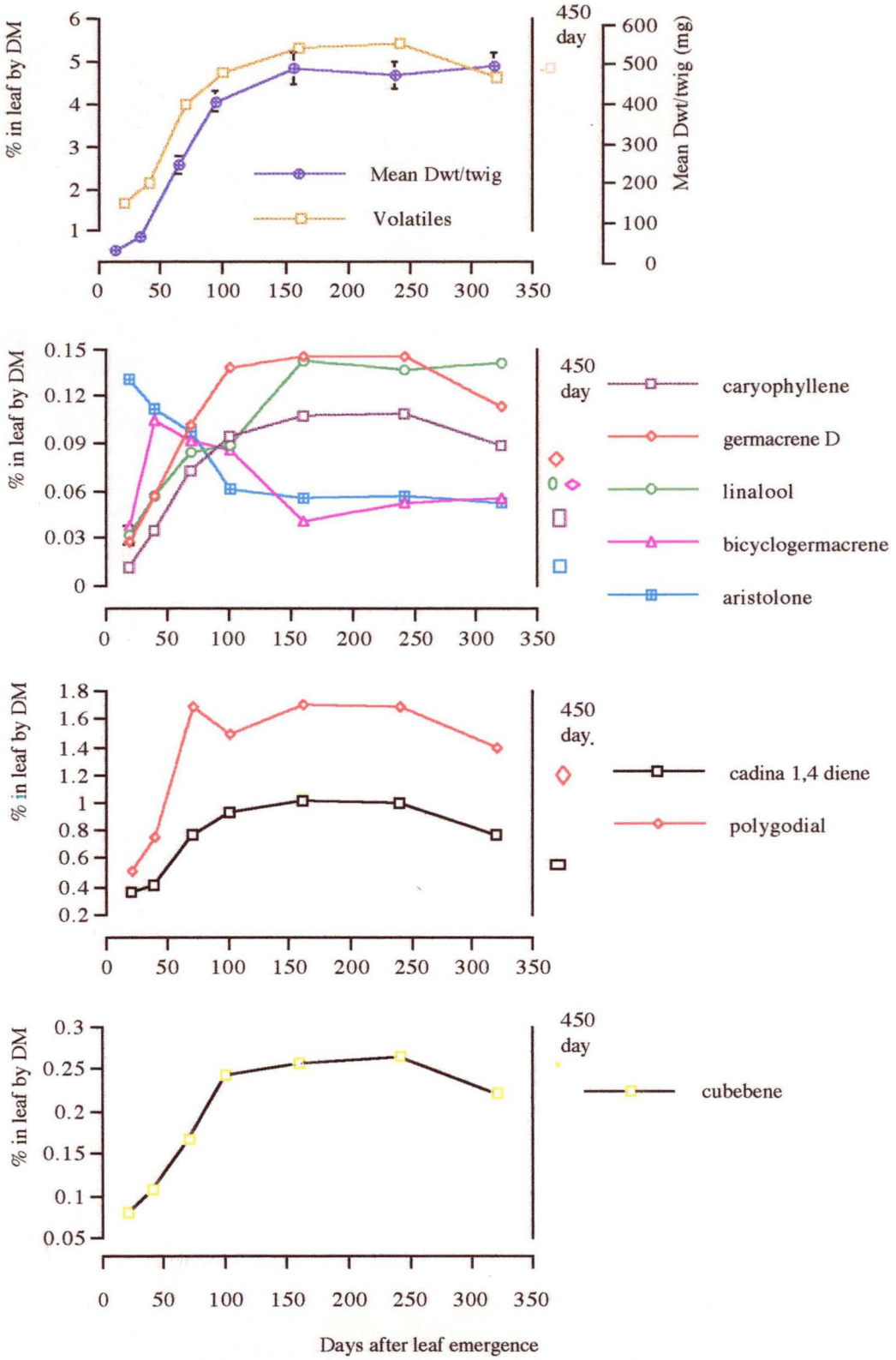


Figure 6.3: Change in % of extract constituents (by DM) in new grown leaf, during development over summer and during the following autumn and winter. Development of total leaf dry matter per shoot is shown in the blue curve on the top graph. The 450 day level ('93 leaf 1yo) is shown in the bar at the right

The estimation of 'per twig' yields of each component was obtained (as explained above) from the raw data for percentage composition and the dry matter per shoot data in Table 6.2. Means and standard errors are summarised in Table 6.3 and depicted in Figure 6.4 below.

Component	Harvest Date (DAB)							
(mg per shoot)	20	40	70	100	160	240	320	*StdErr*
volatiles	0.53	1.47	10.11	19.45	26.64	25.73	23.05	*0.18*
linalool	0.01	0.04	0.22	0.37	0.70	0.64	0.71	*0.02*
cubebene	0.03	0.07	0.45	1.02	1.30	1.28	1.11	*0.01*
caryophyllene	0.01	0.02	0.19	0.39	0.54	0.52	0.44	*0.04*
germacrene D	0.01	0.04	0.27	0.58	0.74	0.71	0.57	*0.01*
bicyclogermacrene	0.01	0.07	0.23	0.36	0.20	0.25	0.28	*0.02*
cadina 1,4 diene	0.12	0.29	2.05	3.91	5.22	4.86	3.86	*0.07*
aristolone	0.04	0.08	0.24	0.24	0.27	0.26	0.25	*0.08*
polygodial	0.17	0.50	4.15	5.99	8.39	7.79	6.94	*0.07*

Table 6.3: Leaf extract composition - estimated total of each component per shoot (LS means for ten trees, 3 samples per tree) derived from raw yield data and Table 6.2.

By transforming the percentage data (Table 6.1 above) into effective 'yield per shoot' it is possible to discern nett trends in accumulation and loss in the shoot as a whole, by volatilisation, metabolic conversion or translocation.

In this regard, for the most part the patterns reflect the trend in dry matter accumulation -the sigmoid curve in which accumulation accelerates at the beginning of the season before reaching a plateau. This was the case even for aristolone, for which the fall in concentration in the leaf was more or less balanced by an increase in total leaf weight. This resulted in an increase to 70 DAB, then maintenance of a very steady level in the shoot for the rest of the growth cycle. For polygodial, cadina 1,4 diene, cubebene, germacrene D and caryophyllene, this plateau was reached by 150 DAB and was followed immediately by a steady decline. In the case of linalool, the decline was not evident, and this compound, together with bicyclogermacrene appeared to still be increasing from the end of summer and through the autumn winter period.

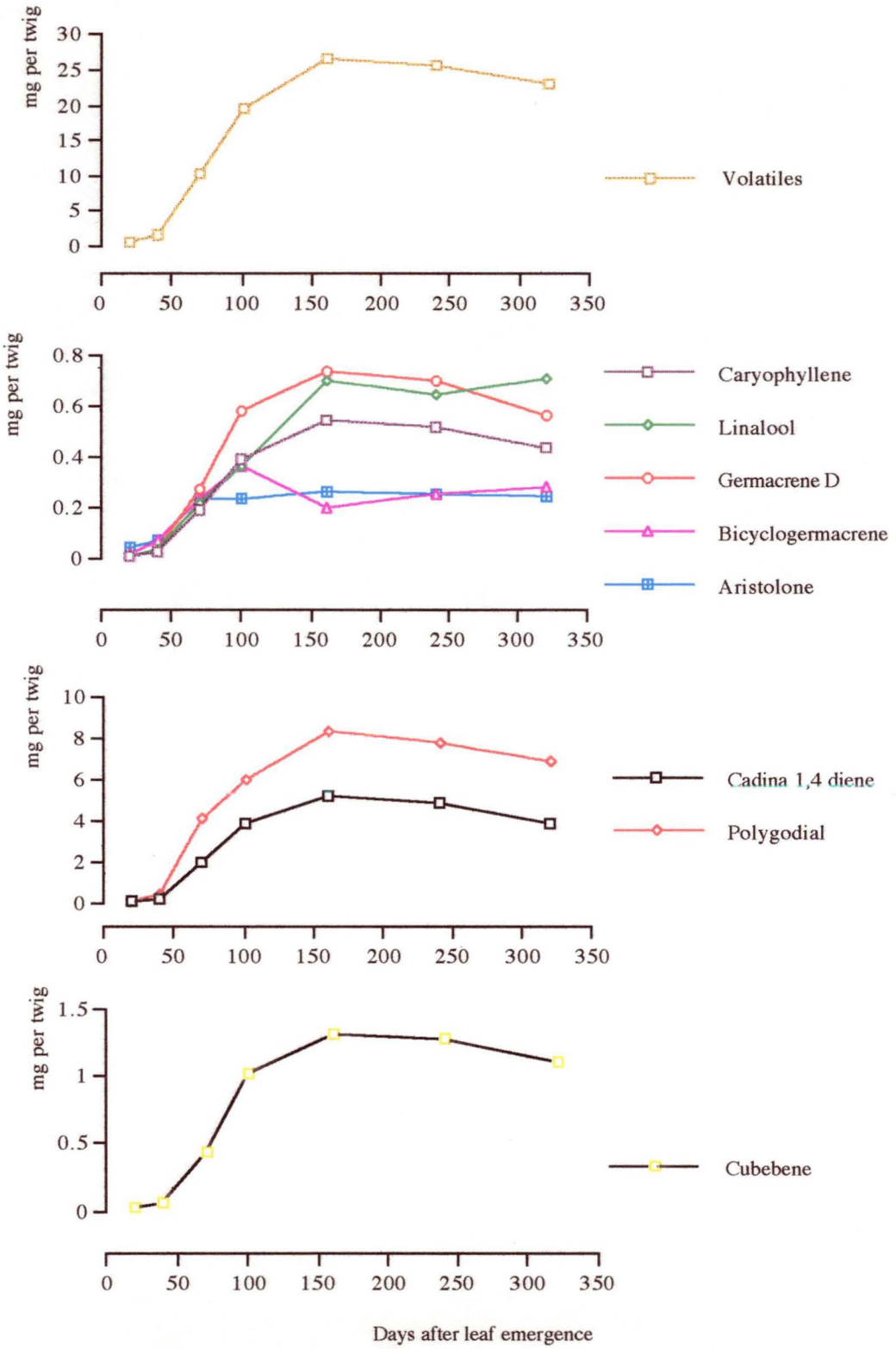


Fig 6.4: Accumulation of extract constituents (mg per shoot) in new grown leaf, during shoot development over summer and during the following autumn and winter.

Mean percentage in dry leaf of the four main parameters ('volatiles', polygodial, cadina-1,4 -diene and cubebene) in mature leaf (calculated from the last four harvests) is shown for each tree in Figure 6.5, demonstrating the between-tree variation at the site.

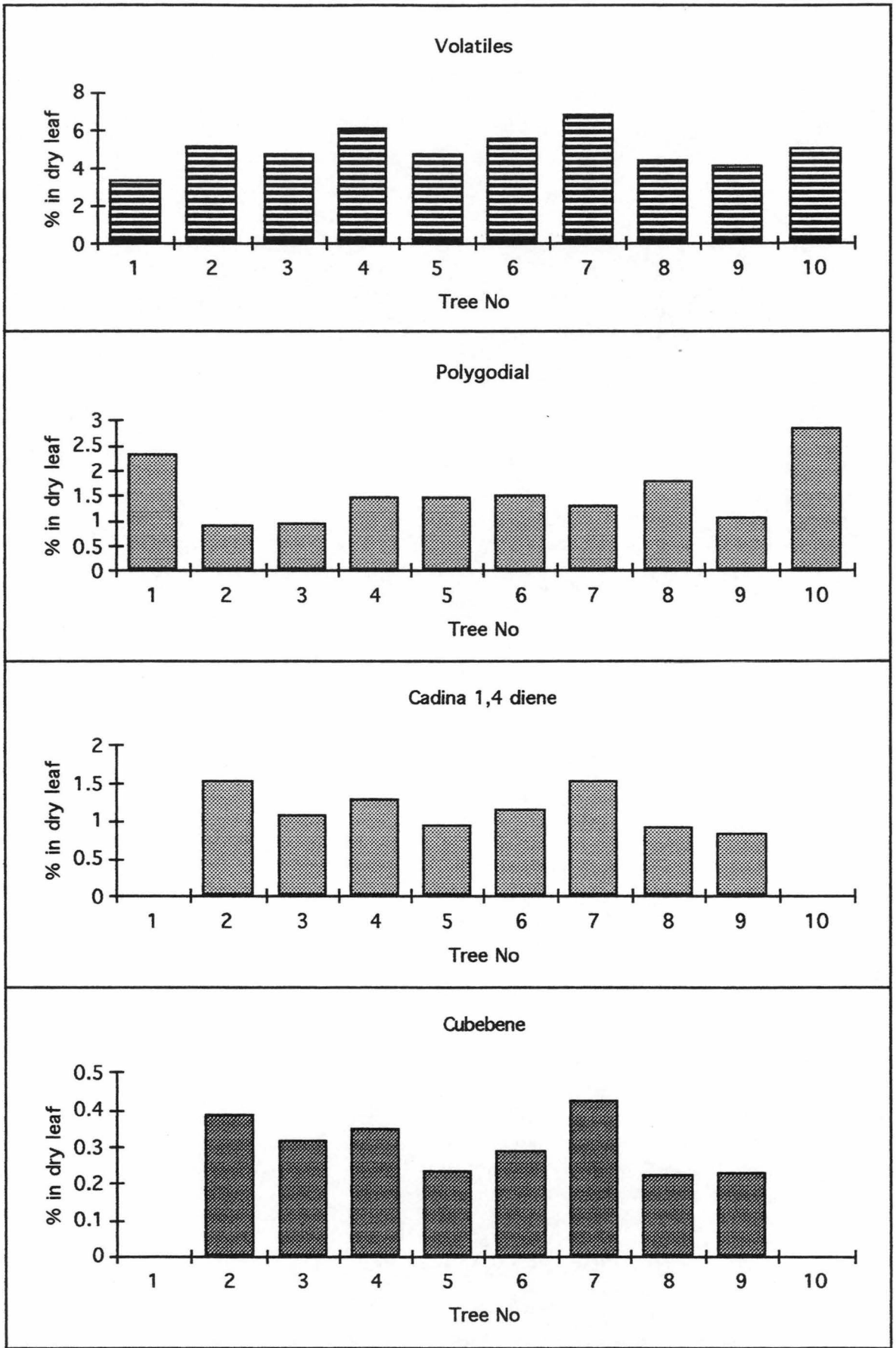


Figure 6.5: Mean % w/w of volatiles, polygodial, cadina 1,4 diene and cubebene in mature leaves >100 DAB (means for ten trees, 4 samples per tree).

The mean percentage of leaf dry weight for 'volatiles' and the eight compounds as determined in leaves from consecutive seasons is shown in the Table 6.4 for '1992' leaf at one and two years old, 93 leaf at maturity and after one year, and 1994 leaf at maturity (see 6.1.1a for the scheme explaining these comparisons). The results show a consistent pattern of slow decline in most constituents from year to year, and of similar levels of each compound in mature leaves produced from one year to the next.

Table 6.4 : Comparison of extract composition (as w/w of dry leaf) in leaves, between seasons - means for ten trees (one sample each except * - four samples per tree)

	92 leaf (1 yo)		92 leaf (2yo)		93 leaf (mature)*		93 leaf (1 yo)		94 leaf (mature)	
% in dry leaf	Mean	*Std Err*	Mean	Std err	Mean	*Std Err*	Mean	*Std Err*	Mean	*Std Err*
volatiles	4.22	*0.18*	4.06	0.377	5.01	*0.32*	4.86	*0.18*	4.41	*0.30*
linalool	-	-	0.09	0.009	0.13	*0.01*	0.10	*0.01*	0.11	*0.02*
cubebene	0.29	*0.02*	0.22	0.053	0.25	*0.05*	0.28	*0.04*	0.28	*0.05*
caryophyllene	0.09	*0.01*	0.06	0.008	0.10	*0.01*	0.09	*0.01*	0.09	*0.01*
germacrene D	0.11	*0.01*	0.08	0.018	0.14	*0.02*	0.11	*0.02*	0.16	*0.03*
bicyclogermacrene	-	-	0.01	0.003	0.06	*0.01*	0.10	*0.01*	0.07	*0.01*
cadina1,4diene	0.66	*0.04*	0.47	0.122	0.93	*0.17*	0.75	*0.10*	0.97	*0.16*
aristolone	0.06	*0.01*	0.07	0.018	0.06	*0.02*	0.05	*0.02*	0.04	*0.01*
polygodial	1.41	*0.09*	1.08	0.160	1.57	*0.17*	1.21	*0.15*	1.31	*0.21*

Table 6.5 compares extracts from unopened buds (one sample per tree) collected in mid and late winter and the previous season's leaf at the late winter stage.
For the nine parameters reported, the composition of bud extract changes rapidly in the 2-3 months prior to budburst. The very high level of percentage volatiles in immature buds when compared with leaf extract reflects a high level of several unidentified compounds as well as elevated levels of the eight compounds listed. Comparing the bud extract immediately before budburst (320 days) with mature leaf on the same trees indicated substantial differences in cubebene, germacrene D and cadina- 1,4 -diene (all much less in bud extract) and bicyclogermacrene (almost three times as much in buds). Interestingly, polygodial levels (as a percentage w/w) in the bud were highest in mid winter, and declined with the onset of spring growth.

Table 6.5: Comparison of extract composition (as w/w of dry leaf) between buds and leaf in late winter

Component (% per leaf DM	**Buds@ 240days**	**Buds@ 320Days**	**Leaf@ 320 Days**
% volatiles	7.15	4.463	4.623
linalool	0.19	0.178	0.141
cubebene	0.05	0.038	0.221
caryophyllene	0.08	0.06	0.088
Germacrene D	0.03	0.022	0.113
bicyclogermacrene	0.17	0.146	0.055
cadina1,4diene	0.19	0.159	0.771
aristolone	0.04	0.038	0.052
polygodial	1.67	1.311	1.400

b) Results for budburst trial

Simple analyses of variance for the two samples per development stage were undertaken for each component. LSD's were calculated where the F test indicated a significant difference amongst the means.

Table 6.6 shows the mean yield of extract components detected in the leaf samples at each developmental stage. In the cases where no result is recorded, an undetectably small yield may be assumed. These results are presented in Figure 6.6, with the bars indicating the least significant difference between means at the 0.05 percent level. It should be remembered that the abcissa in this depiction is not linear, and represents an arbitary series of development 'stages'.

Unfortunately, of the compounds monitored in the field trial only polygodial featured at significant levels in the 'W3' extracts used in the 'budburst trial' (Table 6.6), so it was not possible to follow trends from pre-budburst through to mature leaf. In fact, the pattern of polygodial level in the two experiments was quite inconsistent, field grown buds containing higher levels of polygodial during the dormant period than at budburst, while in the glasshouse experiment, polygodial *increased* rapidly during very early leaf development.

Table 6.6: Changes in percentage by leaf dry weight of compounds detected in the bursting bud (0-3) and the young shoot (4-6).

Development 'Stage'	O	1	2	3	4	5	6	*LSD*
% volatiles in leaf	1.200	1.426	1.878	1.883	1.684	4.213	5.921	*0.199*
cis-ocimene	0.021	0.016	0.027	0.027	0.019	0.007	0.008	*0.01*
linalool	0.041	0.052	0.074	0.070	0.083	0.110	0.211	*0.010*
Unknown I - 0.8	-	0.023	0.029	0.032	0.034	0.036	0.031	*ns*
Unknown II - 1.05	-	-	-	0.019	0.026	0.089	0.242	*0.040*
aristolone	0.029	0.028	0.032	0.031	0.020	0.015	-	*0.008*
polygodial	0.915	1.002	1.338	1.339	1.197	2.748	3.899	*0.188*
kaurene	-	0.027	0.025	0.028	0.022	0.053	0.059	*0.005*

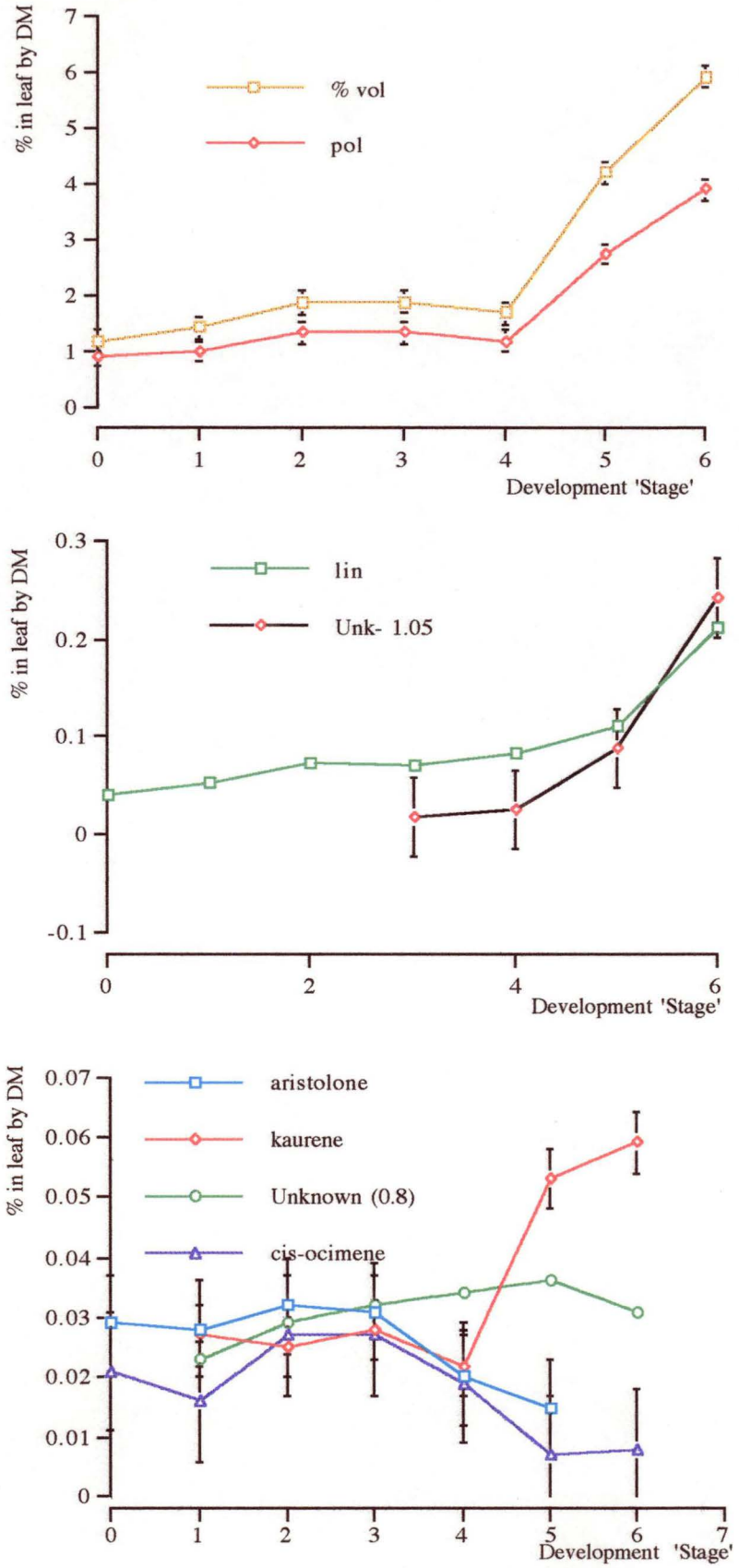


Figure 6.6: Percentage of components detected in leaves in unopened buds and on new shoots - means of duplicate results. See text for description of 'stages'. Error bars represent LSD (p>.05)

6.2 Discussion

The results obtained here concord in a general sense with those of the von Rudlhoff studies in *Pinus* (von Rudlhoff 1972, von Rudlhoff 1975a&b, von Rudlhoff and Nyland 1979, Lapp and von Rudlhoff 1982) inasmuch as the rapid changes are confined to the pre-budburst and shoot growth periods, and are followed by a long period of relative stability with respect to both total quantity and relative proportions of the volatile oil constituents. The gradual decline in levels of terpene compounds over the autumn, winter period observed in the present experiment was also observed for the *Pinus* spp in some of those studies and in this study appeared to continue until leaf senescence began. Such a pattern appears typical for many perennial oil bearing tree and shrub species (Zavarin *et al.* 1971, von Rudlhoff 1972, Bernard-Dagen *et al* 1979, Scora *et al.* 1984, Li 1993). Synthesis and accumulation continue in parallel with dry matter accumulation, followed by a prolonged period of loss, retrieval of these compounds from the storage organ, or their conversion to other metabolites.

These declines (albeit small) occurred after completion of the annual growth cycle and terpene levels continue to fall into the second year and beyond (leaves generally remain on the plant for no more than two summer seasons (see sect. 7.2)). This observation suggests the action of one of three possible agencies in reduction of total leaf terpene content.

Firstly, some further metabolic turnover of secondary compounds from the 'terpene pool' may be occurring, as suggested by the earlier work of Croteau *et al.* (1972) and Burbott and Loomis (1969). Catabolism and transport within Lamiaceae species of several monoterpene compounds as their glycosidic derivatives was reviewed by Croteau (1988) More recently some doubt has been raised as to the generality of the earlier assumptions about metabolic turnover of monoterpenes based on labelling studies in detatched plant structures as opposed to the whole plant (Mihaliak *et al.* 1991, Gershenzon *et al.* 1993). Such retrieval mechanisms have been used in the development of several theories of plant defence (Coley *et al.* 1985) and often appear as explanation of changes in secondary metabolite composition such as those observed here.

Secondly, loss of volatile compounds through emission from the leaf surface as reported by Yokouchi *et al.* (1984) and Monson *et al.* (1994) but rejected as a cause of differential composition with age by Rhodes *et al.* (1976) for oil contained in the superficial and fragile glandular trichomes of *Satureja douglasii.*

A third possibility, not mentioned in any of the publications referred to in the previous paragraphs, could be an ongoing consolidation of leaf dry matter (further development of schlerenchyma, transport tissue, cuticle deposition etc.) without change to the total reservoir of terpene compounds. The results presented appear to support the last process, at least during the initial dormant period, the mean dry weight of leaf per twig remaining unchanged during the winter period despite the observation that insect and herbivore damage was evident on many sample twigs. This suggests that increases in

specific leaf weight roughly compensate for loss of leaf through browsing and 'dilute' the oil in the leaf tissue during this first dormant period.

In the case of *Tasmannia lanceolata* studied here, with the exception of linalool, bicyclogermacrene and aristolone, the compounds showed very similar rates of decline, post-maturity, despite the fact that only one of them, polygodial, is sequestered in a robust and durable oil cell, enclosed by suberin and associated in mature leaf with a degenerate cytoplasmic residue. This suggests to the writer that the agency responsible for the decline in this initial dormant period is not metabolic in nature. Similarly, volatilisation of leaf oil components might be expected to affect low boiling point compounds such as linalool (b.p. 198°C) to a greater extent than compounds such as caryophyllene (b.p. 256°C), and is less likely to affect the contents of the oil cell than compounds stored in less robust structures, but in this case, linalool has maintained its proportion in leaf while caryophyllene has declined, as has polygodial during the dormant period.

Linalool levels are not sustained beyond the subsequent growing season, however, and at 450 days after leaf emergence most compounds, linalool included, have fallen to well below their 'mature leaf' levels. It appears from this that some long term loss of terpenoid compounds from mature leaves must be occurring as well as the short term 'dilution' of these compounds by increases in sclerification and structural development.

With respect to the variation amongst the experimental material, it should be noted that variation between trees was substantial (see Figure 6.5) - leaves on some trees contained more than twice as much polygodial and cadina- 1,4 -diene as others, and some compounds were not detected in some trees at any time during the sampling period. On the other hand, the results obtained for samples taken from the same tree, and from date to date proved very consistent, reinforcing the observation detailed in Sect. 3.5 that the inherent variability in extract composition is substantial, even amongst trees at a uniform site. This suggests a cautious approach in employing this type of information in chemotaxonomic argument (eg Southwell and Brophy 1992).

CHAPTER 7: HARVESTING FOR EXTRACT YIELD AND COMPOSITION

7.0 Introduction

This chapter addresses questions arising from the proposition that commercial control over extract quality and yield will depend upon a managed production system. Such a system will at the least require the use of clonal material, and will incorporate a canopy training and harvest method devised to retain photosynthetic productivity and structural form while maximising the annual dry matter production of leaf.

Dry matter production will depend upon photosynthetic efficiency and the capacity of the plant to regenerate - for new growth points to commence activity and for the remaining leaf area to respond to renewed exposure to the outside of the canopy.

Accordingly, this chapter deals in turn with several matters falling into the general category of 'cultural issues'- the effect on photosynthetic productivity of large changes in light climate on leaf growth and extract yield, a basic examination of canopy response to harvest or pruning operations (specifically shoot growth and the resulting canopy architecture), and finally, a harvest strategy incorporating all of these findings is presented.

