Factors affecting yield and composition of floral extract from *Boronia megastigma* Nees

by

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Hazel Smith MacTavish
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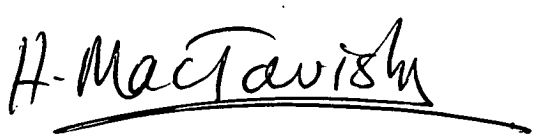

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'There is material enough in a single flower for the ornament of a score of cathedrals'.

John Ruskin, Stones of Venice I. 1851.

I will be the gladdest thing
Under the sun!
I will touch a hundred flowers
And not pick one.

Edna St. Vincent Millay ('Nancy Boyd') 1892-1950.
Afternoon on a Hill.

Abbreviations

A	- antheraxanthin
ABA	- abscisic acid
ADP	- adenosine diphosphate
ATP	- adenosine triphosphate
BSA	- bovine serum albumin
CC	- central cavity
DCM	- dichloromethane
DCPE	- dichloropropene
EDTA	- ethylenediaminetetraacetic acid
ER	- endoplasmic reticulum
GA	- Gibberellic acid
GC	- gas chromatograph
GC/MS	- gas chromatograph and mass spectrometer
GGPP	- geranylgeraniol pyrophosphate
GPP	- geranyl pyrophosphate
HMG-CoA	- 3S-3-hydroxyl-3-methyl-glutaryl-CoA
IPP	- iso pentenyl pyrophosphate
JA	- jasmonic acid
JB–Me	- methyl jasmonate
LM	- light microscopy
LIS	- linalool synthase
MVA	- mevalonic acid
NAD	- nicotinamide adenine dinucleotide
NADP	- nicotinamide adenine dinucleotide phosphate
OPC-4:0	- 3-oxo-2-(2'-pentenyl)cyclopentanebutanoic acid
OPC-6:0	- 3-oxo-2-(2'-pentenyl)cyclopentanehexanoic acid
OPC-8:0	- -3oxo-2-(2'-pentenyl)cyclopentaneoctanoic acid
12-oxo-PDA	- 12-oxo-*cis,cis*-10,15-phytodienoic acid
Pds	- phytoene desaturase
Psy	- phytoene synthase
PVPP	- polyvinylpolypropylene
RNA	- ribonucleic acid
RVE	- rotary vacuum evaporator
SEM	- scanning electron microscopy
TEM	- transmission electron microscopy
V	- violaxanthin
Z	- zeaxanthin

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<u>The Misty Island</u>.
From the lone shieling of the misty island
Mountains divide us, and the waste of seas -
Yet still the blood is strong, the heart is Highland,
And we in dreams behold the Hebrides.

Anon.

Summary

The Tasmanian boronia industry requires detailed information about biological and production factors which affect the yield and quality of floral extract obtained from *Boronia megastigma* Nees. This study included the sites and routes of biosynthesis and accumulation of particular secondary compounds, phenology of flowering and extract accumulation, genetic and environmental yield-limiting characteristics, conjugates between volatile compounds and sugars, the effect of various post-harvest storage conditions and the process of extraction of product.

Cellular differentiation is not a prerequisite for accumulation of volatile compounds; typical floral volatiles are emitted from all floral organs, especially the stigma and anthers. Plastidial droplets and vesicles containing variably osmiophilic material occur within cells surrounding the central cavity of epidermal glands in the petals. The number and size of droplets increase with increasing proximity to the central cavity, and cellular disintegration becomes more apparent. As the content of epidermal glands increases, the cap cell rises above the surface of the epidermis. The septa on the cap cell(s) do not rupture until the petals have lost volatiles, moisture and pigments as a result of senescence, and have abscised and fallen to the ground. This indicates that active release mechanisms are not a prerequisite for fragrance release.

There is evidence for enzyme controlled conversion of carotenoids into β-ionone, most likely by a lipoxygenase. There is a high level of control for β-ionone production which is lost as a result of senescence, when more random degradation of many compounds by other enzymes and via autoxidation occurs.

The activity of biosynthetic enzymes is high in unopened buds and flowers just after anthesis, however activity declines with senescence. The loss of volatiles, pigments and moisture from flowers after anthesis is rapid, and may be dramatic even before obvious visual signs of senescence are apparent. The flowering pattern is an uneven one, and there is good reason not to wait until 100% of flowers are open before harvesting. In late flowering, the loss of flower and extract yield per plant through various catabolic reactions and the action of evaporation on mature flowers is significant. The negative effect on the total yield of product per plant is greater than the effect of reduced extract yield in immature buds which are present in the harvest earlier in the flowering season.

In genetically different plants, the natural variation in extract yield is less than the variation in extract composition. The genetic predisposition to accumulate extract is more important than effects due to the environment, plant age or floral characteristics. There is no correlation between the yield of extract per flower and the number of

epidermal glands per petal, or the weight of petals or stigmas as a proportion of flower weight. Non glandular production of extract components is probably as, if not more significant than glandular production. This indicates that one of the main factors contributing to high yielding clones which produce a good quality product (a high proportion of volatiles, particularly β-ionone) is the enhanced activity or concentration of enzymes responsible for secondary metabolism of interest in these plants compared with lower yielding plants.

Storage of flowers in enclosed boxes after harvest resulted in substantially higher levels of volatiles including β-ionone in the final product. The rate of respiration was highest in boxes that were regularly opened and disturbed. Post-harvest changes of this sort are not caused by hydrolysis of glycosidically bound volatiles, however a large increase in the level of volatile glycosides during extended storage is indicative of the role of glycosides in the catabolism of volatiles or in the transport of volatiles from flowers prior to abscission. Flowers harvested early in the flowering season have a greater potential for post-harvest changes than those harvested later, indicating that controlled biosynthesis of compounds is most likely to be the cause of the increase in volatiles observed. Freezer storage, prior to storage at room temperature, inhibits the increase in volatile content of flowers during this latter storage. During freezer storage for longer than one month, volatiles and extract yield are depleted.

The process of squeezing or partially chopping flowers prior to extraction significantly increases the yield of extract obtained, indicating that some floral tissues are not completely extracted in the absence of this process due to the density of their tissues. A reduction in the solvent volume used during the extraction causes selective extraction of floral compounds such as β-ionone in preference to more volatile compounds, and yields a product of differing organoleptic properties. The duration of extraction and the washing regimen can also be manipulated also to maximise extract yield and vary the nature of the final product.

These results increase the duration of the optimum harvest 'window' and allow for choice of the best harvest date, given that different dates may enable maximal extract yield and increase the potential for post-harvest enhancement of volatile content. The production of an increased range of extracts to suit varying market demands is possible by several means.

I. General introduction

Many plants accumulate secondary compounds such as aromatic volatiles. These plants generally produce such compounds in all their organs (Attaway *et al.* 1967; Hasegawa *et al.* 1986), usually to deter herbivorous animals or to attract potential pollinators (Dicke 1994; Deans and Waterman 1993; Pellmyr and Thien 1986). After extraction, various compounds may be of medicinal or other use, and essential oils or plant extracts are often valuable export products.

Accumulation of volatile compounds usually occurs in specialised cells, organs or areas of the plant which may or may not be synthetically active (Gershenzon *et al.* 1989). It is typical of essential oil-producing plants that various compounds are continually being synthesised, accumulated and catabolised (Burbott and Loomis 1967, 1969). A decline in oil content usually coincides with senescence (Croteau and Martinkus 1979; Kekelidze *et al.* 1989), and this is of great importance when short-lived flowers are considered. Some flowers emit a strongly fragrant mixture of compounds that may function in the attraction of pollinators to the plant (Podolsky 1992). Therefore, the synthesis of such compounds may be directed towards release of volatiles at appropriate times such as flower opening (Watanabe *et al.* 1993).

Oil production is the result of gene-controlled enzyme activity (Murray *et al.* 1980; Perez 1988; Reznickova and Bugaenko 1977). Yield may be influenced by environmental conditions such as water, light, temperature, and nutrition status (Hornok 1988). However, genetic potential may be more significant than environmental factors that affect flower yield, at least in rose (Patra *et al.* 1987). In so-called essential oil crops, the main factors of importance in producing a high quality product are the selection of suitable plant material, the growing conditions, the developmental stage when harvested, any pre- or post-harvest processing, and the extraction of product (Bayrak and Akgül 1994; Verzár-Petri *et al.* 1984).

Boronia megastigma Nees. (Brown boronia) is grown commercially in Tasmania for the extract* obtained from the red-brown and golden coloured flowers. Flowers are harvested in September and October, frozen for transportation and storage, then extracted by solvent to yield a waxy extract. The yield of extract is between 0.4 and 0.7% of fresh weight, and contains 12-20% 'volatiles' of which β–ionone comprises 20-40%.

* 'Extract', rather than 'concrete' will be used when referring to floral boronia products because this is the most suitable term for the purposes of this thesis.

In boronia, flower yield per hectare is proportional to flower size, the number of flowers per node, the number of flowers per plant and the number of plants per hectare (Roberts 1989). The flower index and the yield and concentration of particular components in the floral extract can be increased by application of nitrogen (Reddy and Menary 1989a, 1989b; Roberts and Menary 1994a, b). High nitrogen also increases the vegetative growth (Thomas 1981) and would be expected to decrease floral activity (Day *et al.* 1994). Boronia flower yield is influenced by daylength, light levels and night temperature (Roberts and Menary 1989). Benzyladenine treatment reduces the number of weeks required to reach anthesis (Day *et al.* 1994). In none of these studies were extract yield and composition assessed; it would have been interesting to examine the effect on extract production of a reduced time for flower development. Extract production in boronia is decreased by psyllid infestation (Mensah and Madden 1992), and flower yield is reduced by black scale (Apriyanto 1995).

Leggett (1979) made a small-scale, preliminary study of extract yield in boronia, showing it to be reduced at the beginning and end of the flowering period. He did not consider the composition of the harvest in terms of the proportion of buds and flowers at each harvest, an important factor in boronia. The same work also showed the effects of different solvents on the yield and composition of floral extract, although the study was limited because of the unavailability of large amounts of clonal material.

To date, most of the work on boronia, bar preliminary studies, has concentrated on flower production. Roberts and Menary (1994) have done the only large-scale work examining links between nutrient status, flower yield and extract production. For the production of boronia extract in Tasmania to expand, especially in the face of competition from interstate and overseas enterprises (Smale 1991; Christensen and Skinner 1978), a more detailed knowledge of the factors that affect extract yield and composition is required.

The most sensible approach, given the established industry in Tasmania, involves utilising existing plantations and technology, and increasing their efficiency by altering management practices where necessary. For the purposes of this study, appropriate plantation sites and optimum irrigation, fertiliser and insecticide application regimens are assumed to be in place. The areas where further study were thought to be most likely to produce beneficial increases in extract yield and composition were the timing of harvest, post-harvest flower storage and the extraction process itself. Other studies were directed toward improving existing information about site(s) of synthesis and accumulation of extract, biochemical links between certain pigments and volatiles, and determining any floral characteristics such as flower size that may linked with extract yield. These factors may aid future clonal selection programs or provide a basis for molecular studies. The relationship between the boronia flower and its accumulated

compounds were investigated to determine the role of secondary metabolism in flower production.

II. Literature review

1 Introduction

1.1 *Boronia megastigma* Nees

Boronia megastigma Nees. (Brown Boronia) is a native Australian shrub in the Rutaceae family (Sub-family Rutoideae, tribe Boronieae). In Western Australia, natural stands of boronia occur on swamplands such as those found in the Karri forests near Albany (Christensen and Skinner 1978). In this environment, boronia is a wiry or woody shrub one to two metres high with relatively few flowers. Prolific flowering is achieved in open heath lands with higher light intensities and under cultivation where it forms a more compact bushy shrub up to one metre in height. Boronia is a short-lived seral species that regenerates after fire or soil disturbance from soil-stored seed reserves (Christensen and Skinner 1978). Boronia seeds, like other wild flower seeds, are difficult to germinate (Thomas 1981; Paynter and Dixon, 1991; Whitehorne and M^{c}Intyre 1975). In Tasmania, boronia plants for commercial plantations are established by vegetative propagation to maintain clonal purity.

Boronia is grown for its floral extract under intensive cultivation in Tasmania and to a lesser extent in New Zealand (Smale 1991). In Western Australia, natural stands are harvested (Christensen and Skinner 1978). In Tasmania, a number of selected clonal types are grown under licence. Plants with good flower and extract yield, and desirable extract composition are selected for the suitability of their canopy shape for mechanical pruning and harvesting and disease resistance to *Phytopthora cinnamomi*, *Puccinae boroniae*. (Menary 1989) and *Ctenarytaina thysanura* (Ferris and Klyver) (Hemiptera: Psyllidae) (Mensah and Madden 1992). Most plantations are harvested mechanically using a patented device, smaller acreages and plants in their first year are harvested manually with a mechanical aid (Menary 1987). Boronia is also used in the cut-flower industry (Joyce *et al.* 1993). Stems have a vase-life of three to ten days when put into clean water immediately after cutting and kept at 5°C (Faragher 1989).

Flowers bloom prolifically in spring on the cultivated bushy shrubs. An axillary racemic flowering pattern occurs in boronia. Two or three flower buds initiate in each leaf axil on a current season's lateral in autumn. The resource demands of the inflorescence are such that the subtending trifoliate leaves cease expansion during

flowering (Roberts and Menary 1990), and this is consistent with the general restraint to vegetative growth upon flowering (Lamont 1989; Reddy and Menary 1989b). The most mature flowers usually occur in the axils of the third or fourth leaf below the apex and flower maturity decreases acropetally and basipetally from this point, with basal nodes initiating and maturing earlier than apical nodes (Roberts 1989; Roberts and Menary 1994a). This produces an extended flowering period. After anthesis, flowers remain on the plant until abscission of the petals (about 6-8 days) and subsequent abscission of the remaining stalk (a further 2-7 days) unless fruit set occurs. This aspect of phenology is not well documented.

Boronia flowers droop on one centimetre long pedicels, exposing the epidermis of the abaxial surface of the petals which are usually red-brown or purple and dotted with translucent glands. The petals are orbicular, concave and imbricate, approximately one centimetre in diameter. The epidermis on the adaxial surface of the petal is yellow, sometimes with a green or golden tinge. The actinomorphic flowers are dominated by a large, brown, four-lobed stigma which is sessile upon a superior ovary with only one or two ovules (Weston 1978; Jackson and Jacobs 1985). A nectar secreting disc is situated beneath the ovary in *B. serrulata* (Armstrong 1975), and such structures are common in Rutaceae (Fahn 1979b), however no published documentation regarding the presence or not of such a disc in *B. megastigma* has been noted. Pollen is produced only on the small, yellow, petaline anthers (functional), not on the large, red-brown, sepaline anthers (non-functional). The fruit is a small, ovoid mericarp with one carpel. Very few fruits mature on individual plants (Holliday 1978).

Various colour mutants of *B. megastigma* exist. The petals, stigma and non-functional anthers of 'Lutea' are yellow. 'Harlequin' has a stripe of red on the abaxial surface of the petal, and 'Jacks Red' petals have a more vibrant red colour on the abaxial surface of the epidermis, and a dull suffused gold colour on the inner epidermis (Parry 1963). The attractive odour of the flowers makes Boronia a popular garden plant and to this end growth regulators have been used to increase desirability by restricting plant height and increasing the number of laterals and flowers (Lewis and Warrington 1988; Day *et al.* 1994).

1.2 Extracts from boronia

The vegetation of boronia produces an extract in epidermal glands similar to those described for Citrus (Rutaceae) (Haberlandt 1928; Leggett 1979; Thomson *et al.* 1976). The vegetative extract has a 'green' odour and is not of commercial interest (Leggett 1979). The flowers produce a fine extract, with significant organoleptic qualities. The floral extract comprises monoterpenes, cinnamates, norisoprenoids including ionones, related epoxides and dihydro compounds, fatty acid methyl and

ethyl esters, acetates of decyl, dodecyl and tetradecyl alcohols and methyl jasmonate isomers (Davies and Menary 1983; Guenther 1974; Weyerstahl *et al.* 1994). β–ionone (4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-buten-2-one) is the major volatile. Natural α– and β–ionone were first discovered in boronia extract in 1927 (Penfold and Phillips 1927; Naves 1964, 1971; Naves and Parry 1947).

Boronia extract has an odour that is 'powerful and characteristic; it recalls that of chopped spinach and blackcurrant buds, and after partial evaporation, like that of clove buds and infusion of tea' (Penfold and Phillips 1927). Boronia absolute has an intense floral impact, its odour is reminiscent of cassis and violet. It has a natural fruity-green freshness entwined with the character of ripening hay and sweet tea; undertones of yellow freesias and raspberries emerge before finishing with a slightly spicy-herbaceous (cinnamon and tobacco leaf), woody dry-out (Roberts 1984; Guenther 1974; Weyerstahl *et al.* 1994).

There are distinct chemotypes of *B. megastigma* (Davies and Menary 1983; Leggett and Menary 1980). Quantitative variability in minor components between samples from genetically different plants is quite large. The compounds identified in boronia extract by Davies and Menary (1983) (Table 1.2.1) have recently been increased by Weyerstahl *et al.* (1994) (Table 1.2.2). Davies and Menary (1983) define 'volatiles' as the fraction of extract that elutes from the gas chromatograph (GC) up to the retention time of n-heneicosane. Weyerstahl *et al.* (1994) claim that 50% of boronia absolute is made up of low-volatile compounds that are not important for its fragrance, but may act as fixators or slow-release systems.

Table 1.2.1 Components identified from extracts of *Boronia megastigma* flowers.

1. α–thujene
2. α–pinene
3. camphene
4. myrcene
5. β–pinene
6. octanal
7. limonene
8. δ-3-carene
9. ocimene
10. nonanal
11. linalool
12. methyl octanoate
13. camphor
14. octanoic acid
15. 2,6-dimethyl-3,7-octadiene-2,6-diol
16. ethyl octanoate
17. menthone
18. undecanal
19. methyl nonanoate
20. nerol
21. methyl naphthalene
22. nonanoic acid
23. bornyl acetate
24. ethyl nonanoate
25. decyl acetate
26. acyclic monoterpene
27. methyl decanoate
28. benzyl pentanoate
29. decanoic acid
30. ethyl decanoate
31. α–ionone
32. dihydro–β–ionone
33. 5,6-epoxy ionone
34. phenylethyl pentanoate
35. tetrahydroionone
36. caryophyllene
37. dodecanol
38. β–ionone
39. humulene
40. dihydroactinidiolide
41. n-pentadecane
42. isoamyl salicylate
43. 'sesquicineole'
44. dodecyl acetate
45. methyl jasmonate
46. methyl jasmonate isomer
47. iso-heptadecane
48. (Z)-heptadec-8-ene
49. n-heptadecane
50. methyl-p-hydroxy cinnamate
51. tetradecenyl acetate
52. tetradecyl acetate
53. n-nonadecane
54. methyl palmitate
55. ethyl palmitate
56. iso-heneicosane
57. n-heneicosane
58. methyl oleate
59. methyl stearate
60. ethyl stearate
61. n-docosane
62. iso-tricosane
63. n-tricosane
64. n-tetracosane
65. iso-pentacosane
66. n-pentacosane
67. n-hexacosane
68. iso-heptacosane
69. n-heptacosane
70. n-octacosane
71. iso-nonacosane
72. n-nonacosane
73. n-triacontane
74. iso-hentriacontane
75. n-hentriacontane
76. iso-tritriacontane
77. n-tritriacontane

Source: Davies and Menary (1983).

Table 1.2.2 Recently identified compounds.

Compound	Percentage (%)
β–ionone	15%
7,8-dihydro–β–ionone	1.3%
7,8-dihydro–β–ionol	0.6%
4-oxo–β–ionone	
4-hydroxy–β–ionone	
4-oxo–β–ionol	
3-hydroxy–β–ionone	
3-hydroxymegastigm-7-en-9-one	2.4 - 3%
2 stereoisomers of 3-hydroxymegastigm-7-en-9-one	
8-hydroxylinalyl ester *	10%
methyl (Z,E)-4-(geranyloxy)cinnamates *	10%
methyl (Z,E)-4-(5-hydroxy-geranyloxy)-cinnamates *	10%
N-[2-(4-prenyloxyphenyl)ethyl]tiglamide *	7%

* identified in the distillation residue of the absolute.

Source: Weyerstahl *et al.* (1994).

Extracts from boronia flowers enhance citrus, raspberry, plum and peach flavours in drinks and confectionary (Arctander 1960). The absolute is used in high-class chypre and fougère perfumes (Weyerstahl *et al.* 1994). The increased demand in recent years for natural products has been met by flavour industries (Petrzilka and Ehret 1991; Verlet 1993) and such marketing directions may produce an increase in usage of natural boronia extract. Flavouring compounds and mixtures are described as 'natural' only when obtained from natural sources, or by natural processes such as physical extraction, fermentation or enzymatic conversion (Williams *et al.* undated). The ability of cell cultures to produce or modify natural extracts has been well documented (Banthorpe and Barrow 1983; Mulder-Krieger *et al.* 1988), and the successful *in vitro* culturing of *B. megastigma* (Luckman 1989; Norman 1989) including the formation of flowers *in vitro* (Roberts *et al.* 1993), indicates the potential for further development in the production of extracts from this species.

2 Secondary compounds

The diversity of compounds including pigments and volatile and non-volatile compounds which together make up boronia extract, are all naturally occurring compounds that are generally classified as products of plant secondary metabolism.

Production of a wide range of secondary metabolites is common in plants of the Rutaceae family (Waterman 1990). The importance of secondary plant metabolism in whole plant functioning is stressed because such products perform crucial functions, albeit often non-primary ones. The functions of secondary products include photoprotection (Goodwin 1980a), protection from herbivores (Dicke 1994; Dobson *et al.* 1990) and pathogens (Deans and Waterman 1993; Mikhlin *et al.* 1983), attraction of pollinators (Buttery *et al.* 1982), hormonal control (Herout 1977) and disposal of excess or waste products via degradation into smaller compounds such as amino acids and sugars which are then re-used (Barz and Köster 1981; Croteau and Martinkus 1979).

Plant extracts are most commonly described by their 'flavour' which may be the result of 300 to 400 individual compounds and the interaction of these compounds with sugars and acids present in the extract or foodstuff (McGlasson *et al.* 1987). An array of 'aroma' compounds are directly biosynthesised by some plants (Ohloff 1977a); the yield and total composition of which is determined genetically and affected by the developmental stage of the plant and environmental factors. Factors that regulate rates of primary plant metabolism such as light, temperature, water supply, gas exchange and nutrient levels also have an effect on secondary metabolism because of the carbon flow that occurs between the two systems (Loomis and Croteau 1973). Direct cause/effect relationships are less obvious in secondary metabolism, especially for complex pathways. Commonly, the yield and complexity of an extract increases as the plant matures or initiates flowers, after which both factors decline (Croteau and Martinkus 1979; Pichersky *et al.* 1994).

Some of the extract components may be produced via enzymic and degradative transformations of high molecular weight compounds such as pigments, lipids and proteins during the life of the plant or during post-harvest storage and extraction of product (Ohloff 1977a; Bondarovich *et al.* 1967). One example of direct relevance to boronia relates to the possible production of β–ionone from β–carotene or similar carotenoids (Enzell 1985).

2.1 Secondary compounds in flowers

Compounds responsible for the rich pigmentation and delicate smell of most flowers and fruit are usually distinct from compounds in other plant parts (Wiermann 1981). Both colour and fragrance serve as attractants for pollinating insects and animals. Flowers that open and secrete nectar or emit volatiles at particular times of day may do so in concurrence with periods of activity of pollinating insects (Loughrin *et al.* 1991).

2.1.A Pigments

In flowers, red-violet-blue colours are derived from water-soluble anthocyanins located in the vacuoles of epidermal cells (Greyson 1994). Colour diversity in anthocyanins occurs via conjugation with sugars and complexing with metals, methylation, hydroxylation, oxidation and reduction, and modifications due to changes in pH.

Carotenoids produce yellow, orange and red hues. They are fat-soluble and osmiophilic, and are generally restricted to plastids in the cytoplasm of mesophyll cells where they occur as chromoproteins in the grana of chloroplasts (Goodwin 1980a). Yellow colours in flowers are produced by oxidised carotenoids such as epoxides and xanthophylls, for example, flavoxanthin. Orange colours are produced by lycopene and β–carotene. Colour intensity is reflected in the concentration of carotenoid pigment present (Goodwin 1980a). In vegetative plant tissue carotenoids function as accessory pigments for photosynthesis and are active in oxygen transport and photoprotection. In flowers they may function in pollinator attraction and facilitate seed dispersal by animals; both functions are speculative and difficult to prove (Davies 1976; Pichersky *et al.* 1994). Carotenoids present in anthers may contribute toward pollen development (Wiermann and Gubatz 1992). An increase in carotenoids and a decline in chlorophyll is seen prior to anthesis, and during fruit ripening (Fraser *et al.* 1994; Giuliano *et al.* 1993; Nieuwhof *et al.* 1988), indicative of the development of the plant part concerned.

2.1.B Volatiles

Fragrance in flowers often results from volatile low molecular weight terpenes which occur as small droplets in the cytoplasm of epidermal and neighbouring mesophyll cells of the sepals (Fahn 1988). Like a 'flavour', the 'scent' of a flower is a result of the integration of the main constituents (>1%) with up to several hundred minor constituents (0.1 - 1%), and trace compounds (<0.1%) (Petrzilka and Ehret 1991). Often the richness, complexity, originality and imprint of a scent is brought about by minor and trace compounds and interactions between these compounds. The concentration of a particular compound, especially trace compounds, assessed by analytical procedures such as gas chromatography/mass spectrometry can often under estimate the significance of that particular compound on the overall scent (Ohloff 1977b; Marshall and Moulton 1981; Neuner-Jehle and Etzweiler 1991). Minor compounds may have an intense odour value: the number of times the concentration of the headspace exceeds the detection threshold (Ohloff 1977b). This affects olfactory detection of scent to a greater extent than can be reflected by GC analysis. Most of the

compounds identified in floral extracts are terpenoids, benzenoids, phenylpropanoids, acyl lipid derivatives, nitrogen- or sulphur-containing compounds (Knudsen *et al.* 1993; Pichersky *et al.* 1994).

Differences in volatiles emitted from different species within a genus is a common occurrence, never more obvious than in a comparison of the fragrance of flowers of *B. megastigma, B. serrulata* ('a subtle suggestion of roses), *B. alatior* ('not noticeably fragrant') and *B. citriodora* (green, citrus smell) (Parry 1963; Quick 1963). It is possible that speciation can occur as a result of single mutations changing the floral fragrance compared with species relying on visual cues for pollination (Pellmyr *et al.* 1987).

In foliar secretory cavities of marigold, the concentration of extract increased until early flowering and then decreased, followed by a further increase in late flowering (Russin *et al.* 1988). This may indicate transport of secondary compounds into flowers as they develop, or decreased synthesis within vegetative material because flowers act as a 'sink' for available carbon and energy. The increase in foliar extract during late flowering could represent the transport of compounds out of flowers that will soon senesce (Croteau 1987). Alternatively it may be caused by a depletion in synthetic activity in flowers, leaving more substrates available for synthesis within vegetative plant parts. This increase and subsequent decrease in secondary compounds in flowers and fruit is common (Haagen-Smit 1972; Burbott and Loomis 1969; Kekelidze *et al.* 1989; Menary and Doe 1983).

A rare study of monoterpene biosynthesis in flowers is that of Pichersky *et al.* (1994) on the formation of linalool in flowers of *Clarkia breweri* [Gray] Green (Onagraceae). Linalool is a major component of the scent in this species and is found in many flowers including boronia (Davies and Menary 1983; Kaiser 1991). LIS (linalool synthase) was the only monoterpene synthase active in floral extracts, and phosphase activity was found only in mature pollen grains. High LIS activity accompanied high levels of monoterpenes, and most of the LIS activity (expressed as a concentration of fresh weight) occurred in the pistil organs. Significant emissions of the three monoterpenes in *C. breweri* flowers (linalool, furanoid linalool oxide and pyranoid linalool oxide) begin at anthesis, reach a peak two days later, remain high until after pollination (four days) and then rapidly decline. Low levels of the three compounds were emitted after senescence, until the flowers had abscised. The corresponding levels of monoterpenes in floral tissue were similar to emission patterns, except that monoterpenes were present in, but not emitted from, floral tissue prior to anthesis. Pichersky *et al.* (1994) seem to have been unaware of the studies done by Altenburger and Matile (1990) on emission and tissue levels of different volatiles in *Citrus medica* flowers, of which linalool is also the major monoterpene emitted. Altenburger and Matile (1990) found no relationships between tissue and emission

levels, and no patterns between boiling point and emission. Diurnal rhythms occurred in emission levels but corresponding changes in tissue levels were not observed.

Differences in the spectrum of flower volatiles compared with vegetative volatiles, and compounds emitted from separate flower parts may be important in effecting pollinator attraction and hence pollinator effectiveness (Dobson *et al.* 1990; Hansted *et al.* 1994). Other roles for volatiles present in flowers include interactive responses in pollen germination. Some compounds such as nonanol may stimulate pollen germination (French *et al.* 1979); others, such as jasmonic acid, may inhibit germination (Yamane *et al.* 1982).

All the petals and organs of multi-whorl flowers usually contain all volatiles (Mihailova *et al.* 1977). Quantitative differences are common, and some qualitative differences can occur between attractive, non-food structures (perianth) and attractive food sources such as the androecium and gynoecium (Dobson *et al.* 1990). Francis and Allcock (1969) found only small amounts of free and glycosidically bound monoterpenes in floral organs other than petals in roses. They also found quantitative differences in free and glycosidically bound volatiles between inner and outer petals, tips and bases of petals, and petals and other organs. Generally, petals and female organs have similar oil compositions but a lower yield than stamens which have markedly different compositions (Attaway *et al.* 1966; Mihailova *et al.* 1977). The sepals and green parts of *Rosa rugosa* Thunb. flowers contain mostly sesquiterpenes, monoterpenes and geranyl acetate (Dobson *et al.* 1990). Petals contain benzenoid and monoterpenoid alcohols. The androecium, as in other flowers, contains a far more diverse perfume, including eugenol, methyl eugenol, citronellyl-, neryl- and 2-phenylethyl-acetates and 2-undecanone. Pollen contains mostly fatty acid derivatives: 2-tridecanone, tetradecanol, tetradecyl acetate, hexandecanal, hexadecyl acetate, geranyl acetone, eugenol and methyl eugenol. Some adsorption or translocation of major petal and sepal volatiles to pollen was evident, although only to low levels.

In *Cyphomandra* flowers (Solanaceae), the seven main volatiles were divided into 'vegetative' and 'floral' components (Sazima *et al.* 1993). 'Vegetative' components typical of the leaves (α– and β–pinene, myrcene) occurred to low levels in anthers and were major components of the extract from the 'rest of the flower'. 'Floral' compounds (1.8 cineol/limonene, *trans*–β–ocimene, germacrene D and an unknown), occurring only to low levels in green leaves and the 'rest of the flower', were found at high levels in the anthers. Most of the emission of the main monoterpenes of *Clarkia breweri* flowers comes from the petals; one third of this amount and one tenth came from the pistils and stamens respectively (Pichersky *et al.* 1994). The pistil emitted more linalool per unit fresh weight than other floral organs, however calculations for contribution to the overall scent of particular organs must take into account the weights of each organ as part of the whole flower. The pistil was

almost the exclusive source for emission of pyranoid and furanoid linalool oxides. The method employed by Pichersky *et al.* (1994) involved removal from living flowers of floral organs other than the ones being assessed, and subsequent headspace sampling and GC analysis.

Wilson (1982) found components of boronia extract in all floral organs. β–ionone, dodecyl acetate and (Z)-heptadec-8-ene occurred in extracts from all floral organs, with large amounts of tetradecyl- and dodecyl-acetates in the stigma. Wilson's work was not quantitative but he raised the question of whether oil gland volume or stigma size may be related to extract yield from boronia flowers. Some glands were found in the carpels, although none were found in the anthers or filaments (Wilson 1982).

2.2 Flower and insect interactions

It is thought that insect pollination evolved primarily through meshing of the sexual life styles of phytophagous insects with flowers in which floral odours served as chemical cues for mating sites and food. All the insect groups involved in floral pollination have, as their ancestral condition, phytophagy on other groups of Angiospermae and have since 'specialised' (Pellmyr and Thien 1986). Similarities between floral fragrances and vegetative extracts that serve as feeding deterrants suggest that compounds of this nature served as identification compounds to insects which could bypass the deterrants.

Entomophilous flowers use both fragrance and pigmentation to attract pollinators. Fragrance emission by flowers to attract nocturnal pollinators is more necessary than for diurnal pollinators which are attracted by pigments and use flower volatiles mainly as orientation clues (Jakobsen and Olsen 1994). Insects are most sensitive to coloured light in the range from ultraviolet to green and yellow, and are largely insensitive to red light (Penny 1983). Often colour contrast boundaries act as orientation nectar guides, especially between the petal and reproductive organs (Sazima *et al.* 1993). Flavonol glucosides in the bases of petals of *Rudbeckia hirta* absorb UV light and distinguish the nectar guide to appropriate insect pollinators (Thompson *et al.* 1972). Yellow flowers are often highly reflective and attract an almost unlimited array of insect visitors (Kevan and Baker 1983). It is probably no coincidence that the inner surfaces of *B. megastigma* flowers are richly pigmented with yellow/green, becoming gold-coloured in mature flowers. The tiny functional anthers bear pollen; both anther and pollen are bright yellow and situated between the red-brown non-functional anthers and surrounded by a large red-brown stigma. This would direct any insect visitors to the sexual organs of the flower and any available food rewards.

The positive pollinator response to fragrance alone, and lack of response to colour alone, suggests fragrance is the more primitive means of attraction. Together, colour and fragrance act synergistically: olfactory stimulation induces the visually directed search for food (Pellmyr and Thien 1986). It is assumed that compounds specifically active in pollinator attraction should be emitted only from the flower (Pellmyr *et al.* 1987). Other compounds probably act as general attractants or deterrants. The principal differences in extract of boronia leaves and flowers are the presence of ionone, jasmonates, tetradecyl- and dodecyl-acetates in floral extract (Wilson 1982), although this study was not quantitative nor exhaustive in its identification of compounds. All of these compounds are known insect attractants (Baker *et al.* 1981; Buttery *et al.* 1982).

2.3 Rhythms in fragrance emission

Many flowers emit odour to a greater extent at particular times of day (Joulain 1985; Altenburger and Matile 1988, 1990). Broom (*Spartium junceum* L.) emits most strongly at early morning, and some *Nicotiana* species, at night (Loughrin *et al.* 1990, 1991). Circadian rhythms in fragrance emission may vary between compounds due to different volatilities or diffusion rates, and may be brought about by changes in temperature, activities of metabolising enzymes, or release from bound forms such as glycosides (Altenburger and Matile 1988; Jakobsen and Olsen 1994; Ackermann *et al.* 1989). Presumably, emission of insect pollinator attracting compounds at times when insects are active will increase the likelihood of successful reproduction. Qualitative and quantitative changes in fragrance emission may indicate flower readiness for pollination, or supply of nectar or pollen (Jakobsen and Olsen 1994). In some flowers, the total volume of oil present may fluctuate diurnally although the composition remains constant (Scora *et al.* 1984).

Daily cycling in emission of pollinator-attracting compounds is not a pre-requisite for pollination. Moth-pollinated flowers have been identified that emit volatiles cyclically to coincide with times of moth activity, as have moth-pollinated flowers that do not (Matile and Altenburger 1988; Loughrin *et al.* 1990, 1991). Nocturnal emission of flower volatiles is more often found to be controlled by an endogenous clock (i.e. circadian) than diurnal emission which may be more influenced by prevailing light and temperature conditions (Hansted *et al.* 1994). To demonstrate a circadian rhythm three conditions should be met: 1) the rhythm should continue under constant environmental conditions; 2) the period of the rhythm should not be exactly twenty four hours; and 3) the phase of the rhythm should change under different environmental conditions (Jones and Mansted, quoted in Hansted *et al.* 1994). If any compounds were to be emitted rhythmically, one would expect them to be exclusive to

the flower (Pellmyr *et al.* 1987). Vegetative compounds as general feeding deterrants would be necessary at all times (Dicke 1994).

It is really not known what insect pollinates *B. megastigma.* Many insects may be seen in and around the canopy during flowering. Small moths in the Heliozelidae family are frequently seen around flowers of *B. citriodora* and *Zieria* (Rutaceae) in Tasmania (P. McQuillan pers. comm.).

Summary

Secondary compounds may have diverse structures and functions. They may be the products of biosynthetic enzyme activity, or the result of oxidative or enzyme-mediated reactions acting on larger molecules. The pigments of flowers may be a complex mixture of many different compounds, and together with scent production, may act in the attraction of pollinators. Volatile compounds may be specifically produced in and emitted from sex organs such as anthers, and may be released rhythmically in diurnal or circadian patterns. The complement of pigments and volatiles in a flower may affect pollination by different attraction strategies, and may be linked by metabolic interactions such as the production of the volatile compound β–ionone from carotenoid pigments.

3 Sites of synthesis and accumulation of secondary compounds

Compounds such as monoterpenes usually do not exist free in the cell because they are toxic to normal plant function (Loomis 1974; Scora and Adams 1973; Brown *et al.* 1987). They may be bound to glucose or another sugar and exist as non-volatile glycosides (Francis and Allcock 1969). Glycosides are stored in the vacuole, can move freely around the cell and be transported around the plant (Croteau and Martinkus 1977). In many essential oil plants such as peppermint, the extract is largely contained within specific glands on the leaf surface that may also be active in synthesis of secondary products. In some plants, synthesis and compartmentation of secondary products occurs in otherwise unspecialised cells, within plastids, vacuoles, or membrane systems (Esau 1977; Fahn 1979a). This occurs in flower petals such as rose where no oil glands are found (Stubbs and Francis 1971). However, there are several cited examples of flowers bearing specialised glandular structures (Dobson *et al.* 1990 and references therein; Wilson 1982). In *Calceolaria* flowers, oil is secreted by glandular hairs consisting of a short stalk and a multicellular head (Schnepf 1974). Floret secretory trichomes of *Achillea millefolium* L. ssp. *millefolium* (yarrow)

accumulate osmiophilic secretion in the subcuticular space and this is released by rupture of the cuticle (Figueiredo and Pais 1994).

3.1 Cellular Compartmentation

Glands are distinct groups of highly specialised cells that function in the discharge of materials to the exterior, or into intercellular cavities for accumulation (Schnepf 1974; Menary and Doe 1983). Glands may specifically accumulate hydrophilic or lipophilic secretions, or a mixture of both (Fahn 1979a; Werker 1993). They are made up of secretory and auxiliary cells. There are many different kinds of secretory and gland cells. If a substance is synthesised by a group of cells, but is not actually extruded from the cells, then these cells are excretion cells rather than secretory cells. Lysigenous glands discharge their contents by lysis, and are also classified as (holocrine) glands (Schnepf 1974). Most gland cells exhibit dynamic ultrastructural modifications necessary for their function in secretion. Glands may be of differing complexity, taking the form of storage cells, glands (trichomes) and hairs (Croteau 1977b; Wiermann 1981; Barz and Köster 1981). Since oil glands act as biosynthetic and repository sites for essential oils, their size and number may influence the yield of oil (Henderson *et al.* 1970; Tanaka *et al.* 1989). There may be a limit to production of secondary products per gland or per plant. In hop, reduced gland filling occurred at high gland densities (Menary and Doe 1983).

Usually all oil glands on a leaf (El-Keltawi and Croteau 1987) or a fruit (Scora and Adams 1973) contain all of the compounds found in the extract. Similarly, plants that produce a complex extract in the vegetation, usually produce aromatic compounds in other parts of the plant, although the composition and site(s) of synthesis may vary (Corduan and Reinhard 1972; Henderson *et al.* 1970). There can be quantitative variations resulting from differences between leaf age or position on the plant (Heinrich *et al.* 1983; Werker *et al.* 1993). In cases where more than one type of oil gland occurs, they may contain the same or a different oil (Henderson *et al.* 1970; Venkatachalam *et al.* 1984).

Compounds within oil glands or cells are not static. Synthesis and catabolism are continuous, leading to a dynamic composition and the necessity for the glands to have an interchange of compounds with the rest of the plant. Trichomes are initiated early in the development of a leaf (Postek and Tucker 1983; Werker *et al.* 1993), and glands are most active in synthesis when leaves are young (Croteau 1991). Degenerated cell structure concomitant with gland filling often occurs in young leaves that have hardly begun expanding, however synthesis of secondary compounds continues beyond this time, often until well after expansion has ceased (Loomis and Croteau 1973). In older leaves, many glands are holding their capacity of oil and little

further synthesis occurs, however, rates of catabolism of secondary compounds increases at this time. Storage within glands may be restricted only to free monoterpenes, and loss from gland cells through volatilisation and evaporation may produce a constant demand for biosynthesis. Even after isolation, specialised structures retain their ability to synthesise secondary compounds (Gershenzon *et al.* 1992; Gleizes *et al.* 1987; Russin *et al.* 1992).

It is likely that cells other than gland cells may produce or store essential oil compounds (Fahn 1979a; Heinrich *et al.* 1983; Loomis and Croteau 1973), perhaps as water-soluble glycosides. There is also evidence that cell suspension cultures may continue to synthesise secondary compounds, accumulating them in organelles (Figueiredo and Pais 1994) or expelling them into the medium (Banthorpe *et al.* 1986; Berlin *et al.* 1984; Hong *et al.* 1990).

Oil glands in the Rutaceae family have been described (Haberlandt 1928). Typically they are sub-epidermal glands with a cover of four cells more vertically flattened than the surrounding epidermal cells, making them sunken in appearance. The outer walls of the cover cells are cutinised and usually thinner than normal epidermal cells. It has been suggested that there is an active release mechanism which causes a rupture in the epidermal cap(s) (Haberlandt 1928). The body of such glands comprise one to three layers of flattened cells surrounding a central cavity that is typically oil-filled. Wilson (1982) described similar glands on the petals, sepals and carpels of *B. megastigma* flowers. He did not show sections of glands on the petals, but his light microscope plates of glands in the carpels are similar to those in the ovary of *C. deliciosa* Ten. (Bosabalidis and Tsekos 1982a). Leggett (1979) concluded that ruptures in epidermal cap cells were most likely coincident with senescence.

Secretory cavities of *Citrus deliciosa* are connected to the epidermis by a small conical stalk, the top of which consists of epidermal cells belonging to the glandular structure (Bosabalidis and Tsekos 1982a). Sub-epidermal cavities may initiate from a single epidermal cell (Fahn 1979a), or from a pair of meristematic cells: one epidermal, one beneath the epidermal cell (Bosabalidis and Tsekos 1982a). From the first two divisions of the meristemoid, an upper and a lower strata differentiate. Most of the gland cells develop from the lower stratum, including the future epithelial cells that surround the central cavity and the sheathing peripheral cells which are radially flattened. The cells of the upper stratum divide anticlinally (Bosabalidis and Tsekos 1982a) or periclinally (Fahn 1979a), producing outer cells that remain in the epidermis becoming 'cap' cells, and inner cells that become sheathing peripheral cells.

The expansion of the central cavity in secretory cavities of Rutaceae may develop three ways depending on whose work one is reading. In the leaf of *Citrus sinensis* and *Ruta graveolens*, schizogenous development is described. Oil synthesised by schizogenous secretory cells initially accumulates in the space between

two cells which eventually becomes the central cavity (Thomson *et al.* 1976). As more oil accumulates in this space the cavity becomes spherical, and surrounding cells become flattened. Other work, also with *Citrus* and *Poncirus*, describes lysigenous development in which oily matter formed within plastids remains inside the plastids until they disintegrate. Eventually the cell wall also disintegrates, leaving the oil to accumulate as successive cells lyse (Heinrich 1969). Other work describes schizo-lysigenous development in *Ruta* (Peterson *et al.* 1978). It is possible that entire terpenoid droplets may cross membranes by breakdown of the particles during permeation of the cell wall followed by reaggregation in the lacunae of the gland or duct (Schnepf 1974). Stubbs and Francis (1971) note the presence of small vesicles in cells of rose petals with some evidence for them once having been membrane-bound. They suggest that such vesicles may be involved in the extrusion of monoterpenes through the cell wall of petals.

In secretory cavities on the ovaries of *Citrus deliciosa,* lysigenous development is described (Bosabalidis and Tsekos 1982a, 1982b). Bosabalidis and Tsekos (1982b) describe a process that produces an opening in the centre of the gland, around which lysed cells are radially flattened. Once cellular division has ceased, a cell in the centre of the gland becomes more electron dense, with many degenerated organelles. Surrounding turgid cells protrude into the lysing cell. Degeneration is preceded by dilation of the nuclear envelope, the occurrence of ER elements, and the formation of numerous vacuoles. Once this cell has degenerated, a second cell begins the process. Once this initial space is created, surrounding cells become active in secretion of oily matter into the cavity. As the innermost layer of the secretory cavity (the epithelium) lyses, outer cells may become incorporated into the epithelium (Fahn 1988). Wilson (1982) states that oil glands in boronia flowers probably develop lysigenously because of the evidence for cellular degradation around the central cavity. Wilson did not examine the ultrastructure of the cells.

Peripheral cells of the oil glands of *C. deliciosa* have large vacuoles and thicker walls (600nm) than inner secretory cells (120nm) (Bosabalidis and Tsekos 1982b). Such cells may store assimilates or volatile compounds bound as glycosides that can be further transported into the secretory tissue. In late stages of development of cavities in *C. deliciosa* ovaries, the few remaining plastids contain many starch granules and are true amyloplasts, typical of the gynoecia (Greyson 1994). The many oil droplets have variable electron density.

3.2 Subcellular Compartmentation

The elucidation of organelles active in the biosynthesis of secondary compounds such as monoterpenes is not easy. It is most likely that different organelles may secrete different parts of the extract, and further, that a particular tissue may be involved in secretion of a number of different substances (Fahn 1988). Comparative electron microscopy of different gland types has been used to find relationships between structure and function. The inherent problem with this method is that fixing and dehydrating procedures often extract the majority of the substances of interest, leaving vacuous cavities. The proportion of a cell taken up by particular organelles such as plastids may be indicative of their activity in essential oil biosynthesis (Bosabalidis and Tsekos 1985). Cells of secretory tissues usually have many mitochondria, and accumulate other organelles according to the nature of the secretion (Fahn 1988). Results from various researchers suggests that there are predominantly two organelles active in biosynthesis of secondary compounds contributing to essential oils: the plastidial membranes and the endoplasmic reticulum (Wiermann 1981; Duke and Paul 1993; Frey-Wyssling and Mühlethaler 1965; Heinrich *et al.* 1983; Thomson *et al.* 1976).

3.3 Plastidial synthesis

Plastids are the organelles most involved with synthesis of terpenoids, especially carotenoids (Kleinig 1989; Beyer *et al.* 1980; Soler *et al.* 1992). However, plastids, mitochondria and cytoplasm all have enzymes capable of converting isopentenyl pyrophosphate (IPP) into isoprenoids. There are two separate pathways for isoprenoid synthesis in higher plants; an extra–plastidial site which produces sterols, and another within plastids, producing isoprenoids such as carotenoids (Schulze-Siebert and Schultz 1987). The presence of lipophilic droplets that may or may not be osmiophilic but appear to be composed of terpenoids because of their volatility, and the similarity of these droplets to those formed when plant cells are treated with exogenous citron or d-limonene (Heinrich 1970) support the plastidial theory of terpenoid synthesis (Bosabalidis and Tsekos 1982a). Croteau and co-workers have had much success with various techniques including isolation of glandular trichomes and subsequent biochemical assays with labelled precursors (Gershenzon *et al.* 1992; Croteau 1977b). Radioactive precursors of terpenoids are incorporated within plastids (Treharne *et al.* 1966). One plastid-type linked with monoterpene synthesis are leucoplasts which lack ribosomes and thylakoids (Gleizes *et al.* 1983; Cheniclet and Carde 1985). In schizogenous glands of *Ruta*, methyl ketones accumulate in intercellular spaces (Schnepf 1974); the secretory cells have dense

cytoplasm with many ribosomes, and leucoplasts with a tubular internal structure. Poorly developed ER does not sheath the leucoplasts, although periplastidial ER is common in oil glands (Fahn 1979).

Terpenes may be biosynthesised within plastidial tubuli, and between plastids and the ER envelope which encloses them (Heinrich *et al.* 1983). As the number and size of the oil droplets increases, the plastid structure disintegrates further, the droplets may fuse together, and upon lysis of the plastid, the droplets are expelled into the cytoplasm (Fahn 1979a; Heinrich 1969). It has also been suggested that the membrane permeability of plastids changes during differentiation (Hampp and Schmidt 1976), and that an active process may control the movement of terpenoids from the interior to the exterior of plastids (Cockburn and Wellburn 1974). A close association between oil-filled plastids and ER was seen in cavities of *C. deliciosa* ovaries (Bosabalidis and Tsekos 1982b). It is thought that osmiophilic material from the plastid may get into the central cavity of oil glands by transportation to the ER and thence to the cell wall, where it moves via the plasmodesmata into the periplasmic space (Bosabalidis and Tsekos 1985; Burns *et al.* 1992). In this way, toxic materials remain segregated from the rest of the cytoplasm. Some oil droplets may be membrane-bound (Carde 1976), and this represents another method for protective segregation.

3.4 Chromoplasts

Plastids, being the site of photosynthesis in green tissues, are significant cellular organelles, and their ability to change morphology and function has been noted (Esau 1977). In flowers and fruits which are young, unexpanded and green, chloroplasts are similar to those found in leafy tissues, with densely packed, well-organised thylakoids that are active in photosynthesis (Vemmos and Goldwin 1993). Floral organs such as sepals, receptacles and pedicels may have stomata (Esau 1977) and significant chlorophyll levels, and continue to be active in photosynthesis throughout the life of the flower. Such photosynthesis may be important for the flower during early stages of expansion, since the leaves may not be active exporters at this time. Photosynthetic activity may increase during fruit development in fleshy fruits such as apples (Vemmos and Goldwin 1993).