7.1 The effect of changes in light environment on leaf physiology and oil production in *Tasmannia lanceolata*

Introduction

Where commercial production of leaf material and leaf extract are concerned, light environment and photosynthetic activity in *Tasmannia lanceolata* will influence productivity as primary regulators of CO_2 assimilation, and therefore, of production of leaf dry matter and synthesis of secondary products. Specifically, information regarding the behaviour of the photosynthetic apparatus will aid

- prediction of the effect of light levels on leaf dry matter yield, morphology and oil content and
- interpretation of yield and composition data for leaf collected from plants across the normal range of the species.

In practice this information would apply in

- selection of promising propagation material from wild stands,
- choice of site for commercial production,
- development of a strategy for removal of part of the canopy at harvest.

Comparison of leaf extracts collected from plants growing in the bush will be improved by taking account of the effect of light environment on leaf morphology, particularly where yields and composition are expressed in terms of dry weight of leaf. Trends in specific leaf area, with different local light environment may interact with yield of secondary products by dry leaf weight, and an indication of any tendancy in this regard would enable more careful sampling, or removal of such covariates in the analysis of yield data.

Manipulation of the canopy (for example by removal of new growth during harvesting) will drastically alter leaf age composition for the whole plant and the light environment of the remaining leaves. The carbon balance of the plant depends heavily on the ability of these leaves to adjust to the altered regime and to contribute sufficient photosynthate to sustain renewed growth. This requirement is recognised in commercial tea production systems and pruning to lower the harvest platform is undertaken only at infrequent intervals Barua (1969), and the retention of 'lung' shoots is incorporated in the periodic pruning strategy in some production areas (Mwakha and Anyuka 1990). Barua (1969) also stressed the importance of clonal variation and leaf orientation habit in comparing photosynthetic capability.

The general questions to be addressed in this investigation are:
1) what is the effect of different light levels on leaf morphological and physiological characteristics relating to leaf and oil production and
2) if specific leaf area changes, what is the effect on polygodial content expressed as dry matter, i.e. is there any connection between sun/shade growth and % polygodial and is there any effect on polygodial yield.

Clonal material was used in the following series of experiments in which sets of plants were subjected to different light levels during a growth cycle and the effects of this treatment on several morphological and physiological parameters are compared.

Experimental Methods

Plant material: Healthy plants of clonal material of 'W3', of similar canopy size, shape and density, all held previously for twelve months in a shade tunnel (covered with Rheem green knitted shadecloth admitting 75% sunlight) were selected. Plants were kept in 150mm pots, placed in large saucers and watered every second day, to ensure a plentiful supply of water throughout the experiment. The plants (three per treatment) were moved into four growth cabinets operating at 15/20°C night/day and set to four 'light environments' - 100, 50, 30 and 10% of the maximum available illumination - 300µmol m^{-2} scc^{-1}, (measured by a Lambda Instruments LJ185 recording in the range 400-700nm, with a quantum flux sensor positioned at the midpoint of the portion of canopy to be sampled). Plants were rearranged within each cabinet every week.

Instrumentation

Net assimilation of CO_2 was measured as described in the general methods, sect. 3.7. Calculation of net assimilation rate was based on the method described by Long and Hällgren (1985) in which A is calculated as:

$$A = \text{mole fraction of air} \times \frac{(1-Xe)}{(1-Xo)} \times \frac{\Delta CO_2}{\text{leaf area}}$$

(where X_e and X_o are water vapour at inlet and outlet respectively (mol mol^{-1}) calculated from humidity and temperature, and ΔCO_2 is difference between inlet and outlet (µmol mol^{-1})).

Assimilation measurements

a) Instantaneous response to light level

Two of the three plants were used to determine assimilation parameters prior to commencement of the treatment, at seven days, and fortnightly thereafter, until the new leaves which commenced growth after the plants were placed in the cabinets appeared to have fully developed, or a second shoot extension had begun. At each measurement, the plants were moved to the instrument room with the minimum of disturbance, and

allowed to adjust to the changed conditions for some 30 minutes before fitting of leaves into the leaf chamber. Preliminary checks confirmed that stomatal conductance remained undiminished with this treatment for at least 2 hours after the plants were removed from the light cabinet. After this time, some leaves began to reduce transpiration activity, particularly on those plants treated with the highest light level. This time was, however, sufficient to obtain four reliable determinations per treatment. Leaves were allowed to stabilise in the chamber for 10 minutes before the first readings were taken. The leaf chamber was maintained at 20-22°C throughout.

Stomatal conductance was determined (Delta-T Devices AP3 Automatic porometer) for leaves to be measured for assimilation rate prior to fitting into the leaf chamber, and only those leaves having diffusive resistances less than 4 s cm^{-1} were used.

CO_2 assimilation was first determined at saturating PFD (800µmol m^{-2} sec^{-1}) before various combinations of shade cloth were used to obtain a range of PFD's down to 0. Light saturation curves for each light regime were obtained after 7, 21, 35 and 49 days of treatment. Comparison of the treatments and dates was simplified by use of parameters derived from these response curves, *viz*

A_{max}- the maximum CO_2 assimilation rate observed under conditions of saturating light, taken directly from the mean of readings obtained at 800 µmol m^{-2} sec^{-1}.

Q - apparent quantum yield and R_{dark} -leaf respiration rate under zero photon flux, were obtained from the linear regression for the three points below 150µmol m^{-2} sec^{-1}.

C_p - light compensation point, derived as the ratio of dark respiration to quantum yield, was calculated from the mean values for each time and treatment.

b) Temperature and assimilation rate

The response of maximum assimilation rate to leaf temperature was determined at two degree intervals in the range 8-32°C and held at each measurement temperature for 5 minutes or until the CO_2 differential had stabilised before readings were taken. Instantaneous light levels were maintained at 840µmol $m^{-2}sec^{-1}$ during these determinations. The leaf was stabilised in the chamber at 20°C, and the temperature reduced at approx. 0.5° min^{-1} to 8°, then raised to 32°C at the same rate. This procedure was repeated three times with a plant taken directly from the shade house at the commencement of the experiment.

Other analyses

Samples of leaves produced during the growth cycle ('new'), and of those present on the plant prior to light treatment ('old'), were collected from the lower two thirds of new, and pretreatment shoots at the completion of the treatment period. This material was then subjected to a number of analyses as follows:

a) Extract composition and yield

Duplicate samples were dried and analysed in the usual manner, (see Sect. 3.1-2) and the yield of 'total volatiles' and polygodial calculated as a percentage of dry leaf weight. Two samples of leaves collected from untreated plants were also analysed. These samples are referred to as 'pre-treatment' in the results.

b) Chlorophyll extractions

Six determinations, (two each for three leaves) per category and treatment, of chlorophyll a and chlorophyll b were conducted:

samples comprising two discs taken from the mid section of the lamina of each leaf, avoiding margins and midrib were weighed, and ground in three washes of cold acetone in a small mortar, and the solvent pipetted into a centrifuge tube. The tubes were made up to to 4ml and 0.5ml cold distilled water was added (resulting in approx. 90% acetone) and the tubes sonicate (Branson 5200 sonicating water bath) for 15 seconds each before being centrifuged for 20 minutes (MSE Super minor centrifuge). Samples were assessed for absorbance at 647 and 664nm using a 90% acetone blank and concentrations of chlorophyll a and b calculated by the equations of Jeffrey and Humphrey (1975).

Chlorophyll a and b are expressed as grams metre^{-2} and as milligrams gram^{-1} and means for these values and the ratio between them are calculated for all six samples for each treatment.

c) Specific leaf area and percentage dry matter

Two 'lots' of five leaves of each category and for each treatment were weighed fresh and the total area determined using a Paton Electronic Planimeter, resolving to 0.1mm^2, before the leaves were dried for 24hrs at 70°C and reweighed. From this data, % dry matter of the fresh leaf and specific leaf area (SLA) were calculated.

d) Leaf inclination

The angle of the midrib to the horizontal (classed to the nearest 10°) was estimated for leaves on three shoots ('old' and 'new') on each of the three plants in each treatment. No data for leaf inclination was gathered prior to plants being placed in growth cabinets.

e) Lamina morphology

Leaf discs were taken from fresh leaf material and placed immediately into 3% glutaraldehyde in phosphate buffer, left for 12 hours, then rinsed and stored in fresh buffer. The samples were then sectioned by hand and photographed under the light microscope to show differences in mesophyll and palisade structure.

f) Field measurements

Irradiance at the canopy surface and beneath the leaf layer were determined for ten trees at the field site at Parrawe in late spring (28/11/1995). Following the estimation of the inclination of canopy surface on the northern and southern aspects of each of ten trees, a tube solarimeter (see sect. 3.7) was positioned firstly at 65 ° to the horizontal, facing north, then at 75° to the horizontal, facing south and the total count for each was compared with that of a spot pyranometer positioned horizontally at ground level. Pyranometer integrations of 100 units were then used to set recording periods for subsequent tube solarimeter readings.

Canopy measurements were obtained by fixing the tube solarimeter beneath the leaf layer, parallel to the canopy surface and facing north or south, and incident radiation measured for a period corresponding to 100 counts on the pyranometer. Two readings were obtained and averaged for each position and for each tree. Canopy surface angle for north and south aspects was determined using a precision clinometer, and quantum flux measured using the Lambda LJ185 quantum flux sensor. Conditions during the measurement period (1130 - 1430 hrs) were overcast.

Results

1) Assimilation measurements.

Light saturation curves for each light regime obtained after 7, 21, 35 and 49 days are shown in Figure 7.3.1. The parameters derived from these curves (*viz* A_{max}, Q, R_{dark} and C_p) are tabulated in Appendix 2.13, and shown graphically: (a) -(d) in Figure 7.3.2.

It should be remembered that these results arise from the application of only one set of treatment environments to each group of plants. Replication occurs at the level of plant and leaf selection.

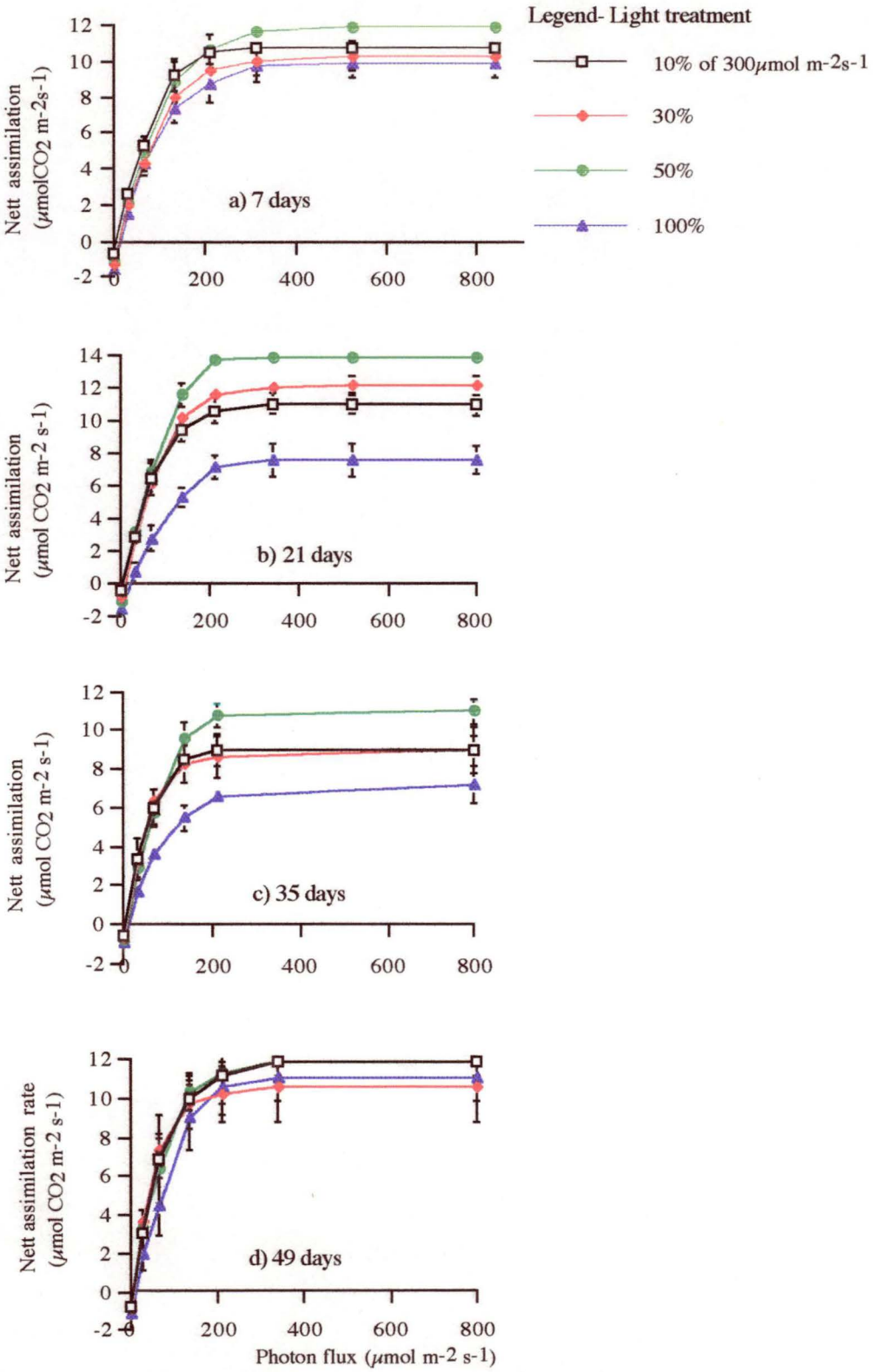


Figure 7.3.1 Light saturation curves for *Tasmannia lanceolata* grown under four light levels- error bars represent ± SE of mean for three leaves (7 days), and four leaves (21,35 and 49 days).

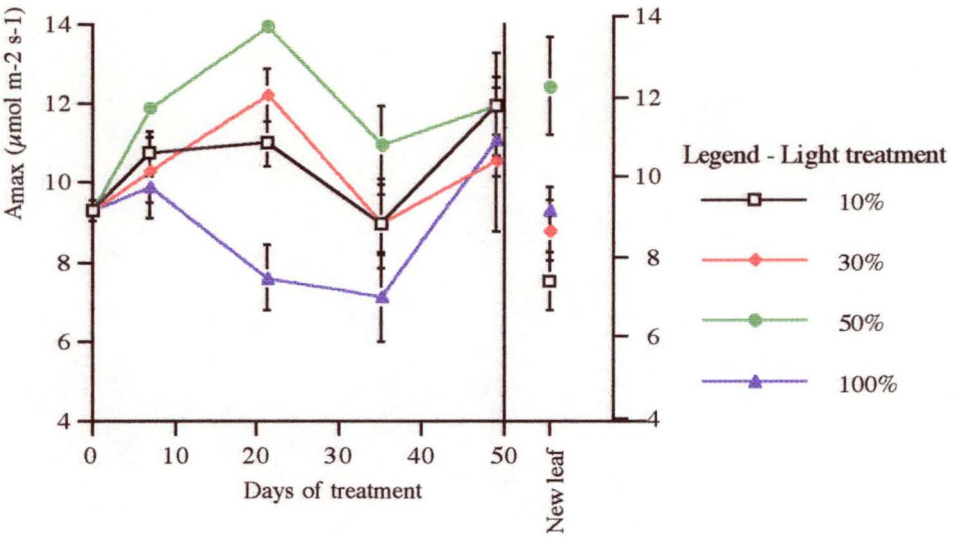


Figure 7.3.2 (a)

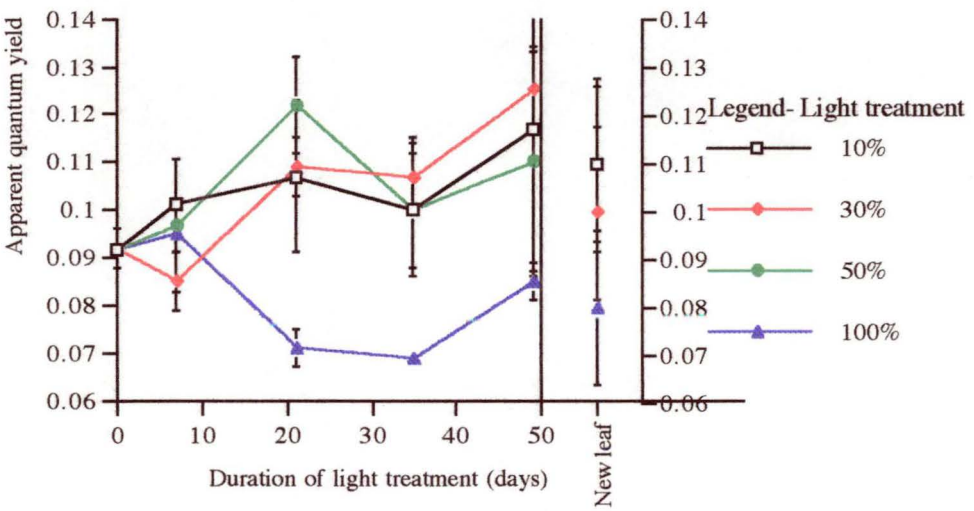


Figure 7.3.2(b)

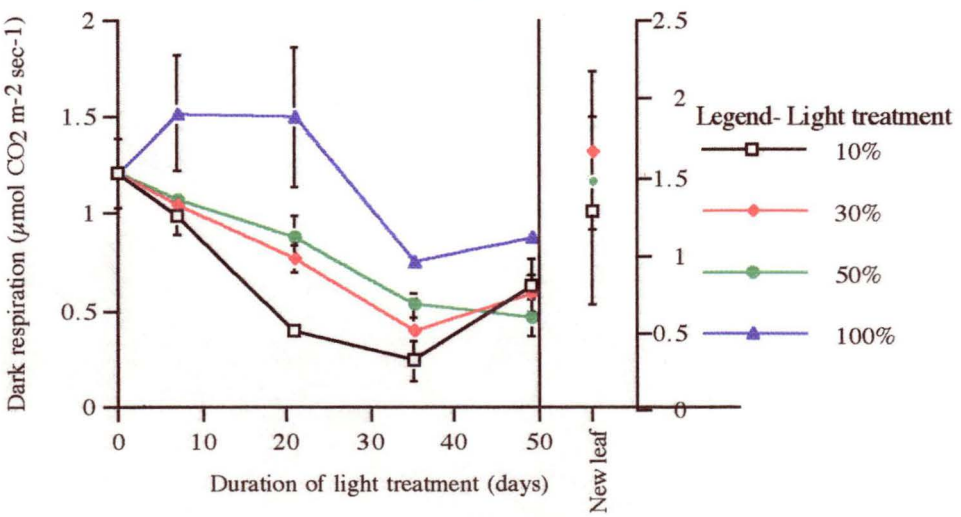


Figure 7.3.2 (c)

Figure 7.3.2 Parameters derived from light saturation curves (a): light saturated assimilation rate (b): apparent quantum yield, (c): dark respiration. Error bars are ± SE of means.

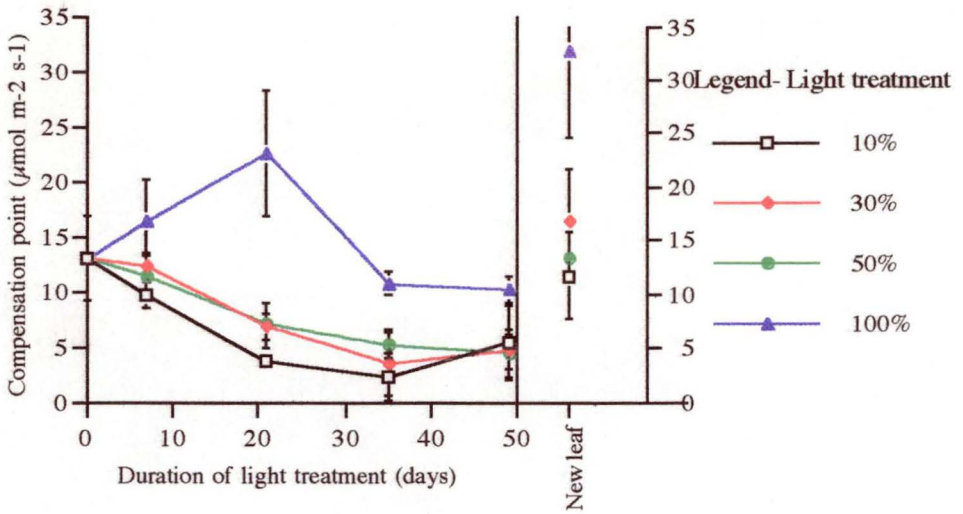


Figure 7.3.2 (d)

Figure 7.3.2 (continued). Parameters derived from light saturation curves- (d): light compensation point. Error bars represent ± SE of means.

All treatments exhibited a small increase in A_{max} in existing leaves after the move to warmer conditions, which in the lower light treatments remained elevated until approximately the 35 day determination, by which time leaves under all these treatments exhibited A_{max} levels similar to those of the untreated plants at the outset, levels which remained the same at the final 49 day determination.

At 300μmol m^{-2} s^{-1} photoinhibition was observed to reduce A_{max} significantly by 21 days, although these leaves appeared to have completely recovered their pretreatment maximum assimilation rates by the end of the experiment. The 35 day readings appeared to suffer from a depression in A_{max} across all treatments, leading one to suspect some undetected water stress effect perhaps due to a missed watering during the period prior to the taking of readings (as discussed by Clough *et al* 1983).

Among the leaves developed under the treatment light regime, only those in the 150 μmol m^{-2} s^{-1} reached the A_{max} level of the pretreatment leaves by the end of the experiment.

Changes in apparent quantum yield were slight over the duration of the treatment, declining under the highest light treatment, and increasing only slightly with the other treatments. This accords with Björkman's (1981) conclusion that quantum yield does not vary markedly across a wide range of light levels, unless plants are subjected to excessively high light levels or other stresses such as low leaf water potential.

A significant increase in dark respiration in plants subjected to 300μmol m^{-2} s^{-1} suggests an explanation for the reduced assimilation rate observed in these leaves in the early part of the treatment period. The intermediate light treatments were not significantly different from one another, respiration rate declining steadily during the course of the treatment. In the case of the very low light treatment, this decline was

exaggerated during the first part of the treatment period, but by the end of the experiment was not different from that of the two intermediate treatments. Similarly, (and as a result of) the pattern of changes in respiration rate, the light compensation point rose sharply and remained high under the highest light treatment, but declined steadily, and to the same extent in all the other treatments.

The temperature response of net assimilation in leaves of plants taken from the pretreatment environment is shown in Figure 7.3.3, for the range 8-32°C. In each case leaves were taken to 35°C, after which assimilation rates were observed to recover incompletely or not at all upon subsequent cooling to 25°C.
This result confirms that of Casey (1991) for low altitude populations of *Tasmannia lanceolata* with the rather broad curve indicating an optimum temperature for net assimilation between 18-25°C and 90% of A_{max} was obtained for temperatures between 10 and 30°C, a typical result for low altitude species inhabiting mesic environments (Berry and Björkman 1980).

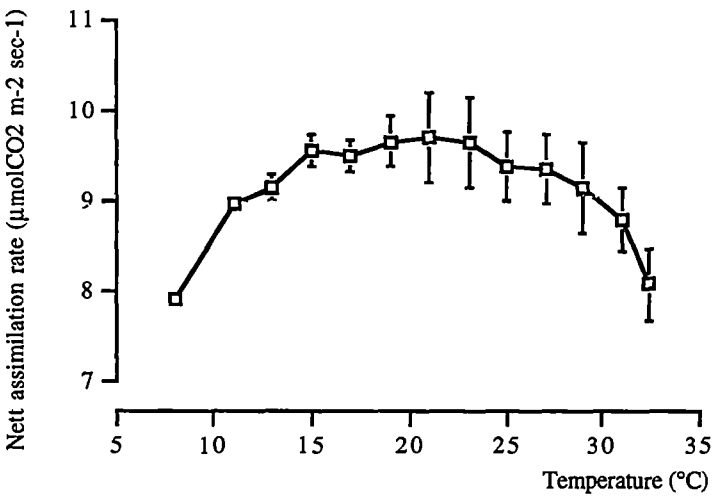


Figure 7.3.3 Response of assimilation rate to temperature in shadehouse grown *Tasmannia lanceolata*, error bars show ± SE of mean of three determinations.

Other results

a) Extract composition and yield - polygodial content and the level of 'total volatiles' expressed as a percentage of dry leaf material are tabulated below (Table 7.3.1) and pictured in the histograms in Figure 7.3.4.
In summary, polygodial percentage (w/w in dry leaf) was *not* affected by the light environment in which leaves grew, despite the effect observed on SLA (see below), while volatile level appeared to be slightly reduced at extremes of high and low light growth environment, and slightly increased at 150µmol $m^{-2}s^{-1}$.
In pretreatment leaves subject to the treatment light levels for 49 days, polygodial and volatile levels were reduced substantially at the high light regime. On the basis of

extract composition alone, it appears that the light environment resulting in the most similar response in new leaves as old, was the 30% (85 μmol m^2s^{-1}) level.

The fall in percent volatiles and percent polygodial observed in pretreatment leaves at 300μmol $m^{-2}s^{-1}$ suggests either a loss of secondary metabolite by volatilisation, transport out of the leaf, or metabolic conversion into some non-volatile product, or an increase in leaf dry matter (relative to oil compounds) arising from changes to the distribution of photosynthate amongst soluble carbohydrate and the structural and photosynthetic components of the mature leaf under this 'extreme' light regime.

Table 7.3.1: Polygodial (P) and volatiles (vols) content (% of dry leaf) obtained from leaves at completion of light treatment and samples taken from untreated plants. Standard errors are for the means of two samples.

	PFD (μmol $m^{-2}sec^{-1}$)	Mean P	*Std Err*	Mean Vols	*Std err*
Pre-treatment	-	3.37	*0.18*	5.05	*0.22*
'Old' leaves	300	2.14	*0.08*	3.18	*0.33*
	150	2.77	*0.14*	4.47	*0.25*
	85	3.33	*0.23*	5.10	*0.27*
	30	3.21	*0.04*	4.59	*0.19*
'New' leaves	300	3.27	*0.15*	4.20	*0.05*
	150	4.00	*0.41*	6.30	*0.47*
	85	3.43	*0.10*	4.92	*0.35*
	30	3.23	*0.07*	4.45	*0.14*

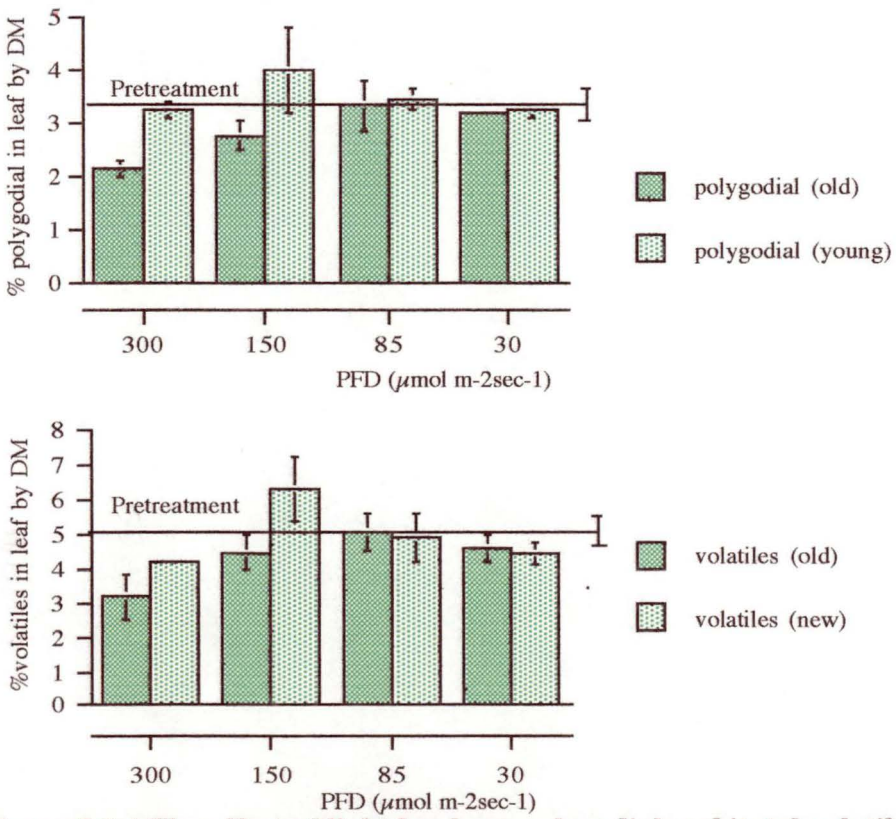


Figure 7.3.4 The effect of light levels on polygodial and 'total volatiles' in dry leaf after 50 days light treatment- mean % of each in leaf dry matter 'new' leaves produced during treatment. Error bars show ± SE of means.

b) Chlorophyll extractions - chlorophyll content is expressed on a leaf area and leaf fresh weight basis and is shown in the two charts in Figure 7.3.5.

Chlorophyll levels in new leaves were all lower than those in mature leaves suggesting that leaf development was incomplete at that time. Chlorophyll a:b ratios were lowest in the 30% light treatment, as a result of the higher levels of chlorophyll b following that treatment when compared with higher and lower light levels.

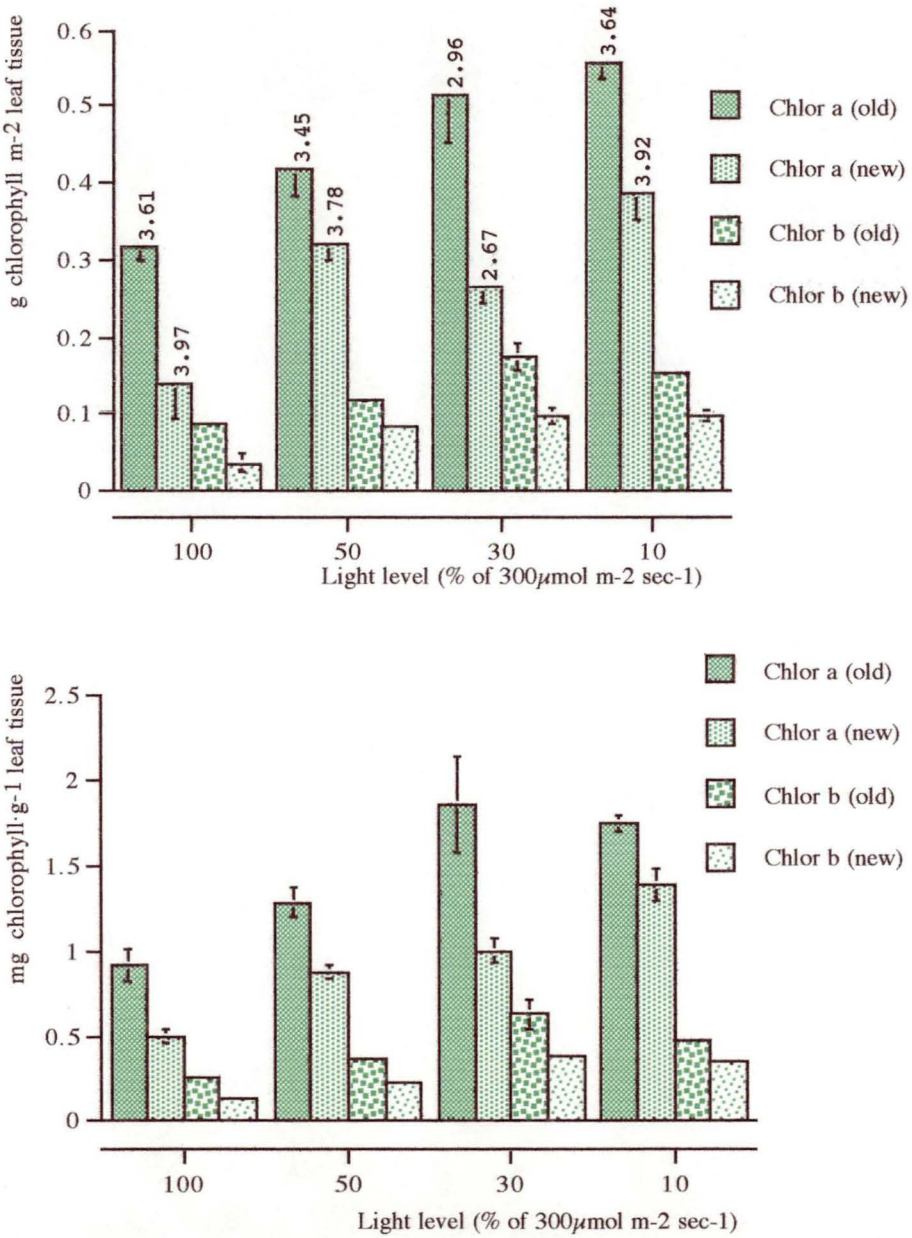


Figure 7.3.5 The effect of light levels on chlorophyll in leaf tissue (w/area -upper figure, w/w -lower figure) following 50 days treatment of pre-existing and newly produced leaves. Figures attached to histogram bars (upper figure) are chlorophyll a:b ratios for each category. Error bars are ± se of mean of 6 determinations.

c) Specific leaf area and percentage dry matter results for old and new leaves are presented in the histograms in Figure 7.3.6.

Inspection of the trends in specific leaf area and percentage dry matter, suggests that, as expected, SLA diminished and %DM increased steadily as light levels increased, even in leaves which were fully mature before treatment. This trend in SLA was more marked in the newly formed leaves, those under low light with significantly higher SLA and high light leaves significantly lower SLA than established leaves on the same plant. There was no significant difference at 30% light.

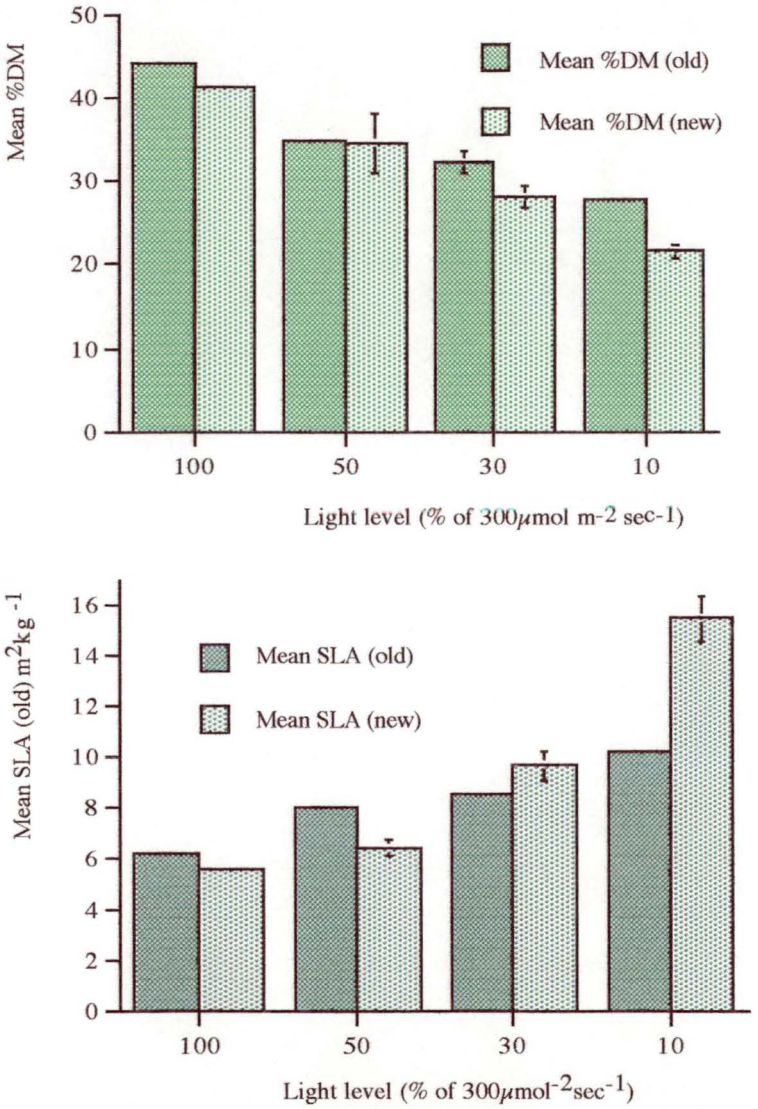


Figure 7.3.6 The effect of light level on specific leaf area (m^2 kg^{-1}) and % dry matter in leaves maintained, or newly grown, under four photon flux densities. Error bars are ± se of means of two samples of five leaves taken from lower two thirds of the shoots.

d) Leaf inclination to the horizontal as measured on all plants at the completion of the light treatment is shown in Figure 7.3.7. The plants responded predictably, low light inducing a more horizontal distribution in the canopy and the effect being more obvious in newly formed leaves.

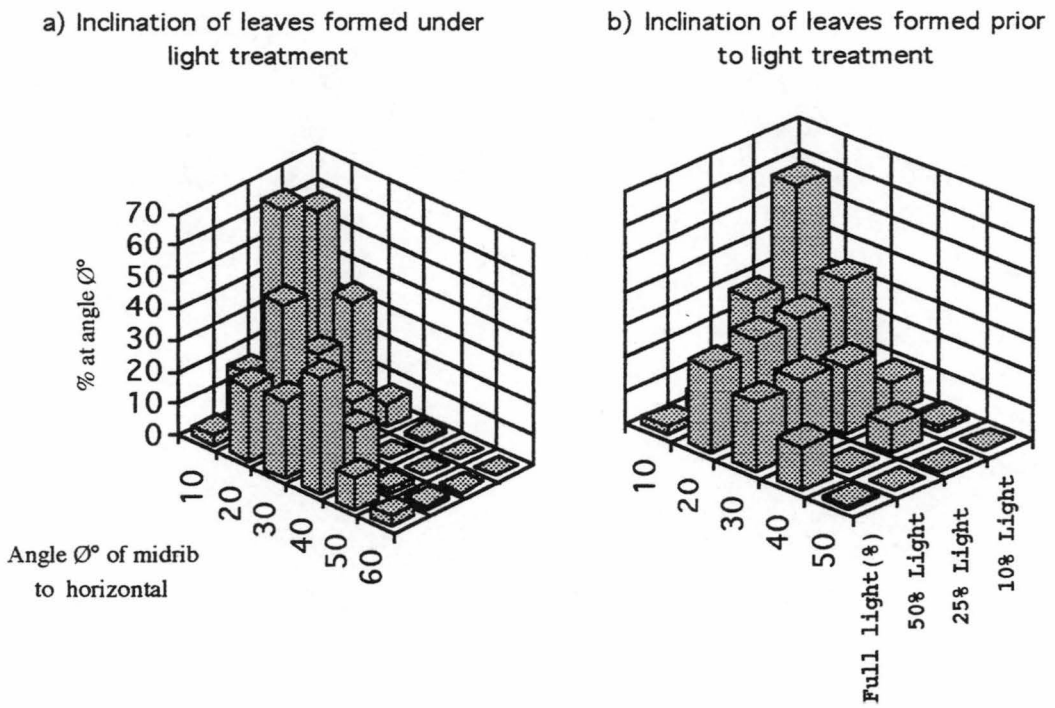
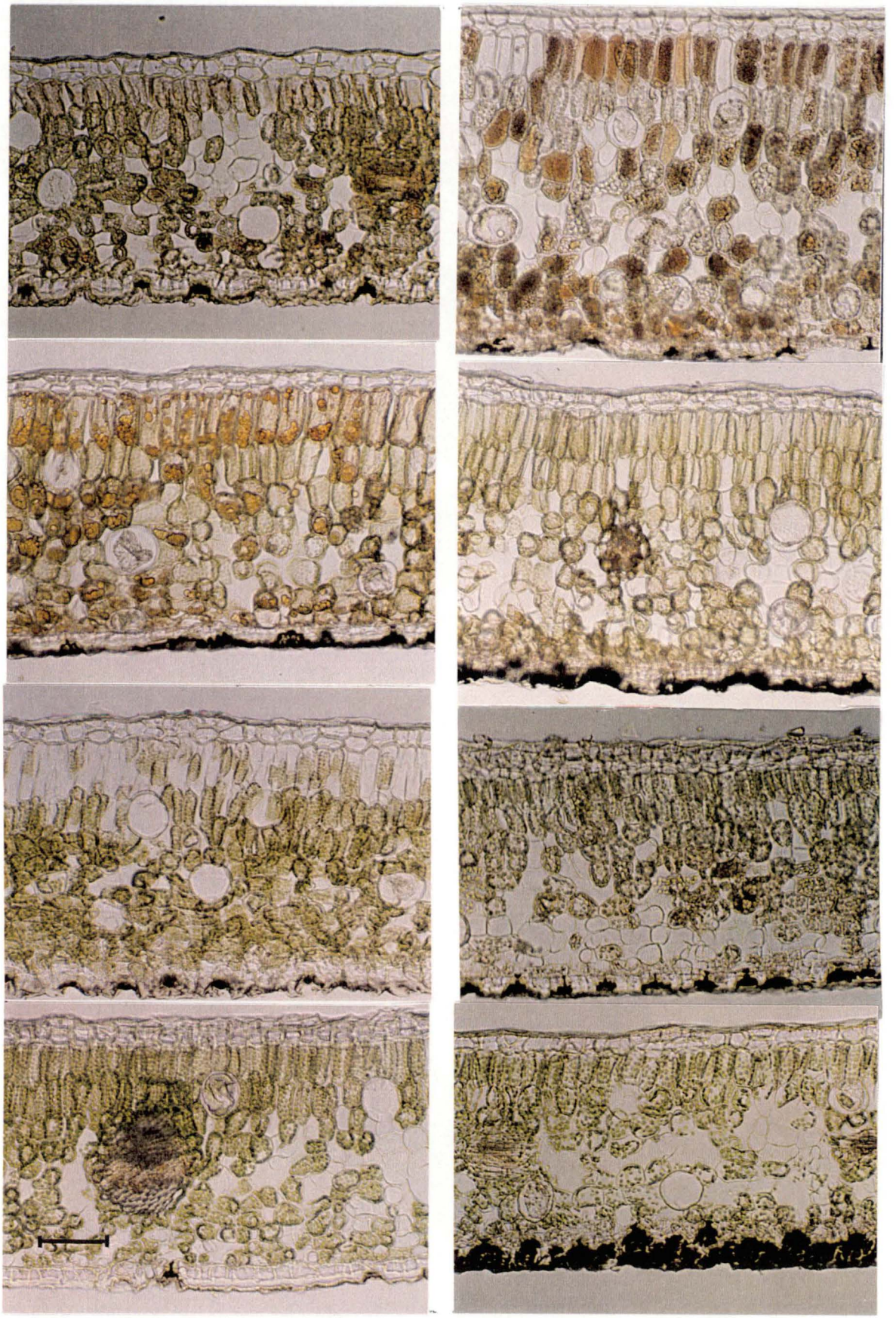


Figure 7.3.7 Histograms showing the distribution of leaf inclination for leaves formed prior to and during the four light treatments. Inclination measured as degrees from the horizontal, for the first half of the midrib of leaves means for three shoots per plant.

e) Lamina morphology.

Photomicrographs taken of leaf sections after treatment are shown in Plate 7.1. While leaves which were fully formed prior to the application of the treatment remain quite similar to one another with respect to thickness (0.35-0.4mm) and lamina morphology (1-2 layers of epidermal cells, 2 layers of columnar palisade, 0.2mm spongy mesophyll), the leaves formed under the four light regimes exhibit characteristics typical of extremes of light environment. Leaves formed at the highest light level were much thicker (>0.6mm), exhibiting three layers of cells having a columnar section, while those grown under the lowest light conditions contained only one distinct layer of columnar mesophyll cells, and consequently were thinner than the pretreatment leaves (< 0.35mm). There was no marked alteration of epidermal development evident in the sections, nor was any trend in oil cell frequency apparent by inspection. Increased leaf thickness under high light levels appeared to arise largely from the increased elongation of upper mesophyll layers, while an increase in spongy cell diameter could be observed at the highest light level only.



Leaf present at commencement of treatment

Leaf grown during treatment period

Plate 7.1: Fresh, hand cut leaf sections taken at completion of the light level treatments; From top: 100%, 50%, 30% and 10% of maximum flux density in cabinets (300μmol m^{-2} s^{-1}); bar = 100μm.

f) Field radiation observations

The angle at the surface of northern and southern faces of the canopies of ten trees was measured using a clinometer and the mean of these used to set the solarimeter tubes for 'canopy surface' measurements (65° to the north, 75 to the south). The proportion of radiation received by the horizontal pyranometer which was detected by the north- and south-facing inclined tubes was 0.78 and 0.52 respectively. For ten mature trees, each at least 3m high and 5m distant from its nearest neighbour, the mean proportion of solar radiation detected inside the canopy was 0.39 and 0.67 of that detected on the north and south facing canopy surfaces respectively, and therefore 0.30 and 0.34 respectively, of the radiation recieved by the horizontal detector. The canopies were observed in almost all cases to approximate to a layer of leaves from 150 -300mm thick.

Quantum flux measured using a sensor positioned horizontally 1m above the ground ranged between 200 and 900 $\mu mol\ m^{-2}\ s^{-1}$ during the measurement period.

Discussion

The four treatment light levels have induced several predictable consequences.

The effect on chlorophyll level is typical of a number of C3 species for which increases in controlled light level have increased chlorophyll content (cited in Björkman 1981), usually with a resultant increase in light saturated assimilation rate, unless some photoinhibitory effect is induced. The association between these trends is complex, and includes the effect of increased leaf thickness and structure (mesophyll size and shape) on absorption, alterations in the proportions of other photosystem components and the (slight) contribution of changes in stomatal conductance with altered leaf morphology (Boardman 1977). In this case, specific leaf area responded to the different light regimes (affecting leaf thickness and mesophyll density) as would be expected from previous reports (Björkman 1981) and reduced SLA was clearly associated with increased dry matter both in new and pretreatment leaves at higher light levels. The relationship between light saturated photosynthesis and specific leaf area reported for some species could not be confirmed due to the limited number of data points (light levels), but in new leaves at least, the decline in A_{max} at high light levels appeared very similar to that reported for several 'shade' species - eg *Fragaria spp* (Björkman and Holmgren 1963) and *Solidago virgaurea* (Holmgren 1968) in which lower SLA (larger, thinner leaves) does not provide increased photosynthetic capacity at high light levels.

The results of Turnbull *et al.* (1993) indicated significant acclimation to reduced light level in several rainforest understorey species, both early and late successional.

If *Tasmannia lanceolata* is to be considered an early successional species (Read and Hill 1983), then it would be expected to demonstrate a capacity to respond favourably to variation in light regime (Bazzaz and Carlson 1982), and the results here would

appear to support this. Acclimation resulted in gradual recovery of pretreatment levels for the net assimilation rate, (after the initial effects of photoinhibition at the highest light treatment), with some suggestion of a slight increase, due probably to the warmer conditions during treatment. Quantum yield was relatively unaffected, and dark respiration declined steadily during the treatment period in all but the highest light level. In this treatment, a large increase in respiration rate over the first half of the treatment period, and a drop in quantum yield contributed substantially to the fall in initial net assimilation rate in this treatment.

The fall in respiration rate over the duration of the other three treatments would have been an important factor contributing to the improvement in net assimilation rate observed at transfer despite the predicted fall. This has had the consequence of masking the expected pattern of a fall in A_{max} after reduction in PFD followed by gradual acclimation to the new light regime as is normally observed following such transfers (Turnbull *et al.* 1993, Sims and Pearcey 1991, Osunkoya and Ash 1991). Another 'masking' influence is the capacity of the foliage to reorient itself in partial compensation for changes in light level. In this case, changes in leaf angle will have reduced the differences in effective light level between treatments. Such 'nastic' responses to short lived changes in light pattern are often observed in plants growing in low flux densities, allowing them to obtain maximum illumination by following the sun (Fitter and Hay 1987).

Lastly the results show an apparent increase in commitment to leaf dry matter in leaves grown at higher light levels. The substantial reduction in SLA in 'old' leaves is attributed to an increase in weight per leaf - Turnbull *et al.* (1993) found small post-transfer changes in leaf area occurred but individual leaf areas were not monitored in this experiment. In higher light treatments this increase might be expected to reduce the yield (by DM) of sequestered secondary products, even without any real change in the total quantity per leaf. In fact, there was a reduction in yield of secondary products, and SLA changes may wholly explain the reduced levels of these compounds at the higher light level. An analysis of yield per leaf of the relevant compounds could have established this - Lincoln and Langenheim (1978) found significant light treatment differences in total monoterpene yield by leaf weight that did not translate into significance in yield per leaf pair in *Satureja douglasii*, but in explanation of significant interactions of light with compositional type and temperature on monoterpene yield per leaf pair, invoked volatilisation and photochemical reactions as possible causes.

Overall, three aspects of the response of the plant - adjustment of the photosynthetic apparatus by acclimation, apparent changes in investment in leaf dry matter (not volatile solvent soluble compounds) and the response of leaf angle will all help to buffer the effect of naturally induced changes in irradiation.

Such agencies might be responsible for the reduction in 'total volatiles' observed in this experiment for newly formed leaves, particularly since the major volatile component, polygodial does not differ from levels found in leaves before treatment. On the other hand, the fact that total volatiles appear to have been enhanced at 50% of the maximum light treatment at the same time as SLA has increased over that of pretreatment leaves suggests an overall increase in yield of extract with growth of new leaves under these conditions. Yamaura *et al* (1989) for example found that monoterpene production in thyme seedlings was induced by irradiation, correlated with number of peltate glandular trichomes in which oil is accumulated. These changes were detected over a short period (up to 8 days), and seem to indicate simply that in the absence, or with very short periods of daily illumination, normal production of secondary compounds is prevented or greatly reduced.

In the context of seasonal changes in irradiation during development of new growth, the picture is more complex than that investigated in this experiment. In open situations in the natural habitat of the species, saturating PFD's of 300^{+} $\mu mol\ m^{-2}\ sec^{-1}$ would be commonplace on the outside of the canopy during the growing season.

The field observation that canopy surface assumes a lower angle to the horizontal on the northern (sun) side than on the shady side of the trees at the site is to be expected in trees growing in an open situation in which diffuse incident radiation contributes less to the whole than it might beneath a forest canopy. No attempt was made to include the effect of the prevailing (WSW) wind direction on the general form of the canopy, although at this and many sites of occurrence of the species, plants tend to occupy sheltered niches in which wind pruning is unlikely to be significant.

Radiation levels detected beneath the shadier side of the canopy were larger (as a proportion of those observed by the horizontal pyranometer) than those found beneath the sunny side of the canopy. This might be expected if a higher leaf area index is assumed for the sunny aspect of the trees, so that a larger contribution is due to diffuse and reflected radiation on the interior of the shady side of the canopy. This result might also be attributable to the combined effects of leaf inclination and orientation, although no data to support this were obtained at the field site.

Long term trends in seasonal light levels will result in a gradual fall in incident radiation for the six months following completion of leaf development, during which time some of the changes detected in this experiment would occur.

Furthermore, interaction between light levels and ambient temperature during this cycle are likely to alter the simplistic responses observed here. For example, the extent of photoinhibition upon increase in light level is reduced at higher temperatures (Björkman 1981) an apposite interaction, since in the natural situation, irradiation would increase gradually as the summer solistice approaches and remain high during the warm summer period which follows (see climate data - Appendix 1.1).

Nunez (1983) presents a solar radiation model to calculate direct and diffuse total daily radiation received on sloping surfaces in Tasmania. The model, using azimuth and elevation of the surface, and by reference to simple diagrams developed for December and June for the Tasmanian situation, indicates (allowing for local variations in cloud cover) mean daily solar radiation accurate to within 10-15%. This information could be used, together with the general indications revealed in the above experiments to aid a choice of aspect and slope suitable for cultivation of the species.

Extension of single leaf photosynthetic parameters to predict total canopy productivity, such as proposed by Sims *et al.* (1994) for the case of *Alocasia macrorrhiza*, or Sands' (1995) generalised algorithm based upon single leaf photosynthetic characteristics require highly detailed information regarding the influence of environmental factors such as diurnal and seasonal temperature variation on photosynthetic response as well as canopy characteristics- LAI, mean leaf angle, canopy thickness and so on. The data gathered here do not provide sufficient detail to undertake such a predictive approach and only provide an indication of the photosynthetic performance of the plant in comparison to other species reported in the literature.

7.2 Canopy architecture and pruning strategies

7.2.1 Introduction

The approach in this section has been firstly to develop, by general observation, a detailed understanding of the cyclic growth pattern in *T lanceolata* in its natural habitat and to describe this cycle, with support from the published literature, with a view to identifying all possible vegetative axes which might respond to harvest or pruning. These observations are then supplemented with the results of some simple trials in which simulation of pruning methods was carried out on a group of mature trees at the field site at Parrawe. The effect of these treatments upon activation of vegetative axes is reported.
Lastly an attempt is made to describe the normal pattern of leaf retention (relevant in considering the consequences of pruning for the photosynthetic capacity of the canopy). No effort is made to predict the form of the canopy likely to result from such methods, since for the natural population, canopy shapes vary widely (see Plate 7.2), reflecting habitat type and differing degrees of herbivorous and insect alteration of the 'ideal' pattern of shoot extension.

7.2.2 Observations of shoot ontogeny and morphology

The developmental sequence observed in *Tasmannia lanceolata* under normal (moderate altitude, field conditions in Tasmania) circumstances may be summarised as follows.

The group of buds clustered at the end of the shoot may be *vegetative*, producing a new shoot flush, or *mixed*, shoots developing from the central meristem, while single flowers develop in each axil of the surrounding floral bracts. All structures in the bud are formed during late summer and autumn, so that flower initiation is complete by the onset of the dormant period in winter (Gifford 1950).
Buds begin to swell in mid spring, with vegetative buds elongating until bud scales no longer cover the outer leaves. Mixed buds assume a globular shape, swelling until the inflorescence of 5 -20 unopened flowers emerges from within the floral bracts. Floral development is described in detail for the carpellate flower by Tucker and Gifford (1966b). Bud break in late spring/early summer is rapid with both bud types. Flowers open acropetaly over a short period and in vegetative buds leaves emerge, bud scales dehisce and shoot extension commences, the first two or three leaves being well developed by the time the shoot emerges. Shoot extension and anthesis may occur together in mixed buds, but more usually, shoot extension is delayed until flowering is substantially complete (Vink 1970). Shoots on mixed buds tend to be shorter and bear fewer leaves than simple vegetative shoots (Plate 7.3).

Plate 7.2: Typical canopy forms observed (left) at an open site and in a sheltered understorey situation (right).

Plate 7.3: Mixed bud, prior to extension of apical vegetative shoot. Note: Several bracts surrounding the central axis have been removed for clarity; the small number of flowers is not typical for most buds.

Vegetative shoot extension, the expansion of 6-10 leaves and elongation of the shoot, commences in late spring (Plate 7.4) and is complete after two to four weeks, (by mid summer), after which the leaves continue to grow and mature and the shoot thickens during late summer. Node length decreases acropetally, while leaf area tends to a maximum with the third or fourth node. The first 1-2 transition leaves are typically small or incompletely formed.

The shoot terminates with an apical bud delineated by bracts or bud scales which continue the spiral of the foliage leaves. Internode length towards the end of a shoot shortens dramatically, resulting in the last two to five leaves forming a false whorl about the terminal bud (Plate 7.5). Consequently the terminal bud may be closely surrounded by several subjacent lateral buds which often develop to substantial size, the tip of the shoot broadening to accomodate them, and often re-orienting the positions of the buds to near- equal elevation. The last, often poorly developed leaf may apear to arise from the terminal bud itself, subtending a very small vegetative bud which usually remains undeveloped in the subsequent season (arrowed in Plate 7.5).

Bract scars remain evident as a short ring around the base of each shoot for many years after the extension of the shoot, enabling identification of seasonal increments of growth for up to 5 years in many cases. In favourable circumstances, (warm, wet conditions) some plants were observed to undergo more than one vegetative extension during a summer season, the bract scars separating them being more difficult to detect in this case. Vink (1970) noted that the lower (and occasionally the upper) few of these scars may subtend vegetative apices rather than floral buds. These buds rarely develop in the normal course of events, unless the shoot above is damaged, when they may produce a short shoot with two or three leaves in the next season, initiating development of a new branch axis.

Lateral shoots arising from the buds immediately below the terminal bud may develop either together with (and sometimes as vigorously as) the terminal shoot (see Plate 7.6), or may develop during a subsequent season in which case they tend to be smaller and carry fewer leaves. Such lateral shoot development is normally associated only with the last 2-4 nodes of the 1 year old shoot. The occasional development of axillary buds (always vegetative) from lower nodes usually results in short shoots bearing 2-3 leaves. Lateral buds in proximal positions on shoots (usually the first 4-5), subtend buds on which the first 2-4 bracts appear decussate, commencing with a 'pair' arranged at 90° to the axes of leaf and stem. With the abscission of the subtending leaf and sometimes the first 2-3 bracts in older wood, the axes of these buds assume a position almost perpendicular to the stem and submerged within the bark. Under the normal cycle of growth these buds rarely develop into active reproductive or vegetative axes, but nevertheless appear to persist long after senescence of the subtending leaves .

Plate 7.4: Emergence and extension of a vegetative bud (spring /early summer). Note shoot carries full complement of leaves as it emerges from within the scales enclosing the bud.

Plate 7.5: Mature shoot after completion of extension and bud development - late summer. Note false whorl about the terminal bud, finishing with a small leaf appearing to arise from the terminal bud itself (arrow). First 4-5 buds below apex will develop shoot systems during next spring- summer.