As petals expand and mature, stomates become non-functional, chlorophyll levels decline, carotenoid levels increase and the thylakoid structure disappears to be replaced by a disorganised membrane structure that may be of several forms (Buvat 1969; Smith and Butler 1971). In flowers, plastids have usually become chromoplasts: carotenoid-bearing, photosynthetically inactive plastids, the function of which (if any) is to synthesise and store carotenoids and, in some cases, osmiophilic material such as essential oils (Buvat 1969; Sitte *et al.* 1980). Chromoplasts are typical

plastids in that they invariably possess an envelope consisting of two separate membranes, and a stroma matrix containing ribosomes which are smaller than the cytoplasmic ones, as well as less dense regions containing slender, uranophilic filaments consisting presumably of DNA. Lipids make up one third of the weight of chloroplasts and one half in chromoplasts. The lipids *trans*-hexadecanoic acid and linolenic acid are much reduced in chromoplasts. Proteins make up one half of the weight of chloroplasts, and one fifth in chromoplasts (Frey-Wyssling and Mühlethaler 1965). Chloroplasts and chromoplasts contain both RNA and DNA (Kowallik and Herrmann 1972; Herrmann 1972).

Chromoplasts are derived from leucoplasts in epidermal cells, and from chloroplasts in internal tissues (mesophyll and mesocarp). They can also form from amyloplasts and the simplest type of proplastid. Chromoplasts can be dedifferentiated and redifferentiated into chloroplasts (Thomson *et al.* 1976). However, usually chromoplasts persist in that state until the cells in which they lie become senescent (Whatley and Whatley 1987). The control of chromoplast development may be hormonal, and does not require the rest of the plant, because similar changes occur when petals are excised (Smith and Butler 1971). Certain observations of chloroplast degeneration in senescent leaves have been extrapolated to the development of chromoplasts in flowers and fruits. There are many differences in the degenerating chloroplasts of senescent mesophyll cells (gerontoplasts) and the plastids of anabolic cells of young flowers and fruits. In fully developed secretory cavities of *C. deliciosa*, plastids undergo a striking increase in number. Their matrix loses its initial high electron density and contains smooth tubular elements (Bosabalidis and Tsekos 1982a). Metabolic activity usually declines in mature, well pigmented chromoplasts (Nichols *et al.* 1967). Chromoplasts occur in flowers and fruits, and their diversity in size and shape is paralleled by pronounced differences in fine structure. There are several different types of chromoplasts:

Globulous type: found in *Citrus sinensis*, *Jasminum nudiflorum* Lindl., and *Lycopersicon esculentum* Mill. (among others). Globulous chromoplasts are lens-shaped or spheroidal, containing carotenoid-carrying lipid droplets in their stroma that vary from 0.05 to more than 1.0 mm in diameter. This is the most common type of chromoplast (Whatley and Whatley 1987).

Tubulous type: found in *Rosa sp.* Characterised by extended carotenoid-carrying internal elements which are more or less circular in cross section. The diameter is about 20nm and can be up to 60nm. 'Tubules' present in the organelles are best seen in ultrathin sections. Chromoplasts seem to be composed of a strongly hydrophobic core and a less hydrophobic superficial layer. The tubules are mostly

unbranched, forming conspicuous paralleled aggregates which appear as birefringent and dichroitic areas under the polarising microscope. Many tubulous chromoplasts preferentially found in flowers are spheroidal in outline, whereas those containing globule-associated tubules are elongated and often spindle-shaped.

Membranous type: Contain a set of up to 20 concentric (double) membranes as their most prominent internal structure. The overall shape is spherical, but heavy deformations are not unusual. Under the polarising microscope, a faint birefringence can be detected, positive in character, attributed to radially oriented lipid molecules. This form is restricted to flowers, particularly the corona of yellow and orange *Narcissus* flowers (Beyer *et al.*. 1980) and rose petals (Stubbs and Francis 1971).

Crystal-containing type found in *Citrus paradisi* Macf., *Lycopersicon esculentum*, *Narcissus sp.* Containing crystalline inclusions of pure carotene; where growth is visible under light microscopes, they dictate the shape of the chromoplast. Carotene crystals can also occur in chromoplasts of flowers and leaves and in certain lycopene-accumulating mutants of corn and barley. Carotene crystals originate not in the stroma matrix, but within thylakoids, i.e. 'intralocularly'.

3.5 Synthesis in other organelles

Other organelles may synthesise components of plant extracts. The accumulation of ER in glandular trichomes on leaves of *Mentha piperita* and *Salvia sp.* suggests involvement in the biosynthesis of lipophilic substances (Schnepf 1974). It appears to be smooth surfaced tubular ER that predominates in mature gland cells; immature glands being rich in rough ER. ER is active in the biosynthesis of sterols and therefore it may also be involved in the synthesis of lipophilic secretions (Schnepf 1974). ER involvement in the synthesis of mono- and sesqui-terpenes has been documented (Gleizes *et al.* 1980).

Dictyosomes (golgi bodies) may mediate transport of neutral and acid polysaccharides as well as glycosylating secondary metabolites such as terpenes in preparation for vacuolar storage or transport (Henderson *et al.* 1970). Glycosidase enzymes may be present within the lysosomal cell compartment, as are other hydrolases responsible for turnover of secondary products (Matile 1974).

3.6 Sites of synthesis in flowers

Typically, flowers do not secrete fragrant compounds into specialised gland cells (Fahn 1979a). Glycosidic conjugation may play a more significant role in the metabolism of fragrant compounds in flowers than in herbage (Francis and Allcock 1969; Watanabe *et al.* 1993).

3.6.A Petals

Flower petals usually synthesise monoterpenes and other such compounds in epidermal cells; a 'glandular' epidermis (Francis 1970). The incorporation of an exogenous precursor for monoterpene synthesis (mevalonic acid) into rose petal monoterpenes was more than ten times greater than incorporation of the same precursor into monoterpenes in peppermint leaves (Francis and O'Connell 1969). This increased rate of incorporation may be a result of the lack of compartmentation of monoterpene synthesis in rose petals (Loomis and Croteau 1973).

Fragrance release from flower petals usually results from volatilisation of accumulated droplets from the cytoplasm of epidermal and mesophyll cells. Increased external temperatures increase the rate of passive diffusion of compounds through the cell wall and cuticle (Fahn 1979a, 1988). In some petals, the adaxial walls may be convex or papillate; this increases the surface area and aids volatilisation of fragrant compounds (Esau 1977; Stubbs and Francis 1971). More papillae occur on the adaxial than the abaxial epidermis and they are not developed at the base of the petals. Epidermal cells of sepals and petals are thin; the anti-clinal walls are folded or undulated and arranged so that the projections of the cells dovetail into one another (Esau 1977).

3.6.B Nectaries

Nectaries may be distinct gland cells or may be specialised areas of parenchyma with a gland-like character through which sap from sieve tubes emerges through modified stomata (Schnepf 1974). Specialised parenchyma cells usually have a dense granular cytoplasm and a relatively large nucleus and many mitochondria (Fahn 1988). Floral nectaries are common in Rutaceae (Fahn 1979b; Weston 1978). They are highly localised sites with distinctive cytology and anatomy: the ER and the golgi apparatus are involved in the process of secretion (Fahn 1979b). Plastids have few thylakoids but usually have abundant starch. Nectaries excrete significant quantities of unmodified or only slightly modified products including amino acids, sugars, proteins, lipids, anti-oxidants and alkaloids (Greyson 1994; Schnepf 1974). The main sugars

found in nectar are saccharose, glucose, fructose and various oligosaccharides (Schnepf 1974). The presence of nectaries complicates the identification of odours from different parts of the flower.

Nectaries, or a nectar-secreting disc are not described in *B. megastigma* flowers (Weston 1978). However, absence of nectar-producing tissue seems unlikely if boronia is pollinated by moths, as believed (J. Plummer pers. comm.), unless the moth visitors eat the pollen (Kevan and Baker 1983).

3.6.C Gynoecium

Secretory cells abound in the gynoecia: complex glandular organs, made up of a wide variety of cell types (Greyson 1994). The stigma may be partly or entirely secretory. The cytoplasmic organisation of the stigma varies between angiosperm families (Knox 1984), manifesting in quantitative differences in organelles important for the secretory process. Some stigma cells accumulate ER, others golgi bodies or vesicles, signifying different secretory product(s) and processes. Many are filled with chloroplasts and contain starch granules that disappear at anthesis (Greyson 1994). In *Petunia hybrida*, the stigma comprises two zones, an outer secretory zone and an inner storage zone (Fahn 1979). There are lots of schizogenous spaces filled with droplets of substance secreted from the ER. High levels of starch are present, which declines with anthesis. Abundant golgi bodies and vesicles are found in *Citrus, Prunus* and *Malus*, and their secretions are mucilaginous, containing carbohydrates and proteins, lipids and polyphenols (Knox 1984; Greyson 1994). Release of glucose from glycosides such as phenolic glycosides provides sugars necessary for pollen germination (Esau 1977; Fahn 1979a).

3.6.D Androecium and pollen

Anthers of *Solanum* have an especially active epidermis that may be active in odour production and is rich in colour (Sazima *et al.* 1993). When water is lost from the endothecium of the anther, the cells become trapezium shaped (Fahn 1979b). The anthers of *Cyphomandra* contain osmophores with many spherosomes, internal secretion droplets and yellow chromatophores (Sazima *et al.* 1993). The cell walls of the osmophores are thick, and have many plasmodesmata indicating active transport, possibly of fragrant materials. Pollen is the sole food reward for insect visitors of nectarless flowers (Dobson *et al.* 1990). Therefore, the fragrance of pollen will be especially significant in the olfactory orientation of insects to such flowers.

Pollen grains exhibit a wide range of form and structure, most lack a cuticle, having instead a complex exine (Knox 1984). The exine is the patterned outer layer,

comprising wall units enveloped in sporopollenin. Within the exine are storage sites for a range of proteins, glycoproteins, carbohydrates, lipids, pigments, volatiles and phenolic conjugates (Knox 1984; Wiermann and Gubatz 1992). Mature pollen has a low water content and can appear dehydrated. Pollenkitt is the oily, pigmented, viscous surface coat of pollen grains, often the source of odouriferous compounds (Knox 1984; Dobson 1988; Dobson *et al.* 1987). A summary of pollen/pollination adaptation strategies may be seen in Table 3.6.

Dobson *et al.* (1990) detected large amounts of tetradecyl acetate and other acetates in pollen, and lesser amounts in empty anthers of *Rosa rugosa.*

Table 3.6. Pollen/pollination adaptation strategies.

Vehicle for pollen dispersal.	Size	Nature of Exine	Colour
Animal	>300 *u* m	massively thickened heavily ornamented	yellow, brown, blue or black
Wind	30 - 50 *u* m	thin	yellow
Water	spherical or filiform grains up to 5mm	reduced exine, thin intine. May be covered in mucilage.	

Source: Knox (1984).

Summary

Various types of glandular and non-glandular sites of production of secondary compounds have been described. The main organelles responsible for terpene production are the plastids and the ER. In flowers, senescent plastids (chromoplasts) occur, and these may be of several types. The production of secondary compounds in floral organs that lack discrete oil glands as in boronia, is of interest, especially when they accumulate compounds similar to those accumulated in organs with glands. Does biosynthesis occur in the same organelles in both cases? Or, does the presence of such compounds in sexual organs lacking oil glands indicate transport from other synthetic sites such as glands on the petals?

4 Volatile glycoside conjugates

Many of the key intermediates in monoterpene biosynthesis including geraniol, nerol, linalol and β–terpineol occur in bound forms as glycosides (Stahl-Biskup 1987). Since Francis and Allcock (1969) discovered glycosides in rose flowers, many researchers have endeavoured to identify instances of glycosidic binding, to isolate hydrolytic enzymes, identify volatiles and sugars, and examine the benefits for the plant in producing such conjugates.

To date, monoterpenes, sesquiterpenes, norisoprenoids, shikimate-derived compounds and pigments have been identified as glycoside conjugates (Hwang *et al.* 1992; Sefton *et al.* 1989). Often the bound fraction within a plant or organ is much larger than the free fraction, and the biological significance for this phenomenon warrants discussion.

4.1 Function

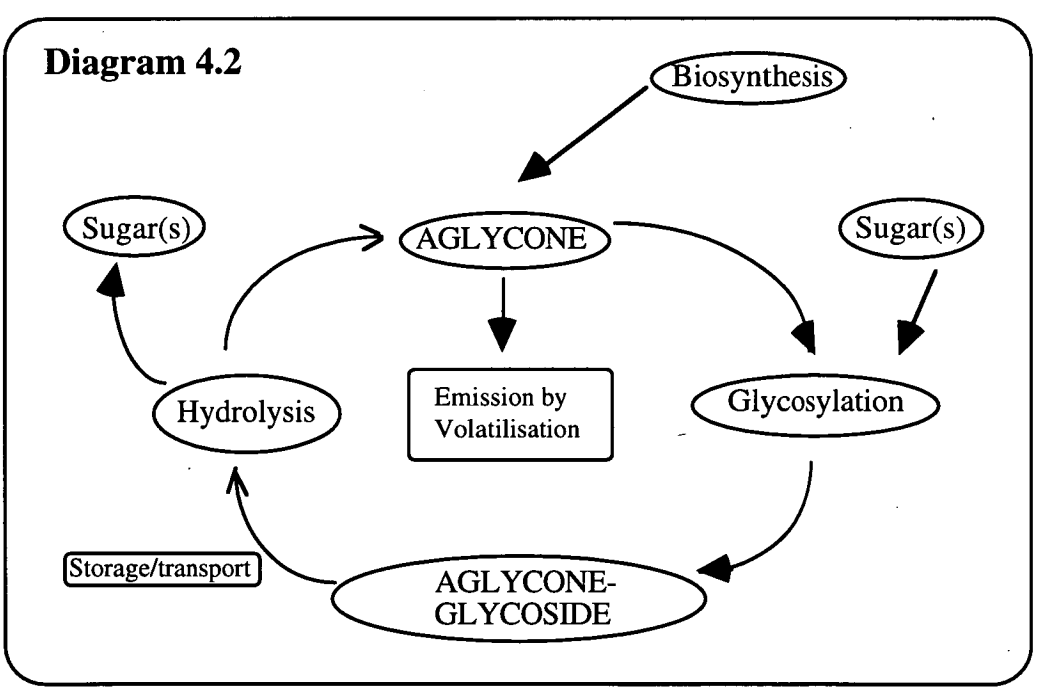
Glycosidic bonding enables aqueous solubilisation, dispersing products into the vacuole (Ackermann *et al.* 1993). Solubilisation may be a prerequisite for catabolism and reutilisation of carbon and energy from monoterpenes by conversion into low molecular weight organic acids and other water-soluble compounds. Monoterpenes are often inhibitory to other plant processes (Brown *et al.* 1987; Scora and Adams 1973) and therefore storage of monoterpenes in a non-toxic form may prevent inhibition of normal plant functions. Free monoterpenes are unable to penetrate cells without destroying membranes, therefore a means of transporting such compounds around the plant is needed. Glycosylation may enable movement of compounds from membrane-bound organelles into the xylem in preparation for transport around the plant (Booth 1960; Valadon and Mummery 1969; Thammasiri *et al.* 1987). Croteau and Martinkus (1979) demonstrated movement of (+)-neomenthyl glycoside from mint leaves to the rhizomes where it was de-glucosylated, hydrolysed to menthone, oxygenated and further metabolised. It has been suggested that monoterpenes in roses are synthesised in the leaf, glycosylated and transported via the phloem to the flower buds (Loughrin *et al.* 1992 and references therein). Bugorskii and Zaprometov (1983) showed that exogenously labelled glycosides were transported upwards through the xylem into the petals where they were hydrolysed into aglycones. This does not preclude the possibility of synthesis, glycosylation and hydrolysis of volatile compounds occurring completely within flowers and flower buds *in vivo*. It is clear that the role of glycosides may vary from time to time, between organs of one plant and between plant species.

It is thought that glycosidases and other hydrolytic enzymes necessary for cleavage of glycosides are newly induced or activated during flower opening (Watanabe *et al.* 1993). In a study of the activity of hydrolytic enzymes and the level of glycosidically bound volatiles in sequential bud stages of *Gardenia*, Watanabe (1993) found that only stages four and five, just prior to flower opening, had appreciable hydrolytic enzyme activity. The level of 'precursor' (aglycone-glycosides) increased prior to this, between stages three and four. This may indicate that a major function of the aglycone-glycosides is to enable rapid release of volatiles at anthesis (Loughrin *et al.* 1992). The presence of diurnal rhythms in emission of volatiles from *C. medica* flowers and the absence of similar patterns in tissue levels of free volatiles is interesting (Altenburger and Matile 1990). It suggests there may be a pool of volatiles dedicated to emission; changes in the amount of volatiles in the pool bringing about patterns in emission. Glycosylation may be the second last step, perhaps occurring during transport of volatiles from storage cells to epidermal cells. Once within epidermal cells, the sugar moiety is removed and the compound rapidly volatilises. The level of activity of glycosylating enzymes, aglycone-glycosides and hydrolytic enzyme activity may have more to do with circadian and diurnal rhythms in emission than the level of free volatiles (Pichersky *et al.* 1994). The release of volatiles by plants after damage by herbivorous arthropods is thought to occur because the insect injects, activates or stimulates the release of glucosidases (Dicke 1994). This may prevent further attack by other herbivores.

The commercial significance of glycosidically bound volatiles is their ability to modify the flavour and aroma of plant extracts, particularly fruit and grape products (Roberts *et al.* 1994; Sefton *et al.* 1989; Williams *et al.* 1982a), and the binding of aglycones in a form not extractable by organic solvents (Ackermann *et al.* 1989). Glycosides in grape juice may be hydrolysed very slightly during the fermentation process and thus they are important potential aroma sources (Gunata *et al.* 1988). Petrzilka and Ehret (1991) hydrolysed rose absolute and waste concrete with acid, glucosidase enzymes and with the microorganisms *Aspergillus, Lycoperdon, Saccharomyces* and *Geotrichum.* They found an increase in volatiles and in usable concrete. If Francis and Allcock (1969) were correct in their assumption that glycosides are biosynthetic precursors for free volatiles, then glycosidic hydrolysis by plant enzymes *in vivo* may explain why essential oil yield can sometimes be increased during post-harvest storage (Bugorskii and Zaprometov 1988; Francis and Allcock 1969; Mihailova *et al.* 1977).

4.2 Precursors?

Glycosidically bound monoterpenes accumulate and decline earlier than free monoterpenes in rose petals (Francis and Allcock 1969) leading to the theory that they are 'precursors' to volatiles. However, glycosides are not obligatory precursors for monoterpene alcohols in roses *in vivo* (Ackermann *et al.* 1989). Schulz and Stahl-Biskup (1991) also doubted any direct connection between glycosidically bound volatiles and the biogenesis of essential oil compounds. Once volatile compounds in flowers are biosynthesised, they may be either volatilised almost immediately, or rapidly bound as glycosides to be stored in vacuoles, or moved to different parts of the organ, or to different organs in the flower. *In vivo* hydrolytic enzyme activity increases prior to a time of 'need' for free volatiles by the flower, such as anthesis or a circadian rhythm, and releases aglycones which are then volatilised and emitted by the flower. Therefore, glycosidically bound volatile compounds are in a state of limbo between biosynthesis of free volatiles and emission of same compounds. Diagram 4.2 may help to clarify this:



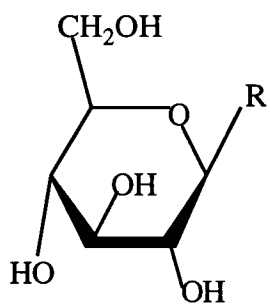
In diagram 4.2, the term 'precursor' relates to the form of volatile (bound or free) preceding the stage being discussed, whether it is the aglycone being emitted or the aglycone-glycoside being stored. Alternatively, glycosidic conjugates may be a means of removal of secondary products from the flower prior to senescence and

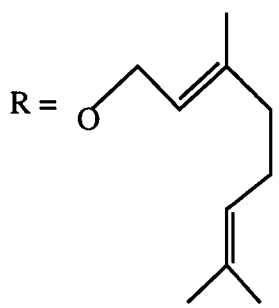
abscission; the route by which carbon and energy are salvaged by the plant (Croteau 1987; Croteau and Martinkus 1979).

4.3 Sugar moieties and structures

The sugar moieties most often bound to secondary metabolites are mono- and di-saccharides including rhamnose, rutinose and glucose (Watanabe *et al.* 1993) and sometimes sugars more polar than disaccharides (Winterhalter *et al.* 1990). Most aglycones are bound to glucose as β–D-glucopyranosides (Stahl-Biskup 1987; Gunata *et al.* 1988). Structures of some typical glycosides may be seen in diagram 4.4.

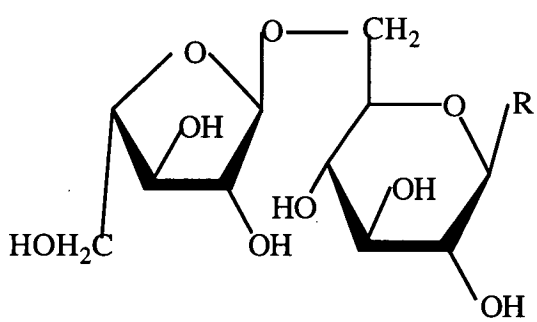
Diagram 4.4





= geranyl

β-D-glucopyranosides



6-O-α-L-rhamnopyranosyl-β-D-glucopyranosides

4.4 Hydrolytic enzymes

Monoterpene glycosides can be hydrolysed by acids to release free monoterpenes, however enzyme hydrolysis is considered to be less likely to modify

the natural monoterpene distribution (Günata *et al.* 1985; Williams *et al.* 1982b). Gunata *et al.* (1988) studied the effects of different glucosidase enzymes on grape monoterpenyl disaccharide glycosides. They showed that the (1->6) linkage is the first linkage to be cleaved, by either β–L-arabinofuranosidase or β–L-rhamnosidase, to release arabinose, rhamnose and the corresponding monoterpenyl β–D-glucosides. Subsequently, the monoterpenol is released by β–D-glucosidase. For cleavage of a mixture of aglycones and sugar moieties, an enzyme that combines glucosidase with rhamnosidase and other hydrolytic enzyme capability may be needed because the degree of ramification of the aglycone effects the cleavage activity (Ackermann *et al.* 1989). For general use pectinase or cellulase are recommended (Humpf and Schreier 1991; Ackermann *et al.* 1989). Agrawal and Bahl (1972) described the preparation of a multi-hydrolytic enzyme preparation from pinto bean cotyledons (*Phaseolus vulgaris*) comprising β– and β–galactosidase, β–glucosidase, β–N-acetylglucosaminidase and β–mannosidase. Watanabe *et al.* (1993) made an enzyme preparation from several flower species; these would be expected to be the most efficient enzyme preparations for hydrolysis of a mixture of glycosidic conjugates extracted from buds of the respective species.

Summary

The presence of glycosidically bound compounds such as monoterpenes and norisoprenoids has been noted. The functions these compounds may have in the plant are storage or transport forms for toxic volatiles, as precursors for release of free volatiles, or substrates for catabolic enzymes. Post-harvest changes in oil composition and diurnal or circadian rhythms may result from *in vivo* hydrolytic enzymes acting on a pool of glycosides. The presence of glycosylated compounds indicates an active role for the compounds bonded in such a way.

5 Biosynthetic Pathways

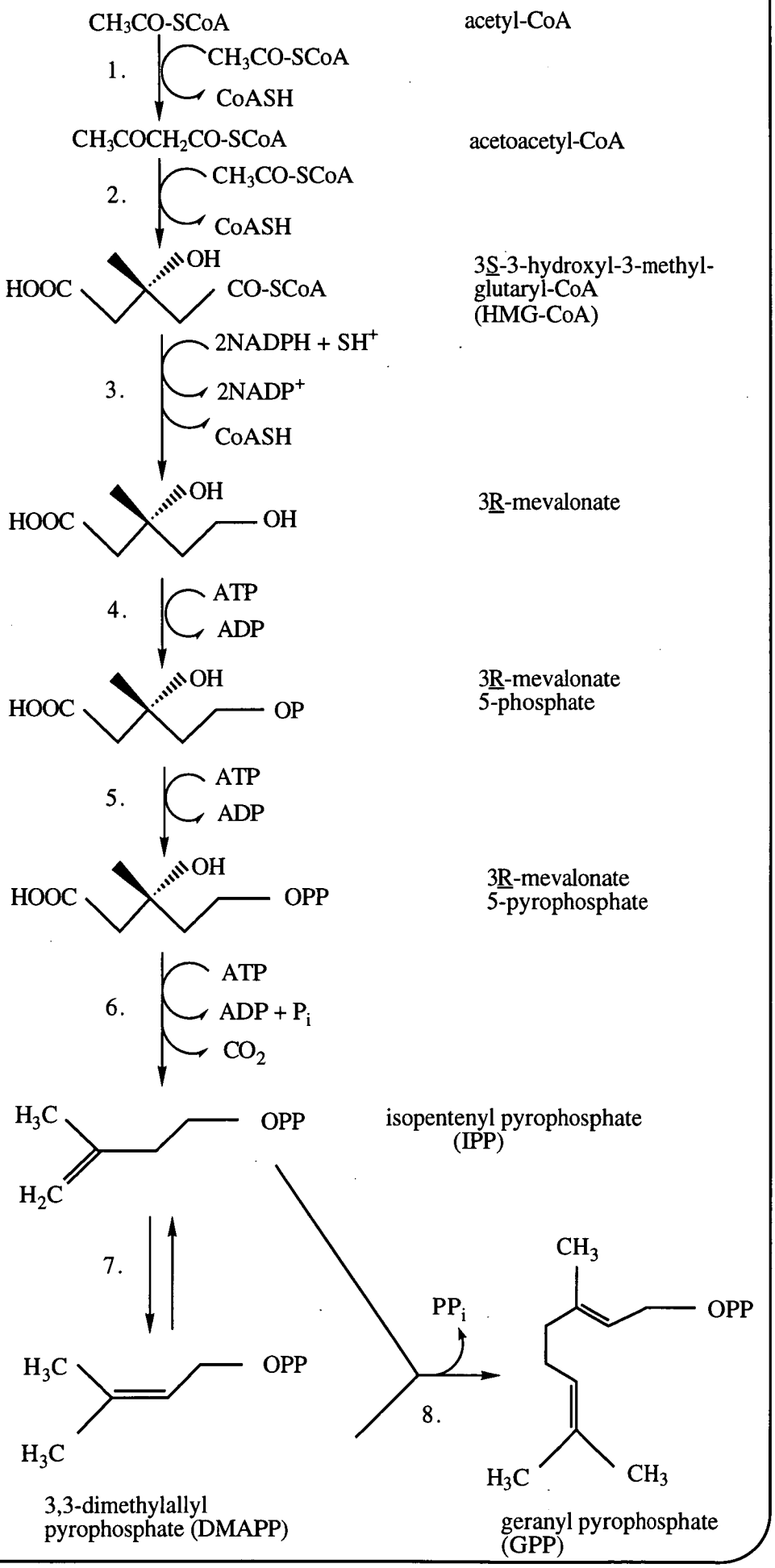
The biosynthetic pathways which will be dealt with in this review are those which produce major compounds found in floral boronia extract (Davies and Menary 1983). The terpenoids and norisoprenoids (ionones) will be discussed not only because they are major components of the extract, but because boronia extract contains a rich supply of orange carotenoids, and it is thought that carotenoids and norisoprenoids are biochemically linked. It should be stressed that the status of our knowledge of secondary metabolic pathways is increasing slowly, but it is at best sketchy, and many of the pathways for products in specific plants are ascribed because

the final products are similar to those for which the specific enzymology is more certain (Wiermann 1981). Very little is known about biosynthetic pathways and catalysing enzymes for the production of specific floral scent compounds. This is because the production of synthetic analogues of unusual floral compounds have generally received more attention from perfumerists and chemists as they formulate relatively inexpensive new perfumes (Pichersky *et al.* 1994; Joulain 1987).

5.1 Terpenoid biosynthesis

Terpenoids are one of the largest groups of secondary compounds, comprising mono-, di- and sesqui-terpenes, carotenoids and a miscellany of similar compounds. A general scheme for the biosynthesis of lower terpenoids has been accepted. The first stage is the activation of mevalonic acid (MVA), a compound derived from three acetyl-CoA molecules (Potty and Bruemmer 1970). Carbon dioxide and water are enzymically removed from MVA–5-diphosphate, producing isopentenyl pyrophosphate (IPP) (Kirk and Tilney-Bassett 1978). Diagram 5.1.1 (over page) shows a representation of the accepted pathway.

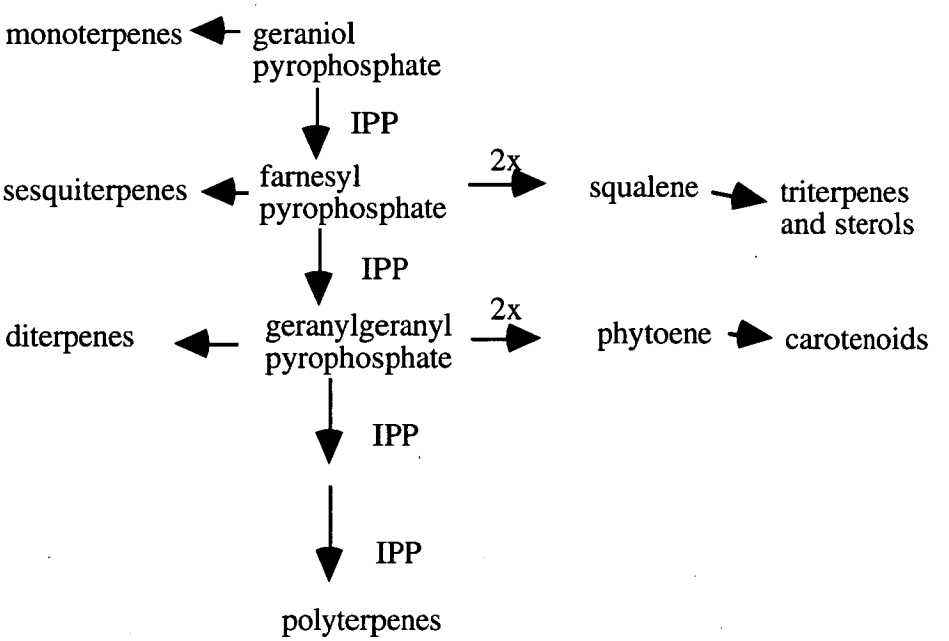
Diagram 5.1.1



Source: Gershenzon and Croteau (1989).

The addition of one IPP molecule to GPP (geraniol pyrophosphate) and to subsequent products continues, producing ever larger polyterpenes by one isoprene (five carbon) unit each time. This is illustrated in diagram 5.1.2.

Diagram 5.1.2



Source: Gershenzon and Croteau (1989).

At various points in this sequence, side reactions can occur producing a series of compounds with a common precursor such as geraniol or citral (Haagen-Smit 1972). Similarly, compounds may branch into other pathways to produce different compounds.

5.2 Carotenoid biosynthesis

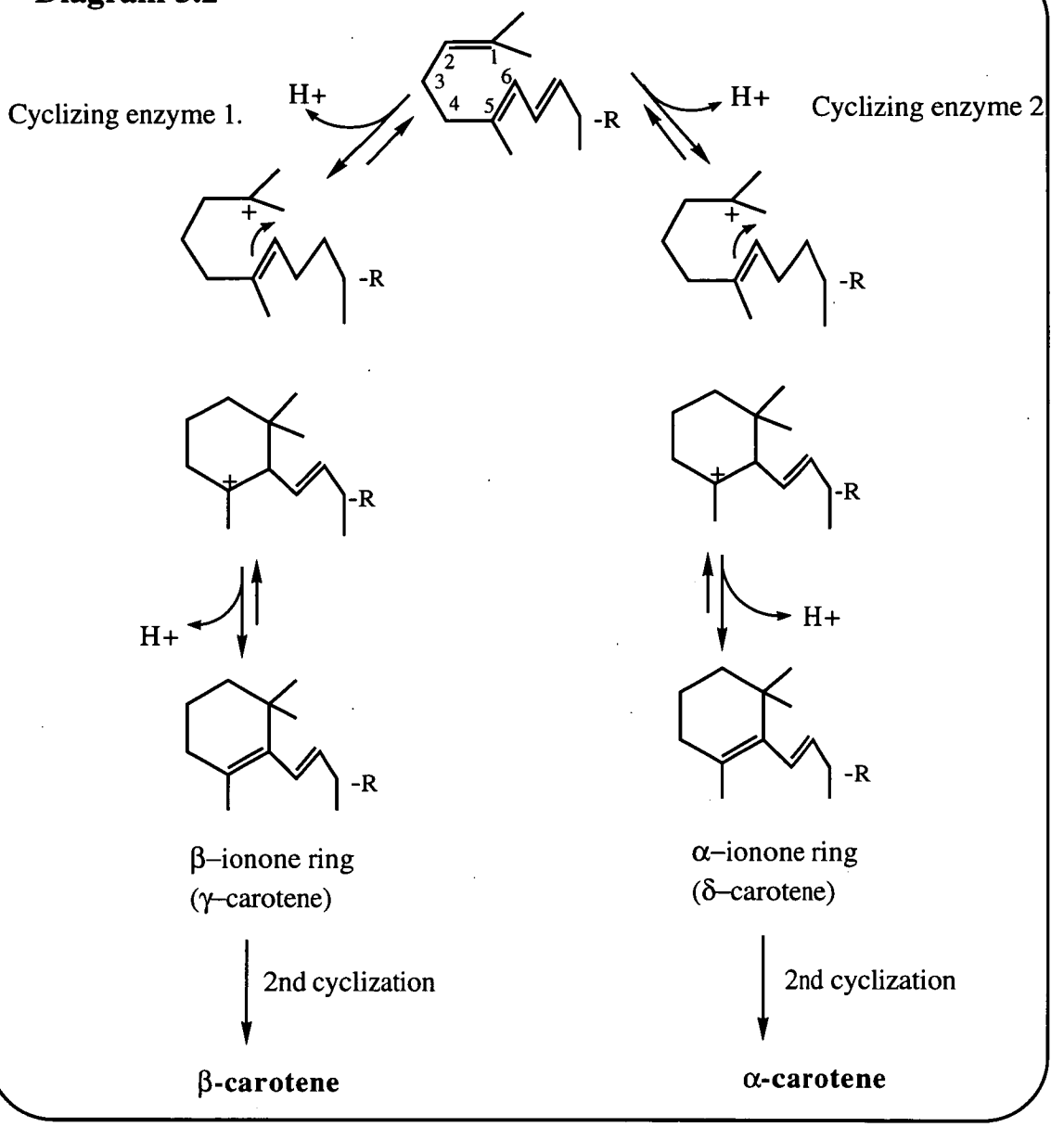
Factors which regulate synthesis and degradation of β–carotene or other carotenoids with a β–ionone ring end group are of interest in plants which accumulate β–ionone and related compounds. Carotenoids are produced after a series of desaturations acting on phytoene, itself comprising two GGPP molecules joined tail to tail. Lycopene is a symmetrical C40 carotenoid with a polyene chain and a sequence of conjugated olefinic bonds with 13 double bonds. Lycopene undergoes cyclisation at one or both ends to form α– or β– rings, yielding carotenoids such as β,β–carotene (Davies 1980; Goodwin 1980a; Bartley *et al.* 1994; Hill *et al.* 1971; Kushwaha *et al.*

1969). The cyclisation of lycopene (diagram 5.2, over page) may involve proton attack at C-2 followed by ring closure to yield a carbonium ion intermediate which then loses a proton to produce an α– or a β–ring.

The enzymes required for such conversions are membrane-bound and found in the plastid (Kleinig 1989; Beyer *et al.* 1985; Camara and Dogbo 1986). Phytoene formation from IPP is mediated by soluble stromal enzymes. The membrane-bound enzymes have been solubilised and reconstituted into liposomes in a functional state by the use of the zwitterionic detergent Chaps (3-[(3-cholamidopropyl)-dimethylamino]-1-propanesulfonate) (Beyer 1987; Beyer *et al.* 1985). This appears to be a very powerful tool for the study of otherwise non-soluble enzymes.

The biosynthesis of carotenoids within plastids is tightly linked with photosynthetic carbon fixation (Schulze-Siebert and Schultz 1987). It is likely that carotene metabolism takes place exclusively in the plastidial compartments and that end products remain there, especially since the plastid is impermeable to isoprenoids and carotenoids (Whatley and Whatley 1987).

Diagram 5.2



Source: Porter *et al.* (1984).

5.3 Alternative pathways to monoterpenes

There have been alternative pathways to MVA and terpenoids suggested and demonstrated, utilising the amino acids valine, leucine and glycine (Goodwin 1980b; Goodwin and Lajinsky 1951; MacKinney *et al.* 1952). Valine and leucine are converted into HMG-CoA and subsequently MVA in *Phycomyces blakesleeanus*, and addition of these amino acids to culture medium greatly enhances β–carotene production. Such alternative pathways for terpenoid biosynthesis are more prevalent in single celled organisms. This may be due to MVA–impermeability of these organisms.

Alternatively, higher plants, while capable of both pathways, may predominantly use the isoprenoid pathway. Further, higher plants may use both systems to synthesise the full gamut of terpenoids they accumulate, and most research has focussed on products of the main route.

Rose flowers are capable of incorporating exogenous labelled sucrose and leucine into monoterpene alcohols at 45-50°C during fermentation in water (Bugorskii and Zaprometov 1988). Experimental conditions may have inhibited enzymes responsible for the conversion of acetyl-CoA to MVA to a greater extent than those enzymes used to convert leucine to MVA. Concurrent assays with labelled IPP and MVA would enable examination of the relative activities of each pathway. The enhancement of extract yield and β–ionone in *B. megastigma* flowers with nitrogen application (Reddy and Menary 1989a, 1989b; Roberts and Menary 1994b) may indicate the channelling of excess amino acids into terpenoid synthesis.

5.4 Norisoprenoid biosynthesis

β–Ionone is frequently found in treated foodstuffs such as tea (Takeo 1983; Hazarika and Mahanta 1983; Sanderson and Graham 1973; Sanderson *et al.* 1971), whisky (LaRoe and Shipley 1969), milk powder (Suyama *et al.* 1983), dry grass (Aii *et al.* 1985), and dehydrated apple (Teule and Crouzet 1994). β–ionone has also been found in the spent culture media of cyanobacteria (Jüttner 1988). In quince oil (Winterhalter *et al.* 1991), β–ionone arises during the extraction process. It was the discovery of a 'violet-like odour' in stored, dehydrated carrot that was the first evidence for linking β–ionone and β–carotene (Ayers *et al.* 1964). Given the labile nature of carotenoids and the types of products β–ionone is found in, such a link seems probable. However, the formation of β–ionone in living plants has not been demonstrated to follow this particular route. β–ionone occurs to low levels in a large number of living plants including *Osmanthus fragrans* (Kaiser and Lamparsky 1981), several *Citrus* species (Kaiser 1991), oat leaves (Buttery *et al.* 1982) and many others. Within *Boronia megastigma* flowers, very high levels of ionones are accumulated. β–ionone is usually associated with flowers or fruit, which indicates a possible role in pollinator attraction and attraction of potential seed dispersal agents. It is a particularly persistent and effective attractant (Donaldson *et al.* 1990).

One way of examining biosynthetic relationships *in vivo* would be to study colour varieties of one species which produces both carotenoids and norisoprenoids while living. Two such studies have been done so far: in freesia (Lamparsky 1984) and in *Osmanthus* (Ding *et al.* 1989). Extracts of freesia flowers contain β–isophorone, α–isophorone, β–cyclocitral, safranal and oxoisophorone. An assessment of the concentration of these compounds in different coloured flowers

(white, yellow and red) showed an increased amount of these compounds in deeper coloured flowers. Only the red flowers emitted the compounds while alive (Lamparsky 1984). Compounds in the headspace and extracts from three colour varieties of *Osmanthus fragrans* (golden, silver and reddish) were examined (Ding *et al.* 1989). The headspace from the three flower types contained different total amounts of ionone compounds including β–ionone, α–ionone, dihydro–β–ionone and 4-oxo–β–ionone. The golden flowers emitted the highest levels, followed by the silvery and red flowers respectively. Extracts from all three varieties contained β–ionone (not quantified). The findings of these two studies are not consistent. Deeper coloured flowers, which presumably contain higher levels of carotenoids, do not always contain the highest level of norisoprenoid compounds. Perhaps the colour of the flowers is not the result of exclusive carotenoid accumulation; red colours may be caused by flavonoids, an accumulation of lycopene, or high levels of β–carotene. Only in the case where colour differences were due to differences in the levels of β-carotene or lutein, would different levels of ionones be expected if ionones are produced by degradation of carotenoids with cyclic end-groups. It would have been interesting to study the carotenoid profiles of the flowers studied.

In vivo studies of carotenoid/norisoprenoid links are difficult. Monitoring degradation of labelled β–carotene *in vivo* is not possible because such large molecules are not usually transported around the plant (Whatley and Whatley 1987). Feeding labelled precursors such as MVA or IPP to plants so that the label will be incorporated into β–carotene and subsequently into β–ionone may produce inconclusive results because the physical processes for extraction of the compounds for analysis may themselves cause cleavage of labile, labelled carotenoids into norisoprenoids (Davies 1977; Winterhalter *et al.* 1991). Studies of this type have been done with success relating to the synthesis of xanthophylls from carotenoids (Grumbach *et al.* 1978). However, there was a discrepancy found in the stoichiometry of label transfer to xanthophylls indicating that β–carotene was metabolised in many ways.

Norisoprenoids such as damascenone and β–ionone may be produced *in vivo* via oxidative or enzyme-catalysed degradation of carotenoids (Croteau and Karp 1991; Roberts *et al.* 1994), and the potential for both methods will be discussed. There are mainly two options for the occurrence of norisoprenoids. Formation may occur through oxidation of carotenes during senescence, death, or post-harvest modifications and is therefore not necessarily enzyme-catalysed. Alternatively, enzyme-mediated degradation of carotenoids may occur within the life of a plant-part or organ, producing norisoprenoids that may function in reproduction and survival. There may also be a case for *de novo* synthesis of norisoprenoids from compounds such as MVA or amino acids. Abscisic acid (ABA) arises via enzyme-catalysed degradation of xanthophylls

(Firn and Friend 1972; Parry and Horgan 1991), and yet this compound has also been shown to be biosynthesised independently from mevalonic acid and isopentenyl pyrophosphate (Britton 1991; Cowan and Railton 1987). Increased valine and leucine resulting from nitrogen and phosphorous fertilisation may have been the cause of enhanced β–ionone levels in tomato fruits (Wright and Harris 1985) and boronia flowers (Roberts and Menary 1994b). Although, once again, corresponding carotenoid levels were not measured in either case, and therefore increased carotenoid levels giving rise to increased amounts of ionones cannot be ruled out. In the example of tomatoes, which accumulate lycopene, the latter alternative is unlikely.

In tobacco, most norisoprenoids abound during and after cell wall degradation, although many are present in the green plant prior to and during the time of most rapid growth (Enzell 1985). This illustrates just how difficult it is to separate *in vivo* directed catabolism of carotenoids (i.e. 'biosynthesis' of norisoprenoids) from random oxidation occurring during senescence or as a result of post-harvest modifications. A further complication arises because β–ionone produced by carotenoid degradation may be rapidly metabolised into other compounds arising by oxidation, reduction, dehydration and cyclisation (Mikami *et al.* 1981). In plants which specifically accumulate β–ionone to high levels such as *B. megastigma* , there may be inherent protective compounds that act as antioxidants to prevent further oxidation. The rate of production of β–ionone by whatever means may greatly exceed its oxidation to other compounds, or β–ionone may be 'stabilised' from further attack by storage as a glycoside.

5.4.A Oxidative production

Photo-oxidation and thermal degradative studies of carotenoids such as β–carotene have been known to produce many compounds including β–ionone, dihydroactinidiolide and damascenone (Berset and Marty 1992; Kanasawud and Crouzet 1990; LaRoe and Shipley 1969; Sakho and Crouzet 1985; Ayers *et al.* 1964; Isoe *et al.* 1969; Onyewu *et al.* 1982, 1986). Mordi *et al.* (1993) observed degradation products from β–carotene produced over 72 hours of oxidation, using a control treated with α–tocopherol (vitamin E) to prevent oxidation. The main products in the early stages of oxidation were 5,6-epoxy-β–carotene, 15,15'-epoxy-β–carotene, diepoxides and a series of β–apocarotenals and carotenones. Later, uncharacterised oligomeric material and the carbonyl compounds became more important, and the epoxides degraded. After 48 hours, the longer chain β–apo-carotenals were oxidised into shorter chain carbonyl compounds, particularly β–apo-13-carotenone, β–ionone, 5,6-epoxy-β–ionone, dihydroactinidiolide and carboxylic acids. The ketones, β–ionone

and β–apo-13-carotenone, predominate over aldehydes at longer oxidation times because aldehydes, with their labile acyl hydrogens, are more susceptible to autoxidation than ketones (Mordi *et al.* 1993). The process of degradation was explained in terms of free radical peroxidation chemistry i.e. non-enzymatic autoxidation of β–carotene. Photo-degradation of β–carotene yields β–ionone as one of the major products, however other work has shown that only small amounts of β–ionone are produced by auto-oxidation of β–carotene (Gloria *et al.* 1993).

5.4.B Enzyme-mediated production

Sanderson *et al.* (1971) describe an enzyme from tea which they believed showed activity in cleaving labelled β–carotene into β–ionone. The method they used however, would adequately oxidise β–carotene even if no enzyme was active, much as in thermal degradative studies (Kanasawud and Crouzet 1990). The frozen tea leaf used for the enzyme preparation may undergo oxidative changes as a result of freezer storage and thawing, enough to cause oxidation of many compounds (Loomis 1974; Benson *et al.* 1992). However, a carotene oxygenase from *Microcystis* which forms β–cyclocitral and 4-hydroxy-β–cyclocitral from carotenoids is activated by freezing and thawing (Jüttner 1988). Sanderson used acidic pH and incubation at 30°C, and made no mention of photo-protection. β–ionone occurred only after flavonol oxidation and drying of the reaction mixture (Sanderson and Graham 1973), both procedures capable of causing autoxidative carotenoid degradation.

β–Carotene is the immediate precursor for vitamin A (retinal), and there are two ways cleavage can occur: by central cleavage (Wang *et al.* 1991) or exocentric cleavage producing a series of intermediate aldehydes (β–apocarotenals) and carboxylic acids (β–apocarotenoic acids) (Glover and Redfearn 1954). Such intermediate compounds are widely distributed in nature (Britton 1991; Gerber and Simpson 1990; Hashidoko *et al.* 1991). Their occurrence in plants is explained on the basis of degradative processes acting on carotenoids, not necessarily the same as those directed at producing vitamin A in animal tissues. It is an open question in nearly all cases whether these other degradative processes are enzymic, for example whether the coupled oxidation of β–carotene and linoleate is accomplished by singlet oxygen or autoxidation (Enzell 1985) or by lipoxygenase (Firn and Friend 1972). Carotenoid depletion by oxidation in flowers occurs as a normal part of senescence; occurring after the control of carotenogenesis has ceased, which in some flowers is as early as the bud stage onwards (Booth 1960; Valadon and Mummery 1969; Thammasiri *et al.* 1987). The incorporation of exogenous MVA into carotenoids in fruits is greatest during initial stages of ripening and declines with senescence (Katayama *et al.* 1971). The

senescence of photosynthetic tissues, incorporating carotene destruction, is under genetic control (Guiamét and Giannibelli 1994).

There appear to be two different enzyme types capable of carotenoid degradation: a lipoxygenase and a haemoprotein-type enzyme (Blain and Styles 1959).

5.4.B.1 Lipoxygenase

Lipoxygenases are aerobic, have an optimum pH of 6.5 - 6.8 and are generally found in cereal grains and legumes, where carotenoids act as antioxidants or secondary substrates to linoleate oxidising systems (Blain *et al.* 1968). Lipoxygenase and a fatty acid hydroperoxide cleavage enzyme system from tomato fruit converted linoleic and linolenic acids into C9 and C6 compounds including hexanal, *cis*-3-hexenal and *trans*-2-hexenal (Galliard and Matthew 1977; Galliard and Phillips 1976; Galliard *et al.* 1976). This enzyme may be sensitive to freezing and thawing (Muller *et al.* 1992). Other researchers have identified the enzyme capable of this conversion as alcohol dehydrogenase (Hatanaka and Harada 1973). A 15,15'-dioxygenase from animal tissue cleaves β–carotene into two molecules of retinal (Wang *et al.* 1991). The same enzyme also cleaves β–carotene non-specifically at several other double bonds to produce a mixture of products, and this may be the more important action in plants (Lutz and Winterhalter 1992). Lipoxygenase also catalyses the conversion of linolenic acid into jasmonic acid.