Plate 7.6: Typical shoot structure on glasshouse - grown *Tasmannia lanceolata*.. One year old shoot (O) bears six new shoot systems, the most vigorous being the apical shoot (A), remaining shoots diminishing in vigour with distance below the apex. Ring of bract scars may be seen at the base of each shoot (B). Previous season's leaf remains present along the whole length of the older shoot.

The frequent insect destruction (in mid to late autumn) of apical buds observed in trees in field situations appears to enable substitution growth by the lateral shoots (above) in place of the terminal shoot. The result is that trees tend to express a sympodial growth habit while all shoots are orthotropic (erect and radially symmetrical) and the underlying morphogenetic habit is rhythmic and monopodial (aspects of the Rauh model of canopy architecture: Sect. 2.6).

In summary, at the end of an annual growth cycle (before the onset of winter), the following potential vegetative axes are observed on the mature plant :

1) terminal apex- in a large, often mixed bud, containing 5-25 flowers, 5-7 leaf primordia
2) a false whorl of three to five distal lateral apices- often more or less identical to 1 above). Any of these may be destroyed by insect attack during the autumn- winter period.
3) Lateral buds in axils of leaves below the terminal 'whorl'. Some well developed, containing 5-7 leaves, though those in the axils of the lower two to three leaves on a shoot are usually very small and undeveloped.
4) Buds in the axils of bract scars - usually the lower few, but occasionally the upper two or three. Normally remain dormant unless apex is damaged.
5) Undeveloped buds in leaf axils and bract scars of all previous seasons' extension growth.

7.2.3 Field trials and observations

i) Response to pruning position in mature trees

Methods

In order to describe the response to application of pruning cuts at various points on the shoot system, the growth points described above were grouped for each shoot increment as follows:
'Type 1': apical and subapical (up to five large buds at the tip of the shoot)
'Type 2': proximal (the axillary buds in the lower 2-4 leaves on the shoot)
'Type 3': bract (in axils of the upper and lower bud scale scars at the base of the shoot)

Pruning cuts were applied at the end of the 1994-95 growth cycle, as detailed below, and the response (size, position and number) of each bud category for at least the most recent shoot cohorts was recorded after three months (late May), eight months (early October) and after the commencement of the subsequent budburst (November 1995) and samples of each taken for dissection. Each treatment was applied to fifteen marked shoots on each of eight mature trees growing at Parrawe in February 1995. The response of uncut shoots on the same tree was recorded as a control. The generalised response is reported. Instances of individual buds breaking in mid winter, and large

differences in behaviour between bud positions on the same shoot or adjacent shoots were noted but are not included here.

The cuts were applied as follows (see Plate 7.7, below) and each shoot marked with a coloured tag denoting the treatment :

A) removal of most of new seasons shoot(s), leaving one proximal bud. This cut removes Type 1 axes for the current shoot, leaving proximal buds, bracts and those on the previous increment.

B- removal of whole of current season's growth to below first node. Only bract buds and those on the previous increments remain.

C- Removal of all of new seasons growth -cut below attachment of lowest of new shoots removing all buds on recent season's growth.

Controls - separated from treated shoots by at least 2 season's growth, not pruned

Figure 7.7: Scheme for application of pruning cuts- A: above first node on current shoot; B: remove all but bract buds on current shoot; C: remove all of current seasons shoots to below insertion point.

Observations

Late autumn- May 21st

Control: All apical buds and subjacent lateral buds swollen (5-7 mm diameter) and contain at least 7 flower primordia. No swollen buds are visible on lower positions.

A: The majority of the remaining axillary buds (1/shoot) had swollen perceptibly when compared to those on untreated shoots.

B: Between 1.6 and 3.4 bract buds (average) per shoot had swollen so as to be visible to the unaided eye (0.5-1.0 mm diameter). On three trees, more than one lateral bud had developed slightly, (diameter 1-2mm) when compared with other dormant buds on the same shoot.

C: No visible change in bud development in previous season's shoot at any of the potential growth points.

Late Spring - 30th November

Control: Fruits well developed (2-4mm dia.) and small apical buds visible at centre of inflorescence. Vegetative shoots beginning to elongate, 2-3 leaves fully emerged from bud in most cases. In the case of trees with heavy flower and fruit set , vegetative bud development (above the inflorescence, or on non-flowering axes) was usually much reduced in comparison with that on other trees at the site.

A: In approximately 50% of cases, the single remaining axillary apex had assumed a stage of development similar to that of the apical bud observed on uncut shoots, while, in the remaining examples, buds were somewhat smaller than the normal apical bud (3-4 mm dia.) and were observed to have just commenced elongation, suggesting roughly a fortnight's delay in budburst. Upon dissection these lateral buds were found to comprise 2-3 bud scales and 4-5 leaves enclosing a terminal bud of 1-2 scales and 3-4 leaf primordia.

In some plants, several bract buds around the base of the shoot had begun to swell, and contained up to 7 well developed leaf primordia but at this time these buds were all less than 2mm in diameter. See Plate 7.8A

B: In almost all cases the group of buds clustered around the base of the (removed) one year old growth in the axils of the lower bract scars had swollen but not yet opened. The largest of these (usually the lowest) contained up to 9 leaf primordia within two to three bud scales. In several cases axillary buds at the last two or three nodes of the previous shoot increment had begun to open, and although quite small, appeared to have begun to elongate. This was the case only in those leaf axils in which no shoot had developed in the previous season, whether or not the leaf was still present. See Plate 7.8B

C: Only occasionally were well developed buds evident in the lateral positions on the remaining shoot. In most cases small buds (up to 1.5 mm diameter) were present and appeared to be developing in the two to three leaf axils beneath the pruning position. These buds contained 4-5 leaf primordia within 2 or sometimes 3 bud scales, and only rarely had these buds begun to elongate or open. See Plate 7.8C.

In summary, a pruning cut above the lowest node on recent season's growth stimulated development of the axillary bud so that in spring it was often able to produce as many leaves as the apical bud on equivalent uncut shoots. Removal of all of the previous

season's growth at its base resulted in commencement of development of the buds in the axils of bracts and bract scars towards the tip of the remaining twig. These buds, although less advanced by the normal time of budburst than those on unpruned new growth, contained a complement of leaf primordia equivalent to buds on that growth. By the time observations were complete (early summer), few of these buds had begun to elongate, although it appeared that some shoot development would occur that season. After removal of the whole shoot and part of the previous season's growth, bud development at the remaining axes was very slow, although sigificantly increased in comparison with the same positions on unpruned shoots.

Removal of shoot growth results in activation of remaining vegetative axes close to the point of pruning. The most rapid and vigorous response follows removal of most of the last season's shoot to leave at least one axillary bud.

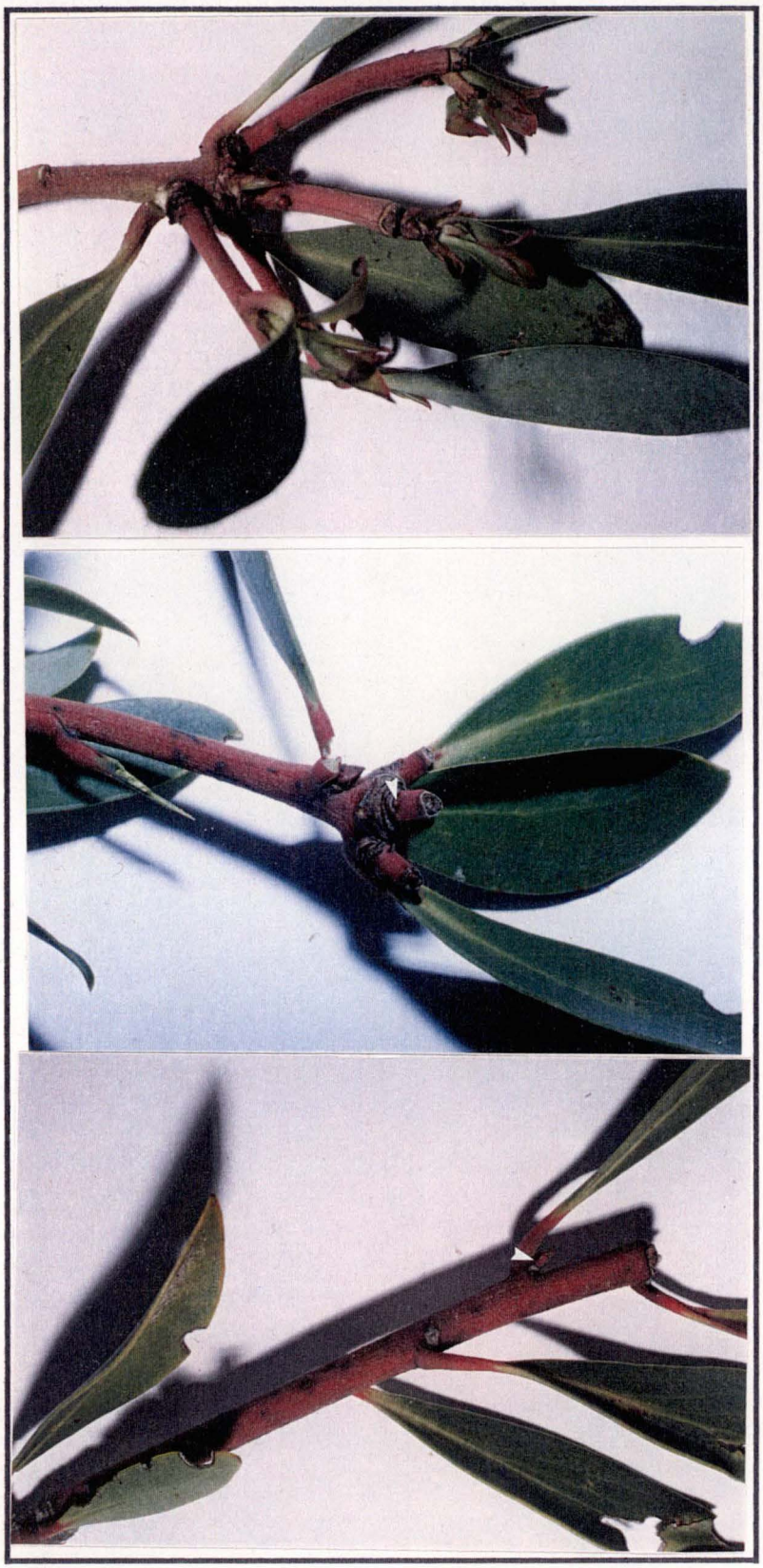


Plate 7.8: Pruned twigs collected in late November;
Treatments: A (top) : last season's shoot cut to above first node;
B (centre) : last season's shoots cut below first node;
C (bottom) : cut below insertion of last season's shoots.
Arrowheads indicate developing buds in bract scars (centre) and lower lateral position on previous season's growth (bottom)

ii) Leaf longevity

With respect to the photosynthetic canopy, leaf longevity appears greatly affected by the agencies of herbivory, and frost and wind damage, so that in general it is unusual to observe leaves older than two years remaining on a mature tree.

Methods

The number of leaves remaining on current and previous seasons' growth on ten trees at the Parrawe site was recorded at the time of termination of the 1994 spring growth . For ten branch systems on each tree, selected at random over the surface of the canopy, the number of fully formed leaves attached to the recent growth extension, the shoot upon which it was inserted, (and in some cases the extension previous to that) were recorded. In each case, where more than one shoot represented the seasonal extension, a mean leaf number for these was recorded. It was usually not possible to determine the true terminal shoot in this case.

Observations

The result of this survey is shown in Table 7.2.1, mean number of leaves present for ten branch systems for each tree. Variance is calculated for the most recent growth increment.

Also presented is the mean number of leaves observed on the '1993' growth increment during the preceding growth period and estimated from fifty twigs collected per tree in February 1994. This provides an indication of the extent of loss of leaves experienced by the 'one year old' twigs.

The number of large limbs constituting the framework of the canopy, that is, arising from the lower 10% of the tree and thicker than about 40mm diameter (the usual size of large branches which continue to attain the full height of the canopy in trees of this size at the site), and the height and breadth of the canopy (expressed as a ratio), are included at Table 7.2.1.

While the ten trees observed varied considerably in the number of leaves per shoot increment in both years for which the data was gathered, the 1994 shoots appear to have borne consistently more leaves than did shoots on the same trees in the previous year. It may be relevant here that in 1994 the species was considered to have been unusually fruitful in Tasmania, (T. Walduck, Tasmanian Forest Seeds. pers.comm.) and in 1995 a late frost resulted in almost no fruit set at the site.

At the time of data collection (by which the new shoots had achieved maturity), a substantial proportion of leaves on the previous shoot increment, and all but a few of those on the 2 year old wood had senesced, been removed by predation, or broken off by wind or frost damage.

Table 7.2.1: Mean leaf number/shoot (and variance for 15 shoots) present on each of the most recent three cohorts observed at the completion of the latest growth cycle (February), and general observations of canopy structure for ten trees at the Parrawe site, 1995.

Tree No	Shoot cohort	Mean No. Leaves present *(variance for new shoots)*	Leaf No (1993)	No. limbs	height/width ratio
1	New	9.8 *(3.96)*		7	1.50
	1yo	4.8	5.7		
	2yo	1.2			
2	New	8.5 *(0.72)*		10	1.15
	1yo	6.0	8.4		
	2yo	0.8			
3	New	7.4 *(1.16)*		8	1.10
	1yo	5.9	5.8		
	2yo	-			
4	New	9.0*(1.56)*		>20	1.20
	1yo	6.3	7.8		
	2yo	0.7			
5	New	5.8 *(3.29)*		20	1.30
	1yo	4.3	5.2		
	2yo	0.7			
6	New	8.7 *(2.23)*		15	1.31
	1yo	5.6	6.4		
	2yo	2.5			
7	New	8.2 *(3.96)*		12	1.31
	1yo	6.2	6.9		
	2yo	4.9			
8	New	8.7 *(3.12)*		20	1.00
	1yo	4.7	5.7		
	2yo	3.7			
9	New	6.8 *(0.79)*		6	1.17
	1yo	3.4	6.1		
	2yo	-			
10	New	8.8 *(3.51)*		14	1.00
	1yo	5.6	6.5		
	2yo	0.5			

Discussion

The observations recorded here provide only the most superficial indication of the response of *Tasmannia lanceolata* to pruning or harvest methods, since the trials were conducted on a small number of trees, and examined response in a relatively small proportion of the whole canopy in each case. A preferable approach would have been to use established clonal material at a field site, to apply a single treatment to the whole canopy and to replicate treatments on large numbers of plants. However the time involved in developing plants to a reasonable size for such a comparison was beyond the scope of the project. The effect of application of each treatment to the whole canopy on source/sink relationships and the resulting stimulation of the various potential growth axes cannot be predicted from this trial. However, the work does serve to describe the common pattern of shoot ontogeny, and identifies potential replacement axes available for continued canopy development in the season following a pruning

event. It also provides an indication of the relative vigour of buds developing at the various sites during the dormant period and spring after several different types of pruning method.

Bud development at all axes on the two most recent shoot systems was observed among the various treatments, distinct from the situation reported for avocado in which only the buds in the bud scale ring remain viable beyond their first year, axillary buds abscising after the first season (Cutting *et al.* 1994). For trees employed in this trial, the most robust replacement occurs when only the upper part of the recent shoot is removed, releasing lateral buds further down the shoot which, by the following spring, have developed substantial leaf and shoot systems and begin to grow simultaneously with the normal undisturbed terminal buds elsewhere on the tree. Where this shoot was removed down to the ring of bud scale scars, the buds in the axils of these scars (and occasionally in previously undeveloped lateral buds nearby) begin development during the autumn and winter following pruning. Development and emergence of shoots arising from these buds was not followed in this experiment, but it appeared that such shoots would be somewhat smaller and emerge later than the control shoots and those subject to treatment A. Leaf primordia numbers given in the results are indicative only, given the evident variation among trees with respect to nodes/annual shoot increment (Table 7.2.1) and a strict comparison would follow the strategy described at the beginning of this discussion.

A study of growth potential of axillary buds in roses by Marcelis- van Acker (1994) found that after release from apical inhibition, shoots arising from axillary buds bore fewer leaves and greater numbers of scale and transitional leaves, and non-elongating nodes with increasing bud age. It would be reasonable from the observations here to presume that, for the initial growth cycle following pruning, the same would be true of replacement axes in *Tasmannia lanceolata.* The development of only a few leaves on shoots at the lateral buds below a 'type A' pruning treatment, together with the presence in some cases of a well advanced (for the time of year) terminal bud at the apex of this shoot suggests that a 'second flush' of growth might arise from these shoots sometime during summer and after maturation of the initial group of leaves, possibly generating *in toto,* an annual shoot increment with as many nodes as the unpruned apices. For the lower growth axes, as with the Marcelis-van Acker (1994) finding, fewer leaf primordia were present at the completion of the experiment, although it is not possible to project this observation forward to future shoot and node development.

To compare the contribution of the successive season's shoots to total leaf number/ canopy, several factors apply:
1) number of shoot axes and the rate of shoot multiplication from year to year
2) number of nodes per axis

3) extent of retention of leaves for more than one full annual cycle.

Firstly, since the normal growth habit tends to result in an increasing number of shoot axes/ canopy each year, and for the trees surveyed in this season at least, the node number per shoot did not appear to fall in any tree (Table 7.2.1: 'new' *vs* 'leaf no.1993'), it might be assumed that total leaf number/canopy increased substantially from 1994 to 1995.

This inference cannot be generalised for all years in mature trees since there are several factors which may result in no increase, or a decrease in number of new leaves per year:

-failure of one or several of the distal buds to produce a shoot, due to a particularly heavy flower or fruit production or severe insect attack,

-seasonal conditions resulting in development of short shoots, bearing few leaves.

Secondly, inspection of the result in Table 7.3.1 for total leaf number present/shoot cohort reveals that in every tree but one, the total number of leaves per shoot on current season's growth exceeds the total surviving 'per shoot' for the two previous cohorts (1yo and 2yo) combined.

A general conclusion for this is that the majority of leaves on the tree at this time (the completion of shoot extension) would be less than four months old, and further, assuming the pattern of leaf loss observed is typical, then at any time it may be assumed that the most recent shoot cohort bears the substantial majority of the leaf area of the plant. The implications of this for harvest and whole plant productivity are clear, since comprehensive removal of the recent shoot cohort will drastically reduce total leaf area for the tree.

The indicative data for limb number and crown shape show only the wide range in both parameters at the site. Trees with a single trunk to 1 metre above ground level are quite unusual in the natural Tasmanian population, reflecting the consequence of loss of the primary apex to external agents, (usually invertebrate) and the propensity for producing multiple, near-equivalent shoots from the cluster of terminal and subjacent lateral buds found at the end of each shoot increment. From a commercial point of view, this habit might be encouraged, perhaps by early pruning in the case of a dominant and persistent terminal apex to stimulate production of multiple stems to aid early canopy closure in an intensive 'orchard' system.

CHAPTER 8: GENERAL DISCUSSION

Clearly from the result obtained in the survey of local populations of *Tasmannia lanceolata*, the scope for adjustment of the extract composition at the plant selection level is considerable. Altering solvent type or extraction protocol were shown to influence the composition of leaf extracts, but among the small group of plants sampled in the survey of twelve sites in Tasmania, levels of many compounds detected ranged over several orders of magnitude. Similarly, overall extract yield exhibited a wide range - from 0.9 to 13.3% of dry leaf weight. As long as commercial production relies on acquisition of leaf from unselected sources, results will fall somewhere between these extremes.

The first step in 'domesticating' selected plant material would not appear to present great difficulties. The species strikes readily from cuttings, and provided parent plants are of reasonable size and vigour, production of a basic stock of several thousand plants from a single parent plant could easily be achieved within a twelve month cycle. The use of semi-hard (autumn) or hard (late winter) cutting material, and application of bottom heat to the rooting medium results in rapid and vigorous root development after ten weeks. Simulation of long, warm days for dormant stock plants resulted in immediate expansion of buds and production of suitable cutting material in early winter, so it would appear that there exists scope for 'doubling up' on cutting cycles if suitable material was limited.

An immediate requirement for a selection programme is the preparation of an inventory of extract 'fingerprints' for as much of the natural population across its range as is feasible, and the development of an industry 'standard' for compositional and organoleptic preference. The first of these may depend upon the sampling and analysis technique developed here. Screening large numbers of plants and generating yield and composition data for each must rely on as few samples as possible per plant, and analysis of the solvent supernatant over finely ground leaf material after a short period of shaking, (used throughout the 'seasonal variation' experiment), was shown to reproducible results with small (less than 200 mg) samples of dry leaf per analysis.

While this analytical procedure proved very reliable for the comparative purposes of the experimental work undertaken here, a number of issues regarding the procedure were not completely resolved. Only a few of the many terpenoid compounds observed using the gas chromatographic procedure were clearly identified, and of these, FID response for polygodial alone, is reported. Secondly, a number of injection artefacts generated from pure polygodial in experimental analyses were not incorporated in the

summation for polygodial used in the seasonal trial. Physical factors affecting these conversions were not examined, and the extent to which the minor peaks were represented in analyses appeared to vary from time to time. These matters are the subject of continuing experimental work.

Lastly, the means of reporting composition does not account for the fraction of extract (up to 50%) which is not detected by the GC method used. The analysis method detects only a limited range of volatile secondary metabolites, predominantly the terpenoid compounds characteristic of most flavour and fragrance products, and does not include the large number of high boiling point compounds which may be present in 'concretes' and 'absolutes' which contribute little to the taste and smell of the product. Should the physical consistency of the extract prove to be of commercial significance, consideration of further preparation techniques may be necessary, for example preparation of a cold ethanol-soluble absolute would remove some waxy compounds and result in a less viscous product.

Consistent with the available market information regarding requirements for flavouring and fragrance extracts, a protocol is proposed which will produce a consistent suite of the solvent soluble compounds found in the leaf. Use of dried leaf material for extraction is indicated for two reasons.

Firstly, separation of fresh leaf from the twigs, and the comminution of green plant material present additional technical problems, and appear unwarranted when the extracts resulting from fresh material (even using a more hydrophilic solvent) are examined. In most cases, slightly less of each of the compounds monitored was obtained using petroleum ether on fresh material than with dry leaf, and still less with a mixed hexane: acetone solvent. Secondly, commercial operations will benefit from the flexibility of using dried leaf material. Fresh leaf may be gathered during a limited period, dried and stored until required. The alternative - freezing of fresh plant material - is expensive and introduces additional technical problems during the freezing and thawing operations. Effective extraction plant capacity is increased by extension of the processing period, and the milling (comminution) of dry leaf requires less specialised equipment.

The drying regimen indicated by the results - approx. 35°C, 48 hours and the use of a circulating fan - provides the most basic guide for the commercial situation. Drying rates are largely dependent on interaction between the equipment used and the nature of the plant material. The experimental oven used here to dry small quantities of leaf resulted in rapid moisture loss over the 48hr period, and probably represents an idealised situation. The system did demonstrate, however, that leaf <u>will</u> dry at 35°C in 48hrs, and that with respect to extract composition, no detectable loss of volatiles occurred under these conditions.

Predictably, the finely comminuted fraction of leaf powder extracts quickly, and the demonstration that an important part of the extract is held in discrete, reinforced

structures embedded in the mesophyll, confirms the need for mechanical disruption of the tough, dry leaf before exposure to solvent. The equipment used to prepare the milled material resulted in a fairly wide spectrum of particle sizes and consequently, an extended extraction period, suggesting that ideally, all leaf material should be reduced to the finest size (less than 0.2mm in this case). In practice, the commercial solution will be a compromise between speed of extraction and the cost of the fine milling. Two aspects of this should be treated cautiously - preparation of finely milled material may result in unacceptable heating of the powder, depending on the equipment used, and preparation of pellets from the fine fraction alone was not attempted and may prove more difficult unless an inert binding agent is incorporated to prevent disintegration during handling. The experiment also demonstrated that solvent saturation by extracted compounds did not limit the extraction process even when concentrations of 2% w/w of solvent were attained.

Pellet preparation, a technique already employed with hops and pyrethrum provides several simple physical advantages - reduced volume of plant material, ease of handling and storage and more complete removal of extraction solvent from amongst the plant material. Extraction of pelletised, rather than loose leaf powder resulted in slight alteration to the proportions of some of the compounds monitored, but a faster, and ultimately more complete recovery of total extract. This last aspect may be due more to reduced loss of extract-rich solvent with the discarded pellets than a failure to dissolve compounds. Repeated washes of powder might retrieve more extract but would certainly increase the cost of extraction markedly.

The confirmation that prepared product remains stable over at least a twelve month period will allow annual adjustment of harvest strategies and an informed commercial choice between strorage of dry leaf material, increased extraction plant capacity, and storage of prepared product when matching supply and demand for product.

As explained in the literature review, it is important to distinguish, for practical purposes, between the contents of specialised glandular structures, and the composition of the essential oil or extract as a whole. This applies whether the structures are specialised multicellular systems within, or external to the main plant body or, as in the case of *Tasmannia lanceolata*, simpler, intracellular structures having the funtional role of separating their contents from the metabolic pool.

In either case, distillation or extraction procedures do not discriminate between the contents of these systems and the range of other susceptible compounds found in the plant. Investigation of biochemical interactions, the production of commercial oils and studies of chemically mediated interactions between plants and their pathogens, competitors and predators all depend upon information about the spatial distribution of the compounds concerned. So long as most of the literature relating to changes in, and the functional significance of secondary compounds relies upon generalised extractions for compositional data, important distinctions may be overlooked - patterns of

synthesis, storage and secretion of these compounds often reveal subtle aspects of the strategies involved.

An example of this is Kobiler *et al* 's (1993) observation that certain antifungal compounds in the mesocarp of avocado fruit are sequestered within oil cells and are ineffective in preventing fungal infection in undamaged ripe fruit, while in the pericarp, containing no oil cells, the same compounds are present and effectively inhibit fungal infection.

Polygodial has been shown to possess powerful (and sometimes destructive) biological activity and the isolation of the compound in impermeable structures might be a self-protective strategy. The compound might then be released by injury of leaf tissue, and serve to prevent further browsing or pathogenic invasion.

As explained in the literature review, oil cells occur in several polygodial-containing species - *Drimys spp*, and *Pseudowintera* , but are also found in many other Magnoliaceous families, in particular members of the Annonaceae and Lauraceae and conform to a similar structural and developmental pattern in most familes in which they are reported. It would seem reasonable to propose a similar functional significance to the structure in these instances as that suggested here for *Tasmannia lanceolata* and by Kobiler *et al* (1993) for avocado fruit - that defensive and potentially phytotoxic secondary products are sequestered within them and are released upon injury.

This proposition then invites an examination of the method of accumulation and storage in other organisms in which polygodial (or any other compounds identified in oil cell systems) has been detected.

As reported in the literature review, in the case of the nudibranch *Dendrodorus spp.* , polygodial is found only in the mantle of the mollusc, where it serves as a chemical defence against predatory fish, but is toxic to the animal if injected directly in similar quantities (Cimino et al 1985). Nudibranch molluscs exhibit a variety of secretory systems in their mantle tissue, specialised arrays of cells often containing active defensive compounds such as sulphuric acid without, apparently, harming the organism, and which are usually ejected when predatory attack seems likely (Thompson 1976).

Similarly, the *Porella* liverwort species belong to an order (Jungermanniales) in which oil containing structures are a feature of the gametophyte leaf, characterised by single or multiple oil bodies within cells with additional thickening at wall junctions, but whose function has not been elucidated (Schofield 1985). An antiherbivory role might be suspected if these cells in fact contained polygodial or any of the related sesquiterpenes found in extracts of these plants.

'Oil cavities' are described in *Polygonum hydropiper* as sites of concentration of polygodial by Hagendoorn et al (1994), although they do not describe any structural specialisation for these features. Lastly, no reference was found to the occurrence of an oil storage system in the Cannellaceae to which the *Warburgia* tree species belong.

The concept of the impermeable oil cell system might lead to detection of analogous structures in each of the above cases, although no direct reference other than those described was found at this time.

In the particular case of *Polygonum hydropiper* several attempts to produce the compound or relevant synthetic enzymes using cell and tissue culture have been reported (Hagendoorn *et al* 1994, Banthorpe *et al* 1989, 1992) in an effort to avoid 'cultivation (which) ....is laborious and inefficient' .
If, as is suggested above, accumulation of this, and other potentially phytotoxic compounds does require specialised storage structures, single cell culture for polygodial production seems unlikely to succeed. Indeed Banthorpe *et al* (1989) report extensive necrosis in cell cultures and maximum levels of polygodial far below those found in intact plant material, suggesting that when synthetic activity occurs but sequestration in special structures does not, physical destruction of the cell system rather than equilibrium dynamics may limit accumulation.