5.4.B.2 Haemoprotein-type cleavage enzyme(s)

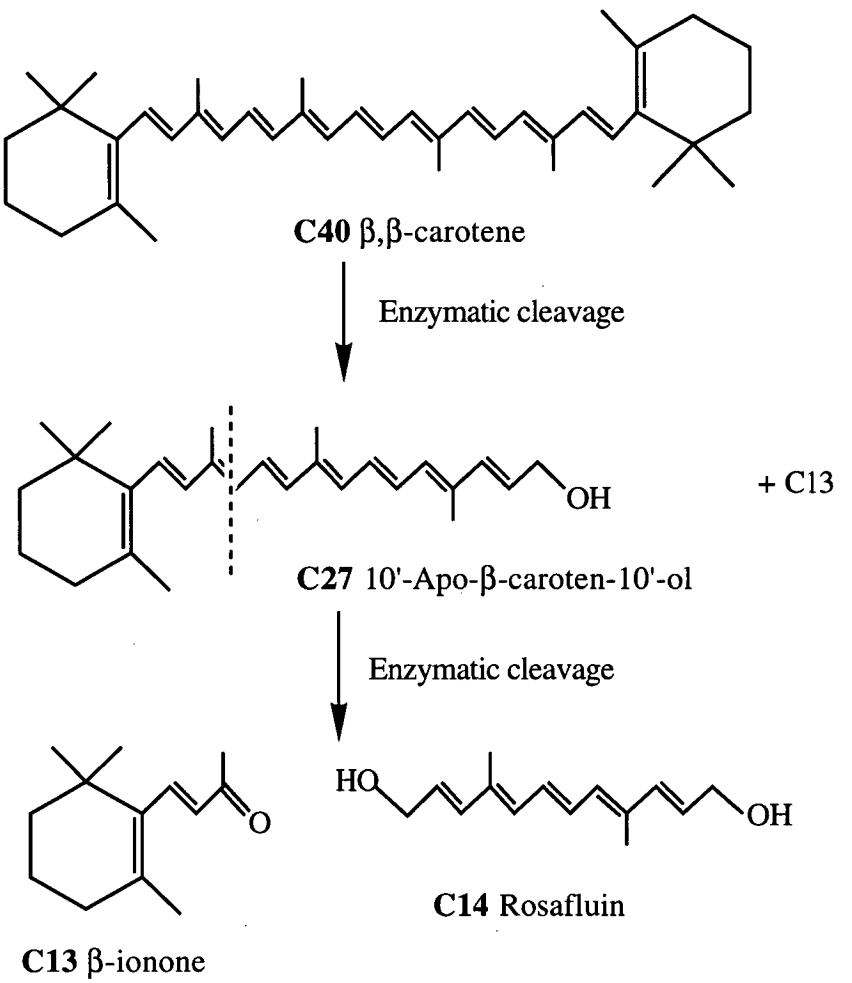
Enzymic carotene destruction in green leaves prior to leaf senescence occurs via a haemoprotein-type enzyme such as chromatin c (Goodwin 1980b). Catalase and peroxidase have been used to show that chloroplastidic destruction of carotenoids is aerobic, and is not coupled with hydrogen peroxide, but utilises pre-oxidised linoleate (Simpson *et al.* 1976). Such destruction is not achieved by a lipoxygenase; the pH optimum in lucerne extract is between 4 and 5 (Simpson *et al.* 1976). A preparation from mitochondria of sugar beet leaves was capable of degrading water-soluble carotenoids; indicating destruction could possible occur in organelles other than plastids (Dicks and Friend 1967), although destruction also occurred with an enzyme from isolated chloroplasts (Friend and Mayer 1960).

In the gibberellin pathway, enzymes involved in the reactions leading from ent-kaurene to gibberellins are either located on the ER and are cytochrome P450 dependent, or are soluble cytoplasmic enzymes (Graebe 1987). If ent-kaurene is synthesised in the plastid, formation of gibberellins requires transport of ent-karuene

from the plastid to the ER for further metabolism (Kleinig 1989). Similarly, transport of carotenoids from the plastids would be required if enzymes cleaving these molecule into norisoprenoids were not present in the plastid. Transport through the plastid of such large molecules is not thought to be possible (Whatley and Whatley 1987), therefore the enzymes producing β–ionone must be plastid-localised, or molecules that are the partial breakdown products of carotenoids are removed from the plastid for further metabolism into compounds such as β–ionone.

A site-specific pathway for enzyme-cleavage of carotenoids into C13, C14 and C27 compounds has been postulated to result from dioxygenase cleavage in the 9,10 or 9',10' position followed by cleavage of the C27 fragment into further C13 and C14 fragments (Lutz and Winterhalter 1992). A representation of the suggested pathway may be seen in Diagram 5.4.2. The apocarotenols may be the point at which carotenoid degradation products are removed from the plastids for further metabolism into β–ionone.

Diagram 5.4.2



Source: Lutz and Winterhalter (1992)

Ionone compounds are highly reactive and occur in virtually all oxidation states (Ohloff 1977a). β–ionone may subsequently be converted into other compounds such as 7,8-dihydro-β–ionone and 7,8-dihydro-β–ionol by enzymes such as carvone reductase (Tang and Suga 1994). The presence of such compounds in extract of boronia flowers (Weyerstahl *et al.* 1994) suggests that similar enzymes are active in these flowers.

5.4.C Norisoprenoid glycosides

Formation of apocarotenoids and related terpenes may be 'biosynthetic', even though they are formed by a 'degradation' step from carotenoids (Simpson *et al.* 1976). The evidence for a biosynthetic purpose behind the production of a particular compound or compounds by degradation of larger compounds may be the occurrence of glycosidic conjugates with the products (Graebe 1987). In plants in which norisoprenoid glycosides are found, for example in raspberry fruits (Pabst *et al.* 1992), starfruit (*Averrhoa carambola* L.) (Herderich *et al.* 1992), quince (Güldner and Winterhalter 1991), *Prunus* species (Krammer *et al.* 1991; Humpf and Schreier 1992), grapes (Sefton *et al.* 1989; Winterhalter *et al.* 1990), *Melia toosendan* (Nakanishi *et al.* 1991) and *Eriobotrya japonica* (De Tommasi *et al.* 1992), rarely is β–ionone itself ever found as a glycoside. There are only one or two references in which β–ionone occurs as a glycoside (Schulz and Stahl-Biskup 1991), yet this compound is one of six methyl ketones supposedly formed directly as a result of 9,10 double bond cleavage of cyclic carotenoids (Enzell 1985). Watanabe and co-workers (1993) hydrolysed extracts containing glycosides from flower buds of various species with an enzyme prepared from the mature flower of the same species. In most species this resulted in a release of volatiles typical of that flower. However, when they examined *Osmanthus fragrans*, no volatiles were released, which is surprising, especially since ionones and related compounds are predominant in this species (Ding *et al.* 1989). Schulz and Stahl-Biskup (1991) hydrolysed extracts of *Hyssopus officinalis* L. (Lamiaceae) with β–glucosidase and Pectinol C (an unspecific enzyme mixture). Only after hydrolysis with Pectinol C was β–ionone released. Perhaps β–ionone is linked (1->6) as an arabinofuranoside or rhamnoside and requires cleavage of this before glucosidase can have any effect (Gunata *et al.* 1988), or perhaps a β–linkage does not occur in β–ionone, or further, that β–glucosidase has a higher affinity for other glycosides. The first suggestion seems more likely. Glycosidic linkage with norisoprenoids may support the theory of their directed production from larger compounds, because linkage will enable movement of such compounds around the cell and plant, or release at appropriate times. However it does not preclude the possibility that glycosylation

enables salvage of secondary compounds in organs such as flowers prior to their abscission from the plant.

5.5 Jasmonic acid biosynthesis

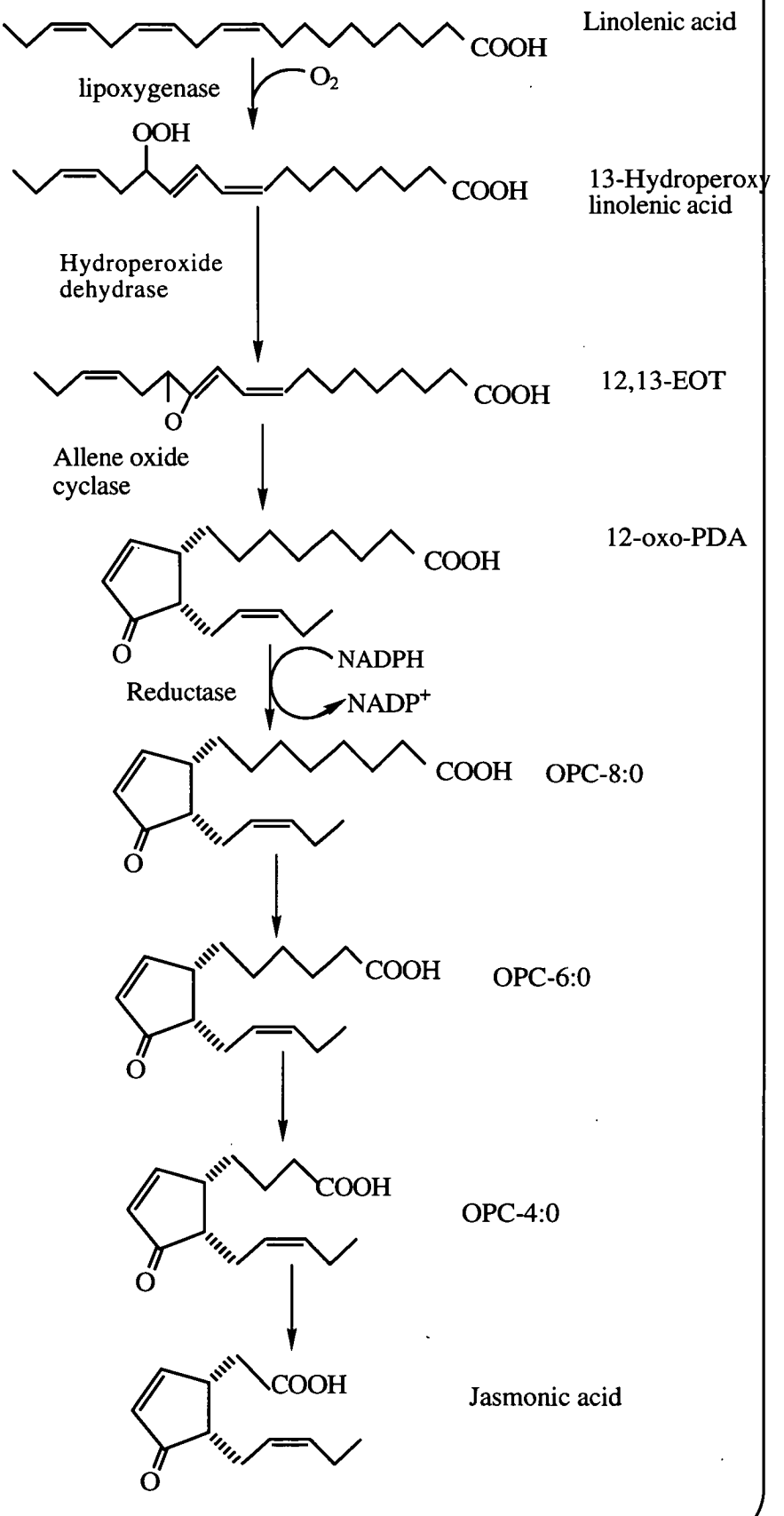
Methyl jasmonate (JAMe) was first isolated as a major component of essential oil of *Jasminum grandiflorum* L. (Demole *et al.* 1962). Both jasmonic acid (JA) and JAMe are important perfume ingredients. Two isomers of methyl jasmonate (JAMe) are found in extracts made from boronia flowers (Davies and Menary 1983).

JA has demonstrated activity as an inhibitor of seedling growth and pollen germination, and as a promoter of leaf senescence (Koda 1992; Yamane *et al.* 1982). JA and JAMe promote senescence in plant tissues including flowers (Puchalski *et al.* 1989) by stimulating ethylene production (Halaba and Rudnicki 1989; Saniewski *et al.* 1987a, 1987b, 1988a, 1988b) and inducing chlorophyll and carotenoid degradation, possibly by impairment of chlorophyll biosynthesis (Hamberg and Gardner 1992). JAMe was not an inhibitor for pollen germination in *Camellia*, whereas JA occurs in the pollen and anthers and is an inhibitor of germination (Yamane *et al.* 1982). The volatility of JAMe permits its access to tissues, and in some cases, to other plants remote from the site of its biosynthesis (Farmer and Ryan 1990; Hamberg and Gardner 1992). Perhaps the conversion of JA into JAMe stimulates germination, or the presence of JA and absence of JAMe in stigmas allows germination. Post-harvest storage of apples with vapours of acetic acid and hexanol increases the content of hexyl esters two-fold (Olías *et al.* 1992), and JAMe inhibited this activity by 50-90%. JA and related compounds have different mechanisms for action on various biological activities; the absolute configurations can determine the effects of JA (Vick and Zimmerman 1984; Koda *et al.* 1992).

The occurrence of JAMe in hairpencils of oriental fruit moths (*Grapholitha molesta*) attracted female moths, however this compound was not synthesised by the moth, but obtained from their food (Lofstedt *et al.* 1989). The production of such compounds by plants may be of significance in their evolutionary development. JA and related compounds are widely distributed among higher plants and their role in growth regulation may be significant (Meyer *et al.* 1984). Amino acid conjugates with JA have been described between phenylalanine, tyrosine, tryptophan, leucine, and isoleucine (Hamberg and Gardner 1992 and references therein). Metabolites of JA include a β–D-glucoside of 3-oxo-2-(4'-hydroxypentyl)-cyclopentane-1-acetic acid and also glycosides of tuberonic and cucurbic acids (Koda 1992; Hamberg and Gardner 1992).

JA is biosynthesised from linolenic acid by lipoxygenase activity. A diagrammatic representation may be seen in Diagram 5.5.

Diagram 5.5



Source: Vick and Zimmerman (1983, 1984).

Summary

The known biosynthetic pathways for production of terpenoids, carotenoids and jasmonates have been discussed. Alternative pathways exist for the formation of some of these compounds, and there is always the potential for yet other pathways to exist in the plethora of plant species that accumulate unusual secondary compounds. The production of norisoprenoids is an area where much speculation and guess-work abounds because of the difficulty in establishing substrates, enzymes and products. The existence of at least two enzyme-types capable of oxidising carotenoids into norisoprenoids are known. Isolating an enzyme from boronia flowers and examining its carotene-oxidising, and β–ionone-producing activity may be possible because of the likely *in vivo* activity of such an enzyme in producing a preponderance of norisoprenoids in this species.

6 Regulation of Secondary Metabolism

6.1 Genetic regulation

Enzymes responsible for the biosynthesis, transformation and degradation of secondary products are genetically determined and synthesised *de novo* from RNA (Luckner 1980; Bartley *et al.* 1994; Cordes *et al.* 1989). Biosynthetic pathways may be activated and inactivated during the life cycle of a plant. The pathway(s) toward production of one or a group of compounds may differ between species, and between organs of one plant (Graebe 1987). Different regulatory strategies may be adopted at different developmental stages and in different organs (Bartley *et al.* 1994; Fraser *et al.* 1994).

The genes controlling secondary metabolic enzyme-mediated reactions are nuclear, and the products are transported into plastids or other synthetic organelles when needed (Bartley *et al.* 1994; Giuliano *et al.* 1993; Jackson *et al.* 1992). Activation of the major genes controlling particular pathways of secondary metabolism may occur via a common regulatory mechanism, however other genes may be triggered independently via a different mechanism (Jackson *et al.* 1992). There are four main genes controlling carotenoid formation and several genes that modify the basic function (Chalukova 1985; Spurgeon and Porter 1980). The activation of gene expression precedes a rise in carotenoid levels such as is seen in ripening fruit i.e. carotenogenic enzymes are most active in green fruit (Fraser *et al.* 1994). The genetically controlled development of chromoplasts from chloroplasts during ripening may increase the

activation of genes Psy (phytoene synthase), and Pds (phytoene desaturase) so that carotenoids such as lycopene are accumulated. Genetic blocks in the pathway of carotenoid biosynthesis as a result of mutation(s) can cause photobleaching and severe alterations in leaf development (Bartley *et al.* 1994).

Control mechanisms for secondary metabolism include the amount and activity of enzymes, transportation of enzymes, precursors, intermediates and products to and from compartmentation sites, and the integration of secondary metabolism into other processes within the whole plant. Within organelles such as plastids and ER, terpenoid biosynthesis may be regulated by the specific impermeability of membranes to substrates and enzymes (Goodwin 1980b; Perez 1988; Rogers *et al.* 1967). The two distinct enzyme types within plastids are 1) soluble enzymes found in the stroma; and 2) membrane-bound enzymes attached to membranes within and enclosing the plastid (Block *et al.* 1980; Camara *et al.* 1982). Enzymes may be membrane-bound because of hydrophobic properties of substrate or products (Kleinig 1989). Both fractions are required for complete synthesis of terpenoids, but also for activation of individual enzymes (Britton 1991; Fraser *et al.* 1994; Heinrich *et al.* 1983; Kreuz *et al.* 1982).

6.2 Environmental regulation

Environmental conditions such as nitrogen availability, inorganic nutrients, temperature and metabolisable carbon sources also have an influence on secondary product accumulation and turnover (Wiermann 1981; Barz and Köster 1981; Roberts and Menary 1994b).

One of the most important regulatory factors for secondary metabolism of many kinds is light, and the regulatory effects vary between organisms. Light has no effect on carotenogenesis in algae and ripening tomato fruit, and enhances carotenoid levels in some other plant species. For Angiosperms, photoinduction is essential for carotenogenesis, initiating synthesis of carotenogenic enzymes (Britton 1991; Rau 1976; Demmig-Adams and Adams 1992; Harding and Shropshire 1980; Spurgeon and Porter 1980). Changes in the regulation of the xanthophyll cycle occur rapidly in response to changes in the incident light levels absorbed (Adams and Demmig-Adams 1992). At high light, violaxanthin (V, a diepoxide) is converted to antheraxanthin (A, a monoepoxide) and thence to zeaxanthin (Z, epoxide-free). The reverse occurs when less light is available. It is thought that zeaxanthin production dissipates excess energy away from the photosynthetic apparatus, thus providing photoprotection. In a similar way, plants exposed to continuous high light have higher levels of V, A, Z and β–carotene than plants in the shade (Demmig-Adams and Adams 1992). Light also stimulates monoterpene biosynthesis (Banthorpe and Njar 1984). Cultures of *Rosa*

graveolens grown in continuous light produced an essential oil similar to oil from the vegetative parts of the parent plant, however oil from dark-grown cultures resembled oil from parent roots (Corduan and Reinhard 1972). Fluctuations in the rates of primary metabolic pathways cause rapid flux in secondary metabolic pools on a diurnal basis (Haagen-Smit 1972; Clark and Menary 1980).

Ultraviolet-B light (280-320nm) and blue light may increase carotenoid accumulation in some plant species (Spurgeon and Porter 1980; Tevini *et al.* 1981, 1982). Ultraviolet-A light (320-350nm) also induced enzymes of the flavonoid glycoside pathway in a dark grown cell suspension culture of *Petroselinum hortense* Hoffm. (Hahlbrock *et al.* 1976).

The pigment phytochrome also regulates monoterpene production (Tanaka *et al.* 1989) and carotenogenesis (Schnarrenberger and Mohr 1970; Spurgeon and Porter 1980) in higher plants, possibly by acting as a structural requirement within the plastid enabling carotenoid accumulation (Goodwin 1980a; Harding and Shropshire 1980) .

Temperatures above 30°C prevent lycopene synthesis in ripening tomatoes (Spurgeon and Porter 1980).

6.3 Hormones, bioregulators and cofactors

Hormonal control of secondary biosynthesis is especially significant. Important changes in biosynthetic directions and accumulated products occur at floral initiation, anthesis, fruit-set and maturation: all developmental stages which are controlled by hormones. Addition of hormones such as GA (Goodwin 1980a, 1980b; El-Sahhar *et al.* 1984), JA, kinetin (El-Keltawi and Croteau 1987) and ABA can produce dramatic and variable responses in secondary metabolism and other growth responses depending on the plant species (Higgins and Jacobsen 1978). Kinetin stimulates the synthesis of anthocyanins in a similar way to stimulation by red light (i.e. phytochrome), possibly by increasing membrane permeability and available substrates at the site of synthesis (Higgins and Jacobsen 1978; Pecket and Hathout-Bassim 1974). Colchicine increased essential oil levels by three times that in control cultures of *M. piperita*, by increasing the number of neoformed secretory glands in the calli (Mulder-Krieger *et al.* 1988 and references therein). Auxins stimulate production of glandular trichomes on blackcurrant (*Ribes nigrum* L.) callus (Joersbo *et al.* 1992). JAMe, applied in a lanolin paste to tomato fruits caused an increase in β–carotene accumulation and a decline in lycopene formation (Hamberg and Gardner 1992), possibly by stimulating cyclising enzymes. JAMe also stimulates production of many groups of secondary compounds in cell cultures, including flavanoids, guaianolides, anthraquinones and various alkaloids, by stimulating phenylalanine ammonia lyase, the key enzyme in the phenyl propanoid pathway (Hamberg and Gardner 1992).

Phytohormones are thought to be involved in secondary metabolism and may cause induction or repression of enzymes, changes in the storage capacity of cells, morphogenetic effects and various other unknown responses (Wiermann 1981).

As well as being a degradation product of carotenoids, β–ionone and abscisic acid are also bioregulators for carotenogenesis, particularly in fungi (Goodwin 1980b; Lewis *et al.* 1990; Yokoyama *et al.* 1984). β–ionone also affects ABA synthesis (Norman *et al.* 1985). Bioregulators produce a response in plants with increasing concentration of bioregulator up to a point, after which no further addition will stimulate a response, and inhibition may occur (Yokoyama *et al.* 1984). The stimulus of carotenogenesis in *P. blakesleeanus* by β–ionone can be inhibited by addition of cyclo-heximide which inhibits protein synthesis (Yokoyama *et al.* 1984). In the past it was thought that citral, α– and β–ionones contributed end-groups for carotenoids (MacKinney *et al.* 1952). It appears that they are actually inducers of enzyme synthesis, launching the plant into carotenogenesis by simulating the results of excessive carotenoid degradation. However, β–ionone inhibits xanthophyll formation in the yeast *Phaffia rhodozyma* (Lewis *et al.* 1990). β–ionone probably blocks the astaxanthin pathway at β–carotene. β–ionone may compete for oxygenation at C-3 and/or C-4 with β–carotene and the xanthophylls.

Various chemicals have been shown to interfere with carotenogenesis and carotenoid degradation, and these may prove to be valuable tools for understanding biosynthetic relationships. Carotenoid-inhibiting chemicals, including diphenylamine, fluorochloridone, nicotine and norflurazon, may act as reversible non-competitive inhibitors of phytoene desaturase, thus causing phytoene accumulation (Cobb 1992; Spurgeon and Porter 1980; Babczinski *et al.* 1992; Giuliano *et al.* 1993). Aliphatic compounds and DCPE (dichloropropene), a soil fumigant, increase carotenoid levels in treated plants (Berry *et al.* 1974). Low levels inhibit lipoxygenase and higher levels inhibit MVA incorporation. It is such selective inhibition that is suspected to cause carotenoid accumulation in treated plants.

The cofactors ATP, NADH, NADP and NADPH are required for enzymic catalysis of the biochemical steps prior to conversion of GGPP into carotenes, and a divalent cation (manganese or magnesium) is necessary for phytoene formation (Block *et al.* 1980; Kirk and Tilney-Bassett 1978; Lee and Chichester 1969). Sizeable levels of all these cofactors (reduced and oxidised) and the divalent cations have been found in oil-producing citrus vesicles (Potty and Bruemmer 1970; Wong *et al.* 1982). The monoterpene synthases that convert GPP into monoterpenes require only a divalent cation as a cofactor (Pichersky *et al.* 1994 and references therein).

Summary

The regulation of secondary metabolism is predominantly controlled by enzyme activity and membrane compartmentation, and is therefore genetically controlled. The influence of environmental conditions, especially light, may be pronounced. Biosynthetic pathways may be stimulated or inhibited by the actions of *in vivo* hormones and exogenous bioregulators. Such compounds represent powerful tools for the study of biosynthetic pathways and their regulation. The effects of light, substrates for terpenoid biosynthesis, and inhibitors and stimulators of carotenoid biosynthesis and accumulation may be of interest in a study of the biosynthesis of compounds in boronia extract. Establishing biosynthetic pathways and their genetic control may enable genetic manipulation to bring about accumulation of increased levels of desired compounds.

7 Yield of secondary compounds

7.1 General effects on yield

Secondary metabolism involves dynamic processes, with compounds continually being synthesised, catabolised and transported around the plant. Rates of synthesis and catabolism may vary between organs during the life of a plant. A large increase in accumulation of secondary products does not necessarily indicate *de novo* synthesis of the compound, but may mean the substance has been translocated from another organ, or converted from bound form(s) (Wiermann 1981).

The turnover of monoterpenes can be observed in diurnal patterns of depletion that result from the effect of photosynthesis and the utilisation of photosynthate on synthetic and degradative enzymes (Croteau 1987; Clark and Menary 1980; Croteau and Martinkus 1979). Such short-term catabolism has been suggested to provide a source of energy during periods of photosynthate deficiency (Loomis and Croteau 1973). On a more permanent basis, turnover results in a decrease in monoterpene content in late development stages, where rates of degradation outstrip those of synthesis (Croteau 1987; Croteau *et al.* 1981). Enzymes responsible for the catabolism of monoterpenes increase their activity at maturity when oil glands begin to senesce (Croteau 1991). Monoterpenes can be degraded into metabolites which are reutilised, and the presence of glycosides may be explained on the basis of the plant mobilising resources for transport out of senescing organs. In the mandarin (*Citrus unshui* Marc), volatile oil content in leaves and fruits increases as fruits develop, but once fruits begin to mature, the oil in the leaf decreases and that in the fruit continues to increase (Kekelidze *et al.* 1989). The decrease in leaves is a result of catabolism of

monoterpenes or transport to other organs. The low leaf content remains constant until bud swelling, when it begins to increase again. Monoterpene turnover is not as rapid as once believed, which indicates that the synthesis of complex mixtures of compounds as a means of natural protection from herbivores etc. is not as costly for the plant as previously thought (Mihaliak *et al.* 1991). The major costs are in energy and carbon for initial biosynthesis, transport and storage.

In vegetative material, essential oils are usually contained within glands or trichomes, and there is usually a positive relationship between the number of glands and essential oil yield (Henderson *et al.* 1970; Tanaka *et al.* 1989). In plants which produce extractable secondary compounds, both the yield and complexity of the extract increases in vegetative and floral tissues during flower development, and then declines with senescence (Arras and Grella 1992; Battaile and Loomis 1961; Holm *et al.* 1988; Putievsky *et al.* 1984). In vegetative material, the process of yield development commences with the formation of new leaves, usually on the upper parts of the plant. Volatile oil content increases with time in the leaves, therefore older leaves lower down the plant usually have more than upper leaves and are eventually shed (Basker and Putievsky 1978). The yield of specific components varies throughout the development of the plant and organ (Cernaj *et al.* 1983; Croteau *et al.* 1981). For example, oxygenated compounds such as linalool in the peel oil of some citrus species decrease markedly as fruit matures, whereas limonene concentration increases (Attaway *et al.* 1967; Kekelidze *et al.* 1989). The composition and yield are both genetically determined, although the influence of environmental conditions may be significant (Hornok 1988; Burbott and Loomis 1967; Bernáth *et al.* 1991). The reactions of different species to the same environmental factor is often different, therefore the interpretation of effects of a particular environmental factor such as light, temperature, moisture and nutrition is difficult, if not impossible (Hornok 1988).

To produce a commercially desirable extract from a crop, a balance between biosynthetic and catabolic processes must be found when the yield and composition are most desirable (Croteau 1991; Cernaj *et al.* 1983). There may be different optimum harvest dates for maximum yield of plant material, oil concentration and oil yield per hectare. Compounds stored within plant cells undergo oxidation, polymerisation and loss of more volatile compounds by evaporation, as does extracted product upon storage in drums and bottles (Guenther 1972; Haagen-Smit 1972; Attaway *et al.* 1967). Some oil mixtures such as clove bud extract can be stored for lengthy periods without significant change and this is thought to be due to natural antioxidants such as eugenol or stabilising phenolics (Nakatani 1988). Alcohols and long chain acids may also influence lasting quality of oils (Dort *et al.* 1993), and such compounds may be active in protection and stabilisation within the plant also.

In clonally propagated plant material, oil quality differences due to plant maturity, regional differences (soil, climate), and light intensity are the result of direct effects on biosynthetic conversions (Clark and Menary 1980; Murray *et al.* 1988). In peppermint, α– and β–pinene, 1.8-cineole, limonene and piperitone exhibit no consistent relationship with region, soil type or harvest time. Light intensity was the most important cause of regional differences in the yield and quality from herbage because high light intensity reduced leaf size and increased leaf number and therefore oil yield per hectare. Oil in the flower had the same composition wherever grown.

Factors which can decrease extract yield in plants include wind which decreases the surface area of leaves (Grace 1977), and typical stress factors to growth, including insect infestation (Mensah and Madden 1992). In field conditions and under increasing temperature and high light intensity, essential oil in *Salvia* is promoted and can alter the complement of compounds accumulated (Bernath *et al.* 1991). Another factor which can increase yield of vegetative oils and may affect yield of floral extracts, is sprinkler irrigation. Croteau (1977a) showed significant decreases in oil yield of peppermint in sprinkler-irrigated compared with furrow-irrigated crops. Hydration and swelling of the cuticle enclosing the oil glands may produce changes in the permeability of the cuticular membrane to the volatile oil. Plant spacing had no effect on oil content or composition in tea tree, *Melaleuca alternifolia* (Small 1981). Age of the plants can also affect the yield of product extracted, for instance yields of linalyl acetate, linalool, 1.8-cineole and limonene were higher during the second year of growth compared with the first year in clary sage (Verzár-Petri *et al.* 1984).

7.2 Effects on yield of extract in flowers

Unlike oil from vegetative material where the composition may vary diurnally as a result of photosynthesis (Clark and Menary 1980), floral extracts are less dependent on environmental influences (Franz *et al.* 1986), due to the non-photosynthetic nature of most mature floral tissue. However, the stage of harvest is more significant (Franz *et al.* 1986). In rose flowers there are no specialised glands for accumulation of extract, the simple glandular epidermal tissue is the site of terpene biosynthesis (Loomis and Croteau 1973; Stubbs and Francis 1971). In such cases, one would expect to find a correlation between yield of extract and factors such as flower and/or petal size and/or weight. Patra *et al.* (1987) examined roses for such correlations and none were found. This indicates that the concentration of extract was constant, and that although flowers with greater mass would have more extract per flower, per gram they are identical to flowers of lesser mass. Sharma and Farooqi (1990) confirmed this in damask rose. The significance of this finding is that chemical treatments which influence flower yield may improve extract yield.

Farooqi *et al.* (1993) showed that kinetin increased flower and oil yield per plant (not oil concentration as a proportion of fresh or dry weight), and selectively increased specific volatiles such as citronellol and geranyl acetate. Treatment of jasmine and rose with Ethrel (2-chloroethylphosphonic acid and related compounds) which is catabolised into ethylene, chloride and phosphate ions, delayed the onset of flowering in jasmine, and increased flower yield in *R. damascena* (Bhattacharjee and Divakar 1984; Sharma and Farooqi 1990). Ethrel also increased the concentration of volatiles in jasmine, and increased flower yield per plant in rose. Cytokinins have been shown to increase vegetative monoterpene production by increasing enzyme activity or amount (El-Keltawi and Croteau 1987).

Variations in the chemical composition of rose oil has resulted from factors such as location, soil, harvesting conditions, fermentation of petals, distillation procedures and handling of oils (Bayrak and Akgül 1994). Roberts and Menary (1994) found increases in the percentage of volatiles, particularly β–ionone content in floral extract from boronia flowers by application of nitrogen. They suggested that a lower wax level as a result of decreased sclerophyll or wax production may have caused the observed increase in the percentage of volatiles. Other suggestions include excess nitrogen being channelled into alternative terpenoid pathways. It is also possible that increased synthesis of secondary compounds, particularly β–ionone, may simply be a result of better plant nutrition and growth; under control conditions plants may have reduced energy available for secondary metabolism.

The length of the flowering period may affect extract yield, and factors which influence the duration of flowering will likewise influence extract yield and/or composition (Hornok 1988). A reduced flowering period in chamomile decreased the oil content significantly (Franz *et al.* 1986). However, other researchers claim no relationship exists between flowering period and yield of concrete from rose, when the latter is assessed by headspace analysis (Patra *et al.* 1987). Day *et al.* (1994) decreased the flowering period of *B. megastigma* with benzyladenine treatment, although concomitant extract levels were not assessed. Extended day lengths and high temperatures have been known to stimulate production of oxygenated compounds (Franz *et al.* 1986).

Summary

The biosynthesis and catabolism of secondary products are genetically controlled and may be regulated by environmental and chemical factors that affect primary biosynthetic pathways or more directly affect activity of enzymes responsible for secondary reactions. Generally the yield and complexity of an extract which at maturity contains many compounds, increases in complexity and yield during

development of the organ(s), and declines during senescence. The yield of an extract which is largely contained within glands may be proportional to the number of glands present; the yield of extract that is produced within non-glandular tissue(s) may be related to the size or weight of the organs, or the activity of biosynthetic enzymes. The yields of floral extracts are less affected by environmental influences such as light, wind and temperature than are vegetative extracts, but floral extracts may be more affected by factors which reduce or extend the flowering period. Because of the short-lived nature of flowers and the depletion of secondary compounds during senescence, the timing of harvest of flowers is crucial, and a careful assessment of the balance between flower maturity and well developed extract complexity, and senescence and concomitant yield depletion must be made.

8 The extraction of secondary plant products

8.1 Headspace entrapment of emitted volatiles

It is a difficult task to capture the essence of a flower; so many compounds that impart notes to the bouquet are present in low concentrations and are frequently lost upon harvesting or during the extraction process (Joulain 1985, 1987; Kaiser 1991). One way to capture their fragrance for analysis is by headspace analysis of living flowers using a concentrating step involving volatile entrapment procedures such as Tenax (De Pooter *et al.* 1984; Ter Heide 1985) or cryotrapping. In many instances, headspace trapping has allowed identification of compounds previously unidentified, or not before described in a particular species (Joulain 1987). One example of use of a cryotrap was in the identification of 2,6-dimethyl-3(E),5(2),7-octatrien-2-ol and its 5(E) isomer in the headspace of flowers of *B. megastigma* (Kaiser 1991). These compounds have not as yet been detected in the extract (Davies and Menary 1983; Weyerstahl *et al.* 1994).

The quantity and quality of scent given out by a species may depend on maturity, biological rhythms and environmental factors (Kaiser 1991). Diurnal rhythms must be analysed from samples given off by a living flower still attached to the plant, and there are many published descriptions of such methods (Altenburger and Matile 1988, 1990; Matile and Altenburger 1988; Loughrin *et al.* 1990, 1991; Pichersky *et al.* 1994). Scented flowers which display the same fragrance profile for half an hour to two hours after cutting can be placed in a glass chamber of suitable volume, and gas can be forced over the flowers and purged out, trapped and analysed (Kaiser 1991).

The concentration of a compound in the vapour phase is a function of its vapour pressure at a particular temperature, and the interactions (additivity, synergism and

suppression) between the odourant molecules in the perfume mix and between the odourants and components of the product matrix (Ter Heide 1985; Neuner-Jehle and Etzweiler 1991). With quantitative headspace analysis there is no need to consider the many factors which influence an odourants volatility (Neuner-Jehle and Etzweiler 1991). The static approach to headspace sampling involves placing the sample in a closed system at constant temperature; any volatile compounds present in the liquid or solid phase will slowly pass from the liquid into the vapour phase until each substance has reached a constant vapour pressure concentration (Neuner-Jehle and Etzweiler 1991). To optimise the concentration of compounds in equilibrium and to minimise degradation or rearrangement of compounds, an equilibration time of at least 45 minutes is recommended for headspace analysis of solid samples such as flowers (Hiltunen *et al.* 1984). There are difficulties in the quantification of compounds in the headspace above flowers because of the unsuitable conditions for addition of internal standards (Kolb 1984).

Cryogenic traps provide one of the easiest methods, because in some cases, no solvent is used at all. Kaiser (1991) analysed the fragrance emitted from *Osmanthus* and found all the normal constituents of the extract, by using a cryotrap that was subsequently extracted by solvent. Such extraction may be necessary if excessive water caused by condensation is collected in the cryotrap also. Some of the problems with methods for entrapment of floral emissions are adsorption of compounds onto the rubber septa used to seal the vials, and leaking of equilibration vials; these are especially significant factors when attempting to quantify emitted volatiles (Drozd and Novák 1979). Low reproducibility (8% in some cases) can also be a problem when using cryogenic traps. Fingerprint comparisons, or the determination of characteristic profiles may be made in such situations by keeping the system under constant conditions and standardising working procedures.

8.2 General extraction considerations

Re-construction of scents by mixing proportional amounts of pure compounds together does not produce perfumes similar to natural scents because of the significant impact trace compounds with a high odour value have on the bouquet *in vivo*. Invariably, scents produced by distillation and solvent extraction are second best compared with real flowers (Calame and Steiner 1982). Better quality extracts, closer to the natural scent, can be prepared using techniques such as carbon dioxide extraction and fractionation. However, the increased cost of extraction may limit the use of such extracts.

Most floral extracts are not essential oils because they are not obtained via steam distillation at atmospheric pressure as are oils of mint, nor by cold-pressing as in citrus

fruit peel oil (Petrzilka and Ehret 1991). Plant extracts as we know them are not necessarily those biosynthesised by the plant, but may be derived through changes taking place as a result of treatments post-harvest and during extraction (Herout 1977). Steam distillation produces artefacts that are pronounced in delicate floral fragrances, including loss of low boiling point compounds and the production of a 'cooked'-smelling product (Haagen-Smit 1972; Moyler and Heath 1988). Many terpenoid esters undergo a variety of chemical reactions and are effectively lost during steam distillation (Pickett *et al.* 1975). Steam distillation normally limits the recovery of components to those with a molecular weight range of 50-220.

Traditionally, flowers were extracted by packing them into layers of cold fat for a few days (Guenther 1972). The nonpolar compounds would diffuse into the fat from the flowers, and after a time the flowers were discarded and the fat was extracted by solvent. This process is called enfleurage and is time consuming and expensive. Since 1835 solvents have been used for extraction of floral fragrances (Guenther 1972). The products of solvent extraction are 'concretes': semi-solid products comprising volatiles, waxes and pigments. An 'absolute' is obtained by alcohol extraction of the concrete to remove waxes and some pigments. Solvent extraction is usually carried out at temperatures less than room temperature for protection of heat labile compounds.

For solvent extractions, a solvent of suitable polarity and boiling point is required. The solvent used should be selective, i.e. it should dissolve fragrant compounds and minimise extraction of other compounds such as pigments (Guenther 1972). It should have a low boiling point to permit easy removal at low temperatures after extraction, but not too low because solvent losses via evaporation during extraction would then be too great. The solvent should not dissolve water and water-soluble compounds, nor should it interact with constituents of the flower oil (Guenther 1972). The solvent used should be free of chemical impurities, especially known toxins such as benzene, chlorinated compounds and compounds that are not registered for use in food products. The process of solvent removal at the completion of extraction by evaporation under reduced pressure, which enables re-cycling and re-using of solvent, effectively concentrates all compounds present: desirable and otherwise. Even compounds such as herbicides and pesticides present on the plant material at the time of extraction can remain in the extract and affect the purity of 'natural' products (Groenewoud *et al.* 1995). The solvent must be readily available, economical and easy to handle, and yield a consistent product.

High purity petroleum ether (pet. ether) with a boiling range of 30-70°C is most commonly used for extraction of flowers because it fulfils most of the ideal characteristics required of a solvent. Pet. ether comprises pentane and hexane, useful because of their chemical inertness and complete volatility (Guenther 1972). Pet. ether extracts low molecular weight oxygenated compounds, and high molecular weight

polar compounds such as waxes and pigments. Benzene was once the second solvent of choice, however health restrictions now limit its use, especially during preparation of food additives. Alcohol is unsuitable because of the amount of water and water-soluble pigments which are extracted, which dilute and contaminate the extract.

During extraction, solvents penetrate the floral organs, dissolving the flower perfume, waxes, albuminous material and pigments (Guenther 1972). The release of these compounds back into the reservoir of solvent surrounding the flowers is by diffusion. The time needed for a complete extraction may depend on the solvent volume used, especially if particular compounds saturate the solvent during extraction. Replacement of solvent may be required after a time to maintain a concentration gradient. Plant material with a high moisture content, such as flowers, cannot be exhaustively extracted, because pet. ether does not penetrate very well through moist tissue (Georgiev and Balinova–Tsvetkova 1977). It has been suggested that the 'caps' of epidermal oil glands on boronia petals may be ruptured upon contact with solvent which dissolves the waxy cuticle (R.C. Menary pers. comm.).

The amount of extract from each of three sequential solvent changes (washes) during extraction of lavender racemes obtained by different solvents was examined (Georgiev and Balinova–Tsvetkova 1977). Pet. ether produced less extract in the first wash than either 1,2-dichloroethane, dichloromethane or chloroform and this caused a lower total yield after three washes for pet. ether compared with the other solvents. The extraction efficiency of alcohol is reduced because of the dilution effect caused by the removal of water from the tissue by this solvent. The highest yield of extract was obtained with a mixture of pet. ether and 1,2-dichloroethane, the second highest yield was obtained by benzene and a lower yield with DCM. All of these solvents and solvent mixtures produced extracts which were ranked higher by organoleptic assessment than the extract produced by pet. ether alone.

Processes such as chopping vegetative material prior to extraction have been shown to increase oil yield (Smallfield *et al.* 1994) and this may be due to better plant/solvent, or plant/steam contact as a result of breaking up tissue and glands in which oils accumulate. The yield and composition of an extract can be influenced by the extraction conditions. For example, distillations with large amounts of plant material produced less oil per unit weight compared with smaller extractions of clary sage (Verzar-Petri *et al.* 1985).

8.3 Carbon dioxide extraction and fractionation

Other methods for extraction include using liquid carbon dioxide which minimises chemical transformations and produces an extract more like the natural scent (Moyler and Heath 1988). The use of carbon dioxide under either subcritical or

supercritical conditions, or both (sequentially), for the extraction of plant material has been known for about 60 years. It has become a commercial alternative only recently. Yields are as good, or better, than those obtained by distillation or solvent extraction (Moyler and Heath 1988). Carbon dioxide extraction is particularly useful for capturing the true note of flowers such as lilac, which cannot be extracted in any other way to yield an acceptable oil (Calame and Steiner 1982).

Compounds with a molecular weight of up to 400 can be recovered by liquid carbon dioxide. Carbon dioxide-extracted oils may be more concentrated than those obtained by steam distillation or solvent extraction because there are less highly volatile monoterpenes produced as artefacts during distillations. At subcritical temperatures (below 31.1°C, 73.8 bars), essential oil-like products are extracted while at supercritical temperatures, a product more like an absolute is obtained. The subcritical fluid at zero to 10°C and pressure of 60-80 bars, maximises the extraction of desirable oxygenated components. It is the presence of such compounds that gives the valued 'top notes', reminiscent of the fresh plant material, to carbon dioxide-extracted oils.

The factors which affect the yield and nature of extracts obtained by carbon dioxide extraction include the solvent power, plant material : solvent ratio, the method of solvent/plant material contact, extraction conditions (pressure, time, temperature), particle size, and the moisture content of the plant material (Adasoglu *et al.* 1994). Essential oils are generally soluble in dense carbon dioxide, however specific knowledge of the solubility behaviour of essential oils in this matrix is required for optimal selection of fractionating conditions (Stahl and Gerard 1985).

Fractionation of extracts by various techniques such as HPLC and supercritical fluid chromatography have been used to produce new flavours by remixing fractions in varying proportions and by removing terpenes (Stahl and Gerard 1985; Yamauchi and Saito 1990).

8.4 Storage of plant extracts

A reduction in the level of volatile compounds in plant extracts is common during storage of oil (Gopalakrishnan 1994). The hazardous effects of light on plant extracts are well known (Georgiev *et al.* 1977; Guenther 1972). Storage of boronia extract, especially under oxidative conditions such as light exposure, causes a decrease in β–ionone levels and an increase in dihydroactinidiolide (Leggett 1979). The presence of carotenoids in boronia extract indicates the potential for large changes to occur as a result of carotenoid autoxidation which causes changes in terpenes and other compounds (Gopalakrishnan 1994).

Summary

Various methods for analysis of the volatiles emitted from plants, and the extraction of these and other compounds present within the plant tissue have been described. Headspace analysis is a powerful tool for analysing the compounds produced by a flower to attract pollinators, and for identifying the degree to which extracts differ from the natural perfume. The yield and composition of the extract obtained depends largely on the method used, and the extraction conditions. Delicate fragrances such as those from flowers are often adversely affected by rigorous extraction methods such as distillation and require methods such as solvent and supercritical fluid extraction.

9 Post-harvest changes to secondary compounds

Senescence of any plant part follows physiological maturity and ends in death, and the study of senescence is the basis for most post-harvest physiology research (Borochov and Woodson 1989). Flower senescence, and especially senescence of petals, is particularly rapid. Harvesting flowers causes the onset of death of the flower, but usually there is a period of time after harvest during which respiration, carbohydrate and amino acid metabolism continue (Bugorskii and Zaprometov 1983; Co and Sanderson 1970; Serrano *et al.* 1992). This 'programmed senescence' is the basis for post-harvest changes in flowers (Romani 1991). Ethylene production, a loss of dry matter due to hydrolysis of macromolecules such as starch, protein and nucleic acids and the redistribution of carbon and nitrogen compounds to other parts of the flower occur during senescence. Loss of membrane integrity and a decrease in phospholipids and bound fatty acids in membranes occurs, although the plasmalemma and tonoplast remain intact until a later stage of senescence. Senescence is a degradative process, but the synthesis of new proteins such as hydrolases, RNase, DNase and glycosidases necessary for senescence also occurs (Borochov and Woodson 1989; Matile 1974). The loss of protein, nucleic acids and other cell constituents is greater than the production of digestive enzymes, causing an unbalanced differentiation that eventually results in cell death (Matile 1974).

The lysosomal cell compartment is largely responsible for autophagy which results in autolysis and death (Matile 1974). The lysosomal compartment comprises the ER and the golgi, as well as vesicles with triple membranes produced by the plasmalemma by pinocytosis and phagocytosis, and the extracellular space. The lysosomal compartment contains hydrolases and glycosidase among other enzymes, which function in digestion of a wide spectrum of compounds contained within vesicles that are brought into contact with lysosomal enzymes. Thus the lysosomal

compartment represents the organisational prerequisite of turnover. In the corolla of *Ipomoa*, rapid wilting is concomitant with a decline in cytoplasmic constituents (Matile 1974). The autophagic activity which characterises senescent cells begins in the mesophyll cells of the mature flower bud, but is not apparent in epidermal cells until the wilting corolla tube is rolled up. Sieve tubes and companion cells also show little change during the early stage of wilting. Delayed senescence in the phloem may allow continued removal of breakdown products from the corolla prior to abscission.

During storage of plant material, gradual evaporation results in losses in extract content; major sources of loss being through oxidative changes and resinification of essential oils (Guenther 1972). Flowers are particularly susceptible to such changes. The storage of such material poses a problem because if left in enclosed containers, ethylene and respiratory activity increases, causing heat to build up and undesirable changes to occur in the flowers (Serrano *et al.* 1992), including changes to secondary metabolism. Given that one must harvest flowers to produce an extract from them, small changes which occur after excision from the plant are inevitable and unavoidable (Mookherjee *et al.* 1986; 1989). What is more significant is the potential for a period of post-harvest respiratory activity to be manipulated by storage under suitable conditions for maximisation of desirable metabolic changes.

Respiratory activity in living tissues is proportional to essential oil yield (Tyutyunnik and Ponomaryova 1977), and it is the absence or slowing of respiration in anaerobic and chilling facilities which protects plant material from deleterious effects of storage for a limited time. Anaerobic conditions, however, have also been attributed with an increase in essential oil content of rose (Tyutyunnik and Ponomaryova 1977). Fermentation alone for four to six hours at 25°C will increase essential oil yield. Anaerobic conditions, as expected due to inhibition of oxidative reactions, increased oil yield by 30-40% in as little as two hours. Aerobic fermentation decreased oil yield significantly and also decreased carbohydrate and amino acid levels. Tyutyunnik and Ponomaryova (1977) examined changes to essential oil content (distilled) of rose petals after post-harvest treatments incorporating exposure to vapours of acetic acid, sulphur dioxide and plumbagene, with heating at 30-40°C. Sulphur dioxide and acetic acid produced undesirable changes to the oil, although they decreased respiration and increased essential oil content. Fermentation in water for more than ten hours at 45°C with plumbagene added at 1:500,000 increased essential oil content by 6-8%. Plumbagene stabilises the rose petals, inhibits oxidative processes and growth of microflora and increases essential oil yield with no change to quality.

High proportions of ethanol in rose oil are an undesirable product of fermentation of petals prior to distillation, although phenylethyl alcohol, an aromatic compound with high floral impact was highest in fermented petals (Bayrak and Akgul 1994). Fermentation also increased citronellol, ethanol, nerol and geranial levels,

while decreasing geraniol, nerol and farnesol (Baser 1992, referred to in Bayrak and Akgul 1994).

It is possible to alter the yield and composition of extract from many plants with simple post-harvest, pre-extraction techniques. One of the classic examples where such storage is actually necessary is with *Iris* : the rhizomes of which have few irones until at least three years of storage during which time irones are generated from cycloiridals (Petrzilka and Ehret 1991). Likewise, the flavours of fruits are increased during a short period of controlled atmosphere storage (Narain and Bora 1992) during which time the climacteric rise in respiration occurs (Olías *et al.* 1992). Orange fruit stored at 33°C for 24 hours under low oxygen and high nitrogen and carbon dioxide develop twice as much important flavour volatiles than control-stored oranges (Shaw *et al.* 1992). Apples held in atmospheres with acetic acid and hexanol vapours increased by two-fold their hexyl ester content (Olías *et al.* 1992).

Storage of coriander herb for 24 hours after harvest produced no change in oil composition, and yet if this herb is chopped and then stored, a decrease in aldehydes and an increase in alcohols ensues (Smallfield *et al.* 1994). Maximum times for storage of chopped material prior to extraction were found, producing oil of different compositions and higher yield. The processes of chopping and storing, both simple post-harvest treatments, produced oils with different compositions thus illustrating a significant tool for the attainment of product diversity to suit changing market trends.

Documented post-harvest changes in other flowers relevant to a study of *Boronia megastigma* include a rapid reduction upon harvest of compounds such as ionones and derivatives in flowers which emit these compounds when alive such as freesia and *Osmanthus fragrans* (Mookherjee *et al.* 1989). Similarly, semi-fermented tea had more jasmonates, β–ionone and indole than fully processed black tea (Takeo 1983) illustrating that these products can be degraded by post-harvest processing.

Freezer storage does not protect plant tissue from enzymic transformation of compounds such as monoterpenes (Burbott and Loomis 1969), although some enzymes lose most of their activity after freezing and freeze-drying (Leino 1992). Processed onions have different, reduced levels of aroma compounds compared with fresh bulb and herb, caused by partial or complete inactivation of enzymes during processing, some nonenzymatic destruction of precursors and enzymatic hydrolysis of precursors (Leino 1992).

10 Production of secondary products in tissue culture

Biosynthesis of secondary products in tissue cultured plants is usually either non-existent, occurs only to a comparatively small extent, or produces an oil which is only vaguely reminiscent (if at all) of the parent plant (Banthorpe and Njar 1984; Falk *et al.* 1990; Mulder-Krieger *et al.* 1988). Where an oil is produced however, tissue culture is a powerful tool in which to study pathways of essential oil biosynthesis (Corbier and Ehret 1986; Chayet *et al.* 1977).

There are few authenticated cases of *de novo* synthesis of monoterpenes in plant cell cultures available (Banthorpe and Njar 1984; Banthorpe *et al.* 1986). One method of proving *de novo* synthesis is by direct injection into callus of isotopically labelled precursors such as [2-14C]MVA (mevalonic acid) or [1-14C]IPP (isopentenyl pyrophosphate) (Banthorpe and Njar 1984).

Morphological differentiation enabling or enhancing essential oil accumulation has been demonstrated in several tissue-cultured species to a small extent. Within cell suspension cultures, some morphological changes concurrent with production of secondary compounds are seen (Ozeki and Komamine 1981; Thomas and Street 1970; Lindsey and Yeoman 1983; Figueiredo and Pais 1994). *Ruta graveolens* excretes oil into schizo-lysigenous secretory glands initiated in the leaves and toward the periphery of the callus, or distributed within the tissue (Corduan and Reinhard 1972; Peterson *et al.* 1978). In this species, secretory passages in tissue culture resemble those of a differentiated plant, wherein oil is also excreted into parenchymatous cells as small droplets. Callus of blackcurrant (*Ribes nigrum* L.) produce glandular trichomes which proliferate with addition of auxins such as 2,4-D (Joersbo *et al.* 1992). Callus cultures of *Matricaria chamomilla* accumulated oil exclusively in idioblasts, oil cells typical of intact plants (Mulder-Krieger *et al.* 1988). Other species excrete oil into the medium in which they are grown (Berlin *et al.* 1984).

More commonly, morphological differentiation is not a prerequisite for the synthesis of mono- and sesquiterpenes in plant cell cultures (Brown *et al.* 1987). Glycosidically-bound monoterpenes are thought to be the preferred accumulation form in morphologically undifferentiated callus and suspension cultures (Mulder-Krieger 1988). Callus cultures are generally more effective than cell suspension cultures with respect to monoterpene biosynthesis (Banthorpe and Njar 1984; Brown and Charlwood 1986). However, suspension cultures do retain the ability to biosynthesise essential oils (Brown and Charlwood 1986). Suspension cultures of *Salvia officinalis* had no detectable levels of monoterpenes, however enzymes capable of converting geranylpyrophosphate into (+)-camphor were present (Falk *et al.* 1990). Strawberry

cell suspension cultures supplied with precursors and mannitol produced comparatively high levels of monoterpenes (Hong *et al.* 1990). When monoterpene accumulation is not seen, it may be that degradative enzymes also occur, with a greater activity level than biosynthetic enzymes (Falk *et al.* 1990), presumably to prevent toxicity of the monoterpenes to the suspended cells (Brown *et al.* 1987).

Transformation of monoterpenes has also been demonstrated in suspension cultures of *Rosa centifolia* (Corbier and Ehret 1986). In *Rosa damascena* cultures there was no accumulation of monoterpenes seen, however a cell-free extract from the culture was able to convert MVA into IPP, and IPP into geraniol and nerol at a rate 300 times more efficiently than the optimum extract from the parent plant (Banthorpe and Barrow 1983).

Secondary metabolite production may be stimulated after maximum growth (cell division) has slowed or ceased, and metabolites and cofactors become available for alternative processes (Banthorpe *et al.* 1986). It was proposed that a two stage process be considered for essential oil biosynthesis in tissue culture: the first stage maximising conditions optimal for fast cell growth, and the second phase incorporating conditions optimal for aroma production (Hong *et al.*. 1990). However, oil production in *Ruta graveolens* usually occurred three weeks before growth ceased (Corduan and Reinhard 1972).

Essential oil production in various species may be affected differently by the addition of oil precursors, hormones and different growth conditions (Mulder-Krieger 1988). The regulation effects are similar to those documented in an earlier section on regulation of secondary metabolism.

11 Summary and rationale for experimental work

The production of secondary metabolites by flowers is usually directed toward pollinator attraction. Volatile compounds are often present in epidermal cells but may be accumulated in specialised structures, and are released by volatilisation, sometimes in distinct diurnal rhythms. Secondary products such as monoterpenes may be biosynthesised within distinct cellular organelles, and may be conjugated with sugar molecules for storage or transport around the plant. There are several distinct pathways for biosynthesis of a multitude of different compounds, some of which may be produced by degradation of larger compounds by enzymes or by autoxidation. The biosynthesis of secondary compounds is controlled genetically and is heavily regulated by factors such as light and hormones. The seasonal conditions and stage of development of the plant can have a large influence on the yield and complement of secondary products accumulated, however genetic predisposition to accumulate such compounds is often more significant.

Continued synthesis of extract components in harvested flowers has been documented and is a valuable tool for development of new or improved products. Similarly, the ability of plant cell cultures to accumulate or modify secondary compounds is a useful phenomenon in the establishment of biosynthetic pathways. Floral fragrances are often adversely affected by rigorous extraction methods, and therefore solvent extraction or supercritical fluid extraction are used to produce an extract reminiscent of the live flower.

The areas covered in this study commence by gathering biological and chemical information, on a quantitative basis where possible, of the volatile compounds contained within and emitted from each floral organ, over time, and from different developmental stages. This enables us to predict which organs contribute significant amounts of extract components. On an ecological front, it may allow identification of key compounds responsible for attraction of pollinators. The cellular and subcellular sites of synthesis and accumulation of secondary compounds were studied; identification of active organelles may further biosynthetic studies and allow for isolation of active fractions or enzymes. Identification of glandular sites and organs may indicate the potential for yield to be related to the number of structures, or the relative size of glandular organs, and these characteristics may become selection criteria. A study of the carotenoid pigments of boronia flowers has not been published, and given the high levels of β-ionone and the potential biochemical link between carotenoids and this compound, a study is warranted. Attempts were made to identify enzymes capable of carotenoid cleavage into β-ionone, and whether links could be drawn between the levels of both groups of compounds in genetically

different plants. Work on the effects of terpenoid regulators and environmental conditions on flower bearing plants and tissue cultured boronia calli was attempted to verify, or at least study, biochemical relationships.

On a more commercial front, a study of the phenology of boronia flowers and extract accumulation was undertaken to identify the most suitable time for harvesting this unevenly flowering plant. There are various suggested roles for glycosidically bound volatiles in the accumulation and release of volatile compounds, and levels of these compounds were studied to identify precursor/product relationships, and their biological role in boronia flowers, if any. The importance of genetic and environmental regulation of extract accumulation has not been quantified for boronia, and attempts were made to discern between the two to enhance future selection of clones and growing sites. As a result of previous work, the effect of various floral characteristics on the yield of extract was assessed.

The fragile nature of flowers suggests that stringent post-harvest practices be adopted to ensure floral quality and protection of secondary compounds, and the effects of several regimens was studied. It was found that room temperature storage for a number of hours actually increased the percentage of volatiles, and the reasons for this were examined. It was thought that this might be a role for volatile glycosides, however it seems more likely that continued biosynthesis of product occurs, and this has large implications both biochemically and commercially. All of these studies required optimum, precise methods for extraction of product(s), and various aspects of the extraction process were studied. Commercially, the implications from these studies indicate potential areas for improved efficiency and product diversity.

III. General materials and methods

1 Consumable materials

1.1 Solvents and reagents

1.1.A All reagents were analytical grade, and were obtained from Sigma-Aldrich Pty. Ltd., Unit 2, 10 Anella Ave. Castle Hill, NSW, 2154, unless stated otherwise.

1.1.B DCM was HPLC grade, obtained from Mallinckrodt.

1.1.C Hexane was HPLC grade, obtained from Millipore.

1.1.D Petroleum ether was technical grade, redistilled prior to use.

1.1.E Gases used for GC analysis included high purity nitrogen, instrument grade compressed air and high purity nitrogen, obtained from CIG, Hobart, Tasmania.

1.1.F Whatman cellulose filter papers were obtained from Selby Scientific.

1.1.G Tween 80 was used as a monoleate syrup, containing 70% oleic acid; the other 30% comprising linoleic, palmitic and stearic acids.

1.1.H β-Glucosidase (β-D-glucoside glucohydrolase) (from almonds) contained 4-12 units per mg; one unit will liberate 1.0 μmole from salicin at pH 5.0, 37°C. Obtained from Sigma-Aldrich Pty. Ltd.

1.2 Flower material

Flower material used in these studies came from commercial plantations around Tasmania (Appendix 3 for site locations). For most studies, clone 5 plants from Longley were used. Some experiments utilised clone 5 plants grown for three years outside in pots in a composted bark : sand (50 : 50, v:v) mix with additional Osmocote and Micromax.

2 Apparatus

Balances	4 decimal places: Mettler AE200
	2 decimal places: Mettler PM4600
	1 decimal place: 1-200kg arm balance.
Centrifuge	Beckman J2-21ME; Beckman Instruments Australia Pty. Ltd., 24 College St, Gladesville NSW, 2111.
Distilled water	Still from Labglass, Brisbane, Qld, Australia.
Freezer	-18°C, Reuco ULT 1386 W-O-D, Rheem Manufacturing Company, Scientific Products Division, Asheville, NC.

Homogenisers	Stainless steel Semak Vitamizer, CVS-3L, 750 W, 50 Hz, Australia.
	Ultra-Turrax probe. TP18/2, 75 W, 50 Hz, Janke and Kunkel KG.
pH meter	Radiometer, Copenhagen
Spectrophotometer	Shimadzu UV-160
Water bath	Orbital shaking water bath, OW 1412, Paton Industries, Pty. Ltd., 35 Henry St. Stepney SA, 5069 Australia.

3 Extraction methods

3.1 Laboratory-scale extraction for yield analysis (20g flowers)

Twenty grams of frozen flowers were placed (in triplicate) into a 250ml BUNZL plastic flask. 40 ml of petroleum ether (B.P. 40-60°) was added, and flowers were homogenised for 10-15 seconds using an Ultra-Turrax. A cover was placed on the flask which was then left at room temperature for two hours. All traces of solvent were then removed by pouring into an Erlenmeyer flask through a cotton wool plug. Solvent was replenished, and the extraction process repeated for a further two hours. After removal of the second solvent wash solvent, 15ml of solvent was added to the flowers to remove any extract still present on the flowers. The solvent from all three washes was mixed together, and poured into a 250ml round bottomed (RB) flask. The solvent volume was reduced to 25ml under vacuum at 60°C. The remaining solvent was poured into a pre-weighed 50ml RB flask. All traces of solvent were removed under reduced pressure at 60°C; the flask remained on the RVE for a further five minutes after the last drops of solvent had been removed. The cool, dry flask was weighed to four decimal places. The yield of extract was calculated as a percentage of the fresh flower weight as below:

$$\frac{(\text{Wt of 50ml RB flask + extract}) - (\text{Wt of 50ml RB flask})}{\text{weight of flowers used}} \times 100 = \text{extract yield (\%)}$$

3.2 Laboratory scale extraction for yield analysis (100g flowers)

One hundred grams of frozen flowers were placed in triplicate into a glass jar. 260mls of petroleum ether was added, and the extraction was left for 15 minutes at

room temperature. The extract/solvent was poured into a 1L round bottomed flask through a cotton wool plug; 200mls of fresh solvent was added to the flowers for 15 minutes. After two further 15 minute washes with 200 ml of solvent (four washes in total), from which the solvent was bulked together after removal, the flowers were discarded. All but 50ml of the solvent was removed under reduced pressure at 60°C, the remaining solvent was poured into a pre-weighed 50ml round bottom flask which was dried down completely at 60°C, and for a further five minutes after removal of the last visible traces of solvent. Calculations are as in Section III.3.1.

3.3 Micro-DCM extraction of volatiles (five flowers or similar)

Plant material to be extracted (five flowers, or parts thereof) were weighed and placed into a 7ml screw-cap glass vial in triplicate. 4ml of DCM or hexane was added, including 1.25mg of an internal standard (C18). The extractions were left for 12 hours at 4°C. Subsequently, the plant material was removed, and if necessary, the DCM was concentrated under nitrogen; this method allowed rapid concentration at low temperature to minimise any loss of highly volatile floral compounds relative to the internal standard. Samples were analysed by GC (Section III.4.2).

4 Gas chromatography

4.1 General

All samples were analysed on a Hewlett Packard 5890 Series II gas chromatograph fitted with an SGE 15 metre bonded phase, fused silica BP1 column with internal diameter of 0.22 mm. The head pressure was 12psi, with a flow rate of 2 ml/minute and a 50 ml/min split vent flow. The oven temperature was held at 50°C for one minute, then increased by 10°C/minute until it reached 250°C where it remained for 14 minutes. Compounds were detected using a flame ionisation detector set at 280°C. Peaks eluting after between four and 20 minutes were integrated.

4.2 BORDCM method

Between three and five microlitres of sample (Section III.3.3; already containing a C18 internal standard) was injected onto the column. Chromatography was as per Section III.4.1. The integrated peaks were those of area greater than 20 units, and with peak width greater than 0.10mm, with an initial threshold of 0.

Peaks were initially identified by GCMS. The weight of volatiles in the liquid extract was calculated from the combined peak areas relative to the C18 standard;

specific volatiles were calculated as a percentage of the total volatiles. Volatiles were calculated as the fraction of the GC-analysable material eluting before n-heneicosane (Davies, 1984), expressed as a percentage of the amount of extract injected.

4.3 BOR2 method

Solid samples (Sections III.3.1 and III.3.2) were prepared for GC analysis by dissolution of a known amount (30 - 50mg) of boronia extract in 1.0 -1.5 ml of hexane, directly into a small GC vial with addition of a C18 internal standard. One microlitre of the sample was injected onto the column. The integrated peaks were those of area less than 500 units, and with peak width greater than 0.10mm, with an initial threshold of -1.

Peaks were initially identified by GCMS. The percentage of volatiles in the extract was calculated from the weight of the extract and the combined peak areas relative to the C18 standard; specific volatiles were calculated as a percentage of the total volatiles and as a percentage of the extract. Volatiles were calculated as the fraction of the GC-analysable material eluting before n-heneicosane (Davies, 1984).

5 Organoleptic assessment

One percent solutions of boronia extracts were made up in re-distilled ethanol, two drops were added to 100ml of distilled water in 200ml wine glasses. The tests were performed in a 'double blind' situation with glasses simply labelled A, B etc.; none of the participants knew their contents. The glasses were sniffed and ranked according to their floral, citrus and green notes. After 15 minutes, the fragrance faded and one more drop was added to each glass. The glasses were re-tested and ranked differently if necessary.

6 Oil gland enumeration

Fresh petals were mounted abaxial surface uppermost in glycerine, and flattened using a coverslip. Petals were examined under a binocular dissecting microscope/camera apparatus with transmitted light, and photographed in colour. Oil glands were enumerated manually from photographs; the oil glands appeared as non-pigmented areas in the dark red-brown abaxial epidermis. For some petals, natural pigmentation obscured enumeration, and pigments were extracted with acidified ethanol, and then re-stained with crystal violet. Petals were photographed and oil glands were enumerated as above.

7 Petal surface area calculation

Photographs of petals (Section III.6) were photocopied, and from the photocopy, the area of petal was cut out and weighed. By comparison between the weight of toner-blackened photocopy paper of a known area and the weight corresponding to the surface area of the petal, the area of the petal was calculated, taking magnification factors into consideration.

8 Acetone powder preparation and resolubilisation

8.1 Preparation

Acetone powders were prepared by homogenisation of fresh flowers with chilled acetone (-18°C) in a stainless steel blender. The homogenate was filtered using a Buchner funnel and a Whatman No. 1 filter paper. The solids were washed several times with chilled acetone until most of the pigment was cleared from the filtrate. This process removed phenolics that were not covalently bonded to proteins from the remaining solids. After a period of time drying on the Buchner funnel, the solids were spread over filter paper and left to air dry until the smell of acetone disappeared. The powders were stored in plastic BUNZL flasks at 4°C until required. In some cases, acetone powders were used without resolubilisation.

8.2 Resolubilisation and protein estimation

Approximately 1:1.5 g/g acetone powder : PVPP was resuspended in appropriate buffer with 0.1% Tween 80 overnight at 4°C. This mixture was used directly, or filtered and the solubilised protein was used.

Protein levels were assessed using the Bio-Rad Protein Assay (BioRad Laboratories, 32nd & Griffin Ave, Richmond, CA, 94804), which utilises the method of Bradford (1976); involving the shift in Amax at 595nm caused by the binding of Coomassie Brilliant Blue G-250 to protein. The kit was used as per the manufacturer's instructions. A standard curve was prepared with BSA.

8.3 Enzyme purification

In some cases, crude enzymes were prepared as described in Section III.8.2 and then further purified by addition of ammonium sulphate to the supernatant until a proteinaceous precipitate formed. Excess ammonium sulphate was dissolved by dialysis against distilled water. The enzyme solution was then centrifuged at 18,000 rpm for 30 minutes at 4°C, and the pellet resuspended in buffer. Enzyme prepared in

this way had protein levels of between 0.05% and 0.2% (weight of protein/weight of acetone powder used), with a single absorption maxima of 301nm.

9 Glycoside/glycosidase analysis

9.1 Preliminary investigations and results

Various methods for the preparation, purification and quantification of glycosidically-bound volatiles were examined. The methods included:

1) a methanol extraction followed by aqueous solubilisation (Watanabe *et al.* 1993);

2) an aqueous extraction at 90°C followed by purification through a column of Amberlite XAD-2 non-ionic polymeric adsorbent (Gunata *et al.*1985);

3) hydrolysis of glycosides with β–glucosidase, a multi-enzyme preparation from pinto beans (Agrawal and Bahl 1972), crude acetone powders, and resuspended and purified enzymes from acetone powders from boronia flowers; and

4) extraction and hydrolysis of glycosides at different pH values, temperatures, times, concentrations of enzyme(s) and with inclusion of PVPP and Tween 80 at various stages.

The important factors and results of preliminary experiments were:

1) addition of PVPP* at the rate of 5g per 50g of flowers, and 2 drops of Tween 80** during homogenisation of frozen flowers with buffer was required for subsequent activity;

2) At least 0.5g of PVPP was required during incubation of glycoside preparations with enzyme(s) to absorb additional phenolics;

3) extraction of glycosides with a citrate buffer of 0.05M concentration and pH 5.0, chilled to 4°C produced the highest activity during incubations with glycosidase;

4) using an incubation at 37°C for between 12 and 24 hours allowed maximum cleavage of glycosides;

* PVPP prevents inhibition of glycosidases (and other enzymes) which are extremely sensitive to phenolics (Loomis 1974; Loomis and Battaile 1966; Potty and Bruemmer 1970).

** Tween 80, a non-ionic detergent, increases the extraction of enzyme activity from acetone powders (Galliard and Phillips 1976; Kirk and Tilney-Bassett 1978). High levels of Tween 80 inhibited cleavage of β–ionone from β–ionone-glycosides.

5) when β–glucosidase was used for hydrolysis, at least 50mg per 10ml aliquot of the 20g/75ml glycoside preparation (0.3g flower equivalents) was required for activity;

6) when crude enzyme(s) from boronia flowers were used, at least 300mg of acetone powder was required, and use of the acetone powder without resolubilisation or purification showed the highest activity;

7) Glucose levels were assessed prior to and after incubation of glycoside preparations with various enzymes by means of a Glucose Diagnostic Kit (Sigma-Aldrich Pty. Ltd, used as per manufacturer's directions). Glucose levels declined during incubations. This was probably due to usage of sugars by microflora which were not specifically inhibited during the incubation. Therefore glucose analyses are not presented for correlation with release of volatiles from glycoside moieties;

8) there was no significant glycosidase activity in precursor solutions when incubated without addition of β–glucosidase or acetone powder from boronia flowers; and

9) there were no glycosides present in the acetone powders.

9.2 Rapid, precise method for preparation, hydrolysis and quantification of glycosidically-bound volatiles

9.2.A Extraction of glycosides

20g of frozen flowers were homogenised in a stainless steel blender with 75 ml of 0.05M citrate buffer, pH 5.0, chilled to 4°C. Tween 80 (2 drops) and PVPP (5g) were included during homogenisation. The slurry was passed through eight layers of cheesecloth, and the crude precursor solution so made was kept at 4°C until use (within one hour).

9.2.B Incubation of glycosides with β–glucosidase (assessment of quantity of glycosides present)

To 10 ml of the precursor solution (Section III.9.2.A), 100-150 units of β–glucosidase in 2 ml of the same buffer, and 0.5g of PVPP was added. Incubation was for 24 hours at 37°C.

9.2.C Incubation of glycosides with acetone powders from boronia flowers (assessment of glycosidase activity of flower enzyme(s))

300mg of acetone powder was incubated with 10ml of a 'standard precursor solution' prepared from frozen clone 5 flowers (Section III.9.2.A), and 1g of PVPP. Incubation was for 24 hours at 37°C.

9.2.D Estimation of glycosides or glycosidase activity

Duplicate samples at zero time and after 24 hours at 37°C from Sections III.9.2.B and III.9.2.C were 'stopped' and extracted by addition of 4ml DCM containing an internal standard (C18), vials were shaken vigorously, and left at 4°C for 12 hours. After extraction, the DCM was removed, concentrated under nitrogen gas and analysed by GC (Section III.3.3). The level of glycosidically-bound volatiles present in the precursor solution or released from a standard precursor was calculated from the difference between levels of free volatiles before and after incubation with β–glucosidase.

IV.1 Results
Accumulation in, and emission from different floral organs

A quantitative examination of the volatiles contained within, and emitted from different floral organs was done to ascertain the quantity and composition of compounds that each floral organ contributes to the overall extract. An understanding of the relationship between the flower and its extract production may aid future studies to maximise and manipulate floral extract production and composition.

Diurnal rhythms in levels of volatiles in tissues were assessed to examine any patterns that may be confluent with activity of potential pollinators, and therefore to identify which compounds may possibly be active in attraction of pollinators. The effect of temperature changes over short periods of time on floral volatiles was assessed to establish if temperature fluctuations are the cause of diurnal patterns *in vivo*. This was intended to be a preliminary study primarily of interest for natural populations of boronia and to further research into native flora/insect interactions.

1.1 Materials and methods

1.1.A Extraction of volatiles from floral organs

Extracts of all floral organs were made (Section III.3.3) using pooled, dissected organs from 40 flowers (2.0g). The plant material for these samples was weighed prior to extraction and the percentage weight calculated for each organ in the flower. Samples were analysed for volatile levels by GC (Section III.4.2).

1.1 B Headspace analysis

Twelve flowers for headspace analysis were selected from one lateral of one plant and dissected into their component organs. Other flowers (five) and large buds (five) from another lateral of the same plant were analysed whole. Floral organs were placed into 10 ml glass headspace sample vials and the vials were sealed with a teflon septum. Vials were left at room temperature for an equilibration time of 30 minutes. A 10 ml sample of the headspace was removed with a Hamilton syringe, another needle was placed into the septum simultaneously to prevent the development of a vacuum. The 10 ml sample was then injected slowly onto a 25 m HP1 GC column of which the first 10 cm was immersed in a beaker of liquid nitrogen/acetone slurry at approximately

-70°C. Air within the vial was replaced, and after a short equilibration time (1-2 minutes) another 10ml headspace sample from the same sample vial was injected onto the cryotrap. The cryotrap was then removed, the oven was closed and the GC analysis program commenced.

A Hewlett Packard 5890 GC, and an HP 5970 mass spectrometer (MS) were set for split-less gas flow, with an injection temperature of 100°C. A 50m BP1 column was used. The detector was set at 260°C. A 70eV ion source was used. The oven was set for an initial temperature of 30°C for 2.0 minutes, with a gradient of 6.0°C per minute until 150°C, and then a gradient of 10°C per minute until the oven reached 250°C. The GC/MS was set for selective ion monitoring to increase resolution:
Group 1 ions - 200msec - ions in group 67.05 and 82.05. 1-3 minutes.
Group 2 ions - 100msec - ions in group 93.05, 41.05, 68.05, 69.05 and 136.10. 3-11 minutes.
Group 3 ions - 180msec - ions in group 67.05, 71.05 and 82.05. 11-17 minutes.
Group 4 ions - 300msec - ions in group 177.1 and 43.0. 17-20 minutes.
Group 5 ions - 80msec - ions in group 83.05, 151.1, 224.15, 61.05, 140.10 and 43.0. 20-32 minutes.

A small number of peaks typical of the boronia flowers were seen in blank samples (empty headspace vials made up at the same time as the sample vials). The flower-bearing plants remained in the room during analysis, and contamination in the blank was probably due to the high volatility of such compounds at room temperature causing contamination of the sampling syringe between samples. The area of volatile peaks in blank samples were subtracted from all sample analyses as 'background' levels. No internal standards were used because of the lack of appropriate standards for the solid-sample type used. Calculations were made on the basis of the area of each peak, less background levels.

The GC peak area for each compound present in the headspace above each organ type was summed with the area resulting from emission from other organs, producing a total area for each compound from the 'whole flower'. The area resulting from emission of particular volatiles from each organ was then calculated as a percentage of the area calculated as being emitted by the 'whole flower'.

1.1.C Extraction of volatiles from flowers over 24 hours

Flowers were excised from mid-lateral positions from clone 5 pot-grown plants (Section III.1.2) at 6pm, 11.30pm, 6am and 12 noon. Throughout the experiment, plants were subjected to normal environmental conditions: during the first day (sampling at 6pm and 11.30pm) the maximum temperature was 18°C, the minimum was 9.4°C and there were 8.8 hours of sunlight with no rainfall. During the second

day (sampling at 6am and 12 noon) the maximum temperature was 20.9°C and the minimum was 6.7°C, with 11.4 hours of full sunlight; once again there was no rain and very little wind.

At each sampling time, 20 flowers (approximately 1 gram) selected from 4 plants were placed in to each of six vials that contained hexane and an internal standard. Flowers were selected on the basis of their similar maturity, and showed no signs of ageing (no bleaching of pigments in the stigma or petals) After four hours extraction, flowers were removed from the hexane, and a sample of the solvent was analysed by GC (Section III.4.2).

1.1.D Treatment of flower-bearing plants to different temperature regimens

Flower-bearing, pot-grown (Section III.1.2), clonal plants were treated with several temperature regimens for 6 hours. The pre-treatment conditions were normal environmental conditions: Three plants were placed at each temperature. The treatments were:

1) 10°C with light *
2) 20°C with light *
3) 20°C in the dark
4) 25°C with light *
5) 32°C with light *

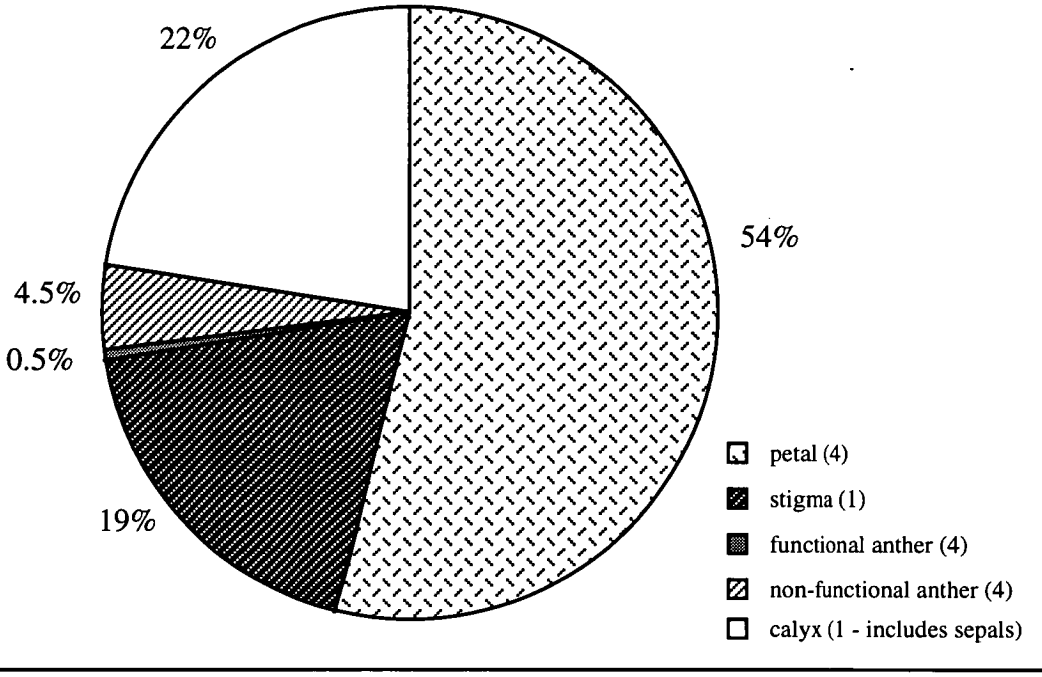
* at least 100 $\mu Em^{-2}s^{-1}$

After six hours exposure to the treatments, all flowers were harvested from each of the three plants at each temperature and extracted in triplicate over 12 hours (Section III.3.3). The solvent was concentrated under nitrogen gas and analysed by GC (Section III.4.2).

1.2 Floral organs: tissue extracts and emission of volatiles

A *B. megastigma* flower comprises 4 petals, 4 sepals, 1 calyx, 4 functional (petaline) anthers, 4 non-functional (sepaline) anthers and a large stigma. Figure 1.2.A illustrates the relative weight(s) of the component organs of the flower. The 'calyx' represents the sepals, calyx and stalk of the flower.

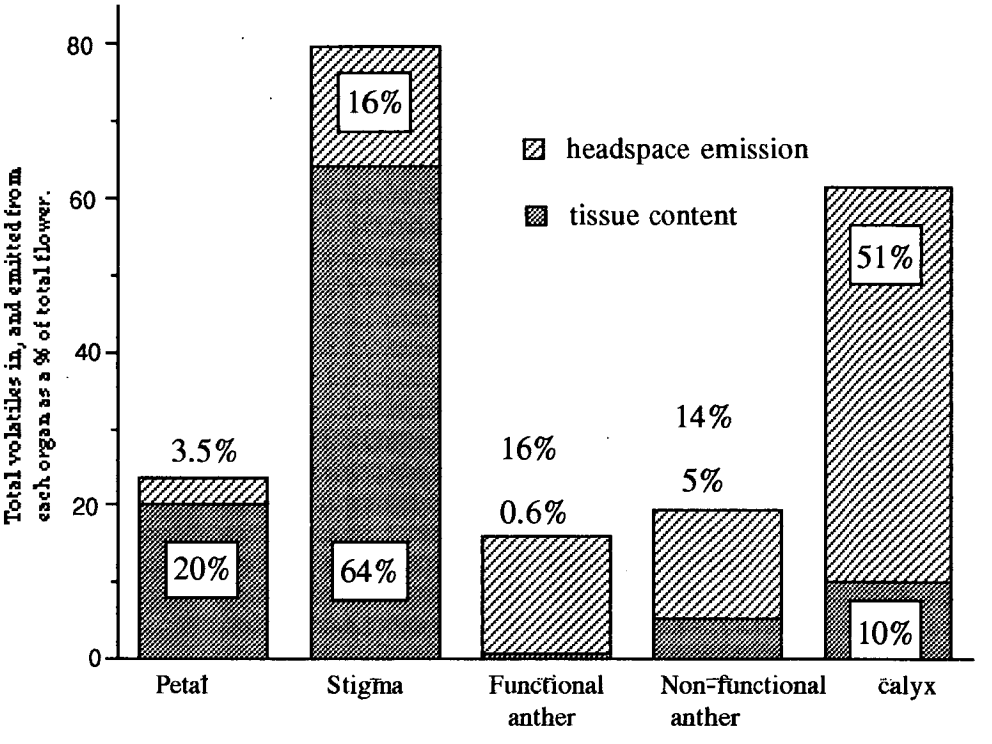
Figure 1.2.A Weight of each organ as a proportion of the whole flower.



The petals comprise more than 50% of the whole flower weight; the stigma and calyx are almost equivalent in weight and comprise about 22% each. The two types of anthers contribute least to the flower weight, the large non-functional anthers weighing at least five times more than the functional anthers.

The total amount of volatiles extracted from floral organs, and emitted into the headspace above each organ type is presented in Figure 1.2.B., expressed as a proportion of the extract and emission levels from the whole flower.

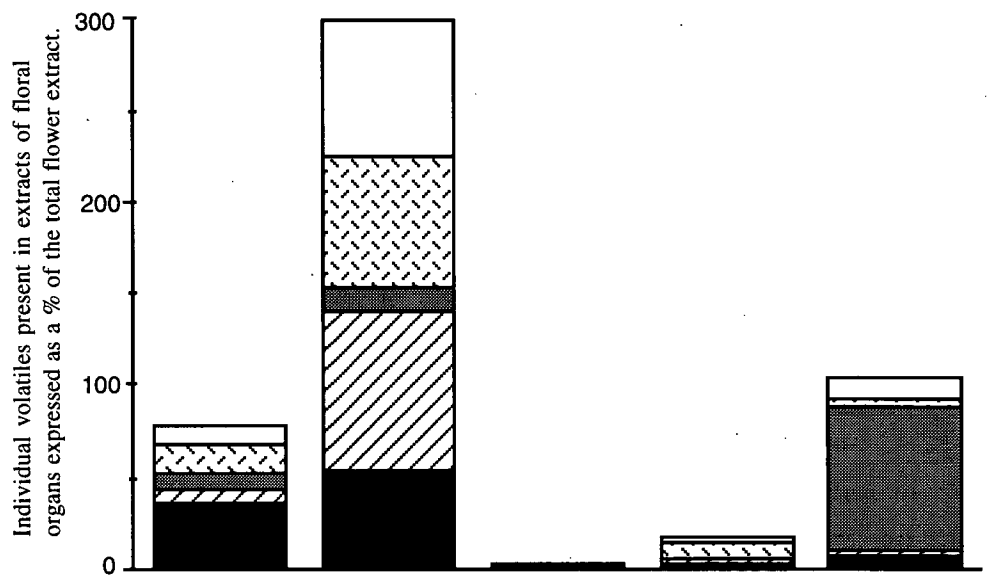
Figure 1.2.B Total amount of volatiles in tissue extractions, and emitted into the headspace from each floral organ, as a percentage of the whole flower levels.



Almost 65% of the total volatiles extracted from the flower is contributed by the stigma. Only 20% is contributed by the petals. The calyx contributes about 10% of the total volatiles, and the anthers, negligible amounts (less than 5%). However, the emission levels follow a different pattern. The calyx contributes the largest proportion. The stigma, functional and non-functional anthers emit similar levels of total volatiles, the total emission from these three organs is almost the same as that from the calyx. The petals contribute only a quarter as much as any of the sexual organs. A more detailed examination of the different volatiles present in, and emitted from component organs was done.

Figure 1.2.C illustrates the levels of different volatiles in the tissue extracts from each organ, expressed as a percentage of the whole flower extract. The volatiles selected for illustration comprise components of monoterpenes, norisoprenoids, acetates, jasmonates and fatty acids. The compounds shown in Figures 1.2.C and 1.2.D were selected on the basis that they covered a range of different compounds distributed throughout the volatile part of the chromatogram; some are significant contributors to the typical floral perfume. Some of these compounds may be active in attraction of insects to floral species, for example, dodecyl acetate, β-ionone and jasmonates.

Figure 1.2.C Selected volatiles in extracts of each floral organ expressed as a percentage of the whole flower extract.



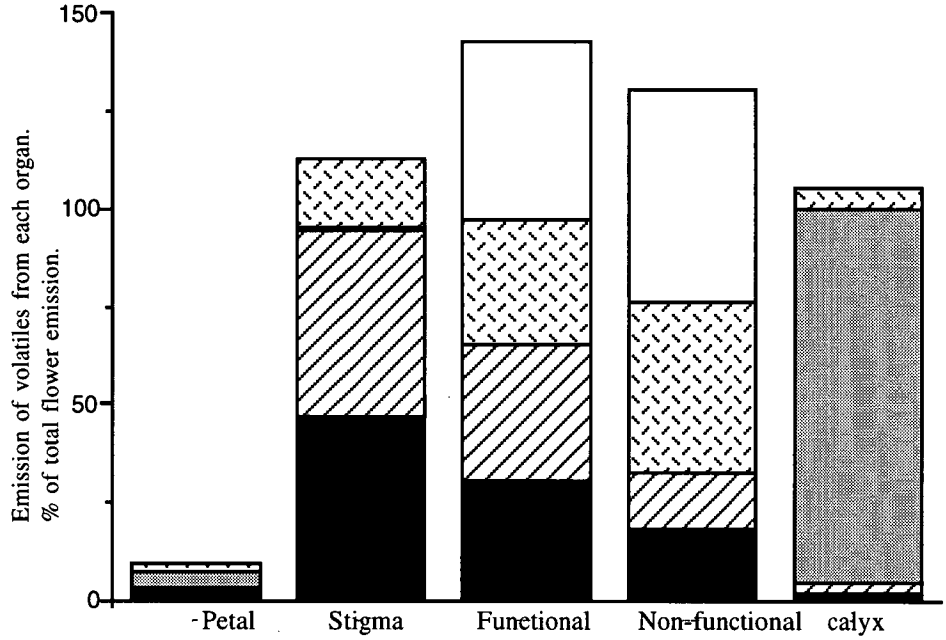
	Petal	Stigma	Functional anther	Non-functional anther	calyx
me. jas. I & II	10%	74%	1%	3%	11%
(Z)-hep-8-ene	15%	70%	1%	8.5%	5%
alpha-pinene	9.4%	13%	0%	0%	78%
dodecyl acetate	6.8%	88%	0.1%	3%	2%
beta-ionone	37%	53%	0.3%	2%	8%

All floral organs contain all of the five volatiles selected for study, except the anthers which lack α-pinene. The stigma and the petals are the main sites of β-ionone accumulation, with the stigma having almost one and a half times the amount in the petals. The stigma contains the most dodecyl acetate, (Z)-heptadec-8-ene, and methyl jasmonate isomers I and II by more than five times compared with the other organs.

The calyx contains most of the α-pinene in the flower and has small amounts of dodecyl acetate and (Z)-heptadec-8-ene, and similar amounts of the methyl jasmonate isomers I and II compared with the petals. The anthers contain low levels of all volatiles, contributing low to almost negligible amounts to the total flower extract.

An examination of the same five selected volatiles in the headspace emitted from fresh floral organs may be seen in Figure 1.2.D.

Figure 1.2.D Selected volatiles in the headspace emitted from individual floral organs. Expressed as a percentage of the headspace emitted from the whole flower.



	Petal	Stigma	Functional anther	Non-functional anther	calyx
me, jas. I & II	0%	0%	46%	54%	0%
(Z)-hep-8-ene	2%	18%	32%	43%	6%
alpha-pinene	4.5%	1%	0%	0%	95%
dodecyl acetate	0.5%	48%	35%	14%	3%
beta-ionone	3%	47%	30%	18%	2%

Anthers play an important part in the emission of volatiles from the whole flower. Although tissue levels of volatiles in these organs were low, the functional anthers contribute almost 30% of the β-ionone, similar amounts of (Z)-heptadec-8-ene and dodecyl acetate, and over 45% of the methyl jasmonate isomers to the headspace of the whole flower. The anthers alone are responsible for the emission of methyl jasmonate I and II isomers. Most of the β-ionone and dodecyl acetate are emitted from the stigma and functional anthers. Lesser amounts of both compounds come from the non-functional anther, which emits most of the (Z)-heptadec-8-ene and methyl

jasmonates. The petals and calyx contribute almost negligible emissions of compounds responsible for the floral bouquet of the flower emission profile. The calyx emits mainly α-pinene.

1.3 Emission from large buds and open flowers: a comparison

A comparison was made between the headspace emitted from five whole, open flowers, and five large buds, the stage just prior to anthesis. The levels of identified compounds in the headspace are compared in Table 1.3, using the levels in the open flowers as the basis for comparison with the levels in the large buds.

Table 1.3 Comparison of some compounds in the headspace of open flowers and large buds.

Compound	% in open flowers (stage #5)	% in large buds (stage #4)
hexenol isomer	0	100
α-pinene	100	9
camphene	100	0
β-pinene	100	7
myrcene	100	0
d-limonene	100	0
ocimene	100	0
C11 hydrocarbon	100	71
unknown, related to a dimethyl octadienediol	100	166
β-ionone	100	204
dodecyl acetate	100	22
(Z)-heptadec-8-ene	100	45
Total	100	12

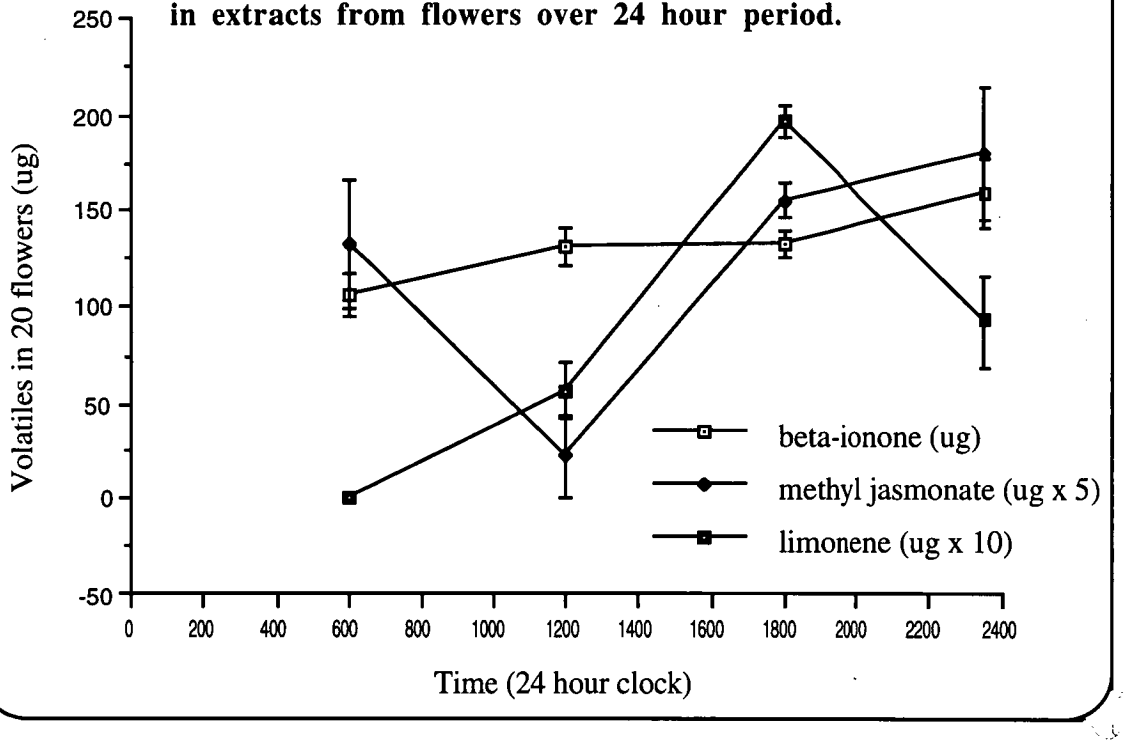
The main differences between the headspace from the two developmental stages is that the large buds only emit 12% of the total amount of volatiles emitted by the open flowers. Despite this, marked compositional differences in emissions from the two stages are apparent, including the emission of an isomer of hexenol, higher levels of

the unknown compound, and a significantly higher emission of β-ionone, by more than two times, from the large buds compared with the open flowers.

1.4 Volatile levels in tissues over 24 hours

There were no significant changes in the total amount of volatiles contained within flowers during a 24 hour period. There were, however, significant changes in the amounts of particular volatiles during this period. Figure 1.4 illustrates the changes in methyl jasmonate, limonene and β-ionone during 24 hours.

Figure 1.4 Beta-ionone, limonene and methyl jasmonate in extracts from flowers over 24 hour period.

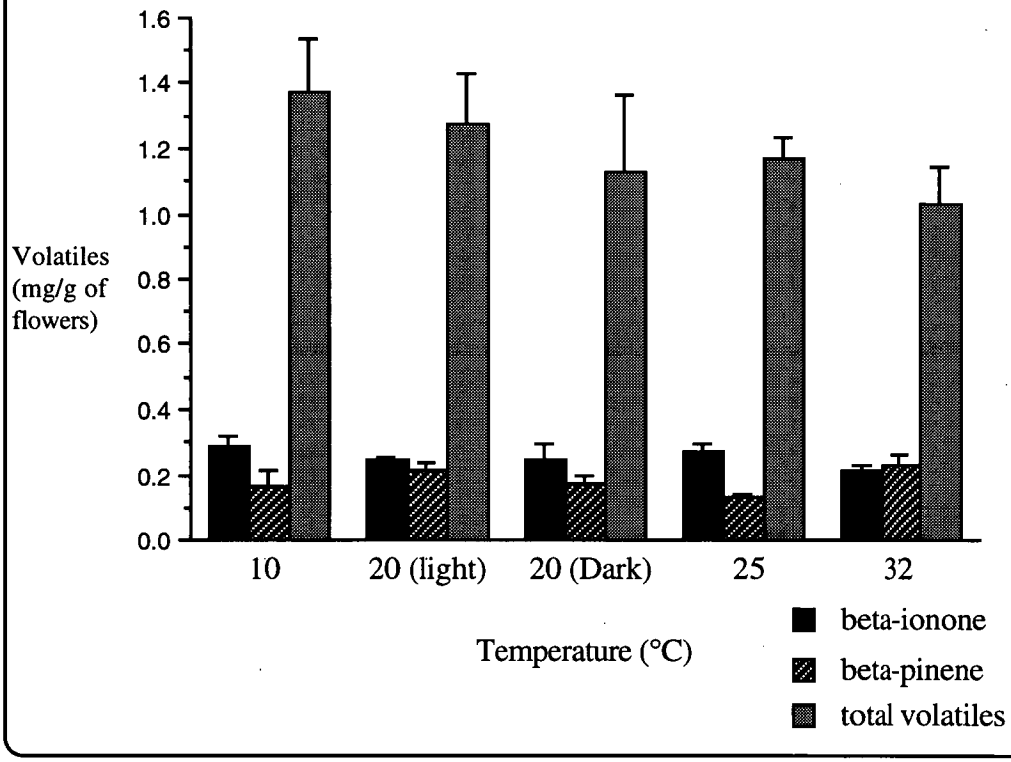


There is significantly less methyl jasmonate at 12 noon compared with all other times. There is no limonene detectable at 6am; this volatile increases in concentration during the day, reaches a peak at 6pm and then declines again. There is significantly more β-ionone in flowers at 11.30pm compared with 6 am. There is a gradual increase in β-ionone between 6am and 11.30 pm.

1.5 Effect of temperature on volatile levels

Different temperature regimens and their effect on volatiles in flowers *in vivo* was assessed. Some of the results are illustrated in Figure 1.5.

Figure 1.5 Effect of Temperature on volatiles in flowers in vivo.



There were no significant differences in the concentrations of each volatile under different temperature regimens. However, there is a trend toward decreasing concentration of total volatiles at high temperatures. There were no differences between light and dark treatments.

1.6 Pollinators?

Information about the pollinator(s) for boronia may be of significance, particularly in situations where boronia is grown under cultivation. *B. megastigma* is an introduced species to Tasmania. For this reason, and because plantations comprise clonally propagated material produced under licence, it would be undesirable for

boronia plants to set seed which may germinate. Information about the pollinator may also aid studies on the control of extract accumulation, i.e. is the attraction of pollinators the driving force for production of some compounds?

Boronia plantations which had been established for over 15 years were observed for the distribution of insects capable of pollinating the flowers. Casual observation had shown that more insects were active in late afternoon, and so observations and samples were collected at this time. Several small moths were found in the vicinity, as were numerous flies and bees. The latter were attracted to co-incident flora and were not seen visiting boronia flowers at all. Samples of insects were collected and examined for pollen, however no boronia pollen was identified. The moths were identified by P. McQuillan (pers. comm.) as being in the family Decophoridae, and thought to be too large for effective pollination of boronia. P. McQuillan (pers. comm.) has identified small moths (1-2 mm long) in the vicinity of native Tasmanian boronias (*B. citriodora*) and on *Zieria* (Rutaceae), as being in the family Heliozelidae, and he believes this to be the family most likely to be responsible for pollination of *B. megastigma*, if pollination occurs at all in Tasmania. Moths from this family were not found in the vicinity of boronia plantations sampled. Both of the moth families mentioned are diurnally active.

A yellow insect trap was placed on one plantation for one week during the flowering period, the only insects collected by this trap were beetles (Coleoptera) and flies (Diptera).

1.7 Discussion

The stigma and petals are the main organs which contribute significant amounts of extract components, and therefore are of interest commercially. The presence of numerous oil glands on particular tissues is not a prerequisite for extract accumulation. Most of the typical floral headspace is contributed by the stigma and anthers (Section IV.1.2). Compounds which are known insect attractants such as methyl jasmonate(s), dodecyl acetate and β-ionone predominate in the headspace. The relatively high levels of emission of compounds not present in high levels in particular tissues may be a result of adsorption of volatile compounds such as β-ionone and methyl jasmonate from other organs (Farmer and Ryan 1990; Sadafian and Crouzet 1987; Dobson *et al.* 1990). The emission of high levels of α-pinene from the calyx may function in deterrence of herbivorous arthropods. Hexenol has not been detected in extracts or headspace produced from boronia flowers before (Kaiser 1991; Davies and Menary 1983); an isomer was found in the headspace emitted from large buds (Section IV.1.3) and separated floral organs (Section IV.1.3).

Anthesis is not a prerequisite for emission of volatiles (Section IV.1.3); in fact, some volatiles such as β-ionone are present in relatively lower concentrations in the headspace from open flowers than from large, unopened buds (Section IV.1.3). There are higher levels of some volatiles within floral tissues in the early evening (Section IV.1.4); this does not appear to be caused by changes in external temperature regimens, although the period over which external conditions were altered (6 hours) may not be sufficient for the effect of such changes to become apparent (Section IV.1.5). Whether or not such changed levels correspond with differences in emission is not known, neither is the effect of the external environment on such patterns.