Clearly since, of the many extract components obtained from leaves of *T. lanceolata* , only polygodial is stored in the only obvious secretory structure, the question of where other lipid soluble terpenoid compounds are to be found arises. Many will be associated with the normal intracellular synthetic structures- plastidial membranes, dictyosomes, mitochondria etc, in lipid solution or as free oil droplets within the cytoplasm, and accordingly might be expected to remain involved in normal metabolic activity within the cell. However, the finding here, common to many studies of seasonal patterns in levels of terpene compounds in plant tissue, is that following intense synthetic activity during which many compounds gradually accumulate, a prolonged period of relative stability occurs. In this, the patterns parallel that for the sequestered polygodial, suggesting that, while no obvious spatial or structural isolation mechanism is identified, the compounds in question are somehow metabolically isolated- either in equilibrium with precursors and products, or lacking appropriate enzyme systems preventing further changes in concentration after accumulation is complete.
Turnover in certain secondary metabolites obtained from essential oil species is subject to some debate - the many reports of re-utilisation of some of these either within the storage organ, or elsewhere in the plant body are reviewed by Croteau (1988). On the other hand, the recent papers of Mihaliak et al (1991) and Gershenzon et al (1993) question whether the turnover often reported for monoterpenes and determined in radiolabelling experiments may not, in some cases, have been artifactual, and characteristic only for the cutting material often used in such experiments.
Details of the localisation of selected terpene compounds found in extracts such as that of *T. lanceolata* , would help to resolve some of these issues, and perhaps identify other intracellular storage or synthetic systems. With *T. lanceolata*, compounds such as cadina- 1,4 -diene, often found in leaf at more than 1.0 -1.5% of the dry weight of leaf

would appear to be amenable to a histochemical or radiolabelling approach combined with ultrastructural examination.

The second aspect of the distribution of active secondary compounds of interest is the temporal one. The experiments conducted in this project have shown that, as is the case with many examples of plant secondary metabolites, a distinct cycle of synthetic activity followed by storage or isolation of the products and a slow decline in absolute quantity with advancing age of the organ in question may be observed in leaves of *Tasmannia lanceolata* .

With respect to commercial extract production, yield and composition are the parameters of most interest. Manipulation of composition in the manner proposed for peppermint (Clark and Menary 1979) or parsley (Porter 1989a) by adjusting harvest time is unlikely to offer much scope in the case of *Tasmannia lanceolata,* at least in relation to most of the compounds monitored here. Relative proportions amongst these compounds (except for bicyclogermacrene and aristolone) do not alter greatly during the first 150 days of shoot growth and only a temporary and slight increase in levels of linalool was detected during the first dormant period thereafter. Since two year old leaves do not exhibit significantly different relative levels of the compounds, the extract obtained from any or all of the current season's growth and that of the previous year would not be expected to alter greatly with respect to the proportions of these compounds during the annual cycle.

During a short period at the beginning of the growth cycle, the small dry matter contribution from new leaf will contain slightly less of most of these compounds (but in the same relative proportions) than the previous season's leaf or the same leaf at maturity, but this will alter only percentage yield w/w of extract compounds, and hence is relevant only in consideration of cost of extraction. This assumes no contribution to dry matter from buds, flowers and fruit, all of which will, at some time, be present in the harvestable parts of the plant.

Clearly the maximum yield by weight of extract containing the characteristic proportions of the major (reported) components is obtained by harvesting fully mature leaf of the most recent growth increment. This corresponds to late summer, before loss of leaf dry matter (leaf fall and browsing damage) and the slow, steady fall in concentration of these compounds in the leaf commences during late autumn and winter. This experiment monitored only a few of the many compounds present reflecting only that there is presently no specific commercial requirement of the extract. Nonetheless, the data gathered do include GC-FID peak areas for the full range of volatile components between linalool and polygodial, (spanning the range of usual interest to perfume and flavour consumers) for all date and tree loci and could be readily reviewed for identified compounds of interest.

From a chemosystematic standpoint the results are both positive and problematic. Firstly it can be seen that except with very small, undeveloped leaves, a 'typical' extract analysis will result from most leaf samples, such as the *Tasmannia lanceolata* specimens used in the report of Southwell and Brophy (1992). A similar observation by Simmons and Parsons (1987) led to the conclusion for eucalypt species that sampling during autumn and winter would eliminate leaf ageing and seasonal effects and allow meaningful comparisons between trees or locations.

On the other hand the quite extreme variation between individual trees reported in the preliminary survey, and supported by the observations at the Parrawe site suggests that great care should be taken in employing the notion of chemotype with this species, certainly in the Tasmanian context. The report of Southwell and Brophy (1992) lists linalool, 1,8-cineole, caryophyllene, and bicyclogermacrene among the major constituents of the ethanolic extract of *T. lanceolata* and this was also true of the extracts examined in this report with the notable omission of cadina- 1,4 -diene (not found in *T. lanceolata)* and polygodial (not identified by the analytical procedure) in the Southwell and Brophy investigation. That paper notes that high 1,8 cineole levels could be considered characteristic of the species, when compared with *T. glaucifolia* (high safrole), *T. insipida* (high viridiflorol or isomer) or *T. xerophila* (myristicin) for example. Unfortunately, while a peak representative of 1,8-cineole appeared consistently in the analyses conducted in this project, it was not well distinguished from a neighbouring peak by the analytical method and was not included in the routine analyses for seasonal variation.

As with the Southwell and Brophy (1992) investigation, polygodial was not obtained to any extent in the steam volatile oil but remained (albeit at quite low levels) in the extracted leaf material. The distilled oil, rich in monoterpene components including pinenes, linalool, and 1,8 cineole also contained guaiol, found by Stevens (1955) in saturating concentrations in oils from some locations, and also obtained by extraction of dry leaf material in routine analyses, although again, this was not included in the seasonal comparison because of problems with occasional overlapping of peaks.

Changes in the levels of specific compounds in leaf material from budburst to maturity are relevant to considerations of defensive strategies, but for this species, there have been few reported studies of insect and herbivore interactions with the plant. Read and Hill (1983) describe the consumption and regurgitation of ripe fruit by several bird and marsupial species in relation to distribution and establishment patterns. Observation at the study site during the conduct of this investigation showed the presence of a number of leaf mining larvae, stem borers, leaf eating larvae and very small larvae developing in the ripening fruit. In the experimental glasshouse, occasional infestations of common scale and mite species have necessitated chemical control methods. Clearly in these cases some adaptive advantage befalls invertebrates able to eliminate or metabolise the high levels of polygodial present in seed and leaf tissue when the compound has been

shown to deter a range of other common herbivorous insect species from feeding (Schoonhoven and Fu-Shun 1989, Powell et al 1995 and others).
Seasonal and intraspecific variation in antiherbivory compounds is reported for a number of other species and there have been several attempts to correlate this with cycles of herbivore browsing pressure or insect pest ecology (reported in the literature review p. 38). In view of the antifeeding activity reported for polygodial, the pattern of insect attack observed in *T. lanceolata* in the natural setting should be considered in the context of polygodial levels in the bud, new leaves and shoots (personal observations).

Some trees appeared to suffer more insect damage than others but this was not noticeably associated with low levels of polygodial in those individuals. Furthermore the most destructive period of insect attack appeared to occur sometime during the dormant period when many terminal buds were destroyed by an egg laying insect but this coincided with a time of relatively high polygodial concentration, apparently contradicting the simple proposition that the compound protects against insect damage. On the other hand, the occurrence of high levels of polygodial in leaves at the commencement of their period of maximum contribution to photosynthate production would appear to represent a timely defence investment if a deterrence effect could be demonstrated. This was not included in the scope of this work, although a substantial literature relating to the antiherbivory effects of the compound, and the presence of a number of leaf eating insect larvae in the canopy of mature trees suggests the possibility of such a strategy, and may provide a clue as to the pioneering success of the species.

The question of recovery of energy-costly secondary metabolites at the end of the photosynthetic life of the leaf was not fully addressed in the experimental work here. The oldest leaf samples taken in the experiment were attached to the tree, and although in these, most of the compounds monitored were at lower levels than found in new leaves, they still contained more than 4% w/w of volatiles in dry leaf, compared with 5% in newly mature leaf on the same trees. No suggestion of turnover or interconversion of the secondary compounds followed in the course of this experimental work could be discerned.

From the point of view of the commercial interest in obtaining high yields of extract on a regular (probably annual) cycle from managed plantations of *Tasmannia lanceolata*, the work undertaken in this project provides a physiological basis for further investigation of the 'plantation' approach.
In considering the effect of different light levels on leaf morphological and physiological characteristics relating to leaf and oil production, Björkman's (1981) general conclusion is relevant- while in sun species, photosynthetic rate and the ratio of mesophyll area to leaf area tend to vary together, the same is not true with obligate

shade species. The results appear to suggest a situation closer to the latter extreme - the highest light level induced the expected change in mesophyll morphology (increased area/area of leaf) but did not produce a similar increase in photosynthetic rate. This suggests that other aspects of the photosynthetic system are limiting, for example, that enzyme components might be at saturating concentration in the chloroplasts.

Accordingly, the experimental results suggest that *Tasmannia lanceolata* behaves as a shade plant, most of the indicators pointing to improved photosynthetic performance with increasing light level up to about 150μmol m^{-2} s^{-1}. At twice this level of irradiation, increased dry matter content and mesophyll thickness have not improved photosynthetic performance and levels of secondary products (w/w) are reduced.
This leads to the general observation that high irradiance sites will not necessarily aid 'productivity' in the strict sense, since extract is the product being sought. Such sites may indeed lead to some photoinhibition, and alteration of leaf morphology.
More particularly, harvest of the complete outer canopy, bearing almost all of the leaf area fully adapted to the existing radiation levels, may in some situations stimulate an adverse response in previously shaded older leaf upon which total reliance for further photosynthate production will fall.
In practice of course, the change in light level experienced by each leaf will be quite specific to the aspect, time of year, position on the canopy, leaf angle, absorptivity of the harvested canopy etc. The capacity to adjust leaf angle and the photosynthetic apparatus to the new circumstances will dictate the effectiveness of the leaf in its reinstated role. For these reasons, this study can only be regarded as indicative of the capacity of typical leaves for acclimation to altered light levels.
Clearly the experience of vineyard and orchard production is relevant, wherein, in most cases maximum light interception is sought, and much research has been undertaken in comparing plant spacing, density, canopy orientation and site selection for their effects on light interception. The provision of artificial or natural shade might also be considered in this context.
The implication of the above, and the observations with regard to canopy growth response, is that the species, as represented by the mature specimens at the study site, appears quite 'forgiving' in the sense that both physiologically and ontogenetically, established trees are fully capable of recovery after removal of all but the first node of the recent season's growth. The vigour of such recovery growth was not studied in detail, and would certainly vary from tree to tree, so that from this work it is not possible to conclude that an annual harvest could be assumed, but rather that repeated harvest cycles, probably biennial, could confidently be considered sustainable.
Several other issues in relation to canopy development for commercial productivity and harvest should be considered.
Firstly the establishment of a basic canopy framework bearing a full diversity of potential growth points occurs during a non-harvest development period. During this

time certain aspects of the natural canopy architecture might be amenable to promotion of the natural tendancy for branching, increase of the length and node number of the periodic shoot increment and perhaps the number of such increments per year. Secondly, clonal selection will help remove variation in response between individual trees to insect damage, pruning intervention and other cultural practices which could confound attempts to construct a uniform canopy shape for mechanical harvest. Lastly, the phenology of shoot flushing may be altered by a regular harvest regimen, particularly if exhaustion of the reserve of viable axes occurs over several cycles of growth and harvest, and the effect of these techniques on carbohydrate cycling and storage clearly requires further study.

Conclusion

The essential oil industry relies largely on the production of established commodities to strict standards, and represents a diversity of international consumers. Introduction of a novel, and distinctly Tasmanian product has obvious commercial possibilities, and for *T. lanceolata* , the technology for extraction and product preparation, while sharing much established technology, clearly requires further research. Commercial quantities, of consistent composition tailored to market requirements must be produced, both by a combination of suitable plant material and careful control of the extraction and preparation of the product. A strategy for selection of suitable material for extract production from a variable natural resource depends upon the relative importance of yield and composition, the former influencing variable costs of harvest and extraction, the latter the price and volume of extract the market will absorb.

The work reported in this thesis provides a starting point for addressing many of the technical problems which will arise during the development process. The establishment of a 'seasonal' setting for the basic processes of propagation, planting, pruning and harvest will enable the initial step towards a managed production system. Post harvest, the extraction and analytical procedures provide a reliable formula for preparation of a high quality product. Both these areas will undergo further development and 'fine tuning' as market requirements are discerned and practical experience is gained.

From the biological viewpoint, the experimental observations also raise several questions regarding the physiological processes and significance of the oil synthesis and storage system in this and related species and may also contribute towards resolution of some of the ecological issues regarding defence, successional status and the energy budget in *T lanceolata* .

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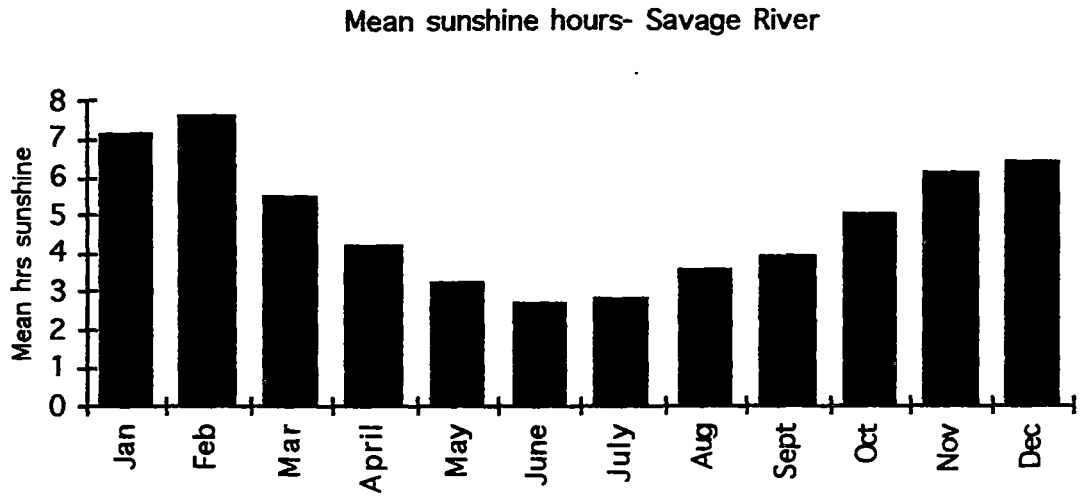
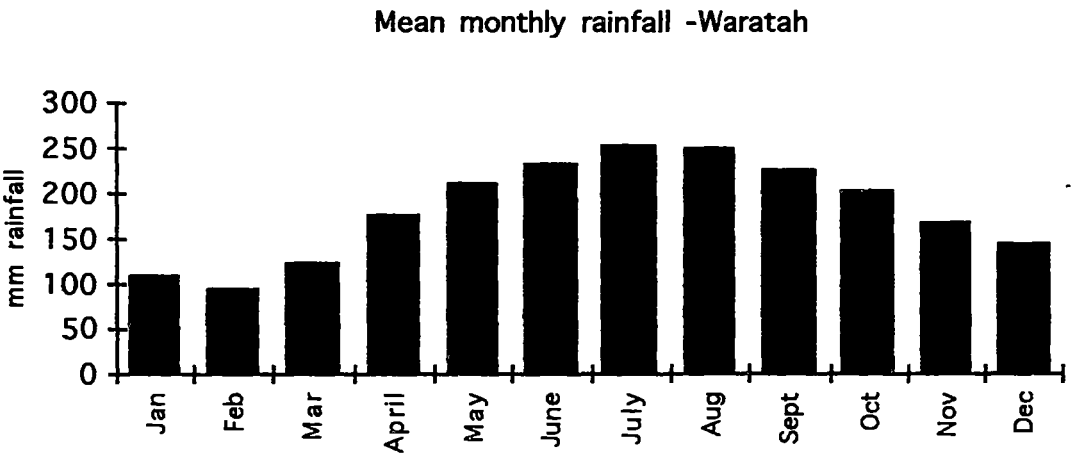
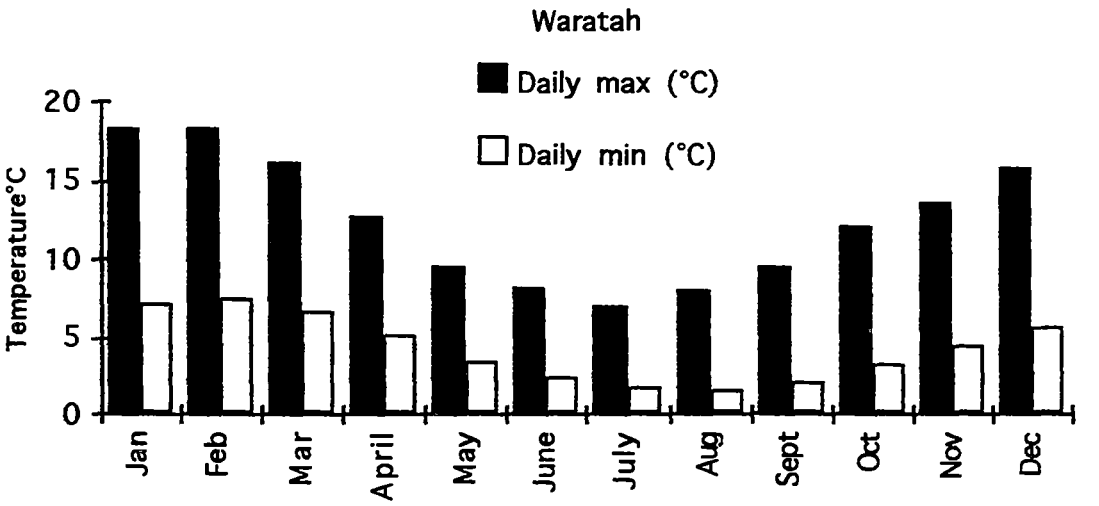
List of Appendices

Appendix 1.0

Appendix 2.0







Appendix 1.1: Climate data - Bureau of Meteorology stations 097047 and 097014. Sunshine hrs- mean for1966-1989; Rainfall data mean for 1882-1993; Temperature data 1882-1973

% w/w leafDM	Retention	N1	N2	N3	N4	N5	N6	D1	D2	L1	L2	L3	W1	W2	W3	W4	W5	W6	G1
Extract yield	Time(min)	3.28	2.58	2.08	2.17	3.41	2.49	1.65	1.36	0.88	2.44	2.83	5.43	9.56	5.44	13.15	10.78	13.3	4.16
1,8 cineole°	5.47	0.030	0.014	0.021	0.034	0.004	0.019	0.004				0.003	0.034	0.008	0.020	0.020	0.027	0.024	0.032
linalool	6.02	0.161		0.002	0.010	0.030	0.030	0.008			0.005			0.039	0.119		0.097	0.219	0.081
a-terpineol	6.91	0.013	0.006	0.007	0.007		0.009	0.002				0.003		0.008	0.011				
piperitone	7.49	0.005	0.009		0.007	0.006	0.004	0.003	0.002		0.005	0.008		0.016	0.008		0.019		0.033
eugenol	8.61	0.048	0.022	0.044	0.038	0.052	0.026	0.009	0.003		0.014				0.028		0.023		0.044
a-cubebene	8.91			0.011	0.003	0.007	0.012				0.004		0.400	0.291	0.013	0.836	0.521	0.602	
caryophyllene	9.92	0.018	0.048	0.008	0.017	0.054	0.019	0.036	0.088	0.021	0.032	0.014	0.196	0.075	0.042	0.224	0.148	0.400	0.021
Unknown I	10.29	0.004	0.007	0.002	0.002	0.004	0.005	0.003					0.047	0.021		0.085	0.054	0.040	0.012
germacrene D	10.76	0.005	0.006	0.003	0.002	0.006	0.004	0.031	0.059	0.002		0.008	0.233	0.173	0.013	0.515	0.315	0.368	0.127
unknown IV	11.00	0.099	0.111	0.043	0.021	0.049	0.039	0.046	0.043	0.029	0.037	0.036	0.214	0.074	0.114	0.072	0.197	0.112	0.221
calamenene°	11.26	0.006		0.016	0.009	0.018	0.004	0.004	0.014		0.004	0.004	0.342	0.509	0.012	1.324	0.745	0.859	0.012
Cadina1,4-diene	11.55	0.006	0.021	0.016	0.012	0.029	0.004	0.002	0.002	0.003	0.030	0.027	1.647	1.425	0.034	3.992	2.345	2.878	0.051
unknown II	12.19	0.010	0.006	0.004	0.002	0.008		0.010	0.022	0.003	0.005	0.006	0.128	0.141	0.016	0.418	0.263	0.302	0.012
unknown III°	12.47	0.161	0.126	0.103	0.098	0.152	0.040	0.017		0.015	0.194	0.175	0.236	0.359	0.215	0.986	0.736	1.096	0.288
guaiol	12.71				0.002	0.005		0.017	0.059			0.006	0.008	0.008	0.006	0.025	0.016	0.028	0.006
aristolone	15.22	0.023	0.013	0.011	0.010	0.006		0.018	0.004	0.006	0.011	0.009	0.021	0.051	0.021	0.089	0.106	0.031	0.014
drimenol	15.44	0.021	0.019	0.014	0.016	0.041	0.016	0.012	0.003	0.004	0.018	0.042	0.007	0.006	0.054	0.024	0.065	0.045	0.027
Polygodial†		0.476	0.705	0.410	0.113	0.829	0.566	0.466	0.053	0.080	0.499	0.817	0.134	0.233	1.279	0.924	0.985	1.479	0.962
kaur-16-ene	20.92	0.046	0.029	0.060	0.006	0.020	0.015	0.005	0.009	0.032	0.040	0.033		0.036			0.089	0.076	0.043
% volatiles*		1.33	1.30	0.92	0.61	1.55	0.97	0.91	0.58	0.34	1.06	1.37	4.25	6.97	2.31	10.84	7.94	9.87	2.59
% non volatiles#		1.95	1.28	1.16	1.56	1.86	1.52	0.74	0.78	0.54	1.38	1.46	1.18	2.59	3.13	2.31	2.84	3.43	1.57
Extract consistency		solid	solid	solid	solid	solid	solid	solid	solid	solid	solid	solid	liq	liq	solid	liq	sol	liq	solid
Shaded habitat?		no	no	no	no	no	no	no	no	part	y	y	y	y	y	y	y	y	n
Fruit in evidence?		y	n	n	y	n	y	y	y	n	y	y	n	y	y	n	n	y	n
Stem colour		orange	red		o	r	ro			o	r	r	o	o			ro		

° Peak area may include other compounds

* Total area integration between peaks at 5.47 mins and 20.92 mins (ex. C18 std)

#% concrete/DM - % Vols/ DM

Appendix 2.1: Composition data and extract characteristics for preliminary collections, Sect. 3.5

% w/w leafDM	G2	G3	G4	G5	G6	P1	P2	P3	P4	P5	P6	H1	H2	H3	H4	Y1	Y2	S1	S2
Extract yield	4.11	4.87	1.14	1.22	1.56	2.82	2.92	4.92	1.72	1.77	2.27	3.38	9.83	6.45	7.04	11.61	13.17	0.88	3.3
1,8 cineole°		0.007		0.006	0.006	0.034	0.055	0.051	0.007	0.013	0.019	0.031	0.021	0.017	0.007	0.021	0.016	0.004	0.013
linalool		0.055	0.008	0.018	0.043	0.006	0.005				0.009	0.020	0.024	0.066	0.030	0.042	0.153		0.005
a-terpineol						0.016	0.015	0.006	0.005	0.009	0.005	0.004							
piperitone		0.015		0.003	0.007	0.003	0.006	0.005			0.015	0.006					0.014		
eugenol	0.047	0.051	0.022	0.008	0.022	0.056	0.057	0.049	0.010	0.007	0.043	0.017		0.035	0.058	0.064	0.051	0.006	0.012
a-cubebene	0.016		0.003			0.010	0.003	0.281		0.021		0.014	0.977	0.443	0.610	0.758	0.869	0.002	0.150
caryophyllene	0.006	0.070	0.038	0.054	0.038		0.072	0.109	0.076	0.013	0.106	0.059	0.173	0.128	0.144	0.193	0.195	0.036	0.069
Unknown I			0.003	0.003			0.012	0.020				0.004	0.354	0.092	0.152	0.134	0.275	0.001	0.031
germacrene D	0.117		0.007	0.024	0.004	0.014	0.026	0.136		0.007	0.004	0.041	0.600	0.199	0.377	0.432	0.524		0.089
unknown IV	0.081	0.094	0.155	0.159	0.071	0.124	0.178	0.092	0.070	0.029	0.090	0.064	0.064	0.103	0.089	0.088	0.103	0.060	0.056
calamenene°		0.035	0.008	0.011	0.003	0.008	0.070	0.543	0.007	0.105	0.018	0.034	0.607	0.439	0.496	0.805	0.953	0.005	0.174
Cadina1,4-diene	0.046	0.104	0.008	0.006	0.009		0.041	0.650	0.009	0.035	0.016	0.062	3.702	1.586	2.367	3.168	3.748	0.013	0.441
unknown II	0.020	0.018				0.008	0.045	0.115		0.010	0.009	0.019	0.289	0.134	0.215	0.355	0.451	0.001	0.051
unknown III°	0.308	0.614	0.076	0.075	0.057	0.007	0.081	0.240		0.010	0.009	0.034	0.457	0.330	0.350	0.829	1.140	0.036	0.181
guaiol		0.015					0.023	0.018				0.030	0.015	0.012		0.027	0.034		
aristolone	0.291	0.036	0.005	0.007	0.016			0.005				0.041				0.204	0.100		
drimenol	0.026	0.035			0.009	0.017	0.043	0.012	0.013	0.013	0.020	0.027							
Polygodial†	0.745	1.080	0.051	0.054	0.231	0.522	0.072	0.019	0.011	0.062	0.031	1.094	0.116	0.271	0.207	1.217	1.389	0.012	0.019
kaur-16-ene	0.051	0.031	0.041	0.028	0.024	0.048	0.039	0.056	0.059	0.072	0.081	0.015	0.065	0.022	0.067	0.052	0.096	0.055	0.123
% volatiles*	1.99	2.71	0.77	0.60	0.72	1.00	1.87	3.23	0.44	0.56	1.06	1.99	8.31	4.57	5.86	9.62	11.17	0.36	1.79
% non volatiles#	2.12	2.16	0.37	0.62	0.84	1.82	1.05	1.69	1.28	1.21	1.21	1.39	1.52	1.88	1.18	1.99	2.00	0.52	1.51
Extract consistency	solid	solid	solid	solid	solid	solid	solid	solid	solid	solid	solid	solid	liq.	liq.	liq.	liq/thin	liq/thin	solid	solid
Shaded habitat?	50/50	50/50		n	slight	n	n	n	50/50	y	y	n	n	n	n	full	full	n	n
Fruit in evidence?	n	y	n	y	n	n	y	n	n	n	n	o				n	y	y	n
Stem colour							crimson	r	r	r	r	r	r	r	r	r	r	bronze	bronze

° Peak area may include other compounds

* Total area integration between peaks at 5.47 mins and 20.92 mins (ex. C18 std)

#% concrete/DM - % Vols/ DM

Appendix 2.1: Composition data and extract characteristics for preliminary collections, Sect. 3.5