There is some evidence for increased activity of potential pollinating species of Lepidoptera in the late afternoon (Section IV.1.6). Direct chemical attractant/pollinator responses are not measurable in Tasmania, where introduced plantations of boronia may be geographically isolated from activity of native boronia pollinators, if indeed they occur at all in Tasmania.

IV.2 Results
Sites of biosynthesis and accumulation of floral extract

Specialised structures potentially capable of accumulating boronia extract have already been described in the carpels, sepals and petals (Haberlandt 1928; Wilson 1982). However, these studies used only light microscopy, and did not ascertain sub-cellular organelles active in biosynthesis. Ultrastructural studies of floral tissues of a Rutaceae species are rare and therefore of interest. The identification of organelles active in the production of secondary products may enable isolation of biosynthetic enzymes; further work may improve knowledge of biosynthetic pathways and allow for future genetic manipulation. Identification of active release mechanisms (if any) of fragrant materials may indicate the necessity for different management practices to maximise extract yield per plant at harvest.

2.1 Materials and methods

2.1.A Microscopy

2.1.A.1 Light microscopy

Fresh petals and sections were mounted in glycerine, because water increased the production of pigmentation artefacts. Transverse sections were cut by hand and oriented with haste to reduce pigment bleaching. Sections 0.5 microns thick were cut both tangentially and transversely from tissue prepared for TEM using an LKB microtome; some sections were stained with toluidine blue.

Various microscopes and cameras were used for light microscopy. Fresh petal sections were photographed on a Zeiss axioplan microscope with brightfield optics using Kodak 100 film. Some photos were taken using a dissecting microscope and attached camera with external light sources.

2.1.A.2 Scanning electron microscopy

Fresh plant material was affixed to stubs with double-sided tape, lightly sputter-coated with gold, observed with a Philips 505 scanning electron microscope set at 15 kV, and photographed using Ilford FP4 120 photographic film. Petals were orientated for photography of oil glands with the epidermis on the abaxial surface

uppermost, and the base downward, at an angle of 44.5°, tilt adjustment 28°. Desiccation prior to coating and observation was minimal.

2.1.A.3 Transmission electron microscopy

Petals from mature flowers and young flower buds were fixed in 3% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2), overnight at 4°C. Post-fixation was in 2% OsO_4/buffer for two hours at room temperature, followed by 45 minutes in 0.5% uranyl acetate. Acetone was used for dehydration. Petals were then embedded in Spurr's resin (Spurr, 1969), and sectioned on an LKB microtome.

Sections for transmission electron microscopy (60-90nm thick) were cut transversely with a diamond knife using an LKB microtome, placed onto Formvar (polyvinylformal 15/95) coated slot grids, and stained with uranyl acetate (4% in water) for 30 minutes, and lead citrate (1.2% in 0.1N NaOH) for three minutes. An Hitachi 300, and a Philips CM100 transmission electron microscopes were used.

2.1.B Plastid isolation

To 200g of frozen flowers was added 200 ml of chilled isolation medium: 1mM mercaptoethanol, 1mM EDTA and 0.4 M sucrose in 50mM Tris-HCl, pH 8.0 (Camara *et al.* 1992). This was homogenised for 30 seconds with an Ultra-Turrax homogeniser. The slurry was passed through four layers of cheesecloth, and the filtrate centrifuged at 900rpm for five minutes at 4°C. The supernatant was centrifuged at 4000rpm for 30 seconds, and the pellet from this centrifugation was washed twice with isolation medium. The pellet was finally suspended in 20 ml isolation medium. 10 ml of the plastid suspension was layered on top of a sucrose gradient of 10 ml solutions each of 0.75 M, 1.0 M, and 1.5 M sucrose in 50 mM Tris-HCl pH 7.6 and 1 mM mercaptoethanol. The other 10 ml of the plastid suspension was layered at the bottom of the same sucrose gradient in a separate tube. Both tubes were centrifuged at 5,000rpm for 15 minutes at 4°C.

After centrifugation, the two coloured fractions from each tube were removed, and fractions of the same colour were pooled together. Samples were observed under a light microscope. Hexane with an internal standard was added to the samples, and they were placed in a bath sonicator for four hours to extract the volatiles. Volatiles were analysed by GC (Section III.4.2).

2.2 Light microscopy of floral tissues

Photographic plates of the observations made from light microscopy of the floral organs and their constituent tissues may be seen in Plates 1 - 3. The epidermis on the adaxial surface of the petal is richly pigmented with yellow, on the abaxial surface, the epidermis is red-brown (1A, 2A). Both epidermis' have epidermal hairs around the apical margin; the abaxial side has more hairs unevenly distributed over the epidermis than the adaxial side. Oil glands are only visible on the epidermis on the abaxial side, discernible as yellow areas of similar size to the normal epidermal cells (2E). In transverse sections of fresh petals (3A) the oil glands can be seen to be in contact with the epidermis on the abaxial side, and are isodiametric structures with a grey interior. Many compressed cells surround the central cavity of the oil gland. The abaxial-most layer of parenchyma stains darker with lead citrate and uranyl acetate compared with other parenchyma cells (3B), possibly as a result of lipid or tannins accumulating in the cells.

The epidermis of the four-lobed stigma is red-brown in colour, the parenchyma is densely pigmented and yellow (1A, 2B). The parenchyma of the stigma has many relatively large isodiametric cells dispersed at regular intervals between other cells. Beneath the stigma are seen the functional anthers (1B), and a disc area that has morphological characteristics (small gland-like areas) suggestive of secretion, probably of nectar (1B, arrows). The non-functional anthers are darkly pigmented (1B, 2D), and the parenchyma is also yellow like that of the stigma (not shown). The non-functional anthers are approximately five times larger than the functional anthers (1B, 2C). Pollen may be seen on the functional anthers in flowers which have just reached anthesis (1B, 2C).

Plate 1.

1A The flower of *B. megastigma*; bar = 0.2cm.

1B Close-up of boronia flower from which the stigma has been removed. Two of the non-functional anthers (nfa) are in various stages of atrophy. Functional anthers (fa) have pollen present. Note gland-like areas on disc (Arrow); bar = 0.25cm.

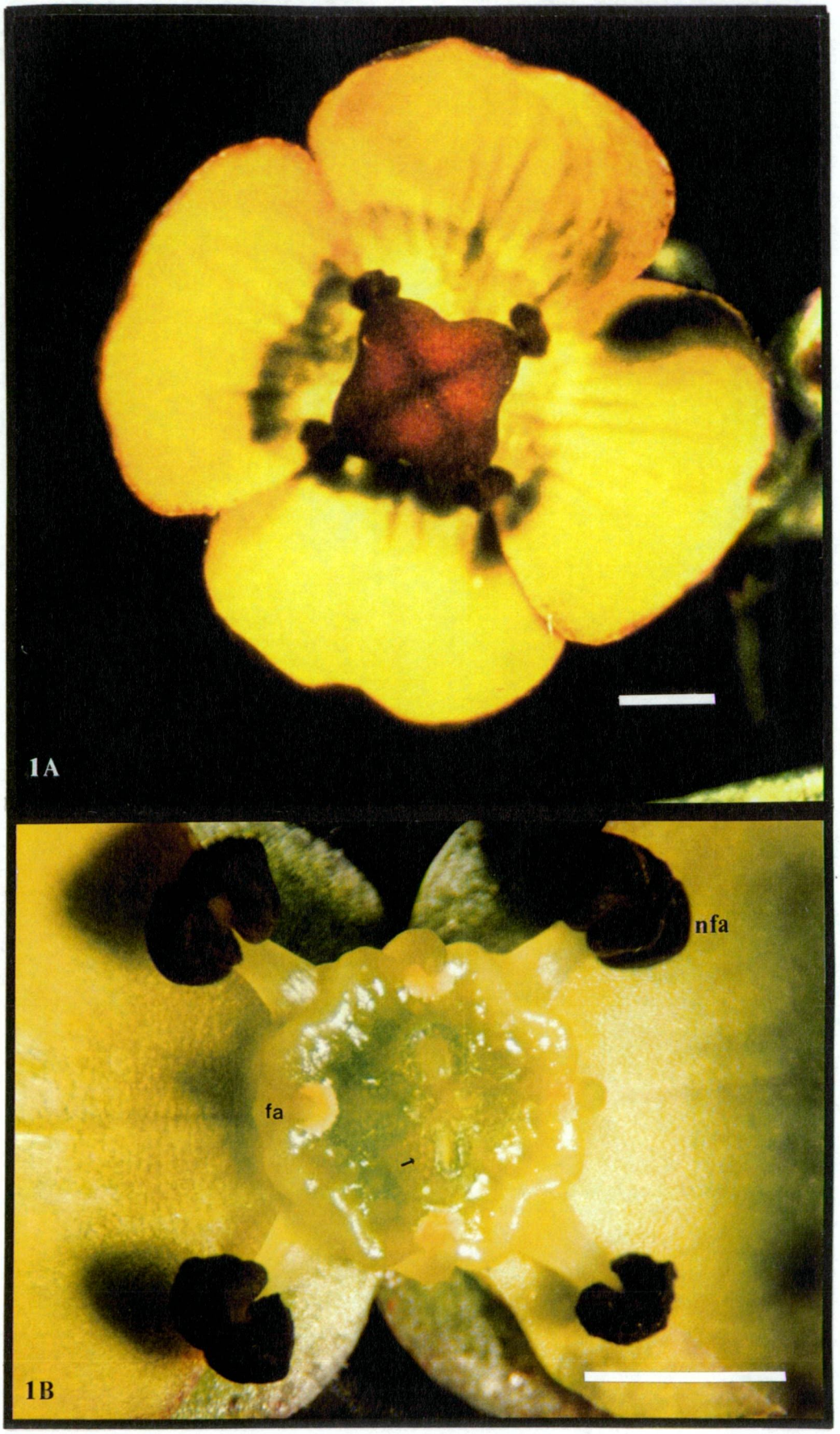

1A
nfa
fa
1B

Plate 2.

2A Epidermis' on the adaxial (yellow) and abaxial (red-brown) surface of petals; bar = 1mm.

2B Abaxial and adaxial surface of stigma; adaxial surface has been cut tangentially to expose parenchyma; bar = 1mm.

2C Functional anthers coated with pollen; bar = 1mm.

2D Non-functional anthers; bar = 1mm.

2E Epidermis on the abaxial surface of petal, with transmitted light; oil glands (og) clearly visible; bar = 0.15mm.

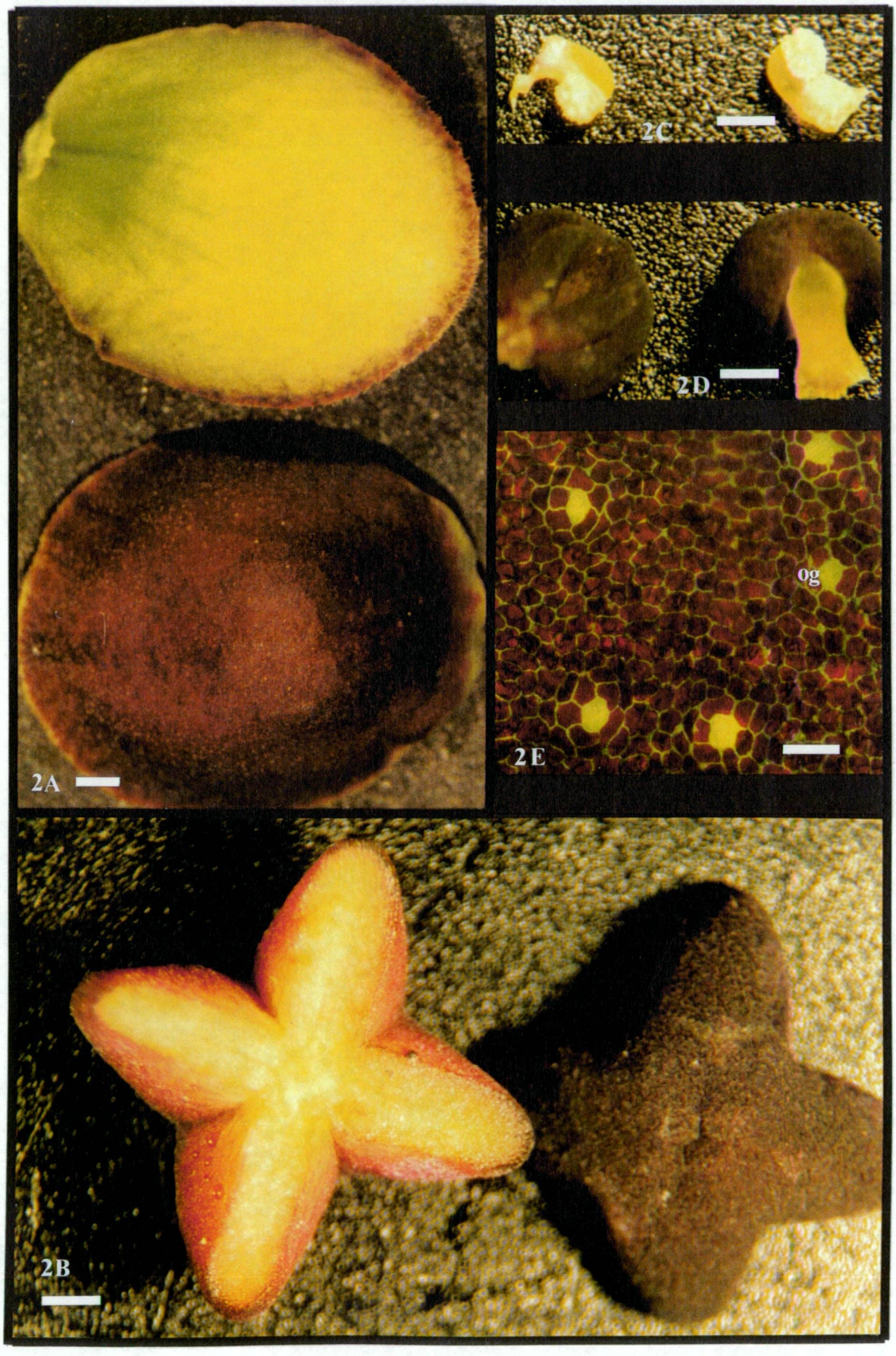

2A
2B
2C
2D
2E
og

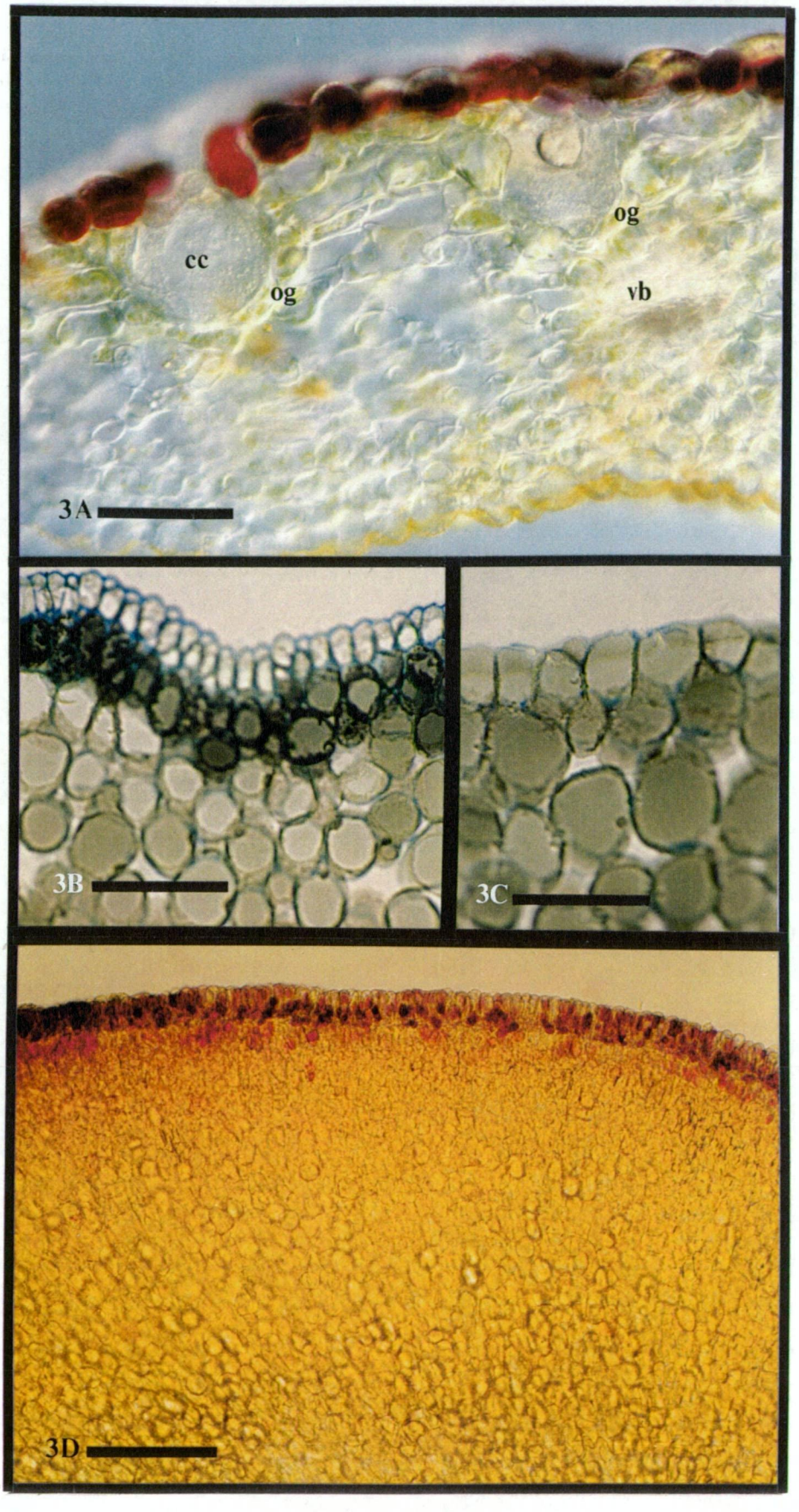
Plate 3.

3A Transverse section through fresh petal. Two oil glands (og) clearly visible showing orientation to epidermis on the abaxial side. Central cavity (cc) of oil gland with grey colouration; vb = vascular bundle; bar = 50 microns.

3B Transverse section through petal near the epidermis on the abaxial side, fixed material, stained with lead citrate and uranyl acetate. Note black material in parenchyma below the epidermis; bar = 25microns.

3C As in 3B, except showing epidermis on the adaxial side; bar = 25 microns.

3D Transverse section through fresh stigma showing dense parenchyma and yellow pigmentation; bar = 0.1mm.


cc
og
og
vb
3A
3B
3C
3D

2.3 Sequential sections through an oil gland

Transverse serial sections (0.5 microns thick) were cut at various depths through an oil gland in the petal. Plates 4 and 5 illustrate some of the sections cut in this study. Plate 5G has been labelled to illustrate the different cell types discussed. This particular oil gland is located adjacent to a vascular bundle (4A, vb). The central cavity (CC) of the oil gland contains droplets of material, presumably oil (5I, arrow). Surrounding the CC are several flattened, thin-walled cells in various stages of lysis: so-called lysing cells (LC). Surrounding the LC are flattened cells with normal cell wall thickness: so-called sheathing cells (SC). The outer-most layer of cells (SC) adjoin normal epidermal cells (EC) abaxially (5G, 5H), and normal parenchyma cells (PC) toward the interior of the petal (5I). The oil gland is isodiametric.

Plates 4 and 5.

Sequential transverse sections through an epidermal oil gland on the petal, stained with toluidine blue.

Distances between sections:

A-B = 4 microns

B-C = 3 microns

C-D = 1 micron

D-E = 3.5 microns

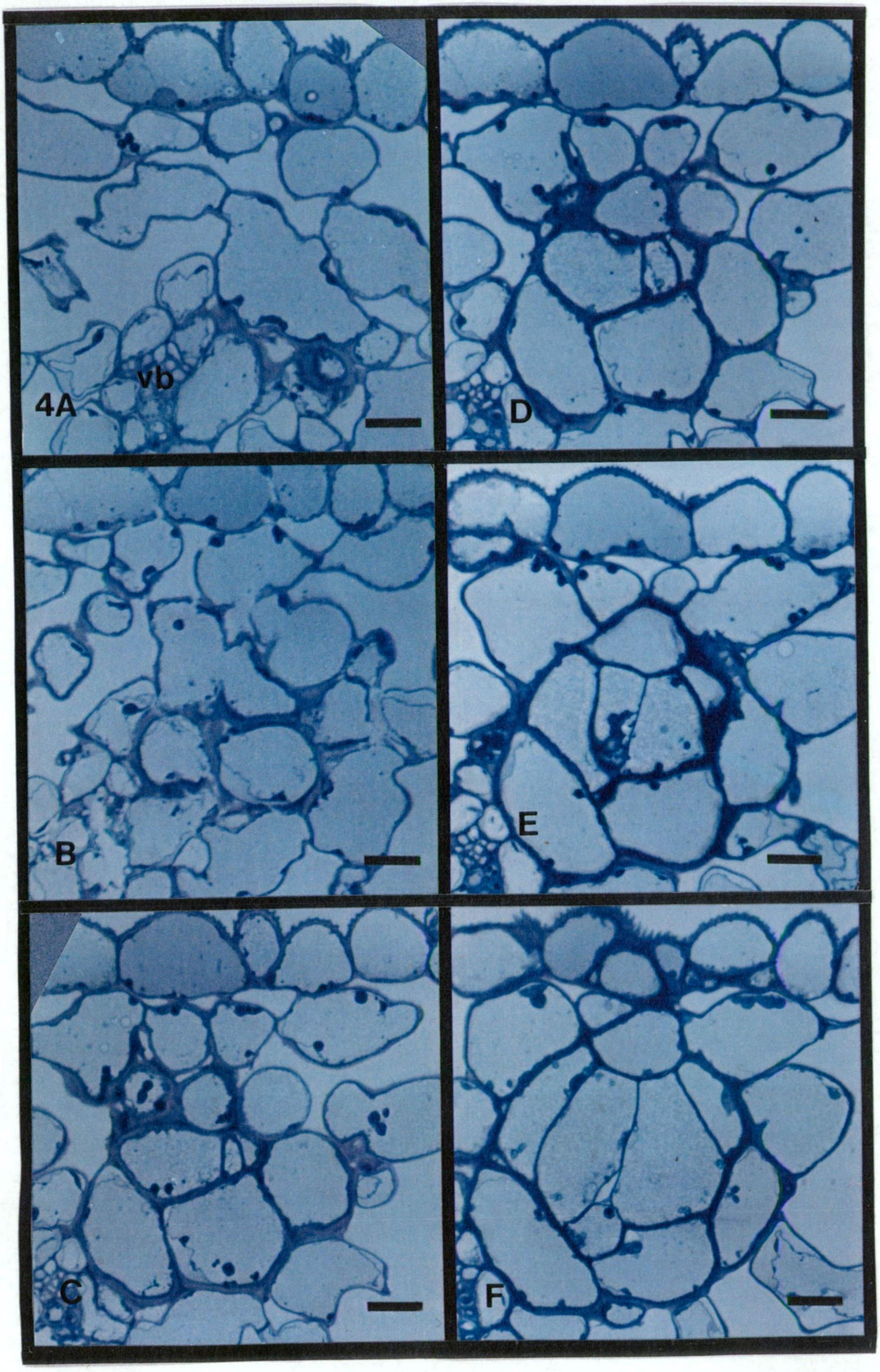
E-F = 5 microns

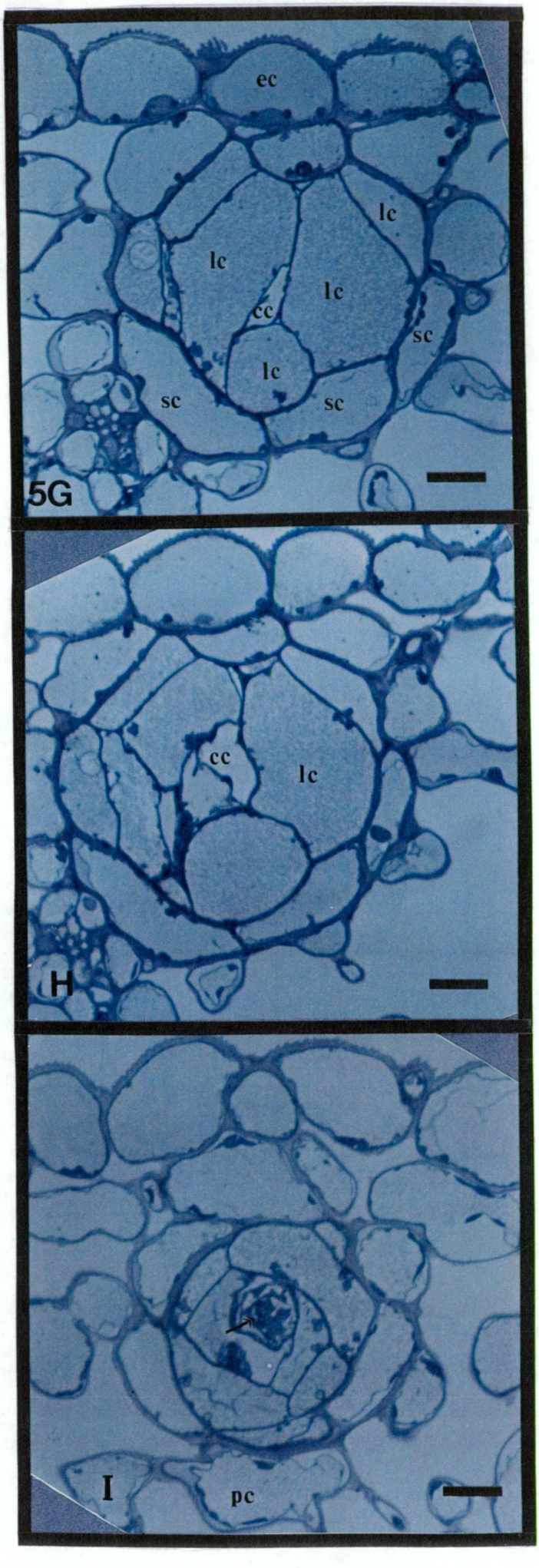
F-G = 3.5 microns

G-H = 6 microns

H-I = 11 microns

All bars = 10 microns. LC = lysing cell, CC = central cavity, SC = sheathing cell, EC = epidermal cell, PC = parenchyma cell, vb = vascular bundle.


4A
vb
D
B
E
C
F


ec
lc
lc
lc
cc
sc
lc
sc
sc
5G
cc
lc
H
I
pc

2.4 Scanning electron microscopy of floral tissues

Micrographs illustrating the results of scanning electron microscopy of floral organs, oil glands and leaves may be seen in plates 6 to 9.

2.4.A Petals and oil glands

The epidermis on the adaxial surface of the petal has a more uniform pattern of striations than the abaxial side (7A, 7B). The former has very few epidermal hairs, except along the acropetal margin, although simple epidermal hairs are common on the epidermis on the abaxial side (7C, 6B). Oil glands are easily discernible on the epidermis on the abaxial surface (7B) and are randomly distributed, with slightly higher density toward the centre and apex of the petal. Stomates are occasionally found on the epidermis of the abaxial side (7B, 7D), similar to those seen on the leaf (8A, 8B). The oil glands on the epidermis of the leaves (8B) and petals (6C) are similar in form, showing a 'cap' comprised of septa that divide the cap into four equal-sized sections; the total area being slightly larger than a normal epidermal cell.

Oil glands on petals of six developmental stages were observed (6A-6H). On very small buds (stage #1*), immature oil gland caps that appear to be depressed (which may be an artefact of preparation) may be seen in apical regions (6A). Such cap areas are approximately 18 microns in diameter. Other glands nearer the centre of the petals of stage #1 buds are more developed (6B), and are over 30 microns in diameter. Such glands are well structured and show some signs of protruding above the epidermis. On petals of stage #2 buds (6C), glands appear sunken below the epidermis, and are between 30 and 50 microns in diameter. Surrounding epidermal cells are also expanded compared with smaller buds. Stage #3 buds have glands of similar size to stage #2 buds. Stage #4 buds (large buds) have glands of diameter 35 to 50 microns also, however surrounding epidermal cells are more expanded than in smaller buds (6D). Oil glands on mature petals from open flowers have glands which are raised well above the epidermis, cells of which are expanded to a greater extent than other stages (6F).. The total area comprising four sections of the caps of oil glands is the same size as one normal epidermal cell. The septa of glands on petals which are found on stage #6 flowers (flowers which are senescing and have lost one or more petals through abscission), have ruptured (6G). The rupture may be in one direction only, as shown, or may divide the septa completely (6H).

* For a description of the different developmental stages see Section IV.4.2.

The epidermal cells in 6G and 6H (senescent petals) are dry-looking and on casual observation appeared to have faded pigments. The gland illustrated in plate 6H is from a petal that has abscised and fallen to the ground. No ruptures were seen in caps of glands on fresh, turgid petals with bright pigments and four complete petals.

2.4.B Functional anthers and pollen

The pollen distribution on the functional anther may be seen (9A), coating the lower surfaces and edges of the anther. The pollen has a 'dimple' on either side dividing it into a symmetrical, pinched, oval-shaped structure, although the dimple may be an artefact of preparation. In plate 9C, pollen is distributed on the surface of a stigma and one pollen grain has produced a pollen tube.

Plate 6.

Scanning electron micrographs of epidermal 'caps' on the abaxial surface of petals of various developmental stages of flowers and buds.

A Very small bud, stage 1; bar = 10 microns.

B Very small bud, stage 1; bar = 50 microns.

C Small bud, stage 2; bar = 50 microns.

D Medium-sized bud; bar = 50 microns.

E Large bud, stage 4; bar = 50 microns.

F Open flower, stage 5; bar = 50 microns.

G Over-mature flower with only one or two petals remaining, stage 6; bar = 50 microns.

H Petal that has fallen to the ground after abscission from the flower; bar = 50 microns.

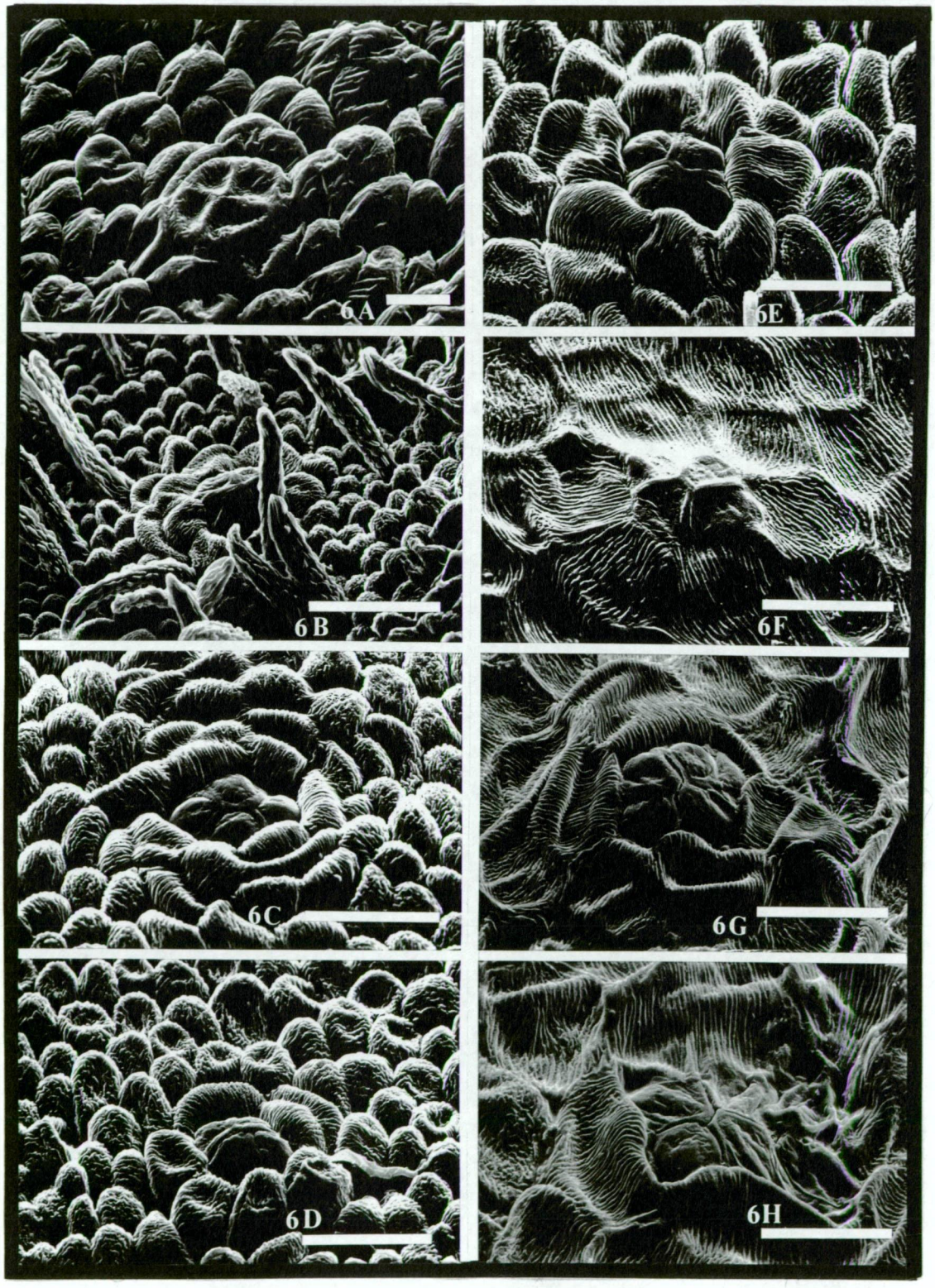

6A
6E
6B
6F
6C
6G
6D
6H

Plate 7.

Scanning electron micrographs.

A Epidermis on the adaxial (yellow) surface of petal; bar = 0.1mm.

B Epidermis on the abaxial (red-brown) surface of petal; bar = 0.1mm.

C Epidermis on the abaxial surface of petal showing three oil glands (og) and stomate (s); bar = 50 microns.

D Stomate on epidermis on abaxial surface of petal; bar = 50 microns.

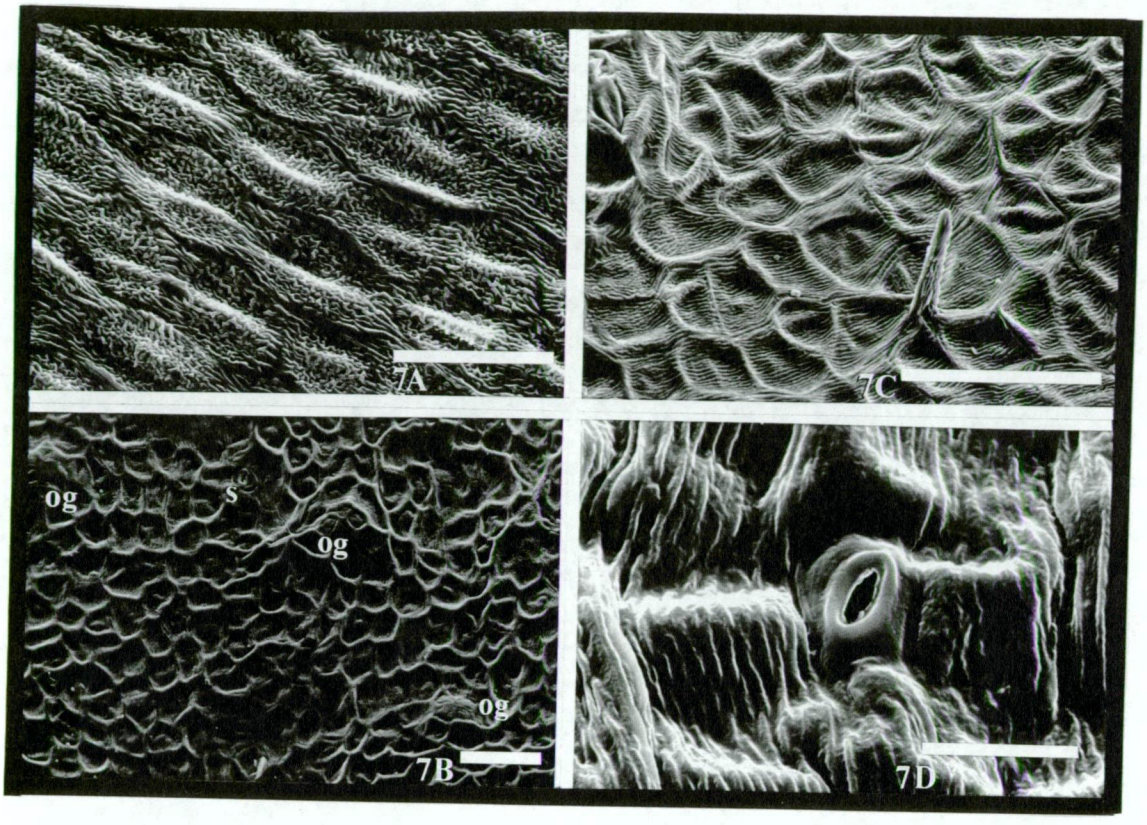

7A
7C
og
s
og
og
7B
7D

Plate 8.

Scanning electron micrographs.

A Epidermis of leaf showing epidermal hairs and stomates; bar = 50 microns.

B Epidermis of leaf showing oil gland and stomate; bar = 50 microns.

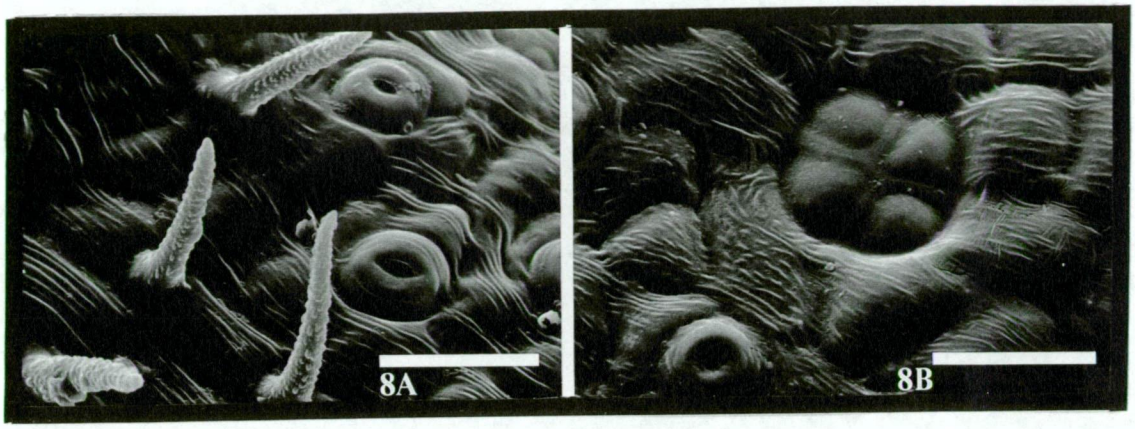
Plate 9.

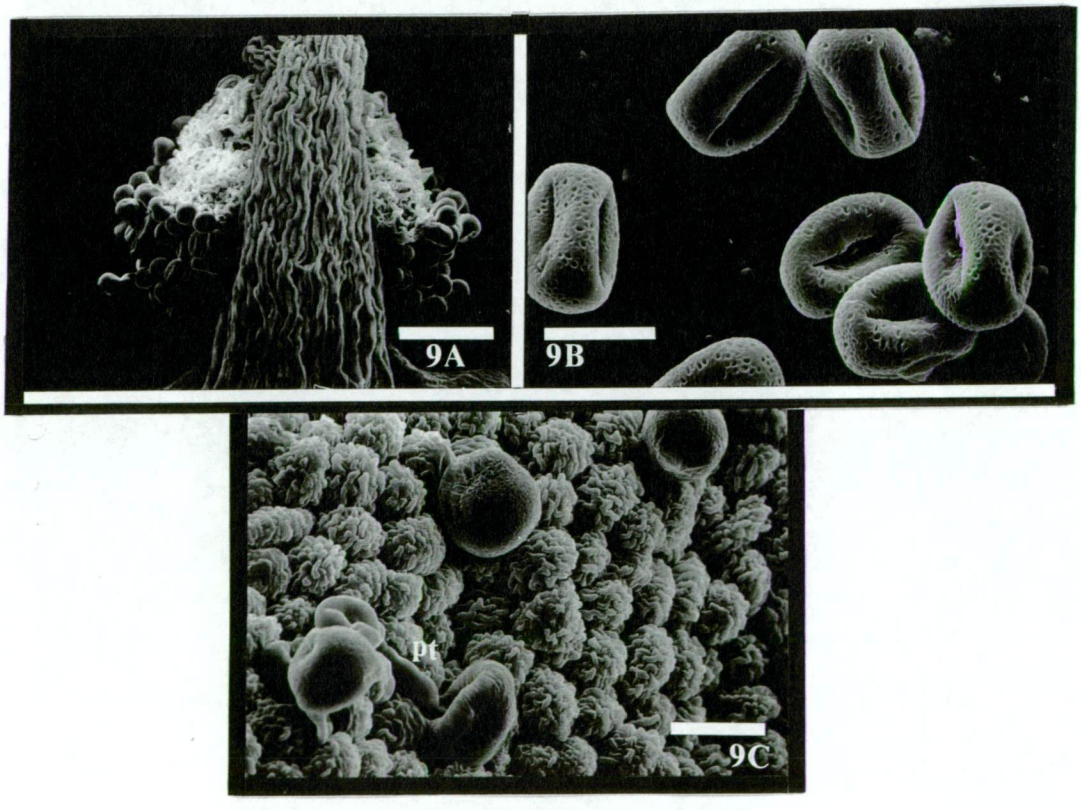
Scanning electron micrographs.

A Functional anther with pollen; bar = 0.1mm.

B Pollen grains; bar = 25 microns.

C Stigma with pollen grains, note pollen tube (pt); bar = 25 microns.


8A
8B


9A
9B
pf
9C

2.5 Transmission electron microscopy of floral tissues

The illustrations of transmission electron microscopy of floral tissues are presented in plates 10-17.

2.5.A Epidermal oil glands on the petal

Epidermal oil glands on the petals are easily distinguished in transverse sections under the TEM. The central cavity (CC) contains large droplets of faintly osmiophilic material (15B), many vesicles and few other discernible organelles. The oil droplets in the CC and in epithelial cells surrounding the CC appear to be membrane bound (11), and have uneven staining (13C, 15B) due to incomplete fixation or extraction of material during fixation and dehydration. The epithelial cells appear to be undergoing lysis, and will be referred to as lysing cells (LC). These cells display obvious signs of advanced autophagy with many vesicles and membranes present in the vacuole (10, 11). The lysosomal cell compartment appears to have been active, indicated by the presence of vesicles with double or triple membrane layers (12A) arising from the ER (12B, 12C, 13B, 13C) which probably contain digestive enzymes. There are structures that may be tubulous or membranous chromoplasts contained both within periplastidial ER (13A, 15A arrows) and free in the vacuole (11, dashed arrow); the latter probably resulting from autophagy of previously sheathed chromoplasts.

The cells between a normal parenchyma cell (PC) (11) and the CC comprise 1) sheathing cells (SC) with thick cell walls adjacent to the parenchyma and thick cell walls adjacent to the lysing cells (LC); and 2) lysing cells with thin cell walls and a more flattened appearance. With increasing proximity to the CC, the size of oil droplets in the plastids increases; membranous elements (lamella) within the plastids are absent, and evidence of autophagy and activity of lytic processes increases (10, 11). In some cells, the plastidial membranes appear to have degenerated, releasing membrane-bound oil droplets into the vacuole (11, complete arrow). Some of the periplastidial ER in lysing cells has been re-oriented away from the cell wall (13C, 15B) and is probably an artefact of specimen preparation.

Between the epidermal cells on the abaxial surface above an oil gland, a thickening of the cell wall may be seen (13C, 15A) which may be part of the septa (S) that dissects the cap of the oil glands into four parts (6). There is sometimes a very small cell present between two epidermal cells (15A, 4F) that may be the septa itself. In sections through petals of immature buds, oil glands were apparent (not shown), although infiltration of fixative and the embedding resin was incomplete in these

tissues. Four epidermal cells above an oil gland had unusual structures (14A) indicating that they may have been immature cap cells. Alternatively, the unusually-shaped cells may result from dehydration after improper fixation or may be a phenomena unrelated to oil gland development.

Parenchyma cells of immature petals have large nuclei, many mitochondria and non oil-filled plastids (14B). Vacuolar contents may be osmiophilic, probably due to tannins, phenolics or starch. In the adaxial-most layer of parenchyma (lower area of 14B), some osmiophilic droplets are present that may be oil droplets.

2.5.B Other tissues

Stigmatic cells from large buds (16A, 16B) have large vacuoles with many vesicles (V), suggestive of a well developed lysosomal compartment. There are many mitochondria (M) present; plasmodesmata (PD) and rough ER are evident (16A). In 16B there are three large, moderately osmiophilic regions on the right hand side that may be oil droplets, although they may also be starch grains or vesicles containing other secreted substances. The stigmatic cells in a mature flower vary in size and contain many vesicles or vacuoles within a granular groundplasm that may contain phenolics or lipids (16C). It is difficult to discern other organelles. The vesicles may contain compounds that contribute to the floral extract, although no firm conclusions can be drawn from the sections observed.

The non-functional anthers from mature flowers have a thick cuticle (17A) and parenchyma cells that contain mainly ER, oil droplets (OD) and vesicles. The accumulation of ER in the centre of the cell rather than around the periphery of the cell is probably an artefact of fixation and dehydration.

The parenchyma cells of functional anthers from immature buds (stage #4) have many mitochondria and vesicles and diffuse structures bearing faintly osmiophilic globules that may be plastids (probably leucoplasts) (17C).

Plate 10.

Transmission electron micrograph of transverse section through epidermal oil gland on a mature petal; bar = 5 microns.

CC = central cavity; LC = lysing cell; SC = sheathing cell; P = plastid.

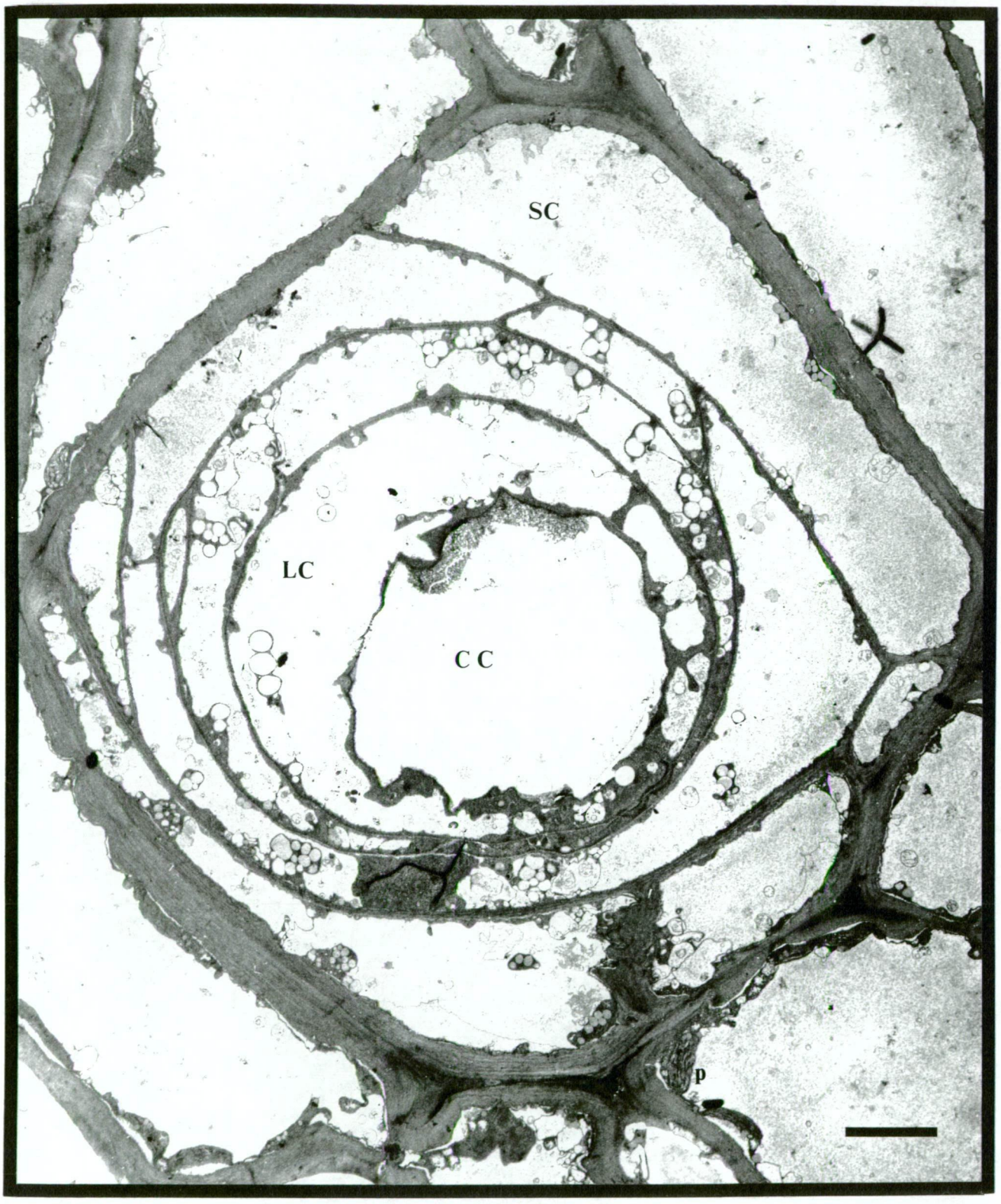

SC
LC
C C
p

Plate 11.

Transmission electron micrograph of transverse section through central cavity and surrounding cells of an epidermal oil gland. Note membrane-bound vesicles (complete, double headed arrow) and membrane whorls (dashed arrow, single head); bar = 1 micron.

CC = central cavity; DP = disintegrated plastid; LC = lysing cell; SC = sheathing cell; P = plastid; PC = parenchymatous cell.

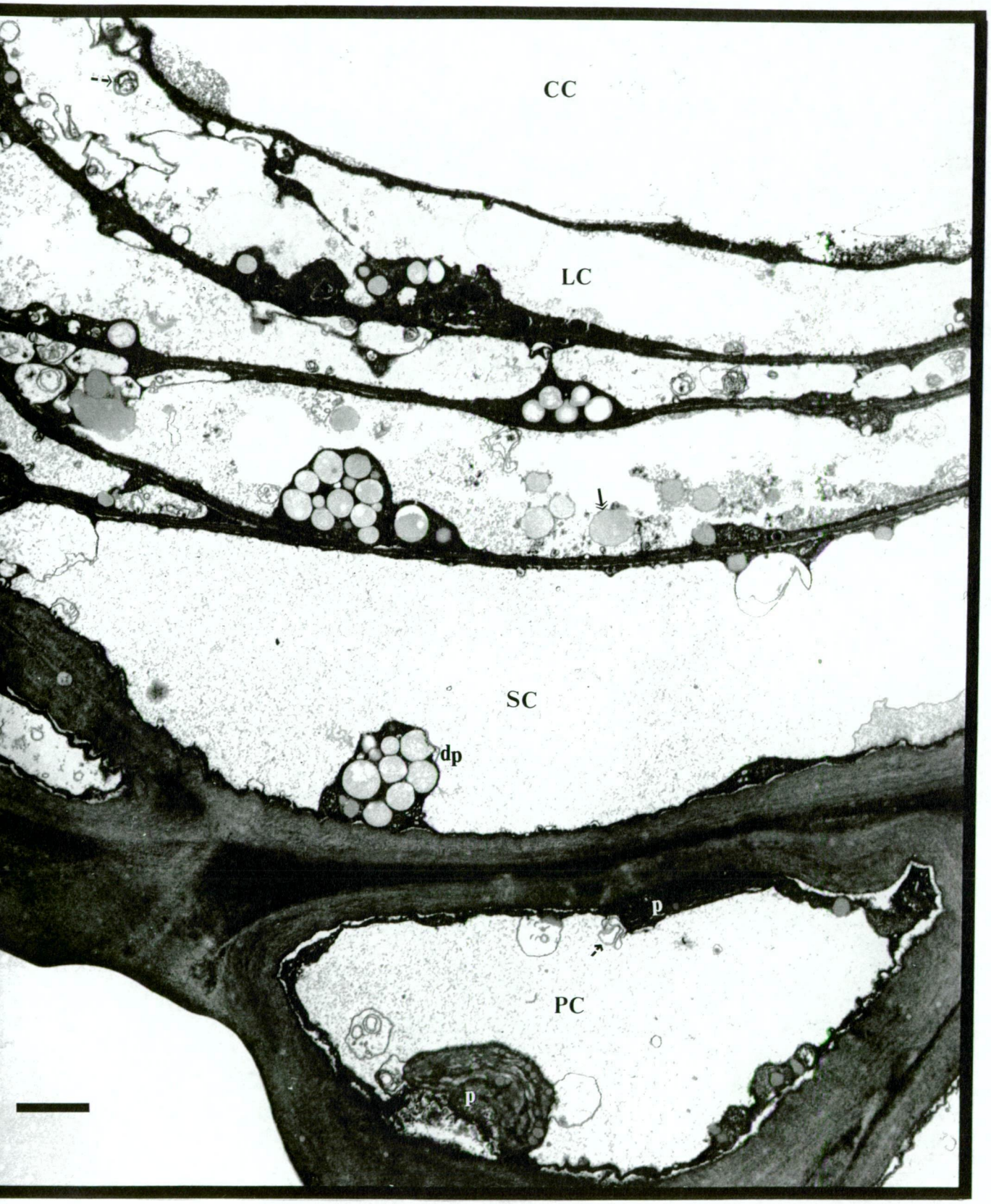

CC
LC
SC
dp
p
PC
p

Plate 12.

Transmission electron micrographs.

A Transverse section through lysing cell surrounding epidermal oil gland. Detail of vesicles arising from ER (dashed arrow); bar = 0.2 microns.

B Vesicle arising from ER; bar = 0.5 microns.

C Further examples of ER production of vesicles; bar = 0.5 microns.

ER = Endoplasmic reticulum; M = Mitochondrion; R = Ribosomes; V = Vesicle.

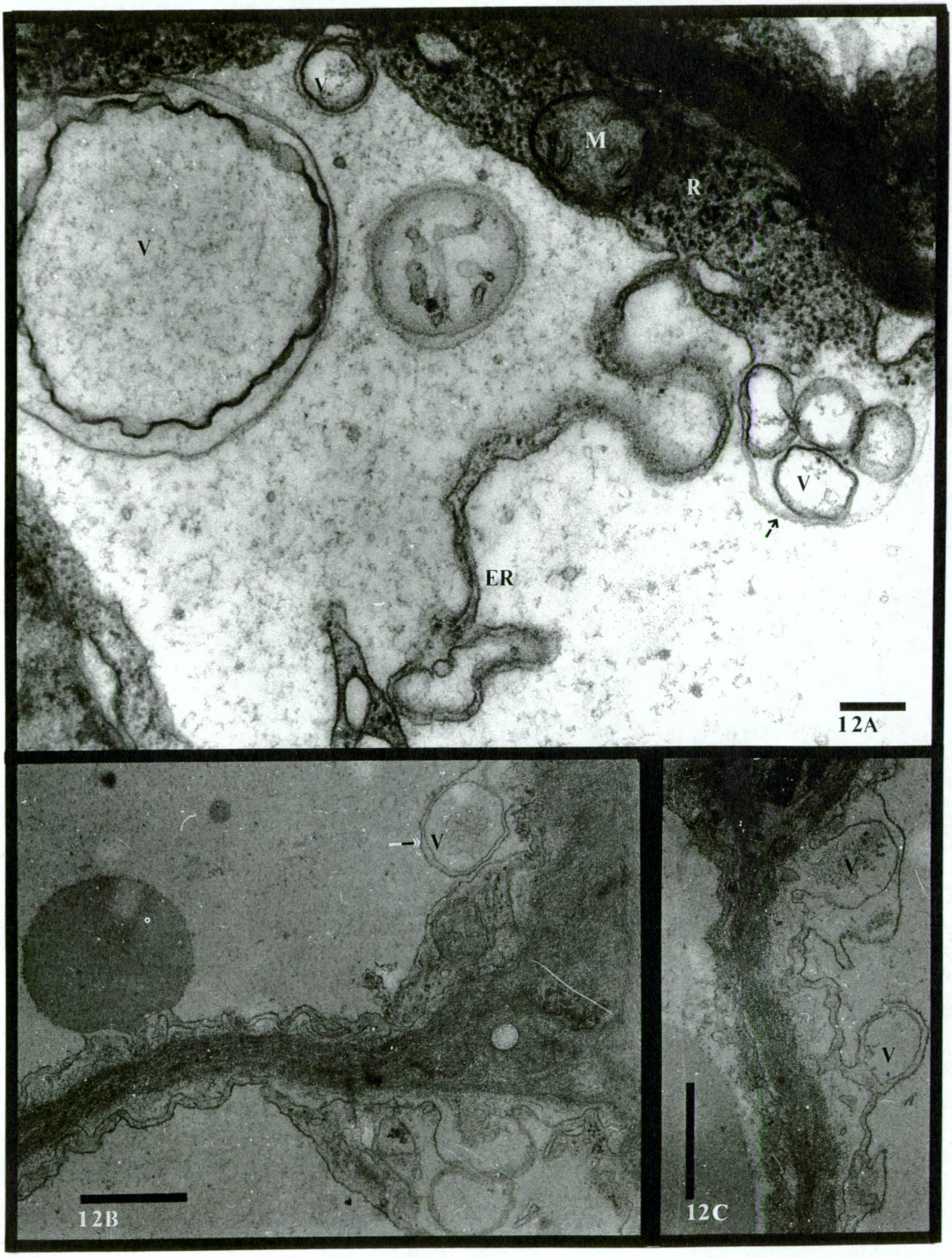

V
M
R
V
V
ER
12A
V
12B
V
V
12C

Plate 13.

Transmission electron micrographs.

A Details in the periphery of lysing cell. Note multi-membrane whorls, or chromoplasts (arrow) within periplastidial ER; bar = 1 micron.

B Detail in lysing cell showing oil droplets and vesicles; bar = 0.5 microns.

C Detail in epidermal cells above oil gland. Note thickening of cuticle between epidermal cell (arrow), which could be the septum that divides the epidermal cap of an oil gland; bar = 2.5 microns.

EC = Epidermal cell; LC = Lysing cell; OD = Oil droplets; S = Septum; V = Vesicle.

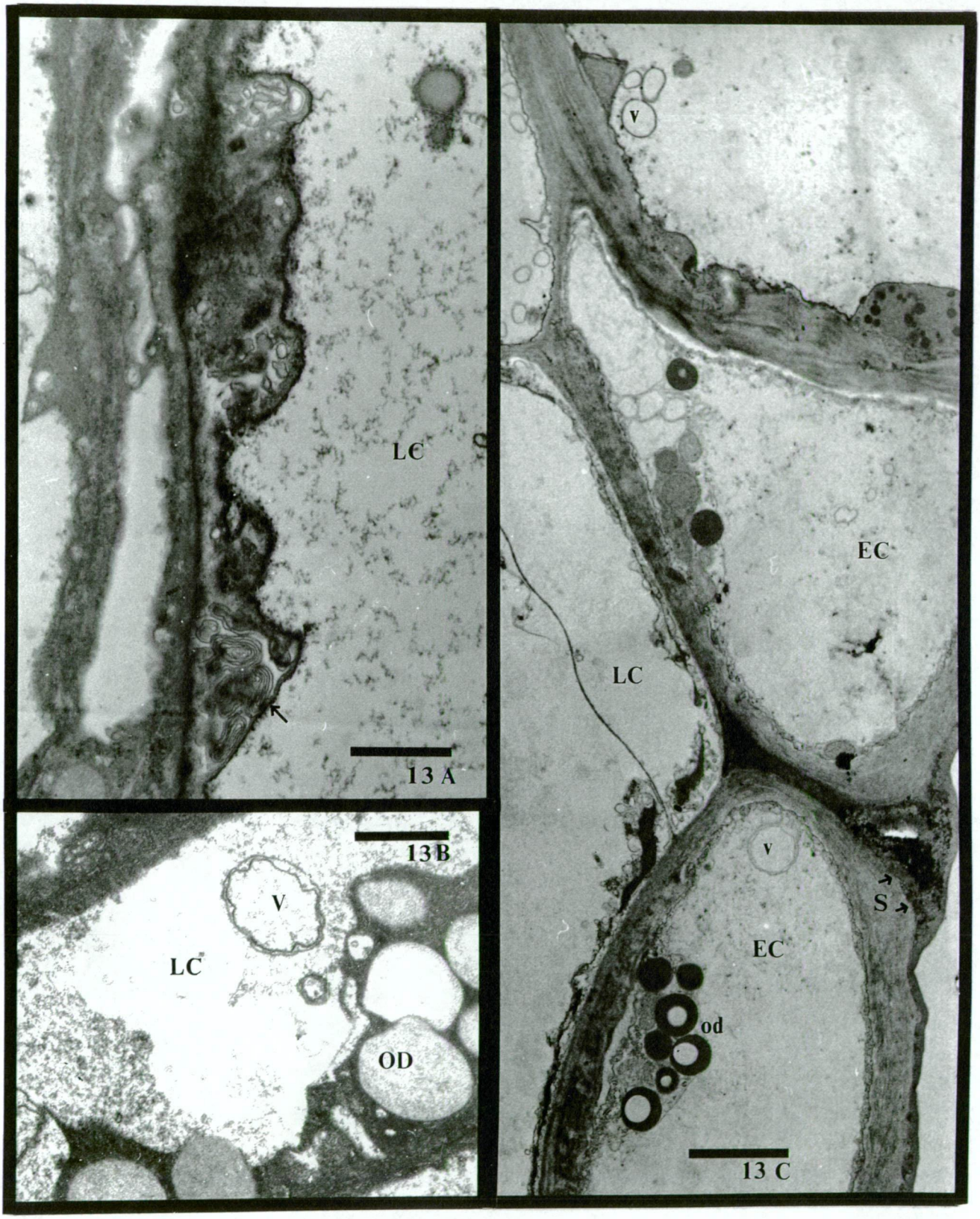

LC
13 A
13B
V
LC
OD
v
EC
LC
v
S
EC
od
13 C

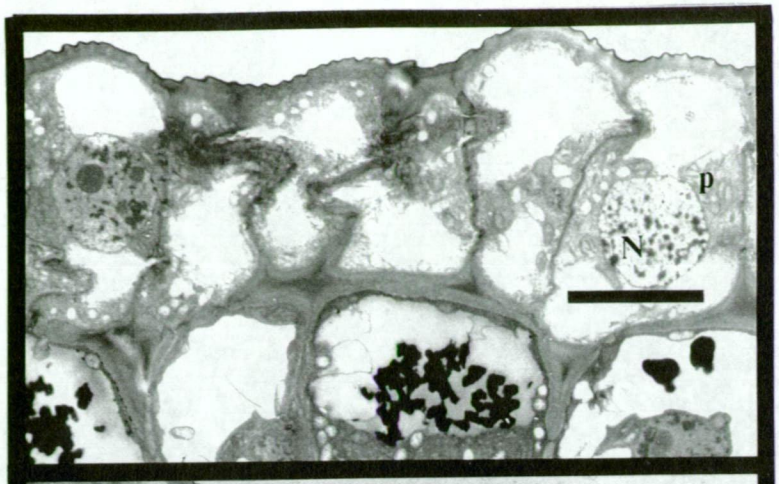
Plate 14.

Transmission electron micrographs.

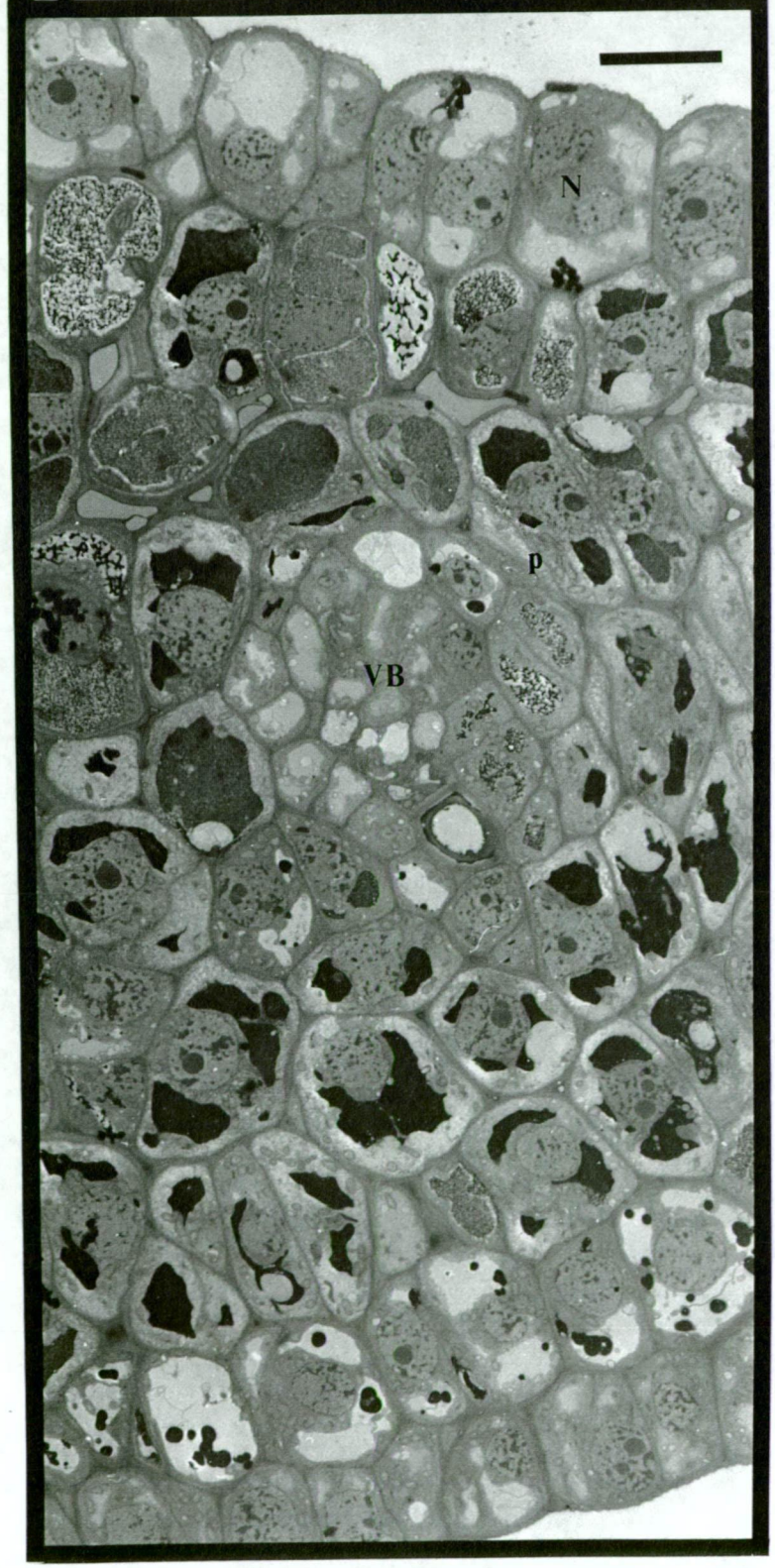
A Transverse section through abaxial epidermis of petal from an immature flower bud (stage #1), above an area that may be an immature oil gland (not shown); bar = 5 microns.

B Transverse section through petal of an immature flower bud (stage #1). Vascular bundle centrally located, abaxial epidermis uppermost; bar = 5 microns.

N = Nucleus; P = Plastid; VB = Vascular bundle.



14A



14B

Plate 15.

Transmission electron micrographs.

A Transverse section through epidermal cells on the abaxial surface showing detail between cells which may be the septa producing the tetra-secting epidermal cap of an oil gland; bar = 5 microns.

B Transverse section showing details in the central cavity of mature oil gland; bar = 5 microns.

EC = Epidermal cell; C = Chromoplast; CC = Central cavity; LC = Lysing cell; OD = Oil droplet; SC = Sheathing cell; V = Vesicle.

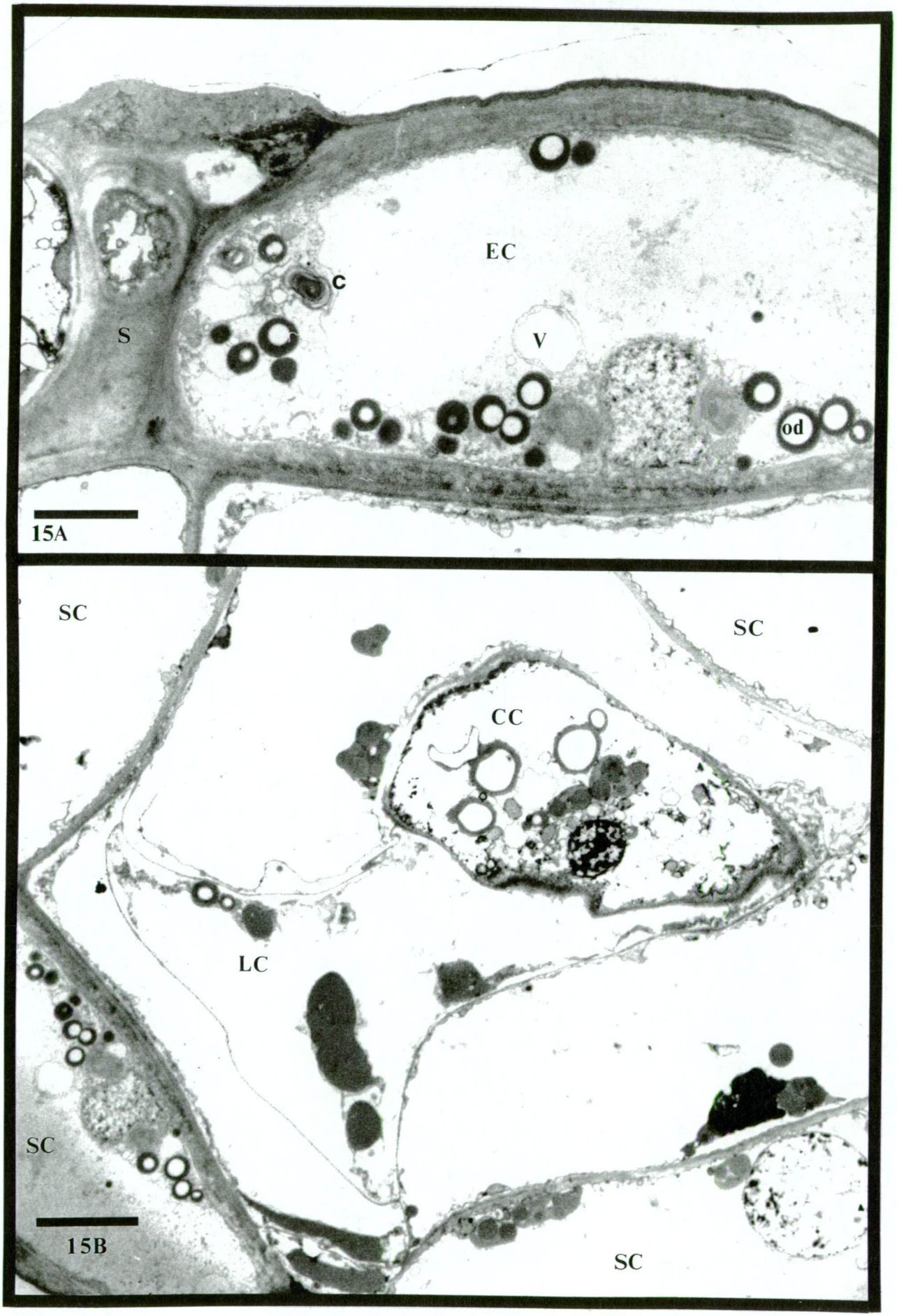

EC
C
S
V
od
15A
SC
SC
CC
LC
SC
SC
15B

Plate 16.

Transmission electron micrographs.

A Detail in cells of a stigma from a large bud (stage #4); bar = 2 microns.

B Transverse section showing details in cells of a stigma from a large bud; bar = 2 microns.

C Section through stigma of mature flower; bar = 5 microns.

M = Mitochondrion; PD = Plasmodesmata; SW = secondary wall; V = Vesicle.

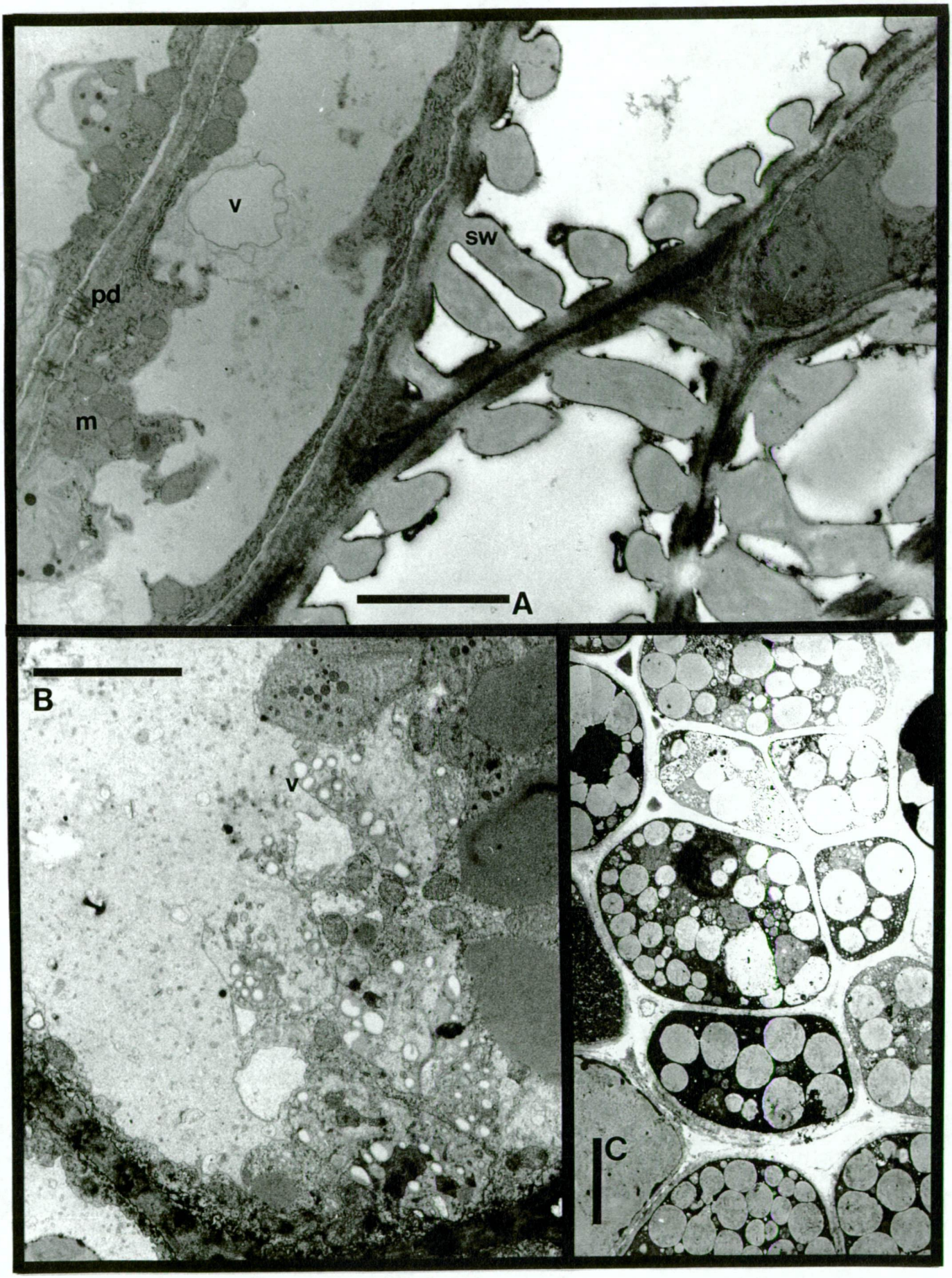

v
sw
pd
m
A
B
v
C

Plate 17.

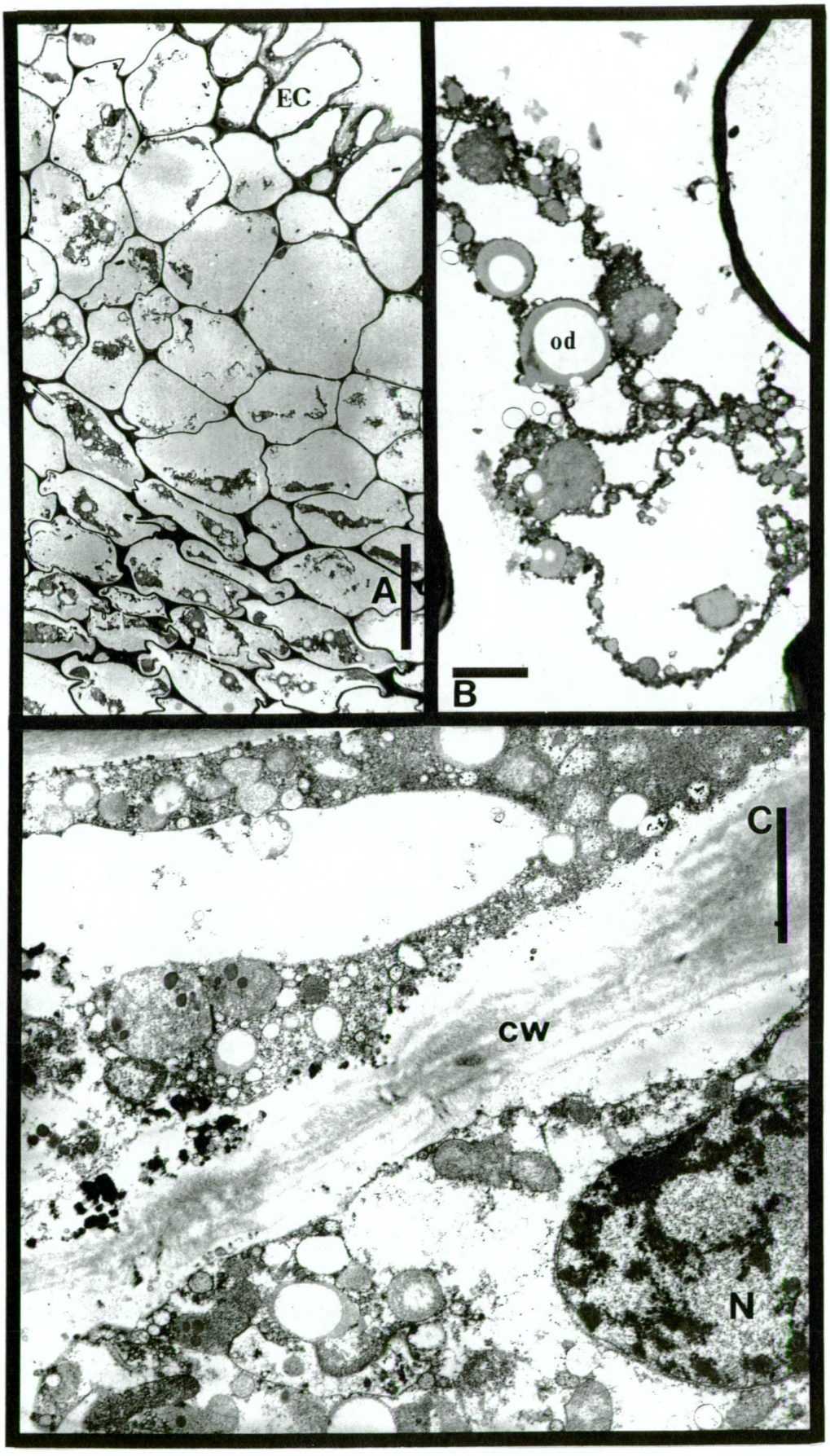
Transmission electron micrographs.

A Section through non-functional anther from mature flower; bar = 2 microns.

B Detail in centre of parenchyma cell of non-functional anther from mature flower; bar = 2 microns.

C Section through functional anther from immature flower bud (stage #2); bar = 2 microns.

CW = Cell wall; EC = Epidermal cell; N = Nucleus; OD = Oil droplets.


EC
A
od
B
C
CW
N

2.6 Isolation of plastids from petals

Plastids were isolated from flowers and extracted for volatiles (Section 2.1.B). After centrifugation of the sucrose gradient with the plastid suspension, two fractions were evident in each tube, one was brown/green and occurred in the 0.4 M - 0.75 M sucrose layers, and the other was green, and occurred in the 1.0 M layer. Under the light microscope, discrete organelles were observed in the green faction, however only broken membranes and a few discrete plastids were observed in the brown layer. There were similar levels of volatiles in both fractions, including β-ionone, dodecyl acetate and (Z)-heptadec-8-ene.

2.7 Discussion

The presence of plastidial oil droplets in parenchyma and gland cells of petals suggests that these organelles are probably responsible for biosynthesis of some of the compounds typically found in floral extract of boronia, particularly monoterpenes and carotenoid pigments. Further evidence for plastidial involvement in oil production is the degradation of thylakoid structure and increase in the number of oil droplets in plastids in cells close to the central cavity in comparison with plastids in normal parenchyma cells, and the occurrence of typical floral volatiles in isolated plastids (2.6).

The occurrence of membrane-bound vesicles that in some cases appear to contain an oily substance in the vacuoles of cells surrounding the oil glands of petals provides a means of protection of cellular contents from toxic cell products even after plastids have degenerated. The preponderance of mitochondria in many cells, particularly in buds, is indicative of active processes such as synthesis and secretion of many products. The cells of most floral organs have undergone various degrees of autophagy, with large amounts of membranous vacuolar contents and vesicles, indicative of activity of the lysosomal cell compartment and flower senescence (Matile 1974). There are organelles and tissues present which are capable of synthesis of other products including nectar, polysaccharides and sugars, and of organelles that may contain hydrolases and digestive enzymes. The production of vesicles filled with faintly osmiophilic material from the ER in cells near oil glands is indicative of active production of various compounds, and the contribution of these organelles to the many compounds present in the extract cannot be ruled out.

The central cavities of oil glands in fresh sections of petals are filled with grey oily droplets (3A), but in fixed and stained sections they are almost devoid of droplets. Because of the removal of lipophilic product during specimen preparation, establishing

the route of secretion of synthesised product into the central cavity is difficult. The lacunae of the oil gland appears to develop lysigenously, as evidenced by epithelial cells with thin walls and advanced stages of internal autophagy (10, 11).

Oil glands on the petals are in contact with the epidermis only at the abaxial most point of the isodiametric structure (4, 5), and this supports the theory that epidermal caps are involved in the activity of the oil gland. The epidermal caps of oil glands are unusual structures, and appear to be produced by tubular structures on the internal edge of each of the four cap cells. Alternatively, the cap may actually be produced by partitions in the abaxial cell wall of one epidermal cell, which upon senescence splits apart. Given the relative size of the epidermal cap and other epidermal cells (6), the latter case seems more likely. One epidermal cell bears internal cross-hatched tubular structures that become the septa (6A); these structures rupture upon senescence when cell walls and other organelles degrade.

The central cavity appears to accumulate increasing amounts of extract as buds mature into flowers (6). The epidermal cap cell expands prior to expansion of other epidermal cells, and protrudes further above the epidermis with increasing maturity of buds. It is unlikely that pressure from oil-filled cavities causes rupture of the septa dividing the cap cell into four, because ruptures were only seen on petals of old flowers in which processes of senescence would already have begun catabolism of secondary compounds in the cells in preparation for retrieval of compounds from flowers back into the rest of the plant prior to flower abscission.

It is difficult to discern the sites of synthesis of extract components in other organs such as the stigma and anthers in the available sections. There are a large number of vesicles present in cells of mature stigmas that may be oil-filled, although these vesicles may actually contain nectar or other substances. There are indications that several cells contain tannins or phenolics in the vacuoles, particularly in the layer of parenchyma directly below the abaxial epidermis. Fresh sections through stigmas showed regular larger isodiametric cells in between smaller parenchymatous cells. These larger cells may be specialised oil-producing cells, or may be related to nectar or polysaccharide production, it is impossible to distinguish in the sections available.

IV.3 Results
Pigments and volatiles

A study of the pigments in boronia flowers may complement a study of the volatile distribution, particularly if there are biosynthetic links between carotenoids and norisoprenoids. Such a relationship, if it exists, may be investigated using *in vitro* techniques, and there is potential for manipulation of the equilibrium position by the use of various post harvest practices. The identification of biosynthetic pathways and control mechanisms for norisoprenoids in boronia flowers may enable future molecular work.

3.1 Materials and methods

3.1.A Carotenoid, anthocyanin and flavonoid extraction

Petals were homogenised in 15 ml of cold methanol (100%), and left at 4°C for 30 minutes in the dark. Particulates were removed by centrifugation. The supernatant was concentrated under nitrogen gas; 10 ml of light petroleum ether and 10 ml of distilled water was added and the solution was thoroughly mixed. The petroleum ether layer was removed and concentrated under nitrogen gas, then re-suspended in ethanol (15 ml) = 'carotenoid fraction'. The aqueous layer was passed through a prepared C18 Sep-pak cartridge (Waters Division of Millipore) which was then washed with distilled water to remove sugars; the anthocyanins were removed from the cartridge by elution in 100% methanol (15 ml) = 'anthocyanin fraction'. Both fractions were analysed spectrophotometrically.

3.1.B Carotenoid extraction and HPLC analysis

90% acetone was added to whole petals which were then left to extract for two hours at 4°C, in the dark. The solvent was removed and analysed by HPLC by Daniel Holdsworth at the CSIRO Division of Oceanography, Hobart, using the method of Wright *et al.* (1991). Details of the method are documented in Appendix 1.

3.1.C *In vitro* degradation of β-carotene

3.1.C.1 Solubilisation of β-carotene and β-ionone

β,β-carotene (95% purity) was dissolved in 1:1:0.1 (wt/vol/vol) β-carotene (mg):chloroform:Tween 80. This solution was concentrated to dryness under

nitrogen gas, and resuspended in 0.067 M phosphate buffer pH 7.0 (10 ml). After centrifugation at low speed, the supernatant was used as aqueous β–carotene (Amax 462nm). This solution was prepared daily.

1 g of β–ionone (1.06 ml), 10 ml of distilled water and one drop of Tween 80 were mixed together (Amax 321.4nm, single peak).

3.1.C.2 Enzyme solution

An acetone powder prepared from clone 5 flowers harvested early in the season was resuspended in 0.067 M phosphate buffer, pH 7.0 to produce the crude enzyme solution. The protein concentration was 0.1 mg/ml enzyme solution. Some of the crude enzyme solution was boiled to deactivate enzymes.

3.1.C.3 Incubation conditions

The incubations included 1 ml of a 1.75 mg/ml β–carotene solution plus either 10 ml of deactivated enzyme solution (control) or 10 ml of active enzyme solution. The incubations were kept on ice in the dark and analysed spectrophotometrically four times during the experiment. Because of the conditions of incubation, condensation formed on the cuvettes when placed in the spectrophotometer, and the reading after 30 minutes was deleted because of this. Subsequently, the incubation was actually left in a spectrophotometer cuvette in the spectrophotometer. At the completion of the experiment 2 ml of DCM (including an internal standard) was added and the vials were left overnight at 4°C in the dark. The DCM layer was removed and concentrated under nitrogen gas and then analysed by GC (Section III.4.2).

Some of the complicating factors that were realised were as follows:

1) carotenoid concentrations suitable for spectrophotometric analysis were too low for detection of maximum possible amounts of product obtained by degradation (β–ionone and other compounds) on the GC;

2) carotenoid present in the GC injection sample may, during GC analysis, produce β–ionone by autoxidative processes in greater amounts than the level of β–ionone produced from enzyme activity during incubation;

3) to stop an incubation which includes aqueous media usually the assay is either heated or frozen, or an extracting solvent is added. Some of these processes may themselves cause degradation of β–carotene. Another problem is that solvent used for stopping the reaction or for extraction of products may be miscible with water as a result of the detergent effect of Tween 80;

4) because carotenoids are labile, exposure to heat, acid and light must be kept to a minimum.

3.1.D Carotenoid and β−ionone levels in flowers after harvest

Flowers from mid-lateral positions from clone 5 plants were excised and placed into empty duplicate vials and left for a period of time at room temperature. In some treatments, flowers were separated into two parts: petals and flowers-without-petals. In other treatments, whole flowers were frozen for a period of time at -70°C, then left at room temperature (to simulate oxidative conditions). Air was removed from vials containing other flowers prior to storage at room temperature (anaerobic). The contents of the vials and their treatment and sampling times are outlined in Table 3.1.D.

Table 3.1.D Details of treatments (duplicated).

Contents	Sampling time
5 flowers	zero time
5 flowers	2 hours (20°C)
5 flowers	4 hours (20°C)
5 flowers	6 hours (20°C)
5 flowers	8 hours (20°C)
Petals from 5 flowers	4 hours (20°C)
Flowers-without-petals (calyx, stigma, anthers etc)	4 hours (20°C)
5 flowers, anaerobic	4 hours (20°C)
5 flowers - frozen at -70°C for 2 hours	4 hours (20°C)
5 flowers	4 hours (4°C)

At the completion of the experiment the flowers were extracted (Section III.3.3), the extracts were analysed by spectrophotometer, and after concentration under nitrogen gas, the extracts were analysed by GC (Section III.4.2).

3.1.E Effect of carotenoid metabolic regulators on carotenoids and norisoprenoids in flowers on excised laterals

The 1,3-Dichloropropene (DCPE) (*cis and trans*) used had a purity of 75-80%. Norflurazon (4-chloro-5-methylamino-2-(α,α,α-trifluoro-m-tolyl)pyridazin-3(2H)-one), or 'Solicam', was of unknown concentration, and for

the purposes of this experiment, a purity of 50% was assumed. This compound is degraded by light. Solutions of each compound were made up in the following concentrations in distilled water with a drop of Tween 80 to increase solubility.

1a. Low DCPE - 20 micrograms/L
1b. High DCPE - 2.4 mg/L
2a. Low Norflurazon - 5 mg/L (crude sample weight)
2b. High Norflurazon - 50 mg/L

Control flasks contained distilled water and Tween 80 in the same concentration as the treatment solutions. Three or four flower-bearing laterals were cut with a scalpel and placed immediately into distilled water before being placed into conical flasks containing one of the above solutions. Four replicate flasks were set up for each solution. The flasks were wrapped in foil to exclude light, and a cotton wool plug was wrapped around the bases of the laterals. The laterals extended well into the solution in each flask. The flasks were placed into a growth cabinet set at 21°C with constant light for 30.5 hours.

Following this, laterals were removed from each flask, flowers were harvested and kept separately from other replicates and other treatments. 16 flowers from each replicate were selected at random and divided into duplicate vials and extracted (Section III.3.3). The absorption spectra of the extract was measured, and the solvent concentrated under nitrogen gas and analysed by GC (Section III.4.2).

3.1.F Effect of exogenous terpenoid precursors *in vitro*

Pre-weighed proliferating tissue-cultured calli (clone 17) were sub-cultured onto 40 ml of solid media (Appendix 2) in 250ml BUNZL plastic flasks. Several flasks were grown under standard conditions of 16 hr light (80-100 u $Em^{-2}s^{-1}$) at 22°C : 8 hr dark at 18°C. Each week for seven weeks, three flasks were 'harvested': the calli were removed, weighed and stored at -70°C. After four weeks growth under standard conditions, calli in some flasks were aseptically treated with exogenous regulators and moved to treatment conditions for a further 7 days, after which they were finally harvested. There were duplicate flasks for each treatment, some had more than two replicates. At the final harvest, calli were removed from the media, dried with a tissue, weighed and stored at -70°C prior to analysis. The treatments included:

1. Addition of exogenous β–Carotene (95%), dissolved in sterilised lanolin (5.6 mg/g); approx. 0.1 g per callus piece was applied (A). Treated flasks were kept in continuous dark at 16hr:8hr, 22°C: 18°C. There were two controls: one included application of sterilised lanolin to normal callus (B), the other, β–carotene in lanolin applied to calli which had been heat-killed (30 minutes at 80°C) (C). Control C llowed for quantification of the effects of the treatment on β–carotene without the action of live calli. After 7 days, no fungus attack was seen on any calli, indicating that aseptic technique was appropriate. Excess lanolin was removed with a tissue prior to freezer storage.

2. Addition of exogenous MVA (+/- MVA-lactone, 97%), dissolved in sterile distilled water (36 mg/ml); 0.1 ml was dropped around each piece of callus.

3. Addition of exogenous DL-leucine (99+%), dissolved in sterile distilled water (19 mg/ml); 0.1 ml dropped onto each piece of callus. Controls for 2 and 3 used distilled water only.

4. Continuous white light at 20°C.

5. Continuous dark at 20°C (Control for dark treatments).

6. 10 hours light (5°C) : 14 hours dark (15°C) (Stress conditions).

Growth rates were calculated using the formula:

$$G = \frac{(M_t - M_o)}{\frac{M_t \cdot M_o}{2}}$$

Where M_o and M_t are masses of explants after the initial sub-culture and at the termination of the experiment after t weeks.

Samples were extracted by addition of hexane containing an internal standard and homogenisation using an Ultra-Turrax. After four hours at 4°C, samples were centrifuged at low speed to remove particulates, and the hexane was concentrated by nitrogen gas. Samples were analysed by GC (Section III.4.2).

3.2 Pigments in two colour mutants of *B. megastigma*

Colour mutants 'Normal', and 'Lutea' were used for this study. Normal flowers have petals that have a yellow coloured epidermis on the adaxial surface, and a red-brown coloured epidermis on the abaxial surface, with red-brown coloured non-functional anthers and stigmas, and yellow coloured functional anthers. Flower petals of Lutea are completely pale yellow-green on both epidermal surfaces, with an orange-coloured stigma and non-functional anthers, and yellow coloured functional anthers. Typical oil glands are present on the epidermis of the abaxial surface of Lutea petals when examined by SEM (not shown).

A normal, fresh petal in transverse section, (Section III.2.Plate 3A), shows the accumulation of red-brown pigments in the epidermal cells on the abaxial side. The parenchyma is dotted with yellow 'droplets'; the first layer of parenchyma below the epidermis on the adaxial surface is densely pigmented with yellow. The yellow 'droplets' in the parenchyma of petals are most likely carotenoid pigments present in the plastids. Red-brown pigments in epidermal cells fill the whole cell, and are most likely water soluble anthocyanins accumulated in the vacuole. The yellow layer on the adaxial side may be due to accumulated yellow flavonoids, or to densely packed carotenoid-bearing plastids. There are no red-brown pigments in epidermal cells on the abaxial surface of Lutea petals.

Pigments were extracted from petals of Normal and Lutea varieties (3.1.A). The absorption spectra are presented in Appendix 4. The absorption due to each pigment-type in the ethanol and methanol fractions is represented in Table 3.2.

Table 3.2 Absorption maxima in ethanol and methanol fractions.

Absorption wavelength	Abs/g/ml 'Lutea' petals	Abs/g/ml 'Normal' petals
Ethanol layer:		
660nm (chlorophyll a)	0.228	0.254
612nm (chlorophyll b)	0.038	0.041
428nm (carotenoids)	0.692	0.925
Other carotenoid maxima at low levels	(405), (450), (470), (370)	
Methanol layer:		
543nm (anthocyanin)	-	0.390
360nm (flavonoids)*	1.03	1.36
346nm (flavonoids)*	1.19	1.58

* samples required dilution to obtain values.

There appear to be slightly higher levels of carotenoids in Normal *B. megastigma* petals compared with petals of the Lutea variety. The complement of carotenoids is quite similar, given the similarity of the absorption spectra from the two extracts (Appendix 4). There are no anthocyanins present in the Lutea extract, in comparison with quite high levels in the Normal petals. The dense layer of yellow on the epidermis of the adaxial surface is of carotenoid origin. Therefore, Lutea petals differ from normal petals only in their ability to produce red anthocyanins.

3.3 Carotenoid content in floral organs

Samples (4 replicates) comprising whole flowers, and flowers separated into petals and flowers-less-petals were homogenised by Ultra-Turrax with petroleum ether and kept at 4°C for 4 hours in the dark. Spectrophotometric analyses of these extracts produced different absorption spectra (Appendix 5). The spectra of extracts from petals alone comprised one major peak with maximum absorption at 423 nm. The spectra of the flowers-without-petals extract had maximum absorption at 423.5 nm, but there were at least four major 'shoulders' between 380 nm and 460 nm. Spectrophotometric analyses of extracts of different floral organs enables a rough comparison of the concentration of carotenoids in each organ. Flowers were extracted in hexane overnight at 4°C without homogenisation. The results are presented in Table 3.3.

Table 3.3. Carotenoid absorption in hexane extracts of floral organs.

Floral organ	Weight of sample used (g)	Abs (426 nm) /g/ml	Weight of organ (% of whole flower) *	Abs(426nm) in each organ (% of whole flower) *
petals	0.2912	0.724	53.84	85.31
stigma	0.2046	0.148	18.73	6.08
functional anther	0.0056	2.589	0.50	2.83
non-functional anther	0.0825	0.182	4.56	1.82
calyx (sepals and stalk)	0.2014	0.081	22.37	3.96

* values for petals, non-functional and functional anthers that each occur four times in each flower are the total amount from all four units.

In a boronia flower, most of the carotenoids are present in the petals, with small amounts in all other organs. The non-functional anther makes the smallest contribution to carotenoids in the whole flower. However, the lowest tissue concentrations of carotenoids (Amax/g/ml) are present in the calyx and the stigma. The highest tissue concentration of carotenoids is present in the functional anthers, which is almost four times greater than the next highest tissue concentration, occurring in the petals.

3.4 Carotenoid and norisoprenoid levels in flowers from genetically different plants

Flowers from genetically different plants from several environments were extracted (Section III.3.3), and analysed spectrophotometrically. Further extractions were made from each sample (Section III.3.2) and these were analysed for extract yield, and by GC analysis (Section III.4.3) for β–ionone and total volatile content.

There were no significant relationships between carotenoid levels and yield of extract, total volatile or β-ionone content in the flowers.

3.5 Identification of pigments by HPLC

An HPLC chromatogram of pigments from whole petals extracted in 90% acetone is presented in Appendix 6. The major pigments identified are presented in Table 3.5. Where absorption maxima from the sample were not recorded, maxima from published works using similar methods are presented.

Table 3.5. Pigments identified in 90% acetone extract of whole petals.

Pigment	Absorbance Maxima (nm)
Chlorophyll *c*	630.9[1]
Neoxanthin	440, 467[3]
Violaxanthin	416, 440, 470[2]
Lutein	420, 446, 476[2,3]
Unidentified (22 minutes)	440[3]
Unidentified (23 minutes)	428[3]
Chlorophyll *b*	646.8[1]
Chlorophyll *a*	664.3[1]
Unidentified	434, 653[3]
α-Carotene	416, 440, 471[2]
β–Carotene	427, 462, 480[2]

[1] Maxima from Jeffrey and Humphrey (1975).
[2] Maxima from Wright *et al.*(1991).
[3] Maxima from eluate of this sample analysis

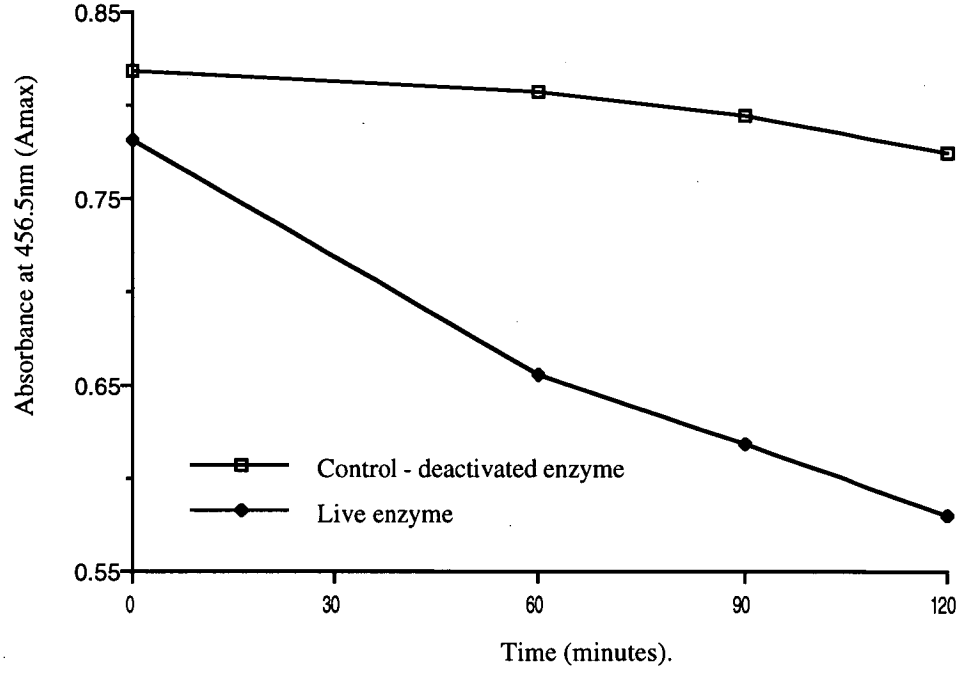
Two unidentified carotenoids are present in large amounts in the extract, both have a single absorption peak. The absorption characteristics of the peak eluting at 22 minutes has a single absorption peak at 440 nm (Appendix 7).