% w/w leafDM	S3	S4	S6	Q1	Q2	Q3	Q4	Q5	Q6	M1	M2	M3	M4	M5	M6	B1	B2	B3	B4	B5	B6
Extract yield	3.68	4.02	7.09	8.9	9.09	7.79	8.45	3.98	4.07	5.970	5.870	5.670	5.000	5.450	5.870	5.020	9.490	7.130	5.220	9.050	6.940
1,8 cineole°	0.021	0.064	0.079	0.150	0.034	0.166	0.013		0.024						0.059						
linalool				0.475	0.148	0.232	0.032	0.063	0.018												
a-terpineol	0.019	0.020	0.023	0.045		0.053			0.009												
piperitone	0.009							0.006													
eugenol	0.011	0.027	0.041						0.018	0.021						0.017		0.028		0.071	
a-cubebene		0.017	0.379		0.321		0.306	0.320							0.088	0.074	0.195		0.020	0.173	
caryophyllene	0.050	0.034	0.135	0.166	0.195	0.118	0.152	0.079	0.041	0.006	0.143	0.081	0.056	0.045	0.048	0.057	0.062			0.064	0.050
Unknown I		0.032			0.059		0.063	0.043													
germacrene D		0.094	0.167		0.175		0.182	0.167		0.008					0.059	0.042	0.081		0.013	0.100	0.078
unknown IV	0.136	0.044	0.063	0.116	0.072	0.181	0.133	0.022	0.109												
calamenene°	0.014	0.265	0.763	0.020	0.470		0.313	0.300		0.137	0.261	0.113	0.062		0.239	0.240	0.554		0.070	0.399	0.155
Cadina1,4-diene	0.041	0.511	0.929	0.046	1.088	0.089	1.137	1.145	0.042	0.125	0.620	0.737	0.244	0.213	0.597	0.607	1.287		0.145	0.989	0.378
unknown II	0.015	0.072	0.123	0.059	0.128	0.032	0.139	0.090	0.018	0.021	0.066	0.054	0.028	0.032	0.059	0.054	0.098	0.046	0.021	0.099	
unknown III°	0.280	0.204	0.330	0.602	0.273	0.587	0.595	0.226	0.247	0.078	0.237	0.133	0.112	0.071	0.229	0.220	0.340	0.133	0.065	0.309	
guaiol	0.006		0.012	0.022																	
aristolone	0.012	0.012	0.008	0.016					0.022												
drimenol	0.035	0.027	0.043	0.103		0.062	0.074		0.046												
Polygodial†	0.151	0.077	0.121	2.857	1.836	2.216	1.447	0.202	1.191	0.782	0.339	0.038	0.480	0.319	0.635	0.438	1.341	2.053	1.223	1.664	1.078
kaur-16-ene	0.159	0.167	0.086	0.041	0.056	0.058		0.012	0.024	0.033	0.038	0.092	0.019	0.083	0.085	0.083	0.116	0.087	0.049	0.062	0.101
% volatiles*	1.42	2.37	4.76	5.54	5.78	4.57	5.43	3.05	2.12	1.80	4.61	2.28	3.09	1.93	2.65	2.99	5.38	3.00	1.94	4.91	2.94
% non volatiles#	2.26	1.65	2.33	3.36	3.31	3.22	3.02	0.93	1.95	4.17	1.26	3.39	1.91	3.52	3.22	2.03	4.11	4.13	3.28	4.14	4.00
Extract consistency	solid	solid	solid	liq/thk	liq/thk	liq/thk	liq/thk	liq/thk	liq/thk												
Shaded habitat?	50/50	n	y	50/50	y	y	y	y	y							y	y	y	y	y	y
Fruit in evidence?	n	y	y	n						y	n	n	y	n	y	y	y	n	n	n	y
Stem colour		rb	r/crmsn						bronze												

° Peak area may include other compounds

* Total area integration between peaks at 5.47 mins and 20.92 mins (ex. C18 std)

#% concrete/DM - % Vols/ DM

Appendix 2.1: Composition data and extract characteristics for preliminary collections, Sect. 3.5

ANOVA AND LSD PROCEDURE - CUTTING PROPAGATION

```
00002 data prop;
00003 input season$ block$ temp$ hormone score length dwt;
00004 cards;
00005 A    I    H    0    14    26.0   14.7.....
00052 C    II   L    3    5     0      0;
00054 run;
00057 proc anova;
00058 class season hormone temp block;
00059 model score length dwt = block season hormone temp season*hormone season*temp
00060 hormone*temp season*temp*hormone;
00061 means season hormone temp/lsd;
00062 means season*hormone season*temp hormone*temp season*hormone*temp;
00063 run;
```

Class	Levels	Values
SEASON	3	A B C
HORMONE	4	0 1 2 3
TEMP	2	H L
BLOCK	2	I II

Number of observations in data set = 48

Dependent Variable: SCORE

Source	DF	SSquares	MSquare	F Value	Pr > F
Model	24	574.3333333	23.9305556	3.83	0.0010
Error	23	143.6666667	6.2463768		
Corrected Total	47	718.0000000			

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLOCK	1	21.3333333	21.3333333	3.42	0.0775
SEASON	2	85.8750000	42.9375000	6.87	0.0046**
HORMONE	3	209.5000000	69.8333333	11.18	0.0001**
TEMP	1	90.7500000	90.7500000	14.53	0.0009**
SEASON*HORMONE	6	88.6250000	14.7708333	2.36	0.0631
SEASON*TEMP	2	34.8750000	17.4375000	2.79	0.0821
HORMONE*TEMP	3	3.7500000	1.2500000	0.20	0.8952
SEASON*HORMONE*TEMP	6	39.6250000	6.6041667	1.06	0.4158

Dependent Variable: LENGTH

Source	DF	SSquares	MeanSquare	F Value	Pr > F
Model	24	2307.891667	96.162153	2.66	0.0109
Error	23	830.738125	36.119049		
Corrected Total	47	3138.629792			

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLOCK	1	36.226875	36.226875	1.00	0.3270
SEASON	2	486.750417	243.375208	6.74	0.0050**
HORMONE	3	398.483958	132.827986	3.68	0.0268*
TEMP	1	1197.001875	1197.001875	33.14	0.0001**
SEASON*HORMONE	6	71.297917	11.882986	0.33	0.9148
SEASON*TEMP	2	45.158750	22.579375	0.63	0.5440
HORMONE*TEMP	3	56.445625	18.815208	0.52	0.6721
SEASON*HORMONE*TEMP	6	16.526250	2.754375	0.08	0.9979

Dependent Variable: DWT

Source	DF	SSquares	MeanSquare	F Value	Pr > F
Model	24	1999.556667	83.314861	1.87	0.0697
Error	23	1026.802500	44.643587		
Corrected Total	47	3026.359167			

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BLOCK	1	19.5075000	19.5075000	0.44	0.5152
SEASON	2	319.4379167	159.7189583	3.58	0.0444*
HORMONE	3	196.0175000	65.3391667	1.46	0.2506
TEMP	1	999.1875000	999.1875000	22.38	0.0001**
SEASON*HORMONE	6	192.5887500	32.0981250	0.72	0.6384
SEASON*TEMP	2	30.9537500	15.4768750	0.35	0.7107
HORMONE*TEMP	3	34.4275000	11.4758333	0.26	0.8555
SEASON*HORMONE*TEMP	6	207.4362500	34.5727083	0.77	0.5981

Appendix 2.2: ANOVA and LSD's for cutting propagation methods- sect. 3.7

Hours @ 35°C	Mean %DM	'Volatiles'	linalool	terpineol	caryophyllene	Rt=12.16	cadina1,4 diene	polygodial
Fresh	23.74	-	-	-	-	-	-	-
24	82.78	3.609	0.220	0.032	0.038	0.216	0.051	2.600
36	96.71	4.102	0.240	0.035	0.038	0.254	0.057	2.949
48	96.00	4.614	0.256	0.044	0.044	0.282	0.067	3.397
72	95.90	4.798	0.352	0.056	0.044	0.327	0.066	3.464
96	97.00	4.059	0.247	0.037	0.042	0.274	0.059	3.028
120	97.68	4.993	0.345	0.059	0.040	0.311	0.067	3.568
Std Err.	-	*0.328*	*0.029*	*0.004*	*0.003*	*0.027*	*0.004*	*0.211*

Appendix 2.3a: Mean percentage (as w/w leaf DM) of selected components after drying at 35°C. Standard errors given are for least squares means for three replicates.

Temp. °C	Mean % DM	Volatiles'	linalool	terpineol	caryophyllene	Rt =12.16	cadina1,4diene	polygodial
Fresh	23.77	-	-	-	-	-	-	-
25	93.62	7.693	0.300	0.050	0.047	0.267	0.067	3.860
35	96.00	4.617	0.257	0.043	0.043	0.283	0.063	3.397
55	95.99	4.530	0.280	0.040	0.047	0.267	0.057	3.330
70	100	2.157	0.067	0.010	0.013	0.093	0.020	1.783
Std Err	-	0.767	0.030	0.005	0.003	0.031	0.004	0.262

Appendix 2.3b : Mean percentage (as w/w leaf DM) of selected components after drying for 48hrs at four temperatures. Standard errors are for means of three replicates

Time (days)	Method (see sect. 4.6)						Std Error					
	1	2	3	4	5	6	1	2	3	4	5	6
alpha-pinene	Peak:Std =0.28											
0	0.86	-	-	-	-	-	-	-	-	-	-	-
14	1.37	1.35	1.23	1.27	1.15	1.03	0.04	0.08	0.03	0.04	0.06	0.04
32	0.86	0.85	0.63	0.85	0.64	0.63	0.19	0.23	0.07	0.08	0.07	0.1
60	1.45	1.3	1.21	1.19	0.87	0.98	0.04	0.09	0.07	0.06	0.04	0.03
84	1.2	1.13	1.21	0.95	1.12	1.17	0.04	0.09	0.04	0.07	0.04	0.02
113	0.84	0.66	0.74	0.64	0.6	0.55	0.13	0.12	0.1	0.03	0.14	0.08
140	1.35	1.23	1.19	1.15	1.22	1.1	0.05	0.07	0.04	0.08	0.02	0.1
175	1.35	1.19	1.22	1.18	1.46	1.37	0.03	0.1	0.06	0.02	0.02	0.02
220	1.3	1.09	0.83	0.79	0.54	0.7	0.02	0.2	0.05	0.01	0.09	0.07
295	1.05	0.92	1.11	0.95	1.18	1.17	0.03	0.23	0.15	0.13	0.06	0.06
Linalool	Peak:std =0.55											
0	1.47	-	-	-	-	-	-	-	-	-	-	-
14	1.7	1.7	1.69	1.65	1.63	1.67	0.03	0.04	0	0.01	0.02	0.03
32	1.64	1.58	1.4	1.55	1.47	1.33	0.08	0.02	0.06	0.04	0.07	0.1
60	1.71	1.64	1.66	1.61	1.62	1.6	0.01	0.02	0.01	0.01	0.01	0.04
84	1.63	1.63	1.65	1.65	1.61	1.66	0.02	0.05	0.01	0.02	0.01	0.04
113	1.55	1.49	1.49	1.52	1.47	1.48	0.02	0.05	0.03	0.02	1.2	0.03
140	1.65	1.66	1.62	1.66	1.65	1.61	0.01	0.02	0.02	0.01	0.02	0.05
175	1.68	1.64	1.63	1.62	1.67	1.67	0.02	0.01	0.02	0.02	0.01	0.03
220	1.71	1.73	1.64	1.66	1.55	1.63	0.02	0.03	0.07	0.01	0.02	0.02
295	1.59	1.57	1.55	1.55	1.55	1.55	0.01	0	0.04	0.03	0.07	0.04
calamenene	Peak:std =0.70											
0	3.49	-	-	-	-	-	-	-	-	-	-	-
14	3.71	3.75	3.75	3.7	3.76	3.79	0.05	0.05	0.02	0.01	0.06	0.04
32	3.92	3.79	3.85	3.76	3.92	3.97	0.16	0.01	0.05	0.14	0.11	0.12
60	3.72	3.67	3.75	3.67	3.81	3.91	0.02	0.05	0.01	0.02	0.02	0.08
84	3.66	3.79	3.79	3.91	3.78	4.08	0.04	0.07	0.03	0.03	0.01	0.07
113	3.87	3.88	3.85	3.97	3.88	4.22	0.02	0.06	0.05	0.04	0.31	0.05
140	3.64	3.94	3.74	3.93	3.88	4.12	0	0.1	0.04	0	0.02	0.06
175	3.73	3.82	3.79	3.91	3.93	4.23	0.04	0.06	0.04	0.04	0.03	0.09
220	3.82	4.11	3.96	4.11	4.02	4.43	0.04	0.2	0.2	0.01	0.01	0.05
295	3.555	3.745	3.71	3.88	3.99	4.26	0.01	0.01	0.07	0.07	0.14	0.04
Cadina 1,4 diene	Peak:std =0.71											
0	4.21	-	-	-	-	-	-	-	-	-	-	-
14	4.64	4.6	4.61	4.43	4.49	4.5	0.06	0.09	0.01	0.02	0.06	0.06
32	4.62	4.42	4.34	4.31	4.41	4.34	0.23	0.08	0.08	0.18	0.17	0.15
60	4.66	4.35	4.49	4.2	4.37	4.2	0.01	0.08	0.02	0.01	0.03	0.1
84	4.53	4.36	4.48	4.42	4.2	4.28	0.05	0.25	0.02	0.03	0.02	0.09
113	4.65	4.37	4.33	4.33	4.21	4.17	0.02	0.19	0.1	0.06	0.42	0.05
140	4.54	4.5	4.35	4.37	4.29	4.01	0.02	0.3	0.05	0	0.04	0.13
175	4.59	4.24	4.28	4.22	4.22	4	0.04	0.2	0.07	0.03	0.1	0.04
220	4.7	4.41	4.42	4.35	4.1	4.01	0.04	0.29	0.1	0.01	0.07	0.04
295	4.5	4.325	4.26	4.14	4.14	3.94	0.01	0.02	0.08	0.06	0.07	0.05

Appendix 2.4: Composition data (obtained by GCanalysis) of extracts stored by six methods as described in section 4.6. Std. Err. is for mean of analyses of three samples per treatment. Continued ....

Unknown III"	Peak:Std 0.77												
	0	2.640	-	-	-	-	-	-	-	-	-	-	-
	14	3.010	3.050	3.000	2.930	2.890	2.870	0.030	0.050	0.020	0.006	0.030	0.040
	32	2.890	2.760	2.460	2.500	2.380	2.240	0.130	0.020	0.040	0.140	0.040	0.160
	60	2.970	2.870	2.900	2.530	2.550	2.500	0.030	0.040	0.020	0.010	0.030	0.040
	84	2.920	2.930	2.930	2.500	2.450	2.500	0.060	0.040	0.010	0.060	0.010	0.050
	113	2.640	2.520	2.620	1.950	2.030	1.970	0.095	0.047	0.045	0.070	0.025	0.046
	140	2.920	2.840	2.790	2.250	2.220	2.180	0.030	0.900	0.070	0.010	0.030	0.080
	175	2.93	2.67	2.69	1.96	2.01	2.01	0.04	0.06	0.03	0.05	0.08	0.04
	220	2.98	2.83	2.71	1.9	1.81	1.87	0.01	0.07	0.1	0.06	0.03	0.02
	295	2.555	2.255	2.28	1.46	1.45	1.49	0.06	0.02	0.01	0.03	0.03	0.02
Total polygodial													
	0	14.86	-	-	-	-	-	-	-	-	-	-	-
	14	15.62	15.89	15.6	15.6	15.7	15.5	0.14	0.24	0.05	0.06	0.11	0.13
	32	16.94	16.03	15.8	16	15.9	15.5	0.92	0.1	0.07	0.27	0.36	0.47
	60	15.92	15.54	15.8	15.3	15.6	15.4	0.07	0.08	0.01	0.08	0.08	0.23
	84	15.7	15.91	15.9	15.7	15.4	15.5	0.1	0.38	0.19	0.16	0.05	0.35
	113	16.26	16.25	16.3	15.8	15.4	15.8	0.09	0.12	0.2	0.35	1.06	0.19
	140	16.05	16.11	16	16.1	15.8	15.4	0.1	0.1	0.2	0.04	0.2	0.5
	175	15.68	15.29	15.3	14.9	15.3	15.2	0.1	0.03	0.2	0.2	0.2	0.2
	220	17.06	17.21	16.8	16.3	15.8	16.3	0.05	0.4	0.6	0.08	0.2	
	295	16.65	16.87	16.7	16	16.2	16.4	0.05	0.3	0.42	0.21	0.06	0.27
kaur-16-ene	Peak:Std 1.27												
	0	1.01	-	-	-	-	-	-	-	-	-	-	-
	14	1.05	1.1	1.1	1.13	1.18	1.15	0.07	0.04	0.02	0.03	0.04	0.02
	32	1.32	1.28	1.21	1.23	1.23	1.2	0.04	0.01	0.03	0.02	0.03	0.02
	60	0.97	1	1.01	0.97	0.95	0.98	0.05	0.03	0.01	0.02	0.01	0.01
	84	0.51	0.82	0.82	0.78	0.85	0.88	0.2	0.04	0.05	0.11	0.02	0.05
	113	0.39	0.46	0.49	0.4	0.42	0.43	0.06	0.1	0.07	0.01	0	0.03
	140	0.4	0.46	0.51	0.51	0.56	0.57	0.03	0.02	0.03	0.06	0.03	0.06
	175	0.425	0.42	0.42	0.4	0.41	0.39	0.01	0.01	0.04	0.01	0.1	0.01
	220	0.46	0.44	0.51	0.43	0.35	0.36	0.01	0.1	0.09	0.1	0.01	0.01
	295	0.355	0.365	0.37	0.36	0.37	0.37	0.01	0.01	0.01	0.01	0.01	0.01

Appendix 2.4 Continued : Composition data (obtained by GC analysis) of extracts stored by six methods as described in section 4.6. Std. Err. is for mean of analyses of three samples per treatment.

Anova

% volatiles

Groups	Count	Sum	Av.	Var.
Pet ether/fresh	5	26.77	5.354	0.271
hexacet/fresh	5	18.06	3.612	0.960
Pet ether/dry	5	31.98	6.396	0.386

Source	SS	df	MS	F
Between Groups	19.785	2	9.892	18.36**
Within Groups	6.4661	12	0.539	
Total	26.251	14	LSD(0.05)=	1.012

1,8 cineole

Groups	Count	Sum	Av.	Var.
Pet ether/fresh	5	0.71	0.142	0.000
hexacet/fresh	5	0.53	0.105	0.001
Pet ether/dry	5	0.77	0.155	0.000

Source	SS	df	MS	F
Between Groups	0.0065	2	0.003	7.88**
Within Groups	0.0050	12	0.000	
Total	0.0115	14	LSD(0.05)=	0.028

aristolone

Groups	Count	Sum	Av.	Var.
Pet ether/fresh	5	0.32	0.064	0.000
hexacet/fresh	5	0.12	0.023	0.000
Pet ether/dry	5	0.40	0.080	0.000

Source	SS	df	MS	F
Between Groups	0.0084	2	0.004	20.87**
Within Groups	0.0024	12	0.000	
Total	0.0109	14	LSD(0.05)=	0.020

adina 1,4 - liene

Groups	Count	Sum	Av.	Var.
Pet ether/fresh	5	6.74	1.348	0.070
hexacet/fresh	5	5.87	1.174	0.058
Pet ether/dry	5	7.76	1.552	0.020

Source	SS	df	MS	F
Between Groups	0.3585	2	0.179	3.64 n.s.
Within Groups	0.5917	12	0.049	
Total	0.9502	14		

alamenene

Groups	Count	Sum	Av.	Var.
Pet ether/fresh	5	4.51	0.902	0.016
hexacet/fresh	5	2.89	0.577	0.046
Pet ether/dry	5	5.09	1.019	0.033

Source	SS	df	MS	F
Between Groups	0.523	2	0.262	8.27**
Within Groups	0.380	12	0.032	
Total	0.903	14	LSD(0.05)=	0.245

aryophylle

Groups	Count	Sum	Av.	Var.
Pet ether/fresh	5	0.50	0.101	0.000
hexacet/fresh	5	0.43	0.085	0.000
Pet ether/dry	5	0.57	0.113	0.000

Source	SS	df	MS	F
Between Groups	0.0020	2	0.001	8.98**
Within Groups	0.0013	12	0.000	
Total	0.0034	14	LSD(0.05)=	0.015

Anova

cubebene

Groups	Count	Sum	Av.	Var.
Pet ether/fresh	5	2.13	0.427	0.002
hexacet/fresh	5	1.85	0.370	0.002
Pet ether/dry	5	2.42	0.485	0.002

Source	SS	df	MS	F
Between Groups	0.0328	2	0.016	8.40**
Within Groups	0.0234	12	0.002	
Total	0.0562	14	LSD(0.05)=	0.061

germacrene

Groups	Count	Sum	Av.	Var.
Pet ether/fresh	5	0.92	0.185	0.001
hexacet/fresh	5	0.78	0.155	0.001
Pet ether/dry	5	1.07	0.214	0.000

Source	SS	df	MS	F
Between Groups	0.0086	2	0.004	4.12*
Within Groups	0.0125	12	0.001	
Total	0.0211	14	LSD(0.05)=	0.044

Polygodial

Groups	Count	Sum	Av.	Var.
Pet ether/fresh	5	4.08	0.816	0.014
hexacet/fresh	5	1.20	0.241	0.072
Pet ether/dry	5	4.56	0.913	0.004

Source	SS	df	MS	F
Between Groups	1.3199	2	0.660	21.93**
Within Groups	0.3611	12	0.030	
Total	1.6809	14	LSD(0.05)=	0.239

Unknown I

Groups	Count	Sum	Av.	Var.
Pet ether/fresh	5	0.56	0.111	0.001
hexacet/fresh	5	0.41	0.083	0.002
Pet ether/dry	5	0.54	0.107	0.001

Source	SS	df	MS	F
Between Groups	0.0024	2	0.001	0.87 n.s.
Within Groups	0.0165	12	0.001	
Total	0.0189	14		

Unknown II

Groups	Count	Sum	Av.	Var.
Pet ether/fresh	5	0.97	0.195	0.001
hexacet/fresh	5	0.54	0.109	0.003
Pet ether/dry	5	1.13	0.226	0.000

Source	SS	df	MS	F
Between Groups	0.0369	2	0.018	12.73**
Within Groups	0.0174	12	0.001	
Total	0.0544	14	LSD(0.05)=	0.052

Unknown III

Groups	Count	Sum	Av.	Var.
Pet ether/fresh	5	2.98	0.595	0.004
hexacet/fresh	5	1.42	0.285	0.028
Pet ether/dry	5	3.42	0.684	0.004

Source	SS	df	MS	F
Between Groups	0.44	2	0.220	18.22**
Within Groups	0.145	12	0.012	
Total	0.584	14	LSD(0.05)=	0.151

ppendix 2.5a: Means and ANOVA for extraction method - solvent type

% vols in leaf Anova: Single-Factor

Summary

Groups	Count	Sum	Average	Variance
Direct sample	5	31.980	6.396	0.386
Total extract	5	35.900	7.180	0.082

Source	SS	df	MS	F
Between Groups	1.537	1.000	1.537	6.56*
Within Groups	1.873	8.000	0.234	
Total	3.409	9.000	LSD(0.05)=	1.116

1,8-cineole Anova: Single-Factor

Summary

Groups	Count	Sum	Average	Variance
Direct sample	5	0.773	0.155	0.000
Total extract	5	0.407	0.081	0.001

Source	SS	df	MS	F
Between Groups	0.0133	1.000	0.013	32.38**
Within Groups	0.0033	8.000	0.000	
Total	0.0166	9.000	LSD(0.05)=	0.048

aristolone Anova: Single-Factor

Summary

Groups	Count	Sum	Average	Variance
Direct sample	5	0.398	0.080	0.000
Total extract	5	0.474	0.095	0.000

Source	SS	df	MS	F
Between Groups	0.0006	1.000	0.001	7.08*
Within Groups	0.0007	8.000	0.000	
Total	0.0012	9.000	LSD(0.05)=	0.021

cadina 1,4 diene Anova: Single-Factor

Summary

Groups	Count	Sum	Average	Variance
Direct sample	5	7.762	1.552	0.020
Total extract	5	8.531	1.706	0.020

Source	SS	df	MS	F
Between Groups	0.0592	1.000	0.059	2.98n.s.
Within Groups	0.1588	8.000	0.020	
Total	0.2180	9.000		

calamenene Anova: Single-Factor

Summary

Groups	Count	Sum	Average	Variance
Direct sample	5	5.094	1.019	0.033
Total extract	5	5.821	1.164	0.019

Source	SS	df	MS	F
Between Groups	0.0528	1.000	0.053	2.02 n.s.
Within Groups	0.2093	8.000	0.026	
Total	0.2621	9.000		

caryophyllene Anova: Single-Factor

Summary

Groups	Count	Sum	Average	Variance
Direct sample	5	0.567	0.113	0.000
Total extract	5	0.619	0.124	0.000

Source	SS	df	MS	F
Between Groups	0.0003	1.000	0.000	5.09 n.s.
Within Groups	0.0004	8.000	0.000	
Total	0.0007	9.000		

cubebene Anova: Single-Factor

Summary

Groups	Count	Sum	Average	Variance
Direct sample	5.00	2.423	0.485	0.002
Total extract	5.00	2.601	0.520	0.000

Source	SS	df	MS	F
Between Groups	0.00	1.000	0.003	3.16n.s.
Within Groups	0.01	8.000	0.001	
Total	0.01	9.000		

germacrene I Anova: Single-Factor

Summary

Groups	Count	Sum	Average	Variance
Direct sample	5.00	1.068	0.214	0.000
Total extract	5.00	1.159	0.232	0.001

Source	SS	df	MS	F
Between Groups	0.00	1.000	0.001	1.54 n.s.
Within Groups	0.00	8.000	0.001	
Total	0.01	9.000		

polygodial Anova: Single-Factor

Summary

Groups	Count	Sum	Average	Variance
Direct sample	5.00	4.563	0.913	0.004
Total extract	5.00	5.583	1.117	0.002

Source	SS	df	MS	F
Between Groups	0.10	1.000	0.104	31.49**
Within Groups	0.03	8.000	0.003	
Total	0.13	9.000	LSD(0.05)=	0.136

Unknown I Anova: Single-Factor

Summary

Groups	Count	Sum	Average	Variance
Direct sample	5.00	0.536	0.107	0.001
Total extract	5.00	0.539	0.108	0.001

Source	SS	df	MS	F
Between Groups	0.00	1.000	0.000	0.0 n.s.
Within Groups	0.01	8.000	0.001	
Total	0.01	9.000		

Unknown II Anova: Single-Factor

Summary

Groups	Count	Sum	Average	Variance
Direct sample	5.00	1.130	0.226	0.000
Total extract	5.00	1.263	0.253	0.000

Source	SS	df	MS	F
Between Groups	0.00	1.000	0.002	4.10n.s.
Within Groups	0.00	8.000	0.000	
Total	0.01	9.000		

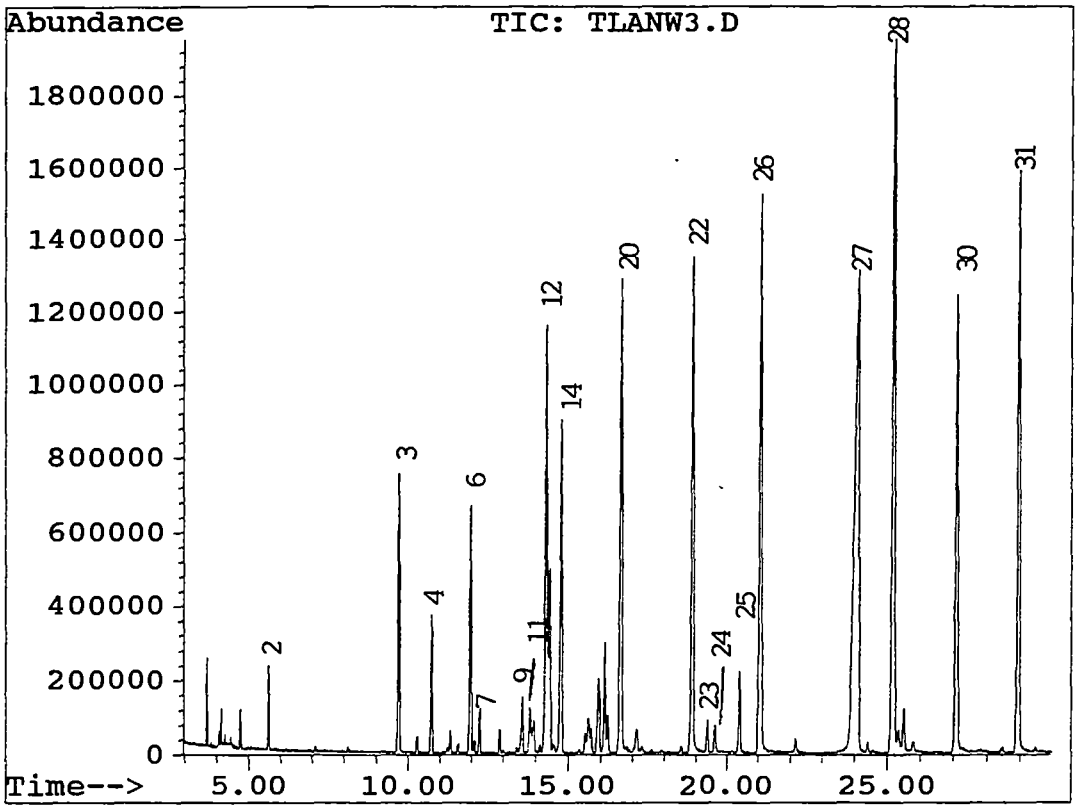
Unknown III Anova: Single-Factor

Summary

Groups	Count	Sum	Average	Variance
Direct sample	5.00	3.422	0.684	0.004
Total extract	5.00	3.932	0.786	0.001

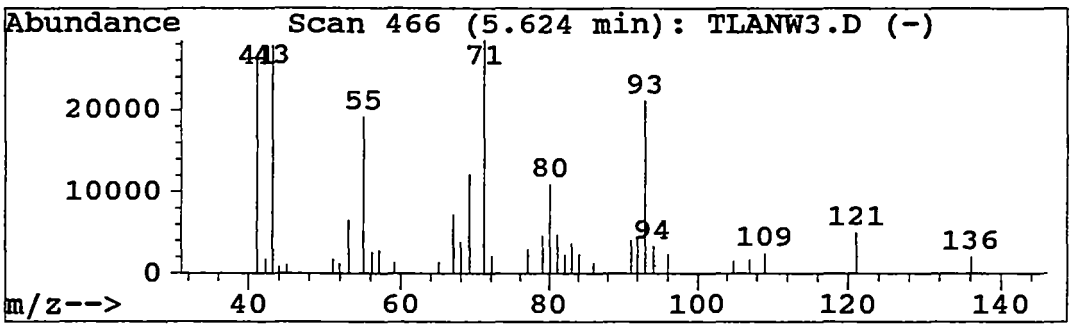
Source	SS	df	MS	F
Between Groups	0.03	1.000	0.026	10.90*
Within Groups	0.02	8.000	0.002	
Total	0.05	9.000	LSD(0.05)	0.115

Appendix 2.5b: Means and ANOVA for direct sample vs drydown method- sect.4.7 - see sect. 4.7

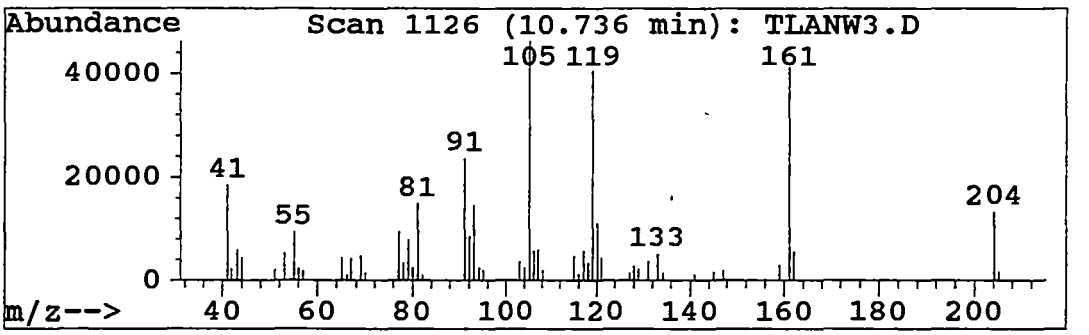


Peak#	Compound name	Ret. Time	Width	Area	Start Time	End Time
1		4.743	0.035	2311696	4.669	4.84
2	linalool	5.627	0.029	4092172	5.51	5.73
3	C13	9.712	0.05	24247895	9.555	9.942
4	a cubebene	10.741	0.049	11341550	10.567	10.816
6	C14	11.974	0.054	22824398	11.793	12.053
7	caryophyllene	12.269	0.05	3795121	12.173	12.36
8		12.908	0.046	1924023	12.792	12.971
9	germacrene D	13.605	0.062	6151512	13.489	13.683
10		13.835	0.066	5279021	13.683	13.897
11	bicyclogermacrene	13.948	0.071	4375756	13.897	14.089
12	C15	14.334	0.062	47893213	14.212	14.39
13		14.439	0.061	19685086	14.39	14.508
14	cadina 1,4 diene	14.794	0.061	36272925	14.642	14.927
16		15.628	0.068	4395305	15.571	15.68
17		15.948	0.079	9900110	15.831	16.03
18		16.138	0.057	10882266	16.03	16.188
19		16.231	0.057	4057130	16.188	16.372
20	C16	16.654	0.065	55436859	16.417	16.789
21		17.147	0.075	2935611	16.933	17.249
22	C17	18.91	0.06	56430295	18.643	19.242
23	aristolone	19.38	0.058	3051747	19.242	19.468
24	drimenol	19.622	0.059	2737628	19.513	19.771
25	polygodial isomer	20.425	0.061	8679973	20.087	20.59
26	C18	21.098	0.064	70479212	20.745	21.345
27	polygodial	24.125	0.123	127214312	23.377	24.338
28	C20	25.217	0.068	93561938	24.867	25.295
30	C21	27.109	0.066	53251346	26.892	27.376
31	C22	28.97	0.065	68978207	28.737	29.266

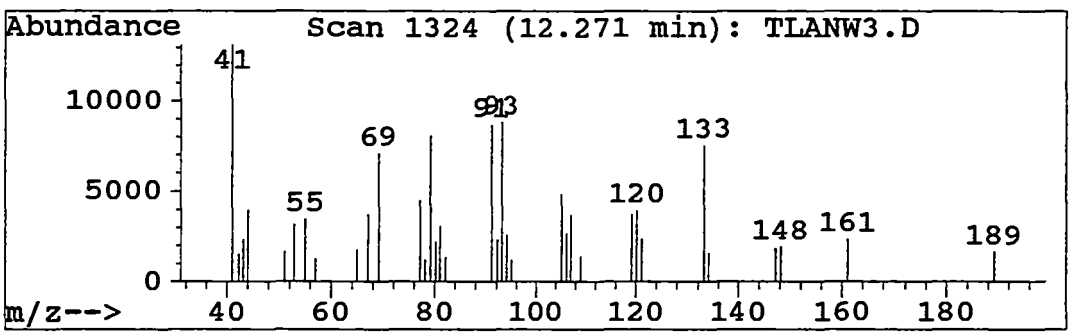
Appendix 2.6: Chromatogram and retention times used for calculation of Kovat's Indices (sect. 4.8).



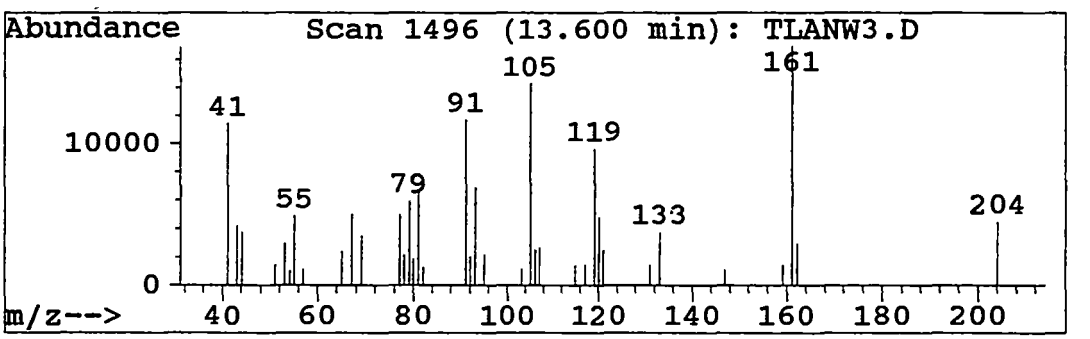
(a)



(b)

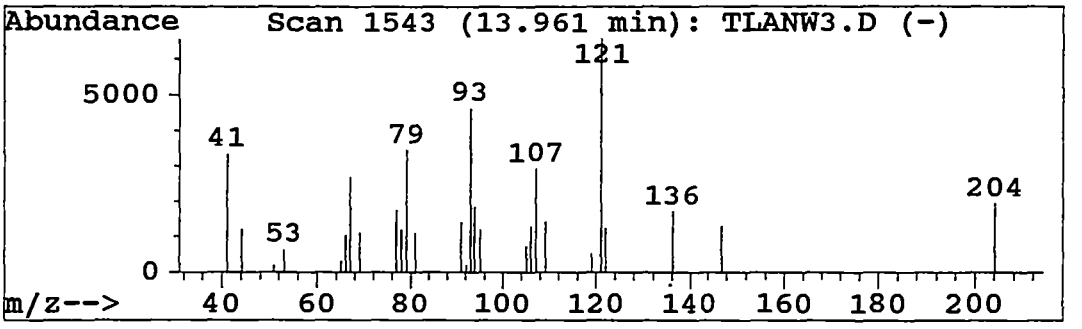


(c)

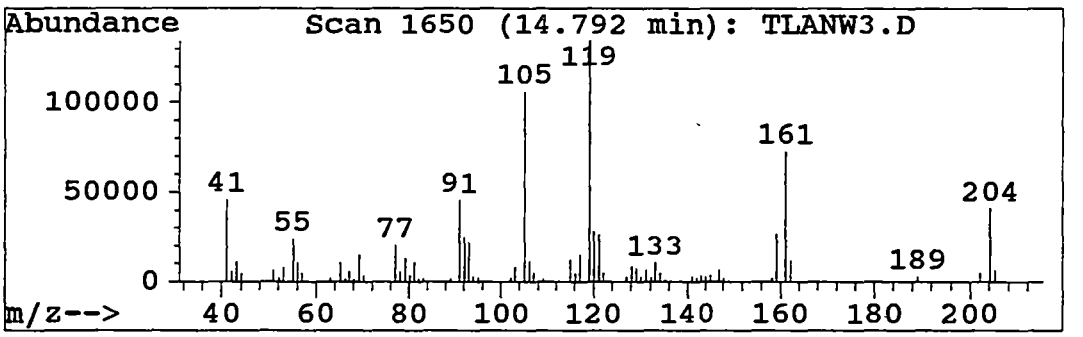


(d)

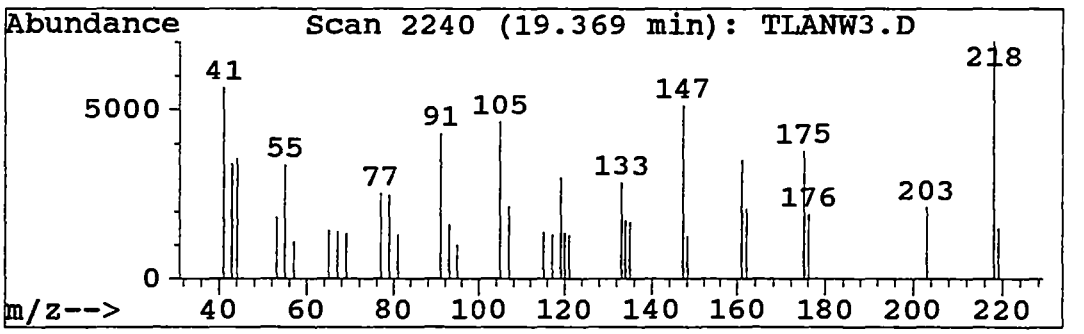
Appendix 2.6 continued: Mass spectral data obtained from GCMS analysis described in Sect. 4.8. a) linalool, b) α-cubebene c) caryophyllene d) germacrene D.



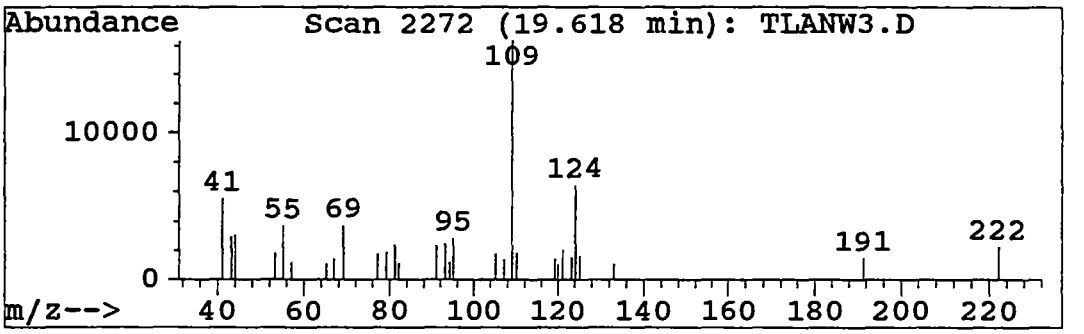
(e)



(f)

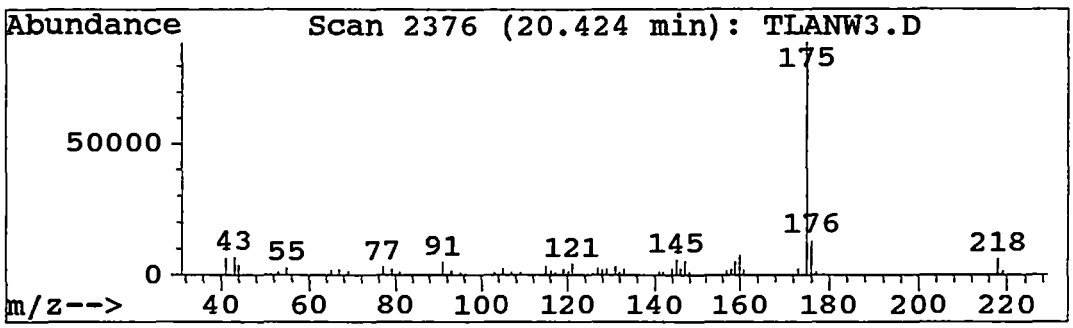


(g)

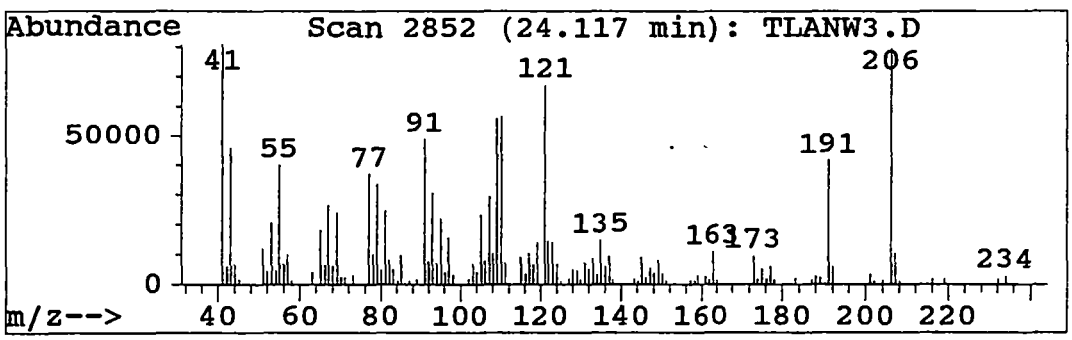


(h)

Appendix 2.6 continued: Mass spectral data obtained from GCMS analysis described in Sect. 4.8. e) bicyclogermacrene f) cadina 1,4 diene g) aristolone h) driminol.

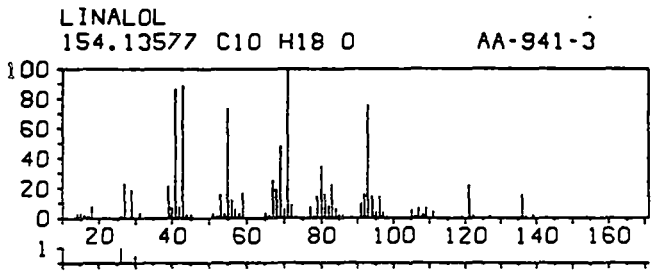


(i)

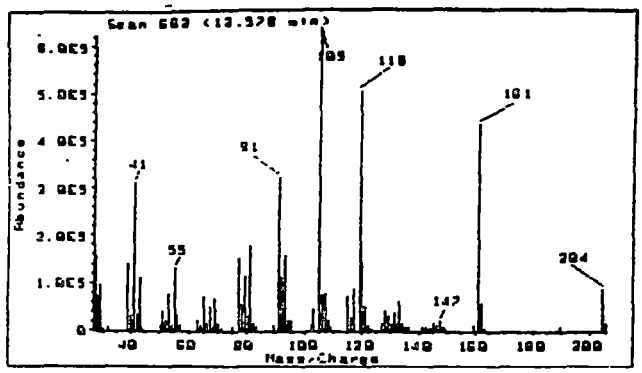


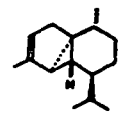
(j)

Appendix 2.6 continued: Mass spectral data obtained from GCMS analysis described in Sect. 4.8. i) polygodial isomer j) polygodial.



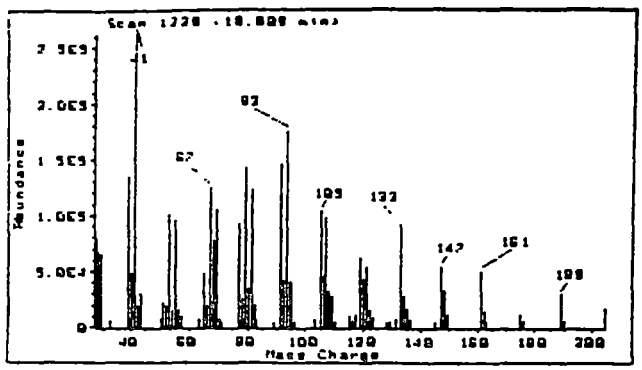
Ref 1

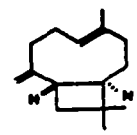




α - Cubebene

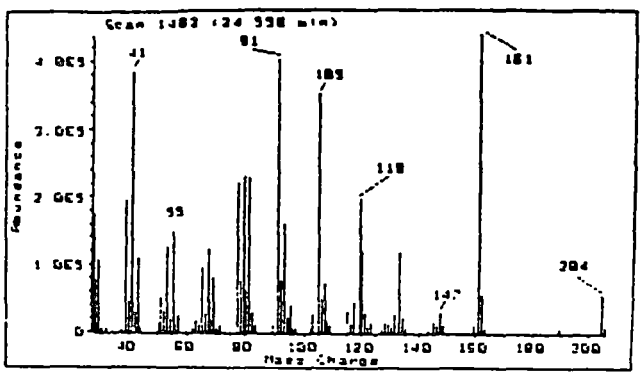
Ref 2

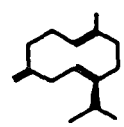




Caryophyllene

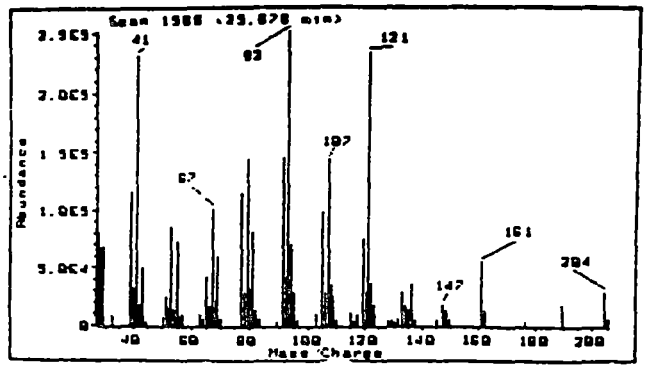
Ref 2

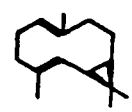




Germacrene D

Ref 2

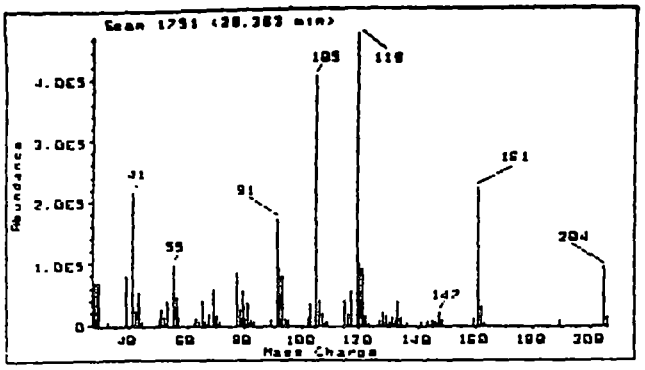


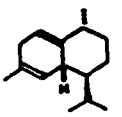


Bicyclogermacrene

Ref 2

Appendix 2.6 continued: Reference mass spectra obtained from library sources (see Section 4.8)

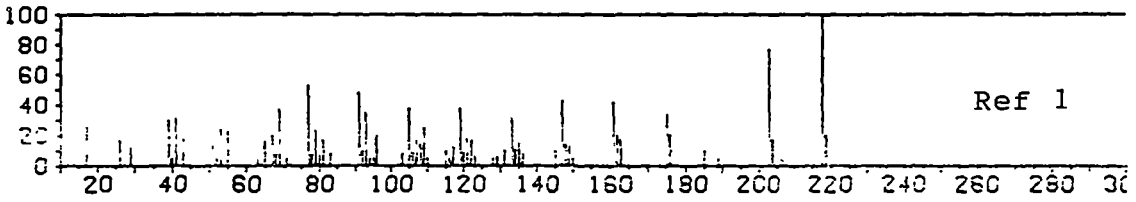




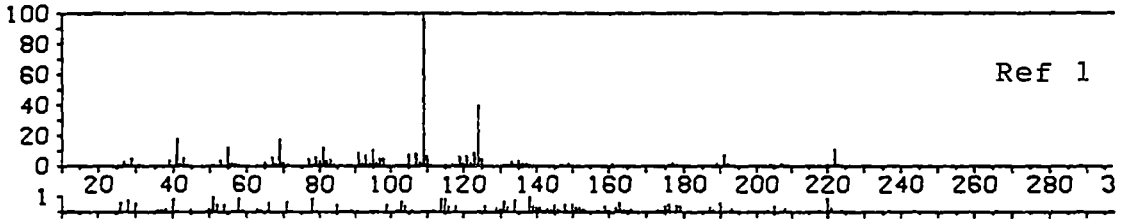
Cadina - 1,4 - diene

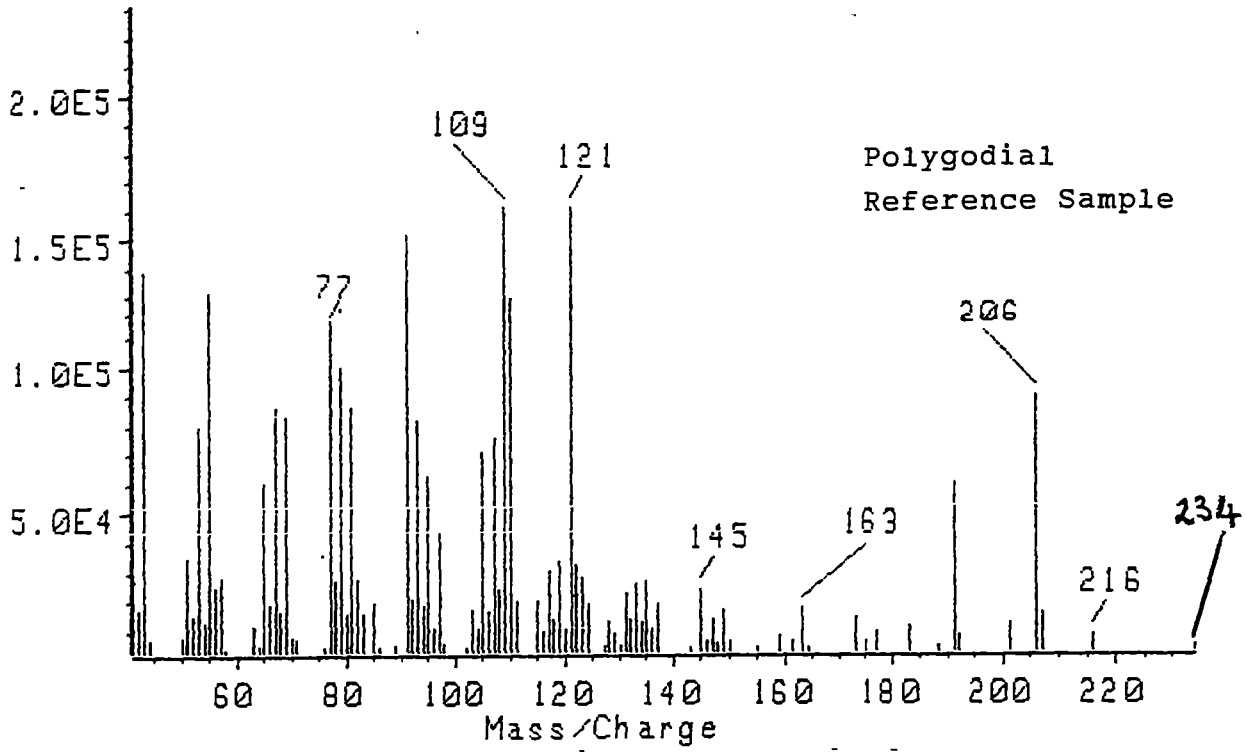
Ref 2

ARISTOLONE
218.16707 C15 H22 O



DRIMINOL
222.19837 C15 H26 O





Appendix 2.6 continued: Reference mass spectra obtained from library sources (see Section 4.8), and from pure polygodial sample.

SAS Program

```
00001 data a;
00002 input no day pos $ vols lin cub car ger bcg cad ars pol; cards;
00004 1 20 A .....4.25 0.176 0.001 0.086 . 0.052 . 0.065 2.356;
00207 data b; set a; if day>=100;
00210 proc glm data=b;
00211 class no day;
00212 model lin cub car ger bcg cad ars pol= no;
00213 lsmeans no/etype=3 pdiff stderr; run;
```

Number of observations in data set = 120

Class	Levels	Values
NO	10	1 2 3 4 5 6 7 8 9 10
DAY	4	100 160 240 320

Least Squares Means

NO	**VOLS** LSMEAN	Std Err LSMEAN	Pr > |T| H0:LSMEAN=0	LSMEAN Number
1	3.42583333	0.06730476	0.0001	1
2	5.14083333	0.06730476	0.0001	2
3	4.76166667	0.06730476	0.0001	3
4	6.15916667	0.06730476	0.0001	4
5	4.71500000	0.06730476	0.0001	5
6	5.57000000	0.06730476	0.0001	6
7	6.88250000	0.06730476	0.0001	7
8	4.39750000	0.06730476	0.0001	8
9	4.07916667	0.06730476	0.0001	9
10	5.01166667	0.06730476	0.0001	10

NO	**LIN** LSMEAN	Std Err LSMEAN	Pr > |T| H0:LSMEAN=0	LSMEAN Number
1	0.16541667	0.00795835	0.0001	1
2	0.11675000	0.00795835	0.0001	2
3	0.11333333	0.00795835	0.0001	3
4	0.13958333	0.00795835	0.0001	4
5	0.15200000	0.00795835	0.0001	5
6	0.09191667	0.00795835	0.0001	6
7	0.13183333	0.00795835	0.0001	7
8	0.05675000	0.00795835	0.0001	8
9	0.13308333	0.00795835	0.0001	9
10	0.16908333	0.00598116	0.0001	10

NO	**CUB** LSMEAN	Std Err LSMEAN	Pr > |T| H0:LSMEAN=0	LSMEAN Number
1	0.00000000	0.03160991	1.0000	1
2	0.38941667	0.00912499	0.0001	2
3	0.31608333	0.00912499	0.0001	3
4	0.34783333	0.00912499	0.0001	4
5	0.23600000	0.00912499	0.0001	5
6	0.28891667	0.00912499	0.0001	6
7	0.42750000	0.00912499	0.0001	7
8	0.22616667	0.00912499	0.0001	8
9	0.22700000	0.00912499	0.0001	9
10	0.00033333	0.00488173	0.9457	10

NO	**CAR** LSMEAN	Std Err LSMEAN	Pr > |T| H0:LSMEAN=0	LSMEAN Number
1	0.07241667	0.00341675	0.0001	1
2	0.09858333	0.00341675	0.0001	2
3	0.09108333	0.00341675	0.0001	3
4	0.10275000	0.00341675	0.0001	4
5	0.06091667	0.00341675	0.0001	5
6	0.17358333	0.00341675	0.0001	6
7	0.13850000	0.00341675	0.0001	7
8	0.06958333	0.00341675	0.0001	8
9	0.08441667	0.00341675	0.0001	9
10	0.10341667	0.00341194	0.0001	10

Appendix 2.7: SAS Output for Tree Means - Harvests 4-7: all positions, 4 dates;

NO	POL LSMEAN	Std Err LSMEAN	Pr > |T| H0:LSMEAN=0	LSMEAN Number	
1	2.32066667	0.19568321	0.0001	1	
2	0.91383333	0.19568321	0.0001	2	
3	0.96816667	0.19568321	0.0001	3	
4	1.47000000	0.19568321	0.0001	4	
5	1.47625000	0.19568321	0.0001	5	
6	1.52516667	0.19568321	0.0001	6	
7	1.33141667	0.19568321	0.0001	7	
8	1.82191667	0.19568321	0.0001	8	
9	1.05558333	0.19568321	0.0001	9	
10		2.83950000	0.02617151	0.0001	10

NO	GER LSMEAN	Std Err LSMEAN	Pr > |T| H0:LSMEAN=0	LSMEAN Number
1	0.03800000	0.01291511	0.0041	1
2	0.21408333	0.00645755	0.0001	2
3	0.17275000	0.00645755	0.0001	3
4	0.18283333	0.00645755	0.0001	4
5	0.12683333	0.00645755	0.0001	5
6	0.15808333	0.00645755	0.0001	6
7	0.23516667	0.00645755	0.0001	7
8	0.13000000	0.00645755	0.0001	8
9	0.12591667	0.00645755	0.0001	9
10	0.00050000	0.00356546	0.8888	10

NO	CAD LSMEAN	Std Err LSMEAN	Pr > |T| H0:LSMEAN=0	LSMEAN Number
1	0.02066667	0.11123685	0.8530	1
2	1.53150000	0.05561842	0.0001	2
3	1.08108333	0.05561842	0.0001	3
4	1.28066667	0.05561842	0.0001	4
5	0.94466667	0.05561842	0.0001	5
6	1.17058333	0.05561842	0.0001	6
7	1.53058333	0.05561842	0.0001	7
8	0.91183333	0.05561842	0.0001	8
9	0.83725000	0.05561842	0.0001	9
10	-0.00000000	0.03436883	1.0000	10

NO	BCG LSMEAN	Std Err LSMEAN	Pr > |T| H0:LSMEAN=0	LSMEAN Number
1	0.03775000	0.01337342	0.0058	1
2	0.07825000	0.00772115	0.0001	2
3	0.05058333	0.00772115	0.0001	3
4	0.08108333	0.00772115	0.0001	4
5	0.05791667	0.00772115	0.0001	5
6	0.07733333	0.00772115	0.0001	6
7	0.08383333	0.00772115	0.0001	7
8	0.05300000	0.00772115	0.0001	8
9	0.04316667	0.00772115	0.0001	9
10	0.04200000	0.00577459	0.0001	10

NO	ARS LSMEAN	Std Err LSMEAN	Pr > |T| H0:LSMEAN=0	LSMEAN Number
1	0.18058333	0.07536925	0.0186	1
2	0.01625000	0.13054336	0.9012	2
3	0.03416667	0.07536925	0.6514	3
4	0.07216667	0.07536925	0.3409	4
5	0.02975000	0.07536925	0.6940	5
6	0.04016667	0.07536925	0.5954	6
7	0.10241667	0.07536925	0.1776	7
9	0.01816667	0.07536925	0.8101	8
10	0.07858333	0.00406026	0.0001	9

Appendix 2.7 (continued): Output for Tree Means - Harvests 4-7: all positions, 4 dates.