3.6 Degradation of β–carotene by an enzyme from boronia flowers

It is generally assumed that genetically directed degradation of carotenoids occurs in boronia flowers *in vivo* to produce norisoprenoids such as β–ionone. Therefore the *in vitro* degradation of suitable precursors into β–ionone was attempted using an enzyme prepared from boronia flowers (Section IV.3.1.3). The results of an incubation may be seen in Figure 3.6.

Figure 3.6

Absorbance changes during incubation of flower enzyme with beta-carotene.



There is a greater loss of carotenoid pigment (456.5 nm in aqueous media) with the live enzyme than with the deactivated enzyme, indicative of enzyme activity causing degradation of β–carotene. GC analyses did not detect any norisoprenoids. A further experiment examined similar reactions using three pH values: 6, 7, and 8, and the addition (or not) of linoleic acid as well as β–carotene. Results were inconclusive, and further *in vivo* experiments were not done.

3.7 Changes in carotenoids and β–ionone in flowers after harvest

Post-harvest changes in pigments and β–ionone were assessed in fresh, whole flowers, dissected flowers, frozen/thawed flowers and flowers that were kept under anaerobic conditions (Section IV.3.1.D). The results of this experiment are presented in Table 3.7.

Table 3.7. Absorbance and β–Ionone in flowers exposed to several treatments post-harvest.

Treatment	Abs 426nm x 10 +/- std. error	β–Ionone (micrograms) +/- std. error
Zero time control (whole flowers)	11.85 +/- 0.35	40.91 +/- 1.79
2 hours (20°C)	13.65 +/- 0.95	48.25 +/- 4.55
4 hours (20°C)	14.15 +/- 0.45	* 74.72 +/- 13.11
6 hours (20°C)	13.8 +/- 1.1	65.39 +/- 5.69
8 hours (20°C)	* 19.25 +/- 0.05	61.33 +/- 1.14
4 hours (petals only) (20°C)	17.55 +/- 0.05	13.01 +/- 0.61
4 hours (flowers-without-petals) (20°C)	5.6 +/- 0	53.69 +/- 8.56
4 hours - anaerobic (20°C)	16.15 +/- 1.45	37.45 +/- 6.67
Frozen flowers, thawed + 4 hours (20°C)	12.6 +/- 0.7	49.96 +/- 10.59
4 hours (4°C)	13.85 +/- 1.15	49.57 +/- 7.76

* Means significantly different to control at 5% level of significance.

There is a significant increase in β–ionone in flowers stored for four hours at 20°C. The trend is for maintenance of this increased level beyond this time, although values are not significant. Flowers stored at 4°C did not undergo a similar increase in β–ionone after four hours. Carotenoid levels in fresh flowers were significantly increased after eight hours at 20°C. Freezing, followed by

storage at room temperature for 4 hours was meant to bring about destruction of membranes and an increase in oxidative processes, did not cause significant degradation of β–carotene, although pigments in the epidermis on the adaxial surface of petals treated in this way were bleached. There was no increase in β–ionone in this treatment. Flowers which were stored anaerobically did not alter during storage. Flowers which were separated into petals and flowers-without-petals had a cumulative (levels in petals + levels in 'flowers-without-petals') β–ionone level of 67 micrograms which is similar to the level in whole flowers after four hours (74 micrograms). However, total carotenoid levels in the separated flowers were greater (17.55 + 5.6 = 23.15) compared with 14.15 in whole flowers after four hours storage.

3.8 Effect of carotenoid metabolic regulators on carotenoids and norisoprenoids in flowers on excised laterals

Carotenoids and norisoprenoids were analysed in flowers attached to laterals that were placed in solutions of 1,3-Dichloropropene (DCPE) which inhibits lipoxygenase, and Norflurazon which is a non-competitive inhibitor of phytoene desaturase (Section IV.3.1.5). After 30.5 hours in the growth cabinet, all flowers and laterals looked turgid and healthy, except those treated with high levels of DCPE which were withered and desiccated. Results of spectrophotometric and GC analyses are presented in Table 3.8.

Table 3.8 Spectrophotometric and GC analyses of flowers treated with carotenoid regulators *in vivo*

Treatment	Amax (417.2nm) of final solution +/- std. error	β–ionone (micrograms) +/- std. error	Total volatiles (micrograms) +/- std. error
Control (distilled water)	0.524 +/- 0.041	39.45 +/- 2.91	267.70 +/- 51.3
Low Norflurazon	0.514 +/- 0.036	34.44 +/- 2.528	182.43+/- 25.8
High Norflurazon	0.573 +/- 0.027	36.64 +/- 3.81	191.51 +/- 26.6
Low DCPE	0.595 +/- 0.022	37.09 +/- 2.865	225.87 +/- 18.5
High DCPE	* 0.662+/- 0.05	* 24.84+/- 1.1	* 168.35+/- 9.1

* means significantly different to control values at 5% level of significance.

There is a significant increase in carotenoid levels, and a significant decrease in total volatiles and β–ionone in flowers treated with high levels of DCPE. There are no other significant results.

3.9 Effect of exogenous terpenoid precursors *in vitro*

The effect of light, exogenous leucine, mevalonic acid and β–carotene on tissue-cultured calli was studied (Section IV.3.1.6). Trichomes and stomates similar to those described on vegetative parts of boronia (Wilson 1982) were seen on sub-cultured calli under the SEM (not shown). After four weeks growth under standard conditions, calli were treated with exogenous compounds and placed under standard or different conditions for a further week. Growth rates, total and specific volatile levels in treated and untreated calli in the fifth week after sub-culturing are presented in Table 3.9.1.

Table 3.9.1 Growth rates and volatiles in calli grown under standard conditions for five weeks and treated with terpenoid precursors and different growth conditions during week five.

Treatment	Growth rate	Mono-terpene (µg / g)	(Z)-Heptadec-8-ene (µg / g)	Total volatiles (µg / g)
Grown under standard conditions: 16hr:8hr, light:dark, 22°C:18°C				
A. 4 weeks	1.60	7.75	9.42	33.80
B. 5 weeks	0.70	5.59	6.98	25.45
C. Mevalonic acid	1.26	9.16	12.02	46.5
D. Leucine	1.12	9.94	10.57	42.97
E. Distilled water	0.79	9.37	8.99	41.5
Continuously dark-grown 16hr:8hr, 22°C:18°C				
F. Dark-grown	1.53	1.46	2.14	11.36
G. Lanolin	4.61	2.62	0.0	21.1
H. Lanolin + β-carotene	1.98	5.79	5.87	37.0
I. Lanolin + β-carotene (heat-treated calli)	1.51	10.35	12.38	51.3
Grown under specified lighting/temperature regimens				
J. 10 hrs light:14 hrs dark 5°C:15°C	1.78	6.78	7.20	26.77
K. Continuous light (20°C)	1.53	3.03	2.78	15.1

The odour of the extracts was very 'green', and no β–ionone was observed in any extracts. An increase in the total amount of volatiles in extracts was found in calli treated with carotenoids, water, MVA and leucine, and a decrease in the calli grown under different light/dark regimens (treatments F, J and K) was seen, in comparison with calli grown for five weeks under standard conditions (control 1).

3.10 Discussion

The carotenoid pigments in boronia flowers have been identified and quantified (Sections IV.3.3 and IV.3.5). The presence of large amounts of carotenoids with single absorption maxima suggests that compounds such as β–apocarotenals may be present (Davies 1976). Carotenoids are present in all floral organs, and it is likely that their synthesis is the result of several genes, as it is in other species. Colour mutants caused by changes in one gene controlling anthocyanin biosynthesis are common (Jackson *et al.* 1992), and it is likely that most colour mutants of boronia are caused by similar changes.

The presence of stomates on, and chlorophyll within petals is suggestive of active photosynthesis. It would be expected that carotenoid levels would decline after anthesis (Valadon and Mummery 1969; Thammasiri *et al.* 1987), and that photosynthetic activity, if present, would also decrease significantly after this time. Concomitant with carotenoid decline, usually as a result of enzyme-mediated degradation, an increase in carotenoid degradation products such as apocarotenals and norisoprenoids should ensue. There is some evidence for a reduction in carotenoid absorption upon incubation of β-carotene with an enzyme from boronia flowers (Section IV.3.6). This enzyme may be a lipoxygenase (Section IV.3.8), however results were not conclusive. Some compounds produced by degradation of carotenoids may actually have a higher absorption than the compound they are produced from (Gloria *et al.* 1993). There may also be other explanations for the increase in carotenoids and decline in total volatiles and β–ionone in DCPE treated flowers. The effects of increased water loss, structural degradation of plastids and general disarray of primary and secondary metabolism caused by compounds such as DCPE may affect relationships such as the carotenoid-norisoprenoid one (if any exists) in many ways.

The process of excision of the flower from the plant increases the rate of senescence in some flowers (Mookherjee *et al.* 1986), and in the case of boronia, post-harvest increases in carotenoid and β–ionone levels in flowers were observed (Section IV.3.7). These increases were inhibited by low temperatures, and an

increase in β–ionone was inhibited by anaerobic conditions. Therefore, there is continued synthesis of both β-carotene and β-ionone in flowers after harvest; accumulation of β-carotene in all floral organs is particularly enhanced by excision into petals and flowers-without-petals prior to storage (Section IV.3.7). Such treatment may disrupt the mechanism(s) responsible for conversion of β-carotene into β-ionone.

There are no relationships between carotenoid and β–ionone levels in extracts from flowers of different plants (Section IV.3.4), however none really should be expected. The many variables such as flower maturity, environmental conditions, extraction rates and precision etc. are probably far more significant than any tenuous relationships that may not even be apparent from substrate-product studies.

Tissue-cultured boronia callus has been shown to accumulate secondary compounds including some typical volatiles, the levels of which were increased by addition of compounds that may act as terpenoid precursors (Section IV.3.9). It is also possible that compounds such as β-carotene acted as precursor(s) to a multitude of volatile compounds that may be produced without interaction with live callus.

IV.4 Results
Phenology of flowering and extract accumulation

The aims of this work were to describe the sequential stages of flower maturation; to describe and quantify the development of extract components; and to quantify the rate at which flowers on a plant reach anthesis. The effect of harvesting at different times throughout the flowering period on the flower yield, the concentration and composition of extract, and the yield of extract per plant is studied. The levels of free and bound glycosides and glycoside-hydrolysing enzymes within flowers are quantified throughout the flowering period.

4.1 Materials and methods

4.1.A Photography

Photographs of developmental stages were taken with a Minolta SRT100X camera, positioned to focus through a binocular dissecting microscope. Photographs of boronia laterals and flowers were taken with Kodak 100 film.

4.1.B Extract in buds and flowers

Extracts of developing buds (Section III.3.1) were analysed by GC (Section III.4.3) and by organoleptic assessment (Section III.5).

4.1.C β–ionone in flower parts during development

Buds of stages #2, #3, and #4 were extracted whole (Section III.3.3), or dissected into petals, and 'buds-without-petals', and separately extracted in triplicate (Section III.3.3). Extracts from all three bud stages and their dissected components were analysed by GC (Section III.4.2).

4.1.D Extraction of flowers at different stages of maturation

Flowers and buds from the three most acropetal nodes ('apical'), the three central nodes ('central'), and four basipetal nodes ('basal') of several laterals from one plant were removed and kept separately. The buds of each developmental stage from each region (apical, central, basal) were enumerated. Triplicates of five representative flowers from each region were extracted (Section III.3.3). The absorbance of the extracts was measured at 426nm to enable relative quantification of carotenoids. The extract was then analysed by GC (Section III.4.2).

4.1.E The rate of flower development

Clone 5 plants from Longley were used for this study, in the 1992 flowering season. Several branches from three plants were harvested completely each week. The total sample (comprising buds, flowers and stalks) was separated into each developmental stage, which were then enumerated and weighed. The proportion of each developmental stage was calculated as a percentage of the total harvest each week by using both the number, and the weight of buds present. Both methods (number and weight) gave the same estimate of the percentage of open flowers each week and either could be used in the field, however estimation by weight is less tedious.

4.1.F Effect of harvest date on flower yield, extract yield and extract composition

One hundred and twenty plants in a uniform area of a plantation of clone 5 plants at Longley were identified by numbered aluminium tags. Each week over a six week period during 1993, 20 plants were randomly selected by number and completely harvested of all buds and flowers. The weekly harvest was mixed together, and a 200g sub-sample was removed. The proportion of open flowers in the sub-sample was assessed by weight. From the separated sub-sample and the remainder of the harvest, three fractions were thus obtained: open flowers, buds and leaf only, and the total sample (flowers, leaf and bud). All samples were frozen for storage at -20°C, and extracted in triplicate within eight weeks of the first weekly harvest (Section III.3.1). The extracts were analysed by GC (Section III.4.3).

4.1.G Protein levels and activity of glycosidase enzymes in buds and flowers: a comparison

Acetone powders were prepared from samples of small buds (stages #1-#3 and some leaf material), large buds (stage #4) and open flowers (Section III.8.1). These were resolubilised and the protein levels were estimated (Section III.8.2). The crude enzyme solutions so produced were then incubated with a standard precursor (Sections III.9.2A, III.9.2.C). The levels of free volatiles before and after incubation were assessed, and the activity of the enzyme calculated as the amount of volatiles released from glycoside bonds per mg of acetone powder over 24 hours at 40°C (Section III.9.2.D).

4.1.H Free and glycosidically-bound volatiles and glycosidase enzymes during the flowering period

Acetone powders were made from samples comprising open flowers, buds and leaf material from Section III.4.1.6 (Section III.8.1). They were then resolubilised and estimated for protein content (Section III.8.2) and subsequently incubated with a standard precursor (Section III.9.2.C). Other frozen samples were made into 'precursor solutions' (Section III.9.2.A), and incubated with a standard, resolubilised acetone powder (Section III.9.2.C). The level of free volatiles in the 'precursor solution' was assessed prior to incubation with crude enzyme, and the levels of glycosidically-bound volatiles were calculated by the difference in the levels of free volatiles before and after incubation (Section III.9.2.D).

4.1.I Activity of glycosidase enzyme(s) in leaves, stems and buds/flowers during the flowering period

Acetone powders were made from samples of leaf, stem (branches and laterals) and buds/flowers from pot-grown clone 5 plants during the flowering period (Section III.8.1). These were resolubilised (Section III.8.2) and incubated with a standard precursor solution (Section III.9.2.C). The activity of the enzymes was assessed by the change in the level of free volatiles in the standard 'precursor solution' due to incubation with the resolubilised acetone powder (Section III.9.2.D).

4.2 Buds and flowers: descriptions

Six main developmental stages of boronia flower buds were identified (Plate 18.1-18.5, 19C). Descriptions of the relative sizes of buds, flowers and stalks, and their constituent organs are presented in Table 4.2.

Table 4.2. Descriptions of buds and open flowers.

	Very small bud, stage #1	Small bud, stage #2	Medium-size bud, stage #3	Large bud, stage #4.	Open flower, stage #5	Stalk, (no petals) stage #6
Maximum width (mm)	1-2	1.5-2.5	2-4	4-6	10	n/a
Petal weight (% of whole bud weight)	10-15	20-25	25-30	35-50	50	n/a
Bud/flower weight (% of open flower weight)	10-15	25-30	35-50	65-80	100	50
Diameter of functional anther (mm)	0.48	0.45	0.67	0.69	0.53	0.50
Ratio functional anther: non-functional anther : stigma *	1/1.4/3	1/1.6/3.3	1/1.6/3.3	1/1.8/3.8	1/2.6/7	1/2/6
Colour of abaxial epidermis of petal	pale green	greenish-yellow	greenish-yellow	yellow-green		
Other observations					pollen on functional anthers	petals have abscised

* Diameter of each organ; stigma diameter measured between apices of adjacent lobes.

Despite some overlap between the sizes of the different bud stages, this classification provides a useful means to describe buds. The developmental stages will be referred to as numbers in most cases; for example #1 is a very small bud, #5 an open flower. During the development of stage #1 buds into open flowers, the weight of the petals slowly increases; the size of the functional anthers reaches a maximum in stage #4, after which the production of pollen (stage #5) reduces the size of functional anthers. The size of the non-functional anthers and stigma increases in each subsequent bud stage, especially between large buds and flowers at anthesis. The process of senescence (stage #6 compared with stage #5) causes a reduction in size of all organs.

Plate 18.

1 Bud stage #1, very small bud. Two petals and one sepal have been excised; bar = 0.5mm.

2 Bud stage #2, small bud. Three petals have been excised; bar = 1mm.

3 Bud stage #3, medium-sized bud. One petal has been excised; bar = 1mm.

4 Bud stage #4, large bud. One and a half petals have been excised; bar = 1mm.

5 Stage #5, open flower. Two petals have been excised; bar = 1mm.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4.3 Buds and flowers: oil glands

The development of external features of oil glands in developing buds have been described (Section III.2.4.1 Plate 6). The number of oil glands present on petals of large buds and open flowers of one clone was assessed (Section III.6). Smaller bud stages could not be enumerated because the oil glands were not easily distinguishable under LM. The number of oil glands on petals at each developmental stage are presented in Table 4.3.

Table 4.3 Number of oil glands on petals of large buds and open flowers.

Developmental stage	No. of oil glands per petal	+/- standard error
large bud	130	6.6
open flower	* 185	6.6

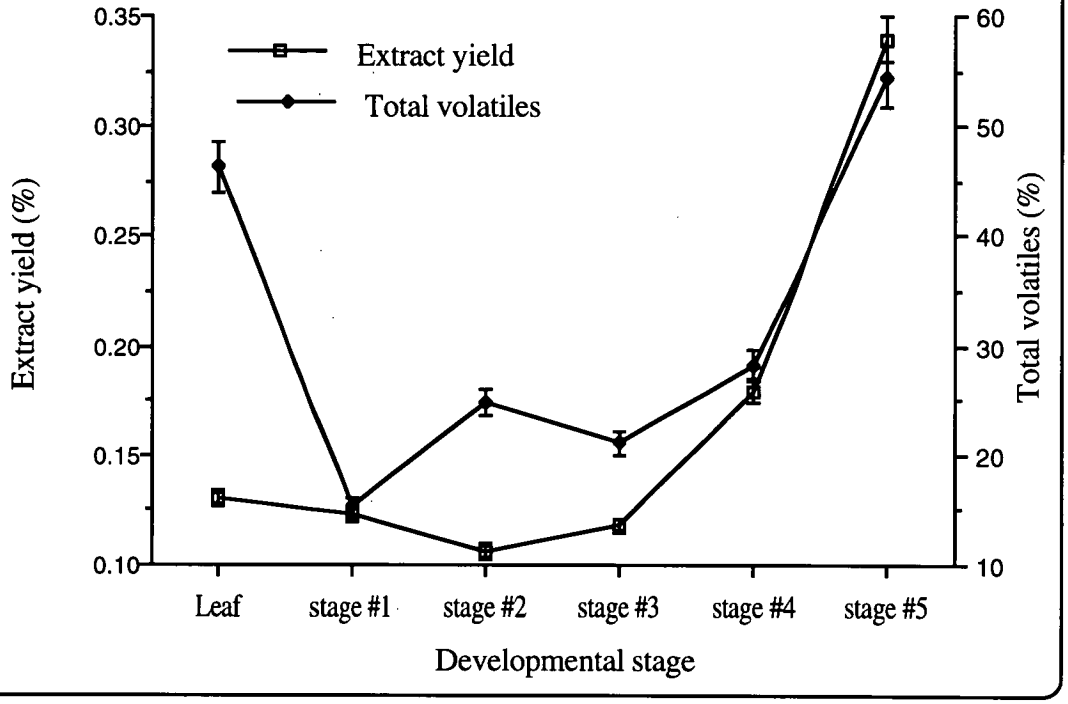
* means significantly different to large bud at 5% level of significance.

There appears to be more oil glands per petal on petals from open flowers compared with large buds. This could be related to the ease of identification of swollen oil glands on petals from open flowers compared with less full glands on petlas from large buds, rather than indicating initiation of new oil glands.

4.4 Buds and flowers: extract yield and composition

The yield of extract was assessed in leaves and flower buds as they develop into open flowers (Section III.3.1). The composition of the extracts was analysed to examine the development of extract components (Section III.4.3). The yield of extract and the proportion of the extract comprised of volatile compounds are presented in Figure 4.4.A.

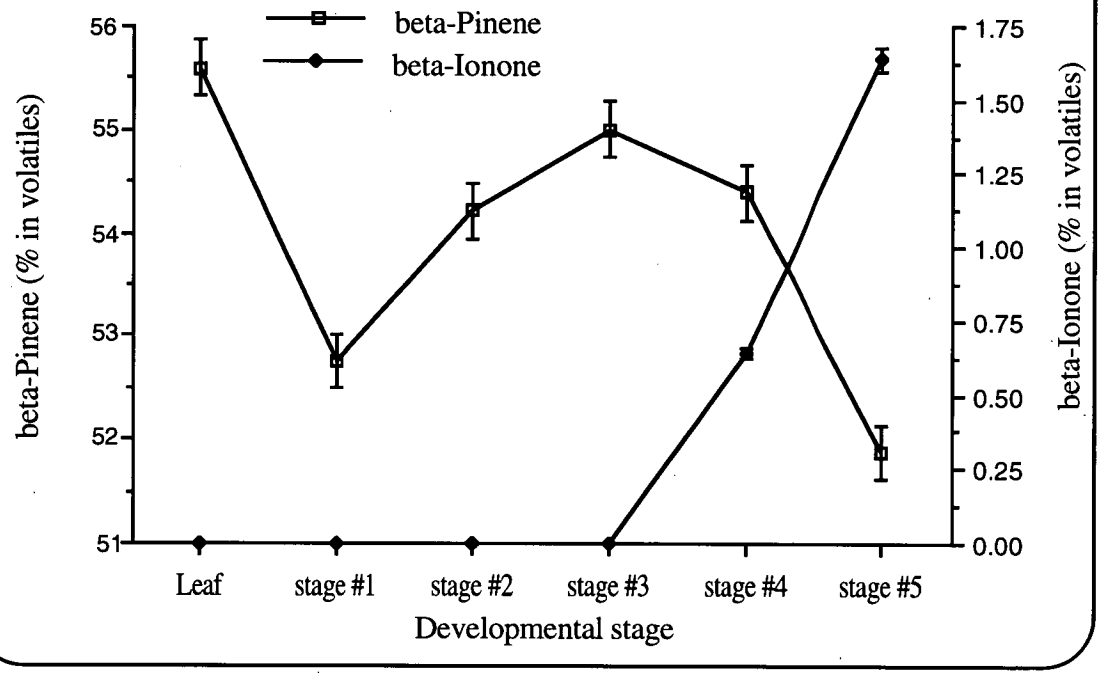
Figure 4.4.A Extract yield and total volatiles in extract from leaves, buds and flowers.



The yields of extract from leaves, and buds between stages #1 and #3 are similar. Between bud stage #3 and stage #5 (open flowers), there is a rapid increase in extract yield; yield from open flowers is three times higher than yield from buds of stage #3 or smaller. The percentage of the extract comprised of volatile compounds is reduced in buds compared with leaves and open flowers, the latter has the highest proportion of volatiles of all the samples examined.

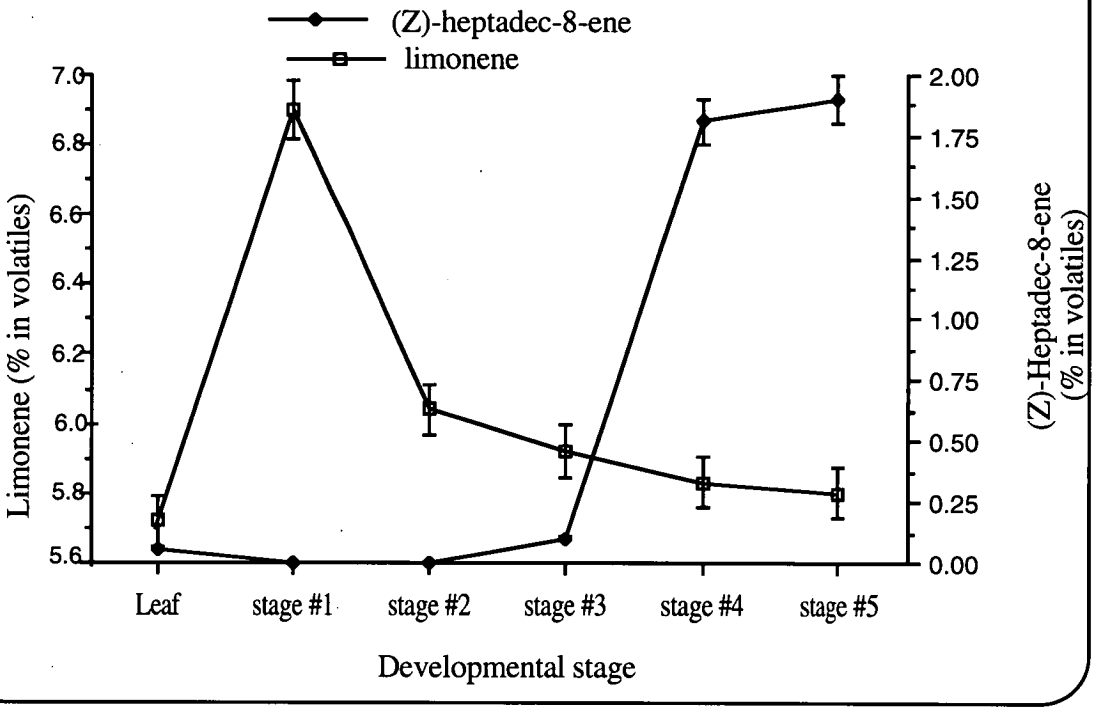
The composition of the extract was studied in more detail to examine the difference in volatiles in a leafy extract compared with a typical floral extract. The proportion of the total volatiles in extracts from leaves and developing buds that is composed of β–pinene (monoterpene, typical of green leaves) and β–ionone (norisoprenoid, specifically in boronia flowers) (Figure 4.4.B); and (Z)-heptadec-8-ene and limonene (Figure 4.4.C) are presented.

Figure 4.4.B beta-pinene and beta-ionone (% of total volatiles) in leaves, buds and flowers.



β–pinene is high in leaves, as would be expected of this 'green-leafy'-smelling volatile, however this compound also occurs in extracts of flower buds. The proportion of β–ionone increases as buds develop from stage #1 to stage #3 buds, after which it declines. β–ionone does not begin to accumulate in flower buds until the buds are larger than stage #3 buds (medium-sized buds), after which it rapidly increases.

Figure 4.4.C limonene and (Z)-heptadec-8-ene (% in volatiles) in leaves, buds and flowers.



Limonene occurs to relatively low levels in leaves. It increases markedly in very small buds compared with in leaves, however the concentration of limonene in subsequent bud stages and open flowers gradually declines. (Z)-Heptadec-8-ene follows a similar pattern to β–ionone accumulation.

Organoleptic assessments of extracts from each bud stage and open flowers was made (Section III.5). The results are presented in Table 4.4.

Table 4.4. Results of organoleptic assessment of extracts from different developmental stages.

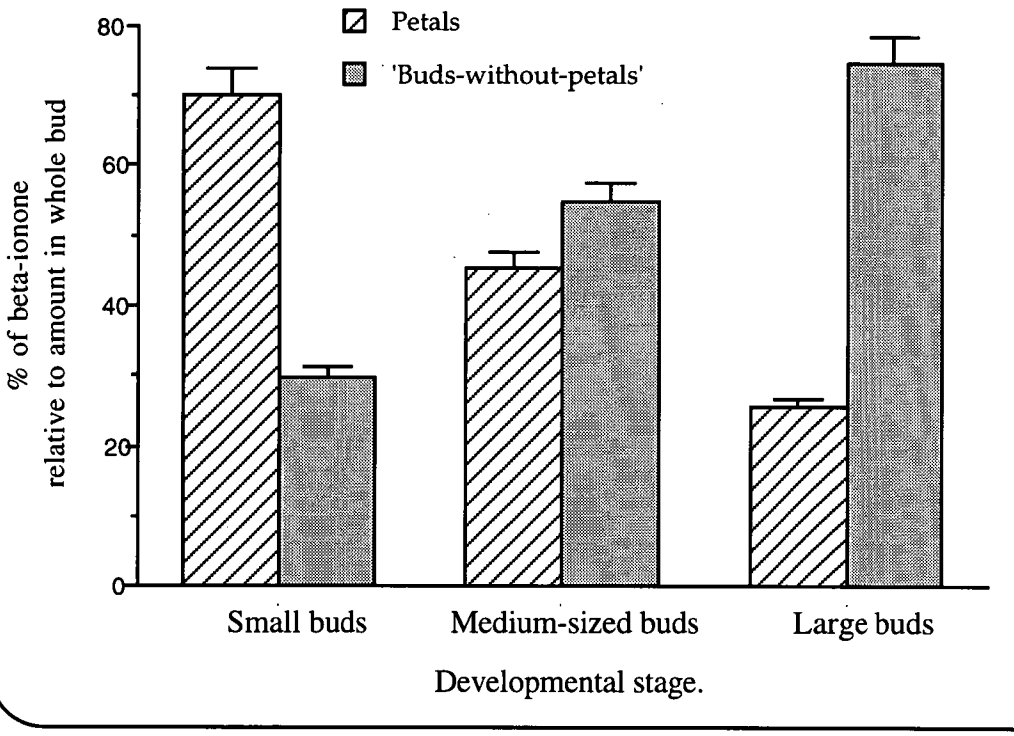
Developmental stage	Organoleptic description of extract
Stage #1 Very-small buds	Very strong and green. Heavy pinene/limonene overtone.
Stage #2 Small buds	Quite green-smelling.
Stage #3 Medium-sized buds	Sweet smelling with muted hay odour, and a green undertone.
Stage #4 Large buds	Soft hay/tea aroma.
Stage #5 Open flowers	Hay and citrus floral aroma.

It appears that the typical odour that is associated with the floral extract begins to develop when the buds reach medium size (stage #3).

4.5 β–ionone in flower parts during development

The β–ionone content in separated petals, and 'buds-without-petals' from three developmental stages expressed as a proportion of the β–ionone in the whole bud, are presented in Figure 4.5.

Figure 4.5
beta-ionone in petals and 'buds-without-petals' in three bud stages, expressed as a percentage of beta-ionone in the whole bud.



As buds develop, the relative proportion of β–ionone in the petals decreases, and the proportion in the rest of the bud increases.

4.6 A typical boronia lateral: description

Boronia flowers occur in clusters of two to three per node along a lateral, with long laterals having ten or more floral nodes (Plate 19). The most mature flowers are found three to four nodes from the tip, and sequentially less mature flowers and buds occur on acropetal and basipetal nodes (Roberts and Menary 1990). In this example the basal nodes are less mature than acropetal nodes because there are more buds on basal nodes. If this particular lateral was harvested, and all buds and flowers were removed (except the very small bud at node #1 which probably wouldn't be removed by the harvester), the yield of flowers would comprise nine flowers (including one senescent stalk) and eight buds, and therefore represents a lateral that has 53% of open flowers. Even at this stage, there are several flowers in which signs of senescence such as pigment fading and abscission of petals has occurred, and there are also stage #3 buds present that will not reach anthesis for another week or more. By that time, the flowers at nodes 3-6 will be in advanced stages of senescence (stage #6). The changes in open flowers during senescence are illustrated (Plate 19B). The most obvious signs are: a gradual reduction in the anthocyanin pigments on the stigma, fading of carotenoid pigments on the periphery of the epidermis on the adaxial surface of the petals (19Ba-e), and abscission of the petals (19Bf).

Plate 19.

A Typical lateral of boronia, with 10 floral nodes. Identification of bud stages at each node:

node 1 = #3, tiny bud;

node 2 = #4, #5a;

node 3 = #5c, #5a-b;

node 4 = #5a, #6;

node 5 = #5a, #5b;

node 6 = #5a, #5a;

node 7 = #3;

node 8 = #4,#4;

node 9 = #4-5, 3;

node 10 = #3-4.

Scale bar = 1cm.

B Open flowers at different stages of senescence. Flowers 'a-e' are stage #5; 'f' is a stage #6 stalk. Note reduction in anthocyanin pigment on stigma and fading of pigments around the periphery of the epidermis on the adaxial surface of the petals. Scale bar = 1cm.

C Developmental bud stages and open flowers. #1 = very small bud; #2 = small bud; #3 = medium-sized bud; #4 (2 examples) = large bud; #5 = open flower; #6 = stalk (petals have abscised). Scale bar = 1 cm.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4.7 A typical lateral: quantification of volatiles and pigments

It has already been demonstrated that at any particular time during the flowering period, a range of developmental and senescent stages of buds and flowers will be present on a lateral (Section IV.4.6). Roberts and Menary (1993) state that the most mature flowers are usually located in the axils of the third or fourth leaves blow the apex, with flower maturity decreasing both acropetally and basipetally from this point. Once most nodes bear open flowers, some flowers will be in more advanced stages of senescence than others. Therefore a study of flowers and buds from apical (nodes 1-3 from the apex), central (nodes 4-6 from the apex) and basal (nodes7-9/10 from the apex) nodes was done to assess pigment and volatile content in flowers that were at different stages of senescence (Section IV.4.1.4). The results are presented in Table 4.6.

Table 4.6. The proportion of buds, and the carotenoids and volatiles in flowers at various positions along a lateral.

Region of lateral	% Buds	Carotenoids Abs 426nm/ml	Total volatiles (μg) +/- std error	β–pinene (μg) +/- std error	β–ionone (μg) +/- std error	(Z)heptadec -8-ene (μg) +/- std error
apical	31%	1.3 ±0.1	132.6 ±4.9	4.8 ±2.3	31.3 ±2.9	40.2 ±3.7
central	1.5%	1.9±0.1	109.2 ±11.1	5.7 ±1.4	34.2 ±4.8	23.6 ±5.0
basal	8%	1.4±0.2	**247 ± 27.1	6.8 ±0.4	**55.7 ± 3.7	*86.9 ± 17.5

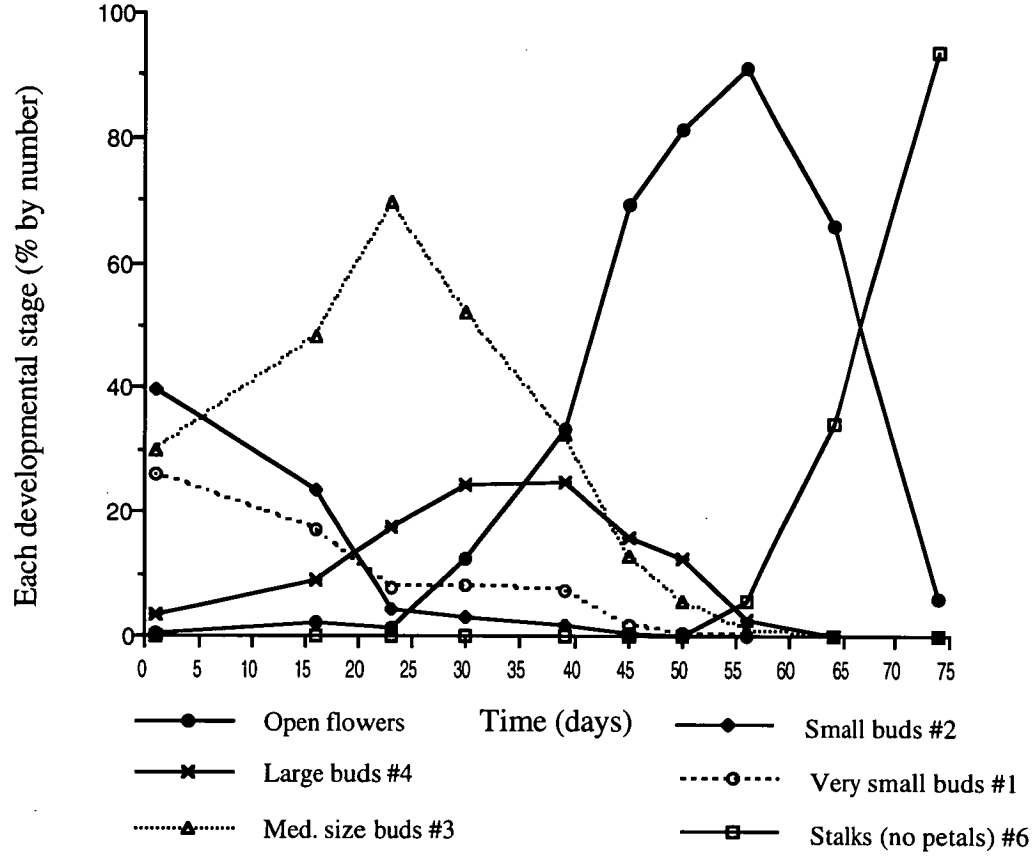
* means significantly different from central region at 5% level of significance.
** means significantly different from apical and central region at 5% level of significance.

In this plant, the proportion of buds at nodes from each region of the lateral illustrates the rate at which nodes mature, and verifies what Roberts and Menary (1993) state. In this case, the most immature nodes bearing the greatest number of buds were at the acropetal end of the lateral. The proportion of buds at the central nodes indicate that the flowers on these nodes were probably the most mature. The

4.8 The rate of flower development

The aim of this work was to monitor the rate of development of buds into flowers, and the rate of senescence of flowers until petals have abscised, leaving stalks with all other floral organs still attached. This has applications for harvest time optimisation. The percentage of each developmental stage (by number) in the total harvest from each week during the flowering season (Section IV.4.1.5) is presented in Figure 4.8.

Figure 4.8 Flower developmental stages during the flowering season



In this graph, the number of buds of each developmental stage may be seen to increase and subsequently decline; the smallest buds reach maximal levels and begin declining prior to larger buds and open flowers, as would be expected. When approximately 65% of the harvest comprises open flowers, there are still small numbers of medium and large buds present on plants. During the next five days the proportion of open flowers increases to 80% and the number of large and medium sized buds decreases. Beyond this (day 56), the number of stalks from which petals have abscised rapidly increases. Following the time when the highest number of open

basal nodes were between the two other regions with regard to maturity. Visual inspection of the flowers showed that yellow pigments were faded in flowers from central nodes, and that in some cases, petal abscission had occurred. However, quantitative extractions showed no significant differences in the concentrations of carotenoids between flowers from different nodes.

Flowers from basal nodes had significantly higher (+50%) levels of total volatiles and β–ionone than flowers from either apical or central nodes, and more (Z)-heptadec-8-ene than flowers from central nodes. If an assumption is made that flower maturity along laterals from this particular plant goes from least mature to most mature as follows; apical flowers < basal flowers < central flowers, it can be said that as flowers mature, the concentration of most volatiles increases and then decreases again, prior to measurable decreases in pigment content and abscission of petals. This is very significant for the production of floral extract from boronia crops because loss of extractable product may have already begun before obvious signs of flower senescence are apparent. Further work was therefore designed to address the question of the most appropriate time for harvest to maximise production of flowers and extract, given the uneven flowering pattern displayed by most boronia plants.

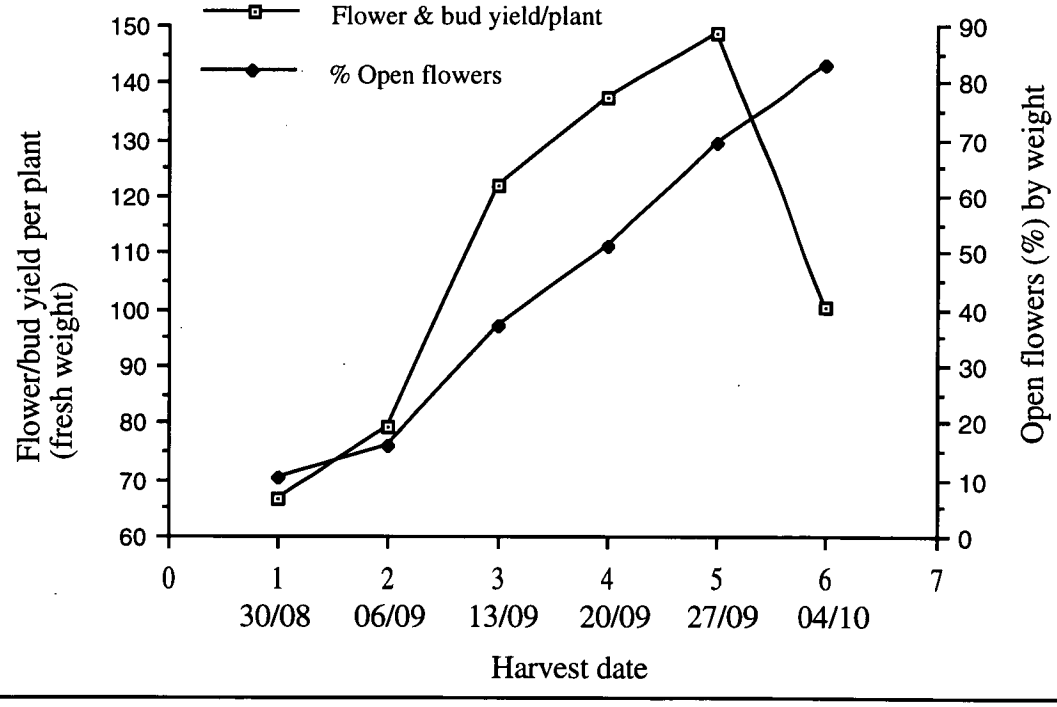
flowers (90%) occurs, there is a rapid decline in the proportion of open flowers due to the high rates of flowers senescing into stalks.

4.9 Flower yield, extract yield and extract composition during the flowering season

It has been demonstrated that when the maximum percentage of flowers have reached anthesis, there are also high levels of senescent flowers (Section IV.4.8) in which the concentration of volatiles may be depleted (Section IV.4.7). For this reason, a study was designed to observe the effects of harvesting at different stages throughout the flowering period on the yield of flowers, buds and extract, and the composition of extract (Section IV.4.1.6). A large plot of clonal plants were used for this study.

The effect of harvest date on the percentage of open flowers in the harvested sample and the total yield (flowers and buds) per plant is presented in Figure 4.9A.

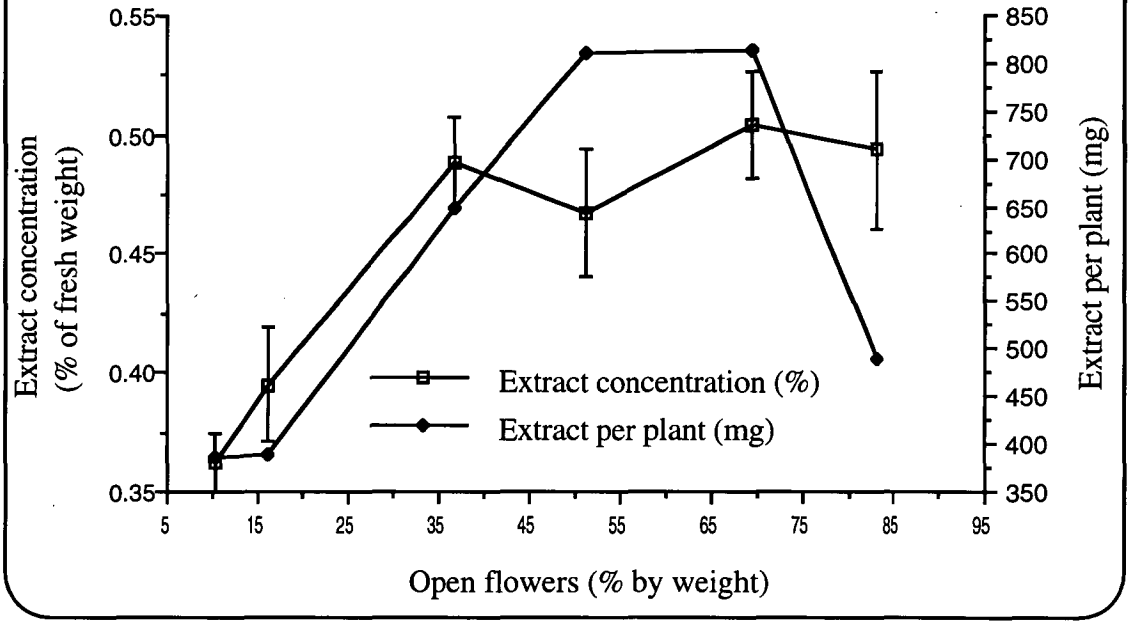
Figure 4.9.A.
Effect of harvest date on the yield of plant material per plant and the percentage of open flowers in the harvest.
Clone 5, Longley, 1993.



As the flowering season progresses, the proportion of the harvest comprising open flowers increases, and concomitantly the yield of flower/bud material per plant increases. Once large numbers of flowers have reached anthesis, increased effects of flower senescence become apparent. The abscission of petals (50% of the weight of a flower) and loss of moisture and volatiles from flowers during senescence produces a decline in the yield of floral material obtained from each plant. The maximum yield of flower/bud material per plant occurs when approximately 75% of flowers have reached anthesis. At this point, there are only small amounts of large and medium-sized buds and stalks (Figure 4.8).

The effects of the percentage of open flowers on the concentration of extract and the extract yield per plant are presented in Figure 4.9.B.

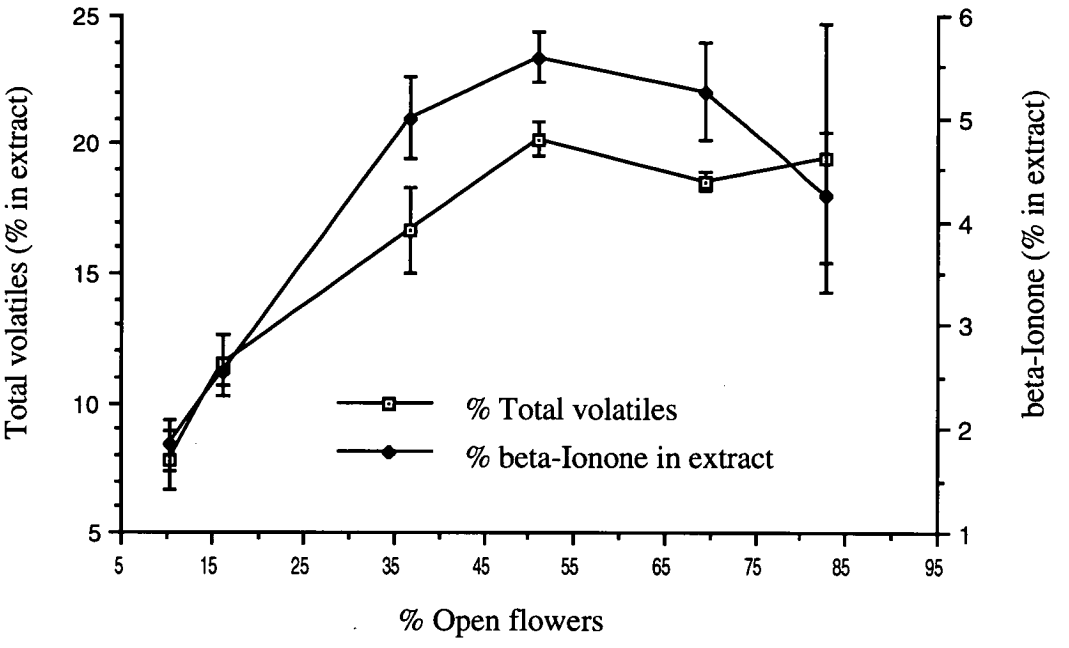
Figure 4.9.B. Extract concentration and extract yield per plant as the percentage of open flowers increases.



The concentration of extract in the harvested sample increases when between 10% and 35% of flowers have reached anthesis, after which it remains constant. The extract yield per plant, however, increases until 50% of flowers are open and remains constant until 75% of flowers are open, after which it rapidly declines.

The proportion of total volatiles and β–ionone in the extract during the flowering season are presented in Figure 4.9.C.