SAS Programme 'posn' - ANOVA for position date by date:

```
00001 data a;
00002 input no day pos $ vols lin cub car ger bcg cad ars pol;
00003 cards;
00004 1 20 A 1.10 0.0 0.0 0.076 0..... ;
00206 run;
00207 data b;set a;
00208 if day= 20;
00209 run;
00210 proc glm data=b;
00211 class no day pos;
00212 model vols lin cub car ger bcg cad ars pol= pos;
00213 lsmeans pos/etype=3 pdiff stderr;
00214 run;
```

20Days

Class	Levels	Values
NO	10	1 2 3 4 5 6 7 8 9 10
DAY	1	20
POS	2	A B

Number of observations in data set = 20

Dependent Variable: VOLS

Source	DF	SSquares	MSquare	F Value	Pr > F
Model	1	0.12333889	0.12333889	0.65	0.4320
Error	16	3.03642222	0.18977639		
Corrected Total	17	3.15976111			

Dependent Variable: LIN

Source	DF	SSquares	MSquare	F Value	Pr > F
Model	1	0.02318422	0.02318422	9.56	0.0070
Error	16	0.03880156	0.00242510		
Corrected Total	17	0.06198578			

Dependent Variable: CUB

Source	DF	SSquares	MSquare	F Value	Pr > F
Model	1	0.00066006	0.00066006	0.22	0.6449
Error	16	0.04787844	0.00299240		
Corrected Total	17	0.04853850			

Dependent Variable: CAR

Source	DF	SSquares	MSquare	F Value	Pr > F
Model	1	0.00192200	0.00192200	3.85	0.0673
Error	16	0.00798400	0.00049900		
Corrected Total	17	0.00990600			

Dependent Variable: GER

Source	DF	SSquares	MSquare	F Value	Pr > F
Model	1	0.00202672	0.00202672	1.66	0.2158
Error	16	0.01952089	0.00122006		
Corrected Total	17	0.02154761			

Dependent Variable: BCG

Source	DF	SSquares	MSquare	F Value	Pr > F
Model	1	0.00291848	0.00291848	2.19	0.1585
Error	16	0.02134306	0.00133394		
Corrected Total	17	0.02426154			

Dependent Variable: CAD

Source	DF	SSquares	MSquare	F Value	Pr > F
Model	1	0.00168200	0.00168200	0.03	0.8549
Error	16	0.77915911	0.04869744		
Corrected Total	17	0.78084111			

Appendix 2.8: SAS output for significance of 'position' for harvests 1 (20 days) and 2 (40 days), continued...

Dependent Variable: ARS

Source	DF	SSquares	MSquare	F Value	Pr > F
Model	1	0.00020000	0.00020000	0.03	0.8682
Error	16	0.11249800	0.00703113		
Corrected Total 17		0.11269800			

Dependent Variable: POL

Source	DF	SSquares	MSquare	F Value	Pr > F
Model	1	0.01462050	0.01462050	0.17	0.6817
Error	16	1.34062978	0.08378936		
Corrected Total 17		1.35525028			

40 Days

Class	Levels	Values
NO	10	1 2 3 4 5 6 7 8 9 10
DAY	1	40
POS	3	A B C

Number of observations in data set = 30

Dependent Variable: VOLS

Source	DF	SSquares	MSquare	F Value	Pr > F
Model	2	0.39462000	0.19731000	0.75	0.4821
Error	27	7.10521000	0.26315593		
Corrected Total 29		7.49983000			

Dependent Variable: LIN

Source	DF	SSquares	MSquare	F Value	Pr > F
Model	2	0.00076580	0.00038290	0.41	0.6659
Error	27	0.02504650	0.00092765		
Corrected Total 29		0.02581230			

Dependent Variable: CUB

Source	DF	SSquares	MSquare	F Value	Pr > F
Model	2	0.00035060	0.00017530	0.04	0.9601
Error	27	0.11612570	0.00430095		
Corrected Total 29		0.11647630			

Dependent Variable: CAR

Source	DF	SSquares	MSquare	F Value	Pr > F
Model	2	0.00014687	0.00007343	0.11	0.8949
Error	27	0.01778610	0.00065874		
Corrected Total 29		0.01793297			

Dependent Variable: GER

Source	DF	SSquares	MSquare	F Value	Pr > F
Model	2	0.00036140	0.00018070	0.16	0.8506
Error	27	0.02997460	0.00111017		
Corrected Total 29		0.03033600			

Dependent Variable: BCG

Source	DF	SSquares	MSquare	F Value	Pr > F
Model	2	0.00375140	0.00187570	0.61	0.5522
Error	27	0.08342730	0.00308990		
Corrected Total 29		0.08717870			

Dependent Variable: CAD

Source	DF	SSquares	MSquare	F Value	Pr > F
Model	2	0.00801927	0.00400963	0.06	0.9419
Error	27	1.80350610	0.06679652		
Corrected Total 29		1.81152537			

Dependent Variable: ARS

Source	DF	SSquares	MSquare	F Value	Pr > F
Model	2	0.00046247	0.00023123	0.03	0.9684
Error	27	0.19434570	0.00719799		
Corrected Total 29		0.19480817			

Appendix 2.8: SAS output for significance of 'position' for harvests 1 (20 days) and 2 (40 days) continued...

Dependent Variable: POL

Source	DF	SSquares	MSquare	F Value	Pr > F
Model	2	0.19252827	0.09626413	0.99	0.3858
Error	27	2.63361760	0.09754139		
Corrected Total	29	2.82614587			

Appendix 2.8: SAS output for significance of 'position' for harvests 1 (20 days) and 2 (40 days)

Dependent Variable: VOLS

Source	DF	Squares	Square	F Value	Pr>F
Model	69	27075.79268	392.40279	420.29	0.0001
Error	128	119.50606	0.93364		
Corrected Total	197	27195.29874			

Source	DF	Type III SS	Mean Square	F Value	Pr>F
NO	9	3976.85712	441.87301	473.28	0.0001
DAY	6	19433.81124	3238.96854	3469.18	0.0001
NO*DAY	54	2821.16691	52.24383	55.96	0.0001

Dependent Variable: LIN

Source	DF	Squares	Square	F Value	Pr>F
Model	69	20.93937059	0.30346914	40.74	0.0001
Error	128	0.95334933	0.00744804		
Corrected Total	197	21.89271992			

Source	DF	Type III SS	Mean Square	F Value	Pr>F
NO	9	3.17127471	0.35236386	47.31	0.0001
DAY	6	14.88687594	2.48114599	333.13	0.0001
NO*DAY	54	2.17386904	0.04025683	5.41	0.0001

Dependent Variable: CUB

Source	DF	Squares	Square	F Value	Pr>F
Model	69	129.7945980	1.8810811	429.60	0.0001
Error	128	0.5604677	0.0043787		
Corrected Total	197	130.3550657			

Source	DF	Type III SS	Mean Square	F Value	Pr>F
NO	9	49.79035476	5.53226164	1263.46	0.0001
DAY	6	48.36647822	8.06107970	1841.00	0.0001
NO*DAY	54	24.69570330	0.45732784	104.44	0.0001

Dependent Variable: CAR

Source	DF	Squares	Square	F Value	Pr>F
Model	69	12.88286298	0.18670816	350.82	0.0001
Error	128	0.06812183	0.00053220		
Corrected Total	197	12.95098481			

Source	DF	Type III SS	Mean Square	F Value	Pr>F
NO	9	2.49096923	0.27677436	520.06	0.0001
DAY	6	8.13399030	1.35566505	2547.28	0.0001
NO*DAY	54	1.62182214	0.03003374	56.43	0.0001

Dependent Variable: GER

Source	DF	Squares	Square	F Value	Pr>F
Model	69	38.45818649	0.55736502	243.34	0.0001
Error	128	0.29317833	0.00229046		
Corrected Total	197	38.75136483			

Source	DF	Type III SS	Mean Square	F Value	Pr>F
NO	9	14.57490418	1.61943380	707.04	0.0001
DAY	6	14.60694269	2.43449045	1062.88	0.0001
NO*DAY	54	7.21014446	0.13352119	58.29	0.0001

Dependent Variable: BCG

Source	DF	Squares	Square	F Value	Pr>F
Model	69	5.61873454	0.08143094	10.53	0.0001
Error	128	0.98953950	0.00773078		
Corrected Total	197	6.60827404			

Source	DF	Type III SS	Mean Square	F Value	Pr>F
NO	9	1.94116616	0.21568513	27.90	0.0001
DAY	6	2.08964125	0.34827354	45.05	0.0001
NO*DAY	54	1.28870557	0.02386492	3.09	0.0001

Appendix 2.9: Output for SAS General linear means - mg per shoot, programme as for Appendix 2.10, using transformed data; continued....

Dependent Variable: CAD

Source	DF	Squares	Square	F Value	Pr>F
Model	69	1932.247822	28.003592	189.77	0.0001
Error	128	18.888096	0.147563		
Corrected Total	197	1951.135918			

Source	DF	Type III SS	Mean Square	F Value	Pr>F
NO	9	756.8443532	84.0938170	569.88	0.0001
DAY	6	684.3674728	114.0612455	772.97	0.0001
NO*DAY	54	386.0464112	7.1490076	48.45	0.0001

Dependent Variable: ARS

Source	DF	Squares	Square	F Value	Pr>F
Model	69	8.46708792	0.12271142	57.84	0.0001
Error	128	0.27155867	0.00212155		
Corrected Total	197	8.73864659			

Source	DF	Type III SS	Mean Square	F Value	Pr>F
NO	9	5.19514408	0.57723823	272.08	0.0001
DAY	6	1.24396355	0.20732726	97.72	0.0001
NO*DAY	54	1.37621677	0.02548550	12.01	0.0001

Dependent Variable: POL

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	69	3096.416539	44.875602	308.86	0.0001
Error	128	18.597616	0.145294		
Corrected Total	197	3115.014155			

Source	DF	Type III SS	Mean Square	F Value	Pr>F
NO	9	784.167824	87.129758	599.68	0.0001
DAY	6	1735.599314	289.266552	1990.91	0.0001
NO*DAY	54	427.025342	7.907877	54.43	0.0001

Least Squares Means - Harvest Date

Dependent Variable: VOLS

DAY	VOLS LSMEAN	Std Err LSMEAN	Pr>|T| H0:LSMEAN=0
20	0.5320000	0.2332147	0.0242
40	1.4653333	0.1738280	0.0001
70	10.1130000	0.1738280	0.0001
100	19.4463333	0.1738280	0.0001
160	26.6433333	0.1738280	0.0001
240	25.7260000	0.1738280	0.0001
320	23.0499000	0.1764125	0.0001

Dependent Variable: LIN

DAY	LIN LSMEAN	Std Err LSMEAN	Pr>|T| H0:LSMEAN=0
20	0.00900000	0.02107020	0.6700
40	0.03966667	0.01570480	0.0128
70	0.21566667	0.01570480	0.0001
100	0.36700000	0.01570480	0.0001
160	0.70133333	0.01570480	0.0001
240	0.64333333	0.01570480	0.0001
320	0.71183333	0.01575652	0.0001

Dependent Variable: CUB

DAY	CUB LSMEAN	Std Err LSMEAN	Pr>|T| H0:LSMEAN=0
20	0.02650000	0.01608692	0.1019
40	0.07166667	0.01199048	0.0001
70	0.44500000	0.01199048	0.0001
100	1.01800000	0.01199048	0.0001
160	1.30266667	0.01199048	0.0001
240	1.27933333	0.01199048	0.0001
320	1.10596667	0.01208119	0.0001

Appendix 2.9: Output for SAS General linear means - mg per shoot, programme as for Appendix 2.10, using transformed data; continued....

Dependent Variable: CAR

DAY	CAR LSMEAN	Std Err LSMEAN	Pr>|T| H0:LSMEAN=0
20	0.00450000	0.00559785	0.4230
40	0.02333333	0.00417239	0.0001
70	0.18733333	0.00417239	0.0001
100	0.38666667	0.00417239	0.0001
160	0.54400000	0.00417239	0.0001
240	0.52166667	0.00417239	0.0001
320	0.43970000	0.00421190	0.0001

Dependent Variable: GER

DAY	GER LSMEAN	Std Err LSMEAN	Pr>|T| H0:LSMEAN=0
20	0.00950000	0.01162802	0.4155
40	0.03733333	0.00866702	0.0001
70	0.27100000	0.00866702	0.0001
100	0.58000000	0.00866702	0.0001
160	0.73566667	0.00866702	0.0001
240	0.70533333	0.00866702	0.0001
320	0.56466667	0.00873776	0.0001

Dependent Variable: BCG

DAY	BCG LSMEAN	Std Err LSMEAN	Pr>|T| H0:LSMEAN=0
20	0.01250000	0.02119607	0.5564
40	0.07233333	0.01579862	0.0001
70	0.23433333	0.01579862	0.0001
100	0.35900000	0.01579862	0.0001
160	0.20000000	0.01579862	0.0001
240	0.25200000	0.01579862	0.0001
320	0.27716667	0.01605281	0.0001

Dependent Variable: CAD

DAY	CAD LSMEAN	Std Err LSMEAN	Pr>|T| H0:LSMEAN=0
20	0.11500000	0.09349599	0.2210
40	0.28466667	0.06968780	0.0001
70	2.04500000	0.06968780	0.0001
100	3.90766667	0.06968780	0.0001
160	5.22266667	0.06968780	0.0001
240	4.86066667	0.06968780	0.0001
320	3.85550000	0.07013398	0.0001

Dependent Variable: ARS

DAY	ARS LSMEAN	Std Err LSMEAN	Pr>|T| H0:LSMEAN=0
20	0.04200000	0.01129782	0.0003
40	0.07533333	0.00842089	0.0001
70	0.23633333	0.00842089	0.0001
100	0.23466667	0.00842089	0.0001
160	0.26466667	0.00842089	0.0001
240	0.25500000	0.00842089	0.0001
320	0.24666667	0.00840942	0.0001

Dependent Variable: POL

DAY	POL LSMEAN	Std Err LSMEAN	Pr>|T| H0:LSMEAN=0
20	0.17000000	0.09100704	0.0641
40	0.49466667	0.06783264	0.0001
70	4.14866667	0.06783264	0.0001
100	5.99366667	0.06783264	0.0001
160	8.39433333	0.06783264	0.0001
240	7.78900000	0.06783264	0.0001
320	6.93596667	0.06959259	0.0001

Appendix 2.9: Output for SAS General linear means - mg per shoot, programme as for Appendix 2.10, using transformed data.

SAS Programme

```
00001 data a;
00002 input no day pos $ vols lin cub car ger bcg cad ars pol;
00003 cards;
00004 1 20 A 1.10 0.0 0.0 0.076 0.0 .....0.0 0.052 0.0 0.065 2.356 ;
00207 proc glm data=a;
00208 class no day;
00209 model lin cub car ger bcg cad ars pol= no day no*day;
00210 lsmeans no day no*day/etype=3 pdiff stderr;
00211 run;
```

Class	Levels	Values
NO	10	1 2 3 4 5 6 7 8 9 10
DAY	7	20 40 70 100 160 240 320

Number of observations in data set = 200

NOTE: All dependent variable are consistent with respect to the presence or absence of missing values. However only 198 observations can be used in this analysis.

Dependent Variable: VOLS

Source	DF	SSquares	MSquare	F Value	Pr>F
Model	69	484.1973177	7.0173524	124.47	0.0001
Error	128	7.2165833	0.0563796		
Corrected Total	197	491.4139010			

Source	DF	Type III SS	Mean Square	F Value	Pr>F
NO	9	103.1677675	11.4630853	203.32	0.0001
DAY	6	327.5621469	54.5936912	968.32	0.0001
NO*DAY	54	36.0047081	0.6667539	11.83	0.0001

Dependent Variable: LIN

Source	DF	SSquares	MSquare	F Value	Pr>F
Model	69	0.49230249	0.00713482	10.56	0.0001
Error	128	0.08646433	0.00067550		
Corrected Total	197	0.57876683			

Source	DF	Type III SS	Mean Square	F Value	Pr>F
NO	9	0.11475675	0.01275075	18.88	0.0001
DAY	6	0.28651008	0.04775168	70.69	0.0001
NO*DAY	54	0.07930044	0.00146853	2.17	0.0002

Dependent Variable: CUB

Source	DF	SSquares	MSquare	F Value	Pr>F
Model	69	3.67376439	0.05324296	210.22	0.0001
Error	128	0.03241867	0.00025327		
Corrected Total	197	3.70618306			

Source	DF	Type III SS	Mean Square	F Value	Pr>F
NO	9	2.26941299	0.25215700	995.60	0.0001
DAY	6	0.82027835	0.13671306	539.79	0.0001
NO*DAY	54	0.34606533	0.00640862	25.30	0.0001

Dependent Variable: CAR

Source	DF	SSquares	MSquare	F Value	Pr>F
Model	69	0.38445615	0.00557183	76.46	0.0001
Error	128	0.00932733	0.00007287		
Corrected Total	197	0.39378348			

Source	DF	Type III SS	Mean Square	F Value	Pr>F
NO	9	0.12721634	0.01413515	193.98	0.0001
DAY	6	0.19488963	0.03248160	445.75	0.0001
NO*DAY	54	0.02950729	0.00054643	7.50	0.0001

Dependent Variable: GER

Source	DF	SSquares	MSquare	F Value	Pr>F
Model	69	1.14727415	0.01662716	104.12	0.0001
Error	128	0.02044117	0.00015970		
Corrected Total	197	1.16771532			

Source	DF	Type III SS	Mean Square	F Value	Pr>F
NO	9	0.62247376	0.06916375	433.09	0.0001
DAY	6	0.30235349	0.05039225	315.55	0.0001
NO*DAY	54	0.14008954	0.00259425	16.24	0.0001

Appendix 2.10 -Output for SAS General Linear Models Procedure- Compound % of DW of leaf material

Dependent Variable: BCG

Source	DF	SSquares	MSquare	F Value	Pr>F
Model	69	0.34321913	0.00497573	10.29	0.0001
Error	128	0.06185182	0.00048049		
Corrected Total	197	0.40507195			

Source	DF	Type III SS	Mean Square	F Value	Pr>F
NO	9	0.13604750	0.01513861	31.28	0.0001
DAY	6	0.11803146	0.01963858	40.71	0.0001
NO*DAY	54	0.07730757	0.00144088	2.97	0.0001

Dependent Variable: CAD

Source	DF	SSquares	MSquare	F Value	Pr>F
Model	69	53.30178994	0.77248971	76.29	0.0001
Error	128	1.29604333	0.01012534		
Corrected Total	197	54.59783327			

Source	DF	Type III SS	Mean Square	F Value	Pr>F
NO	9	34.20160134	3.80017793	375.31	0.0001
DAY	6	10.72692927	1.78782154	176.57	0.0001
NO*DAY	54	5.07093695	0.09390624	9.27	0.0001

Dependent Variable: ARS

Source	DF	SSquares	MSquare	F Value	Pr>F
Model	69	1.04002465	0.01507282	93.89	0.0001
Error	128	0.02054933	0.00016054		
Corrected Total	197	1.06057398			

Source	DF	Type III SS	Mean Square	F Value	Pr>F
NO	9	0.76414127	0.08490459	528.86	0.0001
DAY	6	0.15167696	0.02527949	157.46	0.0001
NO*DAY	54	0.10285492	0.00190472	11.86	0.0001

Dependent Variable: POL

Source	DF	SSquares	MSquare	F Value	Pr>F
Model	69	88.86052450	1.28783369	139.55	0.0001
Error	128	1.18122750	0.00922834		
Corrected Total	197	90.04175200			

Source	DF	Type III SS	Mean Square	F Value	Pr>F
NO	9	41.92867064	4.65874118	504.83	0.0001
DAY	6	33.90820041	5.65136674	612.39	0.0001
NO*DAY	54	7.78954464	0.14425083	15.63	0.0001

Least Squares Means-Harvest Date

DAY	VOLS LSMEAN	Std Err LSMEAN	Pr>ITI H0:LSMEAN=0
20	1.65250000	0.05816161	0.0001
40	2.16300000	0.04335111	0.0001
70	3.97266667	0.04335111	0.0001
100	4.71433333	0.04335111	0.0001
160	5.31100000	0.04335111	0.0001
240	5.40900000	0.04335111	0.0001
320	4.62300000	0.04335111	0.0001

DAY	LIN LSMEAN	Std Err LSMEAN H0:LSMEAN=0	Pr>ITI
20	0.03230000	0.00636633	0.0001
40	0.05730000	0.00474518	0.0001
70	0.08393333	0.00474518	0.0001
100	0.08816667	0.00474518	0.0001
160	0.14236667	0.00474518	0.0001
240	0.13593333	0.00474518	0.0001
320	0.14143333	0.00474518	0.0001

Appendix 2.10 -Output for SAS General Linear Models Procedure- Compound % of DW of leaf material

DAY	CUB	Std Err	Pr>ITI
LSMEAN	LSMEAN	H0:LSMEAN=0	
20	0.08100000	0.00389824	0.0001
40	0.10570000	0.00290557	0.0001
70	0.16733333	0.00290557	0.0001
100	0.24216667	0.00290557	0.0001
160	0.25666667	0.00290557	0.0001
240	0.26410000	0.00290557	0.0001
320	0.22076667	0.00290557	0.0001

DAY	CAR	Std Err	Pr>ITI
	LSMEAN	LSMEAN	H0:LSMEAN=0
20	0.01210000	0.00209098	0.0001
40	0.03436667	0.00155852	0.0001
70	0.07276667	0.00155852	0.0001
100	0.09410000	0.00155852	0.0001
160	0.10676667	0.00155852	0.0001
240	0.10900000	0.00155852	0.0001
320	0.08823333	0.00155852	0.0001

DAY	GER	Std Err	Pr>ITI
	LSMEAN	LSMEAN	H0:LSMEAN=0
20	0.02775000	0.00309545	0.0001
40	0.05600000	0.00230721	0.0001
70	0.10166667	0.00230721	0.0001
100	0.13786667	0.00230721	0.0001
160	0.14510000	0.00230721	0.0001
240	0.14636667	0.00230721	0.0001
320	0.11293333	0.00230721	0.0001

DAY	BCG	Std Err	Pr>ITI
	LSMEAN	LSMEAN	H0:LSMEAN=0
20	0.03261000	0.00519899	0.0001
40	0.10390000	0.00387509	0.0001
70	0.09143333	0.00387509	0.0001
100	0.08573333	0.00387509	0.0001
160	0.03980000	0.00387509	0.0001
240	0.05156667	0.00387509	0.0001
320	0.05480000	0.00387509	0.0001

DAY	CAD	Std Err	Pr>ITI
	LSMEAN	LSMEAN	H0:LSMEAN=0
20	0.36050000	0.02464793	0.0001
40	0.41623333	0.01837148	0.0001
70	0.77096667	0.01837148	0.0001
100	0.92756667	0.01837148	0.0001
160	1.02453333	0.01837148	0.0001
240	0.99390000	0.01837148	0.0001
320	0.77133333	0.01837148	0.0001

DAY	ARS	Std Err	Pr>ITI
	LSMEAN	LSMEAN	H0:LSMEAN=0
20	0.13100000	0.00310363	0.0001
40	0.11183333	0.00231331	0.0001
70	0.09806667	0.00231331	0.0001
100	0.06050000	0.00231331	0.0001
160	0.05476667	0.00231331	0.0001
240	0.05730000	0.00231331	0.0001
320	0.05200000	0.00231331	0.0001

DAY	POL	Std Err	Pr>ITI
	LSMEAN	LSMEAN	H0:LSMEAN=0
20	0.51475000	0.02353084	0.0001
40	0.74926667	0.01753885	0.0001
70	1.69656667	0.01753885	0.0001
100	1.49186667	0.01753885	0.0001
160	1.71086667	0.01753885	0.0001
240	1.68666667	0.01753885	0.0001
320	1.39960000	0.01753885	0.0001

Appendix 2.10 -Output for SAS General Linear Models Procedure- Compound % of DW of leaf material

Light level	Days	Mean Amax	Std Err. Amax(n)	Mean Q	Std Err. Q	Mean Cp	Std Err. Cp	Mean Rdark	Std Err. Rdark
Pretrial	0	9.31	0.14 (3)	0.092	0.002	13.20	1.91	1.21	0.18
10%	7	10.72	0.29 (3)	0.101	0.005	9.80	0.62	0.99	0.10
	21	10.98	0.30 (4)	0.107	0.008	3.81	0.24	0.40	0.02
	35	8.94	0.38(4)	0.100	0.007	2.50	1.05	0.24	0.10
	49	11.95	.64 (4)	0.117	0.015	5.62	1.60	0.63	0.14
30%	7	10.3	0.41	0.085	0.000	12.38	0.56	1.05	0.05
	21	12.21	0.34	0.109	0.003	7.08	0.63	0.77	0.07
	35	8.97	0.57	0.107	0.004	3.73	1.54	0.40	0.16
	49	10.57	0.91	0.125	0.004	4.75	0.86	0.59	0.091
50%	7	11.9	0.06	0.093	0.007	11.57	0.98	1.07	0.01
	21	13.92	0.13	0.122	0.005	7.21	0.94	0.88	0.11
	35	10.95	0.33	0.101	0.006	5.28	0.64	0.53	0.06
	49	11.94	0.36	0.110	0.012	4.56	1.15	0.47	0.10
100%	7	9.86	0.38	0.091	0.008	16.40	2.01	1.52	0.29
	21	7.62	0.41	0.070	0.002	22.69	5.76	1.50	0.36
	35	7.13	0.48	0.069	0.001	10.88	0.60	0.75	0.05
	49	11.07	0.45	0.085	0.004	10.41	0.61	0.88	0.03
10%	New	7.38	0.37	0.110	0.008	11.73	2.13	1.29	0.31
30%	leaves	8.64	0.39	0.100	0.009	16.70	2.38	1.67	0.26
50%	@ 49	12.26	0.61	0.110	0.009	13.36	0.12	1.47	0.13
100%	days	9.18	0.28	0.080	0.008	32.63	4.33	2.61	0.46

Appendix 2.11: Parameters derived from light curves: Amax - light saturated nett assimilation rate; Q - quantum yield (slope of curve below 150μmol m^{-2} sec^{-1}); Cp - compensation point; R_{dark} - estimate of dark respiration.

Tree no	I/Io north	I/Io south
1	0.34	0.44
2	0.38	0.41
3	0.44	0.46
4	0.40	0.54
5	0.39	0.45
6	0.41	0.44
7	0.35	0.41
8	0.39	0.46
9	0.39	0.51
10	0.42	0.46
Mean 10 trees	0.39	0.46
Std. Deviation of Mean	*0.03*	*0.04*

Canopy surface radiation for inclined tubes at 65 and 75 degrees for north and south facing surfaces respectively were 0.78 and 0.52 of that detected by the horizontal pyranometer during four five minute detection periods.

Appendix 2.12: Incident radiation- ratio of canopy surface: canopy interior measured using integrating solarimeter tubes (in canopy) and spot pyrometer (for unobstructed horizontal surface) at Parrawe site 24/11/1995; 1100-1430 hrs