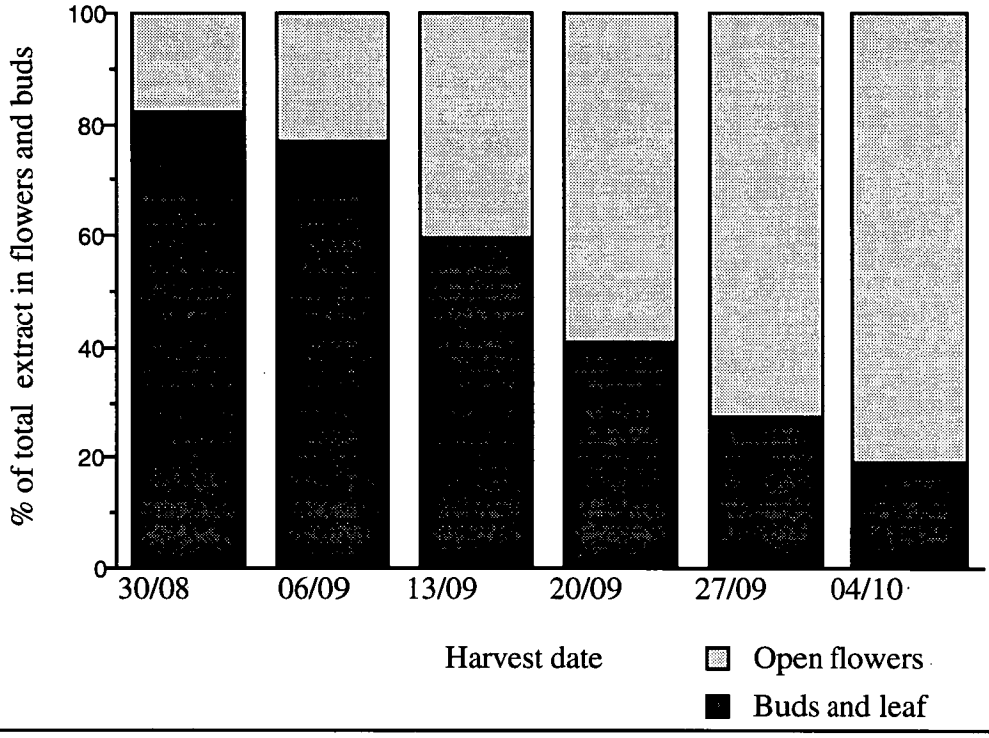
Figure 4.9.C. Total volatiles and beta-ionone in extract as the percentage of open flowers increases.



The levels of total volatiles and β–ionone in the extract during the flowering season follow slightly different patterns. Total volatiles in extract increase when between 10% and 50% of flowers are open, after which levels remain fairly constant. The level of β–ionone in the extract increases when between 10 and 35% open flowers and then remains relatively constant. Total volatiles accumulate more rapidly than β–ionone.

The yield of extract and content of various volatile compounds in flowers and flower buds from harvests throughout the flowering season were assessed. The proportion of the total amount of extract or components contributed by flowers and buds (separately) was calculated. Figure 4.9.D illustrates the proportion of the total yield of extract (per plant) that is contributed by flowers and by buds during the flowering season.

Figure 4.9.D. The proportion of the total extract (per plant) contributed by open flowers and flower buds during the flowering season.



These figures take into consideration the concentration of extract in the buds and flowers, and the proportion of each of these fractions in the total harvestable sample each week. Even when more than 80% of flowers are open, there are buds present which contribute about 20% of the extract yield per plant. The bud stages that would be present at this stage of the flowering period would be mostly medium-sized and large buds (Section IV.4.8), both of which have some extract, but low levels of typical floral volatiles (Section IV.4.4). A more detailed examination of the nature of the contributions to the total extract made by flowers and buds may be seen in Figures 4.9.E and 4.9.F.

Figure 4.9.E. Proportion of total volatiles in extract contributed by open flowers and flower buds during the flowering season.

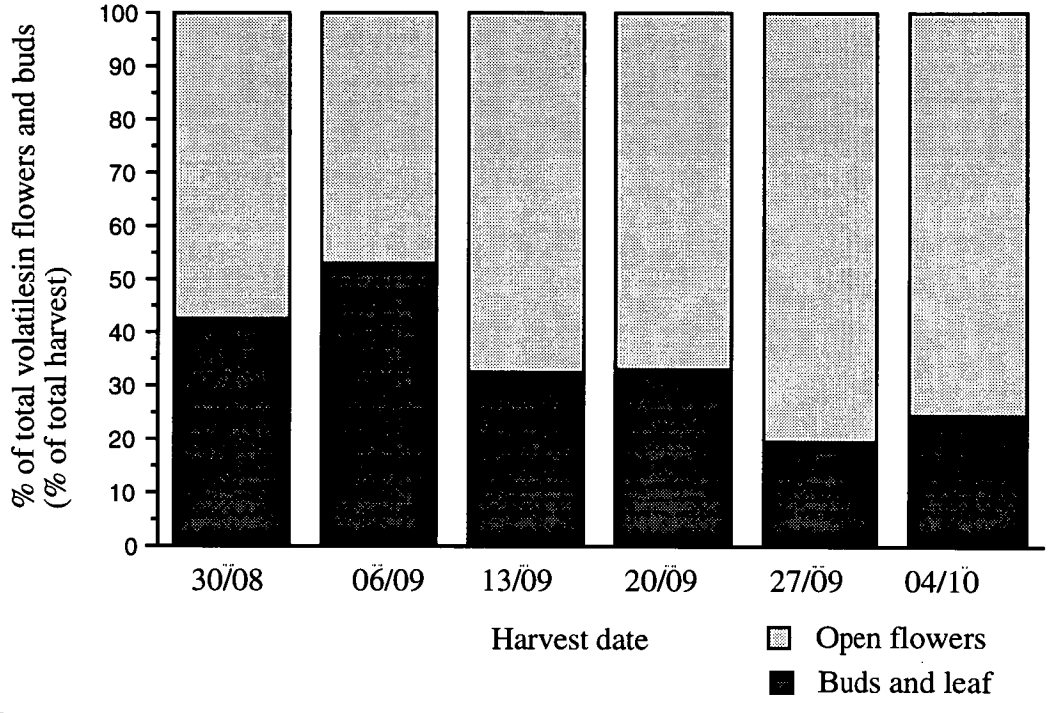
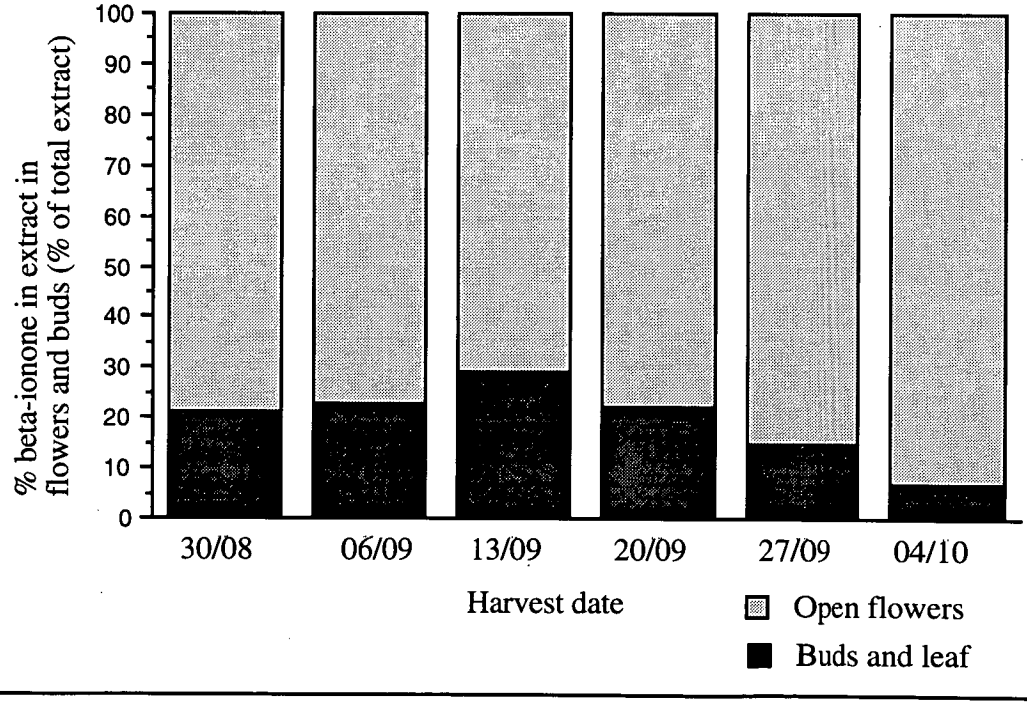


Figure 4.9.F. Proportion of beta-ionone in extract contributed by open flowers and flower buds during the flowering season.



The contributions made by buds to levels of total volatiles in extracts made throughout the flowering period generally declines (Figure 4.9.E). The lowest contribution is 20%, indicating that volatile compounds contained within buds contributes significantly to extract from harvested samples even when 70% of flowers have reached anthesis. The proportion of the volatiles comprised of β–ionone in extract from buds generally increases up to harvest number three (Figure 4.9.F) as the proportion of buds of stage #3 and larger increase, after which time the level of β–ionone in extract from buds declines.

4.10 Protein levels and activity of glycosidase enzyme(s) in small and large buds, and open flowers: a comparison

The levels of protein and activity of glycosidase enzyme(s) capable of cleaving volatile-glycoside conjugates, including glycosides of β–ionone, were compared in samples of small buds (stages #1- #3 and some leaf material), large buds (stage #4) and open flowers (Section IV.4.1.7). The activity of enzymes capable of cleaving total volatiles (Figure 4.10.A) and β–ionone specifically (Figure 4.10.B) are illustrated.

Figure 4.10.A. Activity of glycosidase enzymes from small buds, large buds and open flowers.

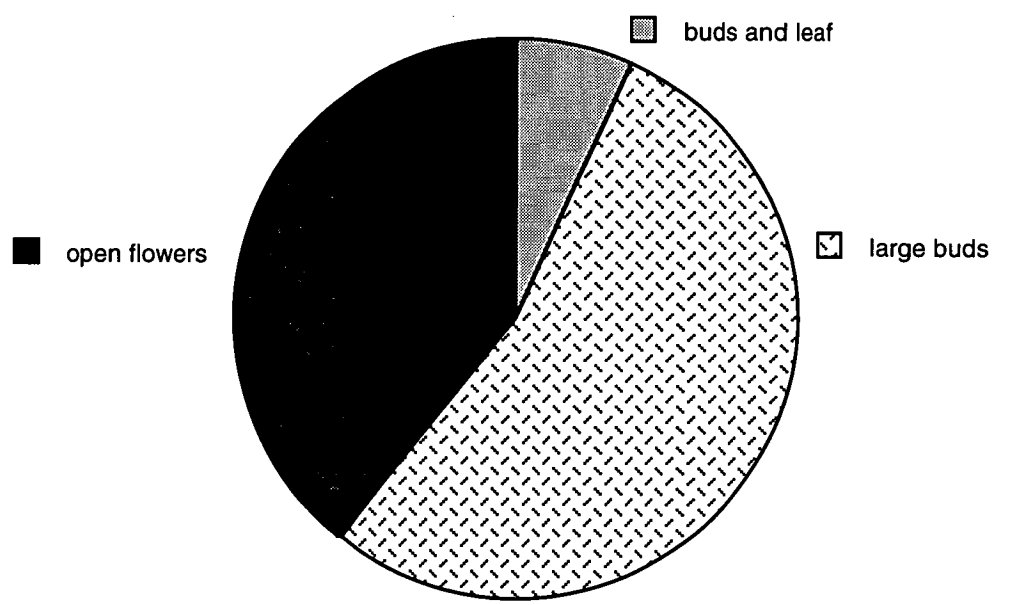
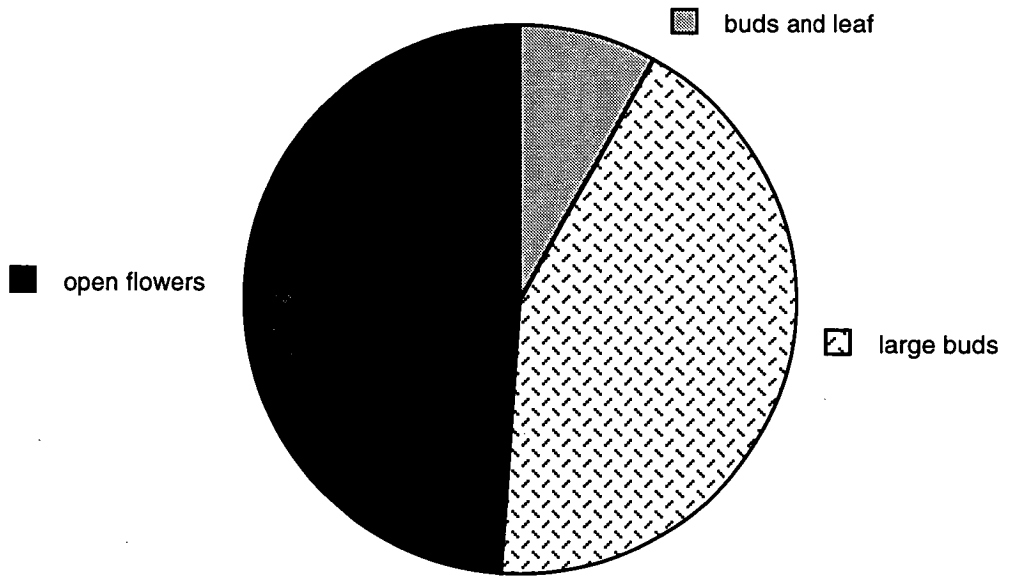
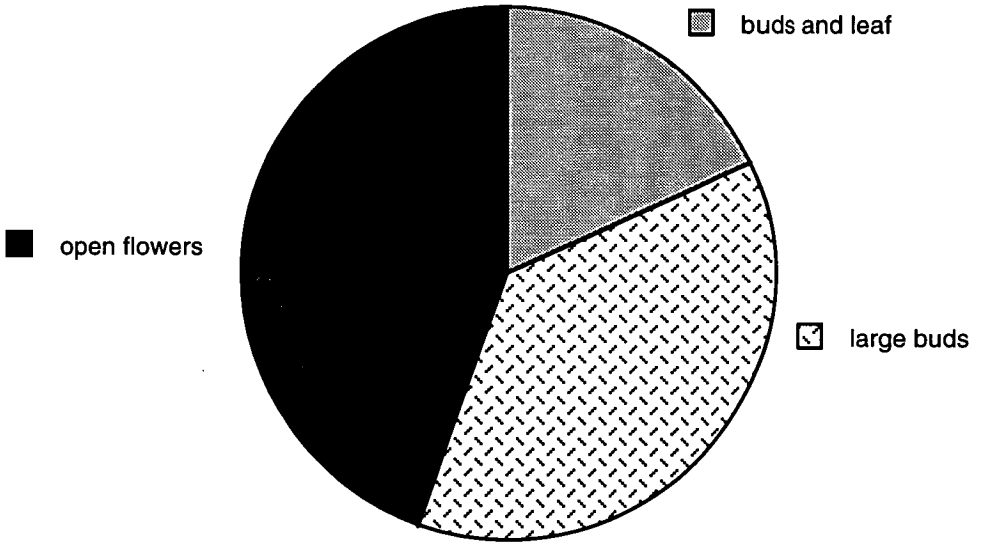


Figure 4.10.B. Activity of beta-ionone-glycosidase in small buds, large buds and open flowers.



There is a small amount of glycosidase activity in small buds and leaf material, but considerably more in large buds and open flowers. The rates at which β–ionone glycosides are cleaved are generally the same as those of other glycosides, although enzymes from large buds have a greater effect on cleavage of β–ionone compared with other volatile-glycosides. The levels of protein in crude enzyme preparations from the three developmental stages examined are presented in Figure 4.10.C.

Figure 4.10.C. Protein levels in small buds, large buds and open flowers.



The level of protein in small buds is relatively higher than the activity of glycosidase enzymes in comparison with the other two developmental stages. The protein levels in open flowers are reduced compared with large buds.

4.11 Free and glycosidically-bound volatiles and glycosidase enzyme(s) during the flowering period

The levels of free and glycosidically-bound volatiles, and activity of glycosidase enzyme(s) in harvested floral material (including buds and some leaf material) throughout the flowering period were studied (Section IV.4.1.8). In this study, β–ionone was also found in significant quantities in both free and bound forms. Figure 4.11.A illustrates the total levels of free and bound volatiles, and activity of glycosidase enzymes; Figure 4.11.B illustrates the levels of free and bound β–ionone, and activity β–ionone-glycoside cleavage enzyme(s).

Figure 4.11.A. Changes in free and glycosidically-bound volatiles and glycosidase enzymes during the flowering season.

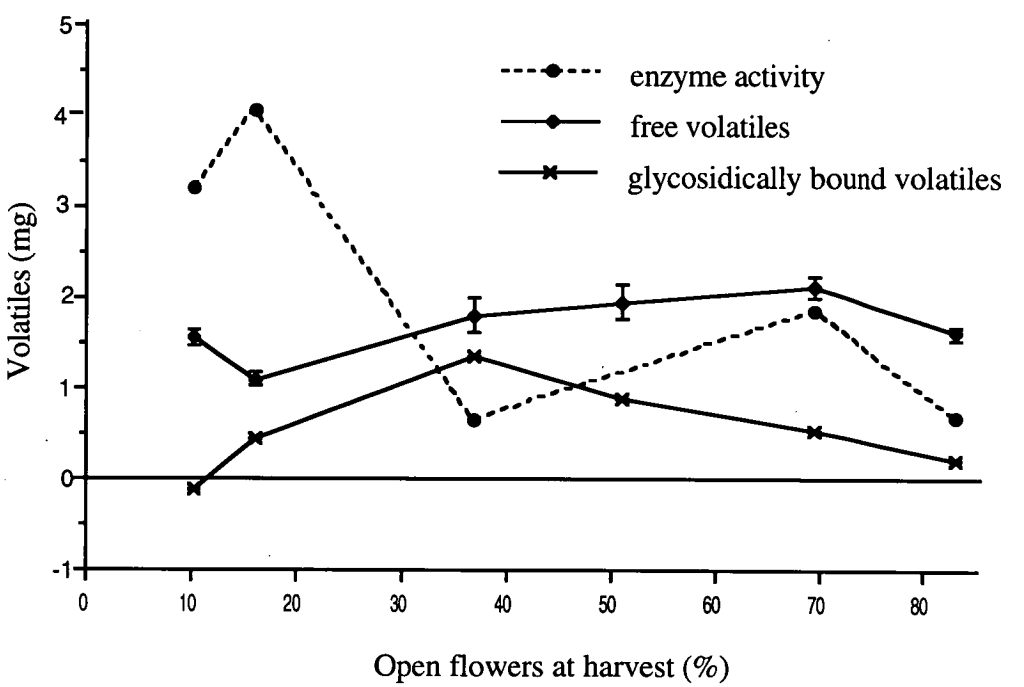
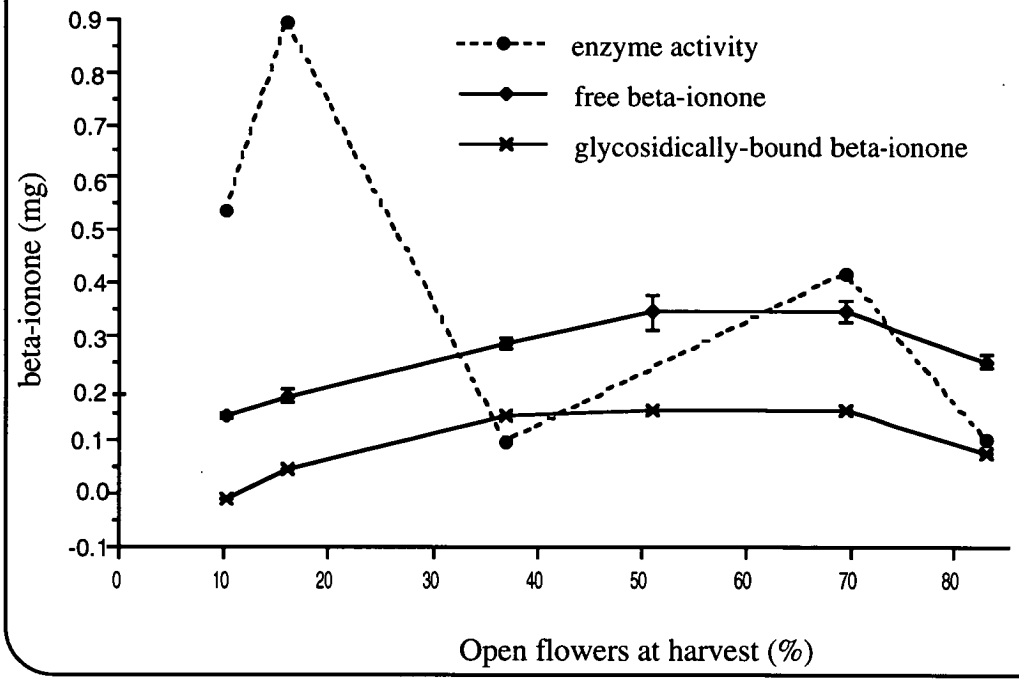


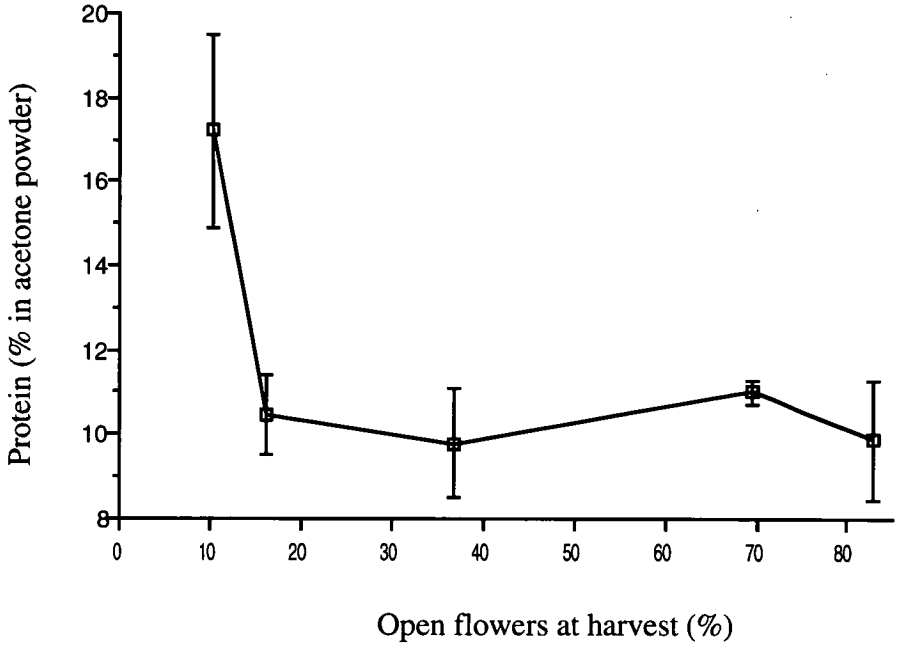
Figure 4.11.B. Levels of free and glycosidically-bound beta-ionone and glycosidase enzyme activity during the flowering season.



The pattern of enzyme activity in the two figures is similar, showing initially high levels that decline until 35% of flowers have reached anthesis, then increase until 70% of flowers are open after which point, activity decreases again. The levels of total volatiles are at their lowest when between 10 and 15% of flowers are open, and decrease again when 85% of flowers are open. The levels of glycosidically-bound volatiles are always lower than free volatiles, however the former increases when between 10 and 35% of flowers are open, and declines linearly after this point. The levels of free and glycosidically-bound β–ionone follow a slightly different pattern to those of total volatiles: both bound and free β–ionone increase in a gentle parabolic fashion and decline when 85% of flowers are open. Bound β–ionone-glycosides also remain consistently lower than free β–ionone levels.

The levels of protein (Figure 4.10.C) follow similar patterns to the level of enzyme activity, indicating that the quantity of enzyme(s) decline as the percentage of open flowers increases.

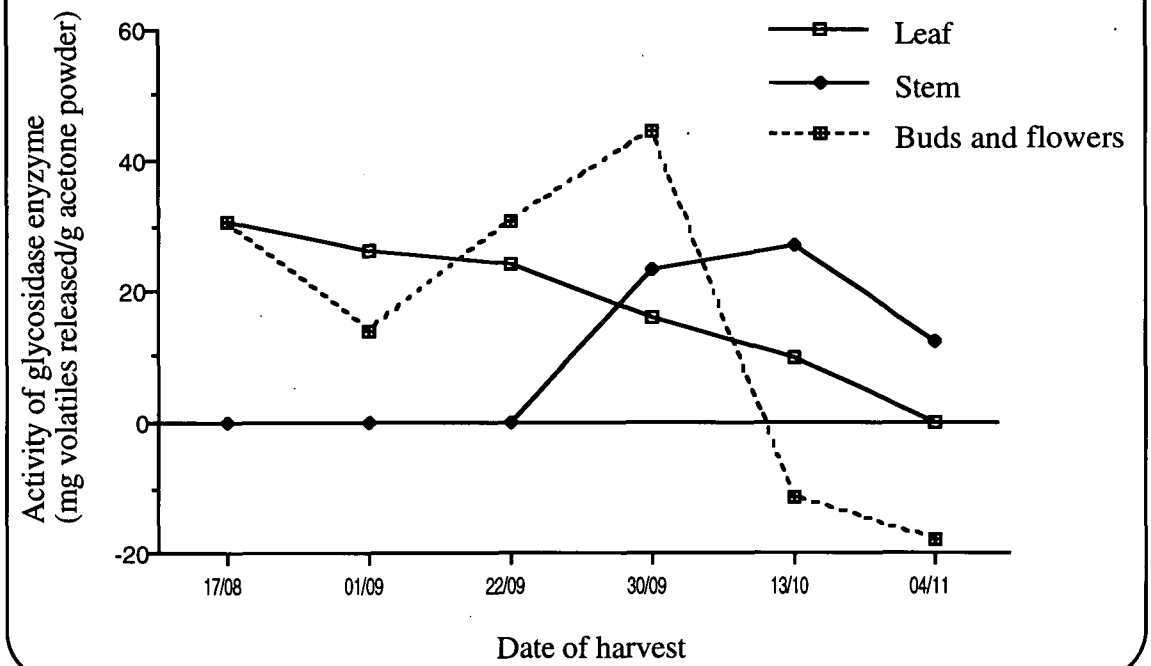
Figure 4.10.C. Protein levels in harvested samples during the flowering season (% of acetone powder).



4.12 Activity of glycosidase enzyme(s) in leaves, stems and buds/flowers during the flowering season

The activity of glycosidase enzyme(s) in different organs of the boronia plant was studied (Section IV.4.1.9) to examine the possibility that glycosidically-bound volatiles could be translocated around the plant and released as bound volatiles or further metabolised in other organs. If this does occur, activity of cleavage enzymes in other parts of the plant may change during flower development. Figure 4.12 illustrates the activity of glycosidase enzyme(s) in three plant parts during a flowering season.

Figure 4.12 Activity of glycosidase enzymes from leaves, stems and buds/flowers during the flowering season.



The activity of glycosidase enzymes in the leaves declines linearly as the flowering season progresses. The activity in the stems (branches and laterals) of the plant is initially negligible, but increases in the second half of the flowering period and then decreases again. Activity of glycosidase enzyme(s) in buds, and eventually buds and flowers, reaches a maximum level when approximately 50% of flowers are open, and then declines markedly in more mature flowers.

4.13 Discussion

This section dealt with the rate at which flowers reach anthesis and accumulate extract, as well as the changing levels of components of the extract during flower maturation and senescence. Although medium-sized buds (stage #3) begin to accumulate an extract that has floral undertones when assessed organoleptically (Section IV.4.4), the yield of extract is low and GC analysis of such extract shows only low levels of typical floral volatiles such as β–ionone and (Z)-heptadec-8-ene (Section IV.4.4). Boronia has an uneven flowering pattern (Section IV.4.6), with flowers at the centre of laterals maturing earlier than flowers situated toward the acropetal or basipetal end. Once all flowers have reached anthesis, flowers at central-lateral positions will be in advanced stages of senescence: they will have lost mass

lateral positions will be in advanced stages of senescence: they will have lost mass (Section IV.4.2), petals may have begun abscission (Section IV.4.6), volatiles will have been depleted (Section IV.4.7), and enzyme activity responsible for metabolism of floral compounds such as β–ionone will be reduced (Section IV.4.10). Loss of carotenoid pigments is a common occurrence during senescence of flowers (Valadon and Mummery 1969), but in the case of boronia flowers, this occurs at a later stage than the onset of loss of volatiles (Section IV.4.7). This means that visible signs of senescence may not be apparent prior to depletion of volatiles by catabolic processes. The rate at which flowers senesce into stalks through abscission of petals increases dramatically once 75% of flowers on a plant have reached anthesis.

The effect of flower senescence on the yield of flowers per plant, concentration of extract in flowers, and yield of extract per plant (or per hectare) is significant and cannot be over-estimated commercially. Even though significant quantities of buds are present when only 55% of flowers have reached anthesis (Section IV.4.8), the yield of flower/bud material per plant is the same as when 70% of flowers are open and declines after this point (Section IV.4.9). Concomitantly, concentration of extract, total volatiles and levels of β–ionone in the extract are at a maximum when between 55 and 70% of flowers have reached anthesis and also decline after this point. Buds present in the harvest during late stages of the flowering period contribute 20% of the extract yield and volatile content to the total yield, and this is probably caused not so much by the presence of high levels of volatiles and extract in the buds, but by relatively low levels in the remaining flowers. Therefore the 'window' during which harvest of the boronia crop is recommended occurs when 50 to 70% of flowers are open. The current practice is to delay harvest until 90% or more flowers have reached anthesis, and at this stage there is a significant increase in the proportion of stalks (flowers from which petals have already abscised) and decreased yields of flowers and extract. There are clonal differences in the onset of anthesis in plants as a whole (Section IV.5.3): in a particular season, flowers on clone #3 plants generally begin to open prior to flowers on clone #5 plants, followed by flowers on clone #250 and clone #17 plants. Variations in this pattern occur depending on the seasonal weather conditions and the site. A flowering period which is lengthened by clonal differences is advantageous for the grower because it maximises the proportion of newly opened flowers which have the highest volatile content at any one time. There may be more than one optimum harvest date. Recommendations based on an in depth study of one clone may be of only limited use in projection of suitable harvest dates for other clones; this is an area of potential for further study.

The process of extract accumulation and development in buds appears to be accompanied by an increase in the number of observable oil glands between developmental bud stages (Section IV.4.3). This is not a common finding; usually all

oil glands (on a leaf) are initiated very early in development (Venkatachalam *et al.* 1984), although an increase in size of the oil glands upon filling with secondary compounds usually continues during subsequent leaf expansion. This also occurs in oil glands on boronia petals. The apparent reduction in the number of glands on petals of buds may be a factor related to poor resolution of relatively un-filled glands in immature buds. The resolution of glands using the method chosen was variable; other methods involving de-staining and re-staining techniques were examined for their use, and proved to be no better. Other work (Section IV.2.4) has shown immature glands on the petals of very small buds; glands which may not be distinguishable by LM.

As buds mature into flowers, the proportion of β–ionone declines in the petals, and simultaneously increases in the rest of the flower (stigma, anthers and calyx) (Section IV.4.5). This may be indicative of the transport of such compounds from sites of synthesis in the petals to sites of accumulation in organs such as the anthers and stigma (Section IV.1.2) where a biological role such as attraction of pollinators requires such compounds. It may also be indicative of the relative growth rates of different floral organs and their inherent biosynthetic capability. Young tissues are usually most active in synthesis of secondary compounds; catabolic processes speed up in older tissues (Croteau *et al.* 1981; Mihaliak *et al.* 1991). Petals and functional anthers have a greater relative increase in size between bud stages #1 and #3 than do the non-functional anthers and stigma (Section IV.4.2). The latter two organs have a relatively large increase in size between large buds (stage #4) and open flowers, whereas petal size remains the same between these stages, and functional anthers actually decrease in size (due to pollen production). Therefore, the decline in β–ionone in petals may be a result of the onset of senescence in these organs, prior to maturation of organs such as the non-functional anthers and stigma, which will undergo senescence at a later stage. Boronia flowers appear to be protandrous: the functional anthers produce pollen and senesce prior to maximum size being attained by the stigma.

Glycosidically-bound volatiles occur to lower levels than free volatiles in buds and flowers and generally decline prior to the decline seen in free volatiles during senescence, probably as a result of increased glycosidase activity (Section IV.4.11). This is similar to the findings of Francis and Allcock (1969). High levels of glycosides in buds may be a result of higher activity of glycoside-forming enzymes (not assayed) than activity of glycosidase enzymes. Although high glycosidase activity was seen in harvested samples early in the flowering season (Section IV.4.11), protein levels were also high at this stage which may indicate that other enzymes such as glycoside-forming enzymes may also be active at this time. Since the level of glycosides peak and decline prior to free volatiles (Figure 4.11.A), it is probable that both glycoside-forming enzymes and glycosidases in flowers are reduced upon

senescence (Figure 4.12). Glycosides may act as storage forms for volatiles synthesised in excess of the amount that can be stored within protective organelles such as plastids, or storage areas such as oil glands. Upon senescence, synthesis of volatiles and volatile-glycosides declines, and for a time, continued 'production' of volatiles occurs by cleavage of stored volatile-glycosides. Eventually stored glycosides become depleted, or activity of glycosidase enzymes declines due to other reasons such as depletion of cofactors or excess sugars, and subsequently the concentration of volatiles in the flower also declines (Figure 4.11.A). Volatile-glycosides may also represent a means by which some of the carbon used in the production of secondary compounds can be retrieved from the flower prior to abscission. Some glycosides, such as β–ionone-glycosides do not decline to the same extent as other volatile- glycosides, perhaps because the activity of enzymes responsible for formation of glycosides with this compound does not decline as early as activity of other glycoside-forming enzymes.

IV.5 Results
Genetic and environmental effects on yield of flowers and extract

Attempts were made to quantify the variability in the yield and composition of extracts due to major differences in the environment and between genetically different plants in one environment. The relationship between extract yield and factors such as plant maturity and floral characteristics including flower yield, the number of oil glands on the petals and the weights of petals and stigmas were studied.

5.1 Materials and methods

5.1.A Extract yield and composition in genetically different plants in three environments

Three populations of genetically different plants were assessed: including 17 different plants from I.'s site at Kingston (1992); 16 different plants from M.'s site at Kingston (1994); and 28 different plants from D.'s site at Surges Bay (1994). A description of each site and its location in Tasmania may be seen in Appendix 3. Where possible, flowers were harvested from plants which had similar number of flowers that had reached anthesis. Extracts were made (Section III.3.1) and analysed by GC (Section III.4.3). Values for the yield and composition of extracts from plants from each site were compared by calculating the mean, the standard error, the minimum and maximum, the range and the coefficient of variation. Pooled results from all three sites were compared by analysis of variance.

5.1.B A comparison of extract yield and composition between four genetically different clones

Four clones are grown commercially, their identification numbers are #3, #5, #17 and #250. Four plants, each four years old, of each of these clones were harvested weekly throughout the 1994 flowering season from a plantation at Longley. The data used as a comparison for extract yield and composition for each clone represents the peak in flower and extract yield per plant that occurs when 70% of flowers have reached anthesis (Section IV.4.9). From other sites around Tasmania (Appendix 3), randomly selected three or four year old plants of each clone were harvested. The harvest dates used were: 1) Longley #3 - 27/09/94; #5, #17 and #250

- 12/10/94; 2) Scottsdale - 14/09/94; 3) Bridport - 14/09/94; 4) Kingston (M's site) - 25/09/94; 5) Surges Bay - 02/09/94; and 6) St. Helens - 08/09/94.

Flowers were frozen at -20°C for storage, extracted in triplicate (Section III.3.1) and analysed by GC (Section III.4.3).

Data for precipitation and temperatures in areas near Kingston (Hobart), Longley (Grove), St. Helens and Scottsdale were obtained from the Bureau of Meteorology, and are presented in an abbreviated form in Appendix 8.

5.1.C Extract production in plants of increasing maturity

Two studies were done:

Study No.1 used clone #5 plants grown at Longley over three successive years; plants were two years old in 1992. During each flowering season, ten or twenty plants were harvested each week. Flowers were weighed, frozen for storage, extracted in triplicate (Section III.3.1), and analysed by GC (Section III.4.3). The data used for extract yield and composition each year represents the peak in flower and extract yield per plant that occurs when 70% of flowers have reached anthesis (Section IV.4.9).

Study No.2 used two, three and four year old clone #3 plants, and three and four year old clone #5, #17 and #250 plants from St. Helens, harvested during the 1994 flowering season. Samples were taken from randomly selected plants. Flowers were frozen for storage, extracted in triplicate (Section III.3.1) and analysed by GC (Section III.4.3).

5.1.D The effect of flower yield per plant on extract concentration

Fifteen individual plants of clone #5 at Longley were harvested in the 1992 flowering season. The flowers harvested from each plant were weighed, frozen for storage, extracted in triplicate (Section III.3.1) and analysed by GC (Section III.4.3).

5.1.E The effect of average flower weight on extract concentration

The weight of individual flowers was assessed by weighing 50 fresh flowers; the extract yield was assessed in triplicate (Section III.3.1) for genetically different plants at two sites: I.'s site at Kingston in the 1992 flowering season, and at Surges Bay in the 1994 season. The results were pooled together.

5.1.F The effect of the density of oil glands on the petal, and the weight of petals on extract concentration

Flowers were harvested from plants generated from seed which were therefore genetically different from each other to allow comparison of a wider range of flower- and extract-producing types than could be achieved by an analysis of a clonal

population. I.'s site at Kingston was sampled in the 1992 flowering season. The weight of petals as a proportion of the total fresh flower weight was assessed by removal of petals from 50 flowers. The weights of the separated petals and the flowers-without-petals were assessed. Oil glands on the epidermis' of petals were enumerated (Section III.6) and the surface area of the petals was assessed (Section III.7). Extracts were made in triplicate (Section III.3.1) and analysed by GC (Section II.4.3).

5.1.G The effect of the weight of the stigma on extract concentration

Flowers from 24 genetically different plants from several environments were used for this study. The stigmas from 25 frozen flowers from each sample were removed with a sharp instrument which caused the stigma to break off at a natural point of abscission (to circumvent errors due to different excision points), and the separated stigmas and flowers-without-stigmas were weighed. The extract yield from whole flowers was assessed in triplicate (Section III.3.1) and was analysed by GC (Section III.4.3).

5.2 Extract yield and composition in genetically different plants in three environments

A comparison of extract yield and some aspects of extract composition from genetically different plants was made (Section IV.5.1.A). A typical population of plants produced from seed (genetically different) may be seen in Plate 20A. The results of the extractions are summarised in Table 5.2, and the ANOVA for the pooled results from the three sites may be seen in Appendix 9.

Table 5.2 Summary of results from extractions and analyses of genetically different plants from three environments.

Value	% extract	% volatiles in extract	% β–ionone in volatiles	% β–ionone in extract
I's site at Kingston, 1992				
Mean	0.424	10.575	21.275	2.275
Standard Error	0.010	0.929	1.129	0.265
Range	0.137	10.39	20.99	4.136
Coefft of Var	10.041	36.226	21.885	47.947
M's site at Kingston, 1994				
Mean	0.4703	13.833	24.196	3.354
Standard Error	0.016	0.7323	1.339	0.251
Range	0.221	11.26	21.623	3.585
Coefft of Var	13.595	21.176	22.129	29.987
D's site at Surges Bay, 1994				
Mean	0.4725	13.505	24.439	3.394
Standard Error	0.014	0.639	1.111	0.286
Range	0.291	18.95	26.753	8.705
Coefft of Var	15.617	25.016	24.059	44.560

N.B. Coefft of Var = coefficient of variation.

A comparison of the coefficients of variation for the values for extract yield and composition illustrates that generally, there is almost twice as much variability in the values for extract composition (% volatiles, % β–ionone in volatiles) as there is for extract yield. There is an even larger variation between β–ionone levels in the extract compared with other values. The coefficients of variation are generally lower for M's site at Kingston than D.'s site at Surges Bay. The results of the ANOVA from pooled

results from the three sites (Appendix 9) also show that there are larger differences between extract composition than extract yield.

If the yield of extract from flowers of all these plants is plotted against the percentage of volatiles in the extracts (Figure 5.2), the relation between the two variables may be seen. The regression coefficient was close to zero. As the yield of extract increases from 0.325% to about 0.45% of fresh flower weight, the percentage of total volatiles increases from 5-7% to about 17%; and as the extract yield increases further, there is a gradual decline in the proportion of the extract comprised of volatile compounds to about 12.5%. The two outlying values (arrows) represent plants which have very high extract yield and high volatile content, and as such, may be valuable plants for further clonal selection.

Figure 5.2 Effect of extract yield on the percentage of total volatiles. Genetically different plants, 3 environments.

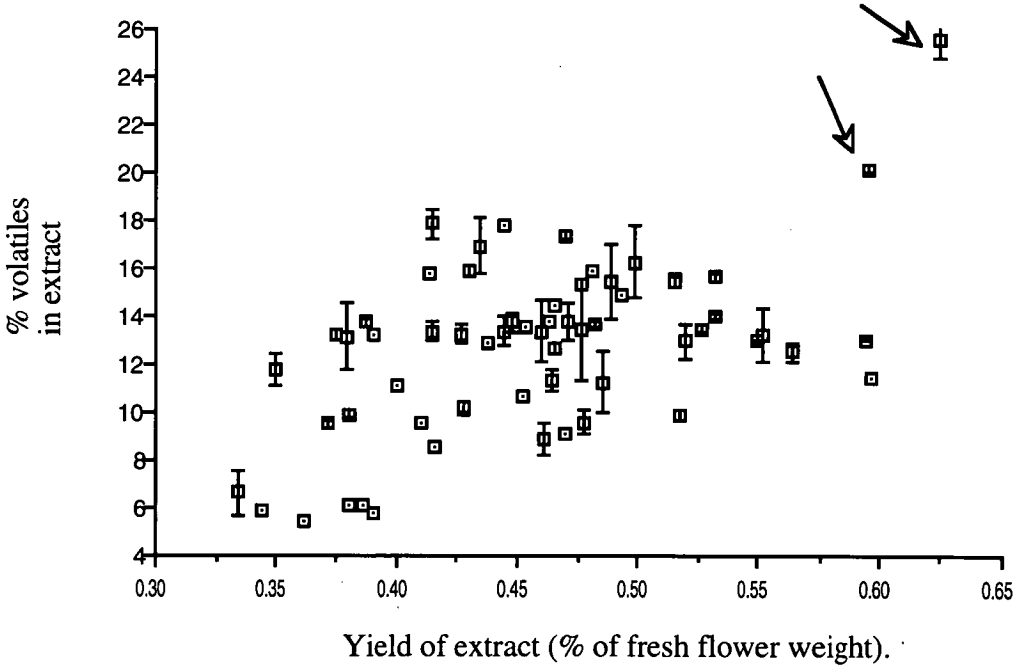
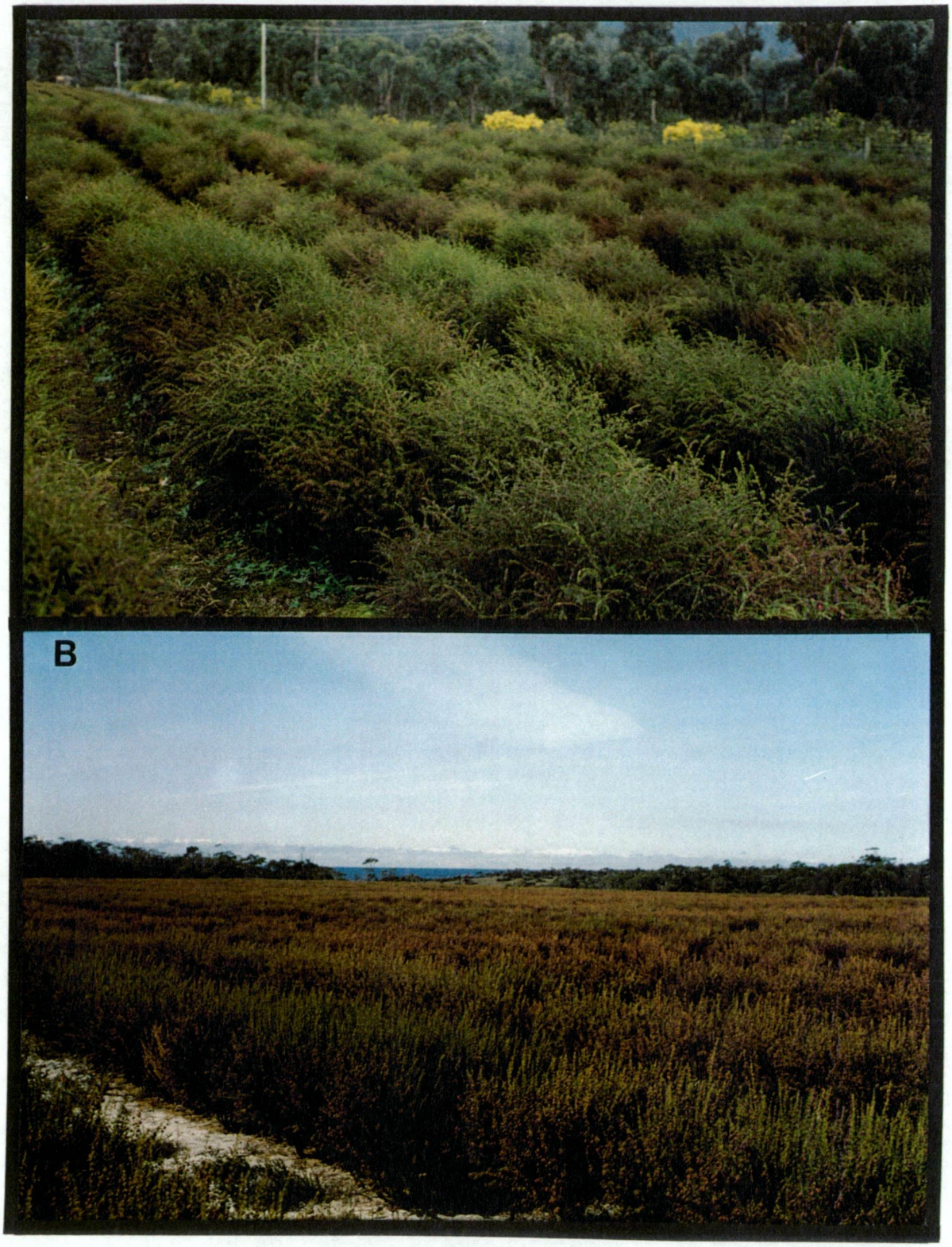


Plate 20.

A Colour photo of plantation of genetically different plants at Surges Bay.

B Colour photo of plantation of clones #17 and #250 at St Helens.


B

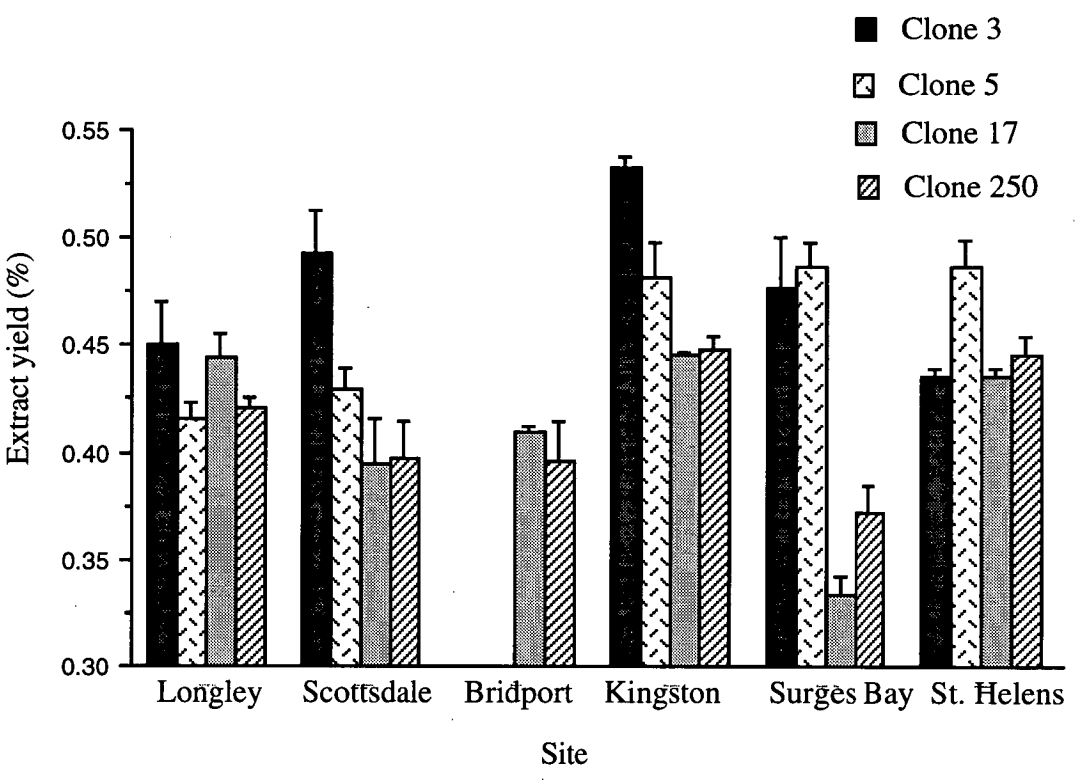
5.3 A comparison of extract yield and composition between four genetically different clones

A plantation of clonal plants (clone #250) may be seen in Plate 20B, in comparison with a plantation of genetically different plants (Plate 20A). Along the nearest row of plants in Plate 20B note the uniformity of habit; laterals project vertically above the plant. There are slight colour differences between rows which are due to differences between clones in separate rows (Plate 20B). In Plate 20A the plants vary in habit and colour of the canopy along a row as well as between rows. Many plants have droopy, horizontal laterals (Plate 20A).

A comparison of the yield of extract, and the content of total volatiles and β–ionone in the extract from four clones at each of six environments harvested in the 1994 season was done (Section IV.5.1.B). The sites are described in Appendix 3. The total precipitation for the months of April to November inclusive, and the mean maximum and mean minimum temperatures during the flowering season (September to November inclusive) during 1992, 1993 and 1994, for several of the regions in which sampling occurred are presented in Appendix 8. Generally, St. Helens experiences higher maximum temperatures during the months of September and October than Kingston, Longley or Scottsdale, which have similar maximum temperatures during these months. Longley and St. Helens have the lowest minimum temperatures during the months of flowering, and Kingston tends to have the highest minimum temperatures during these months. The rainfall during the months of April to November inclusive does not take into account irrigation by individual growers, however it is rare that irrigation would occur during flowering, when attempts are made to reduce vegetative growth to enhance flower growth. The highest rainfall is generally experienced at Scottsdale, and Longley had comparable levels in 1994. St. Helens had a relatively low level of rainfall in 1994. During the three years studied, Longley and Kingston experienced less variation in total rainfall in the months of April to November than other sites; these two sites are geographically close to each other.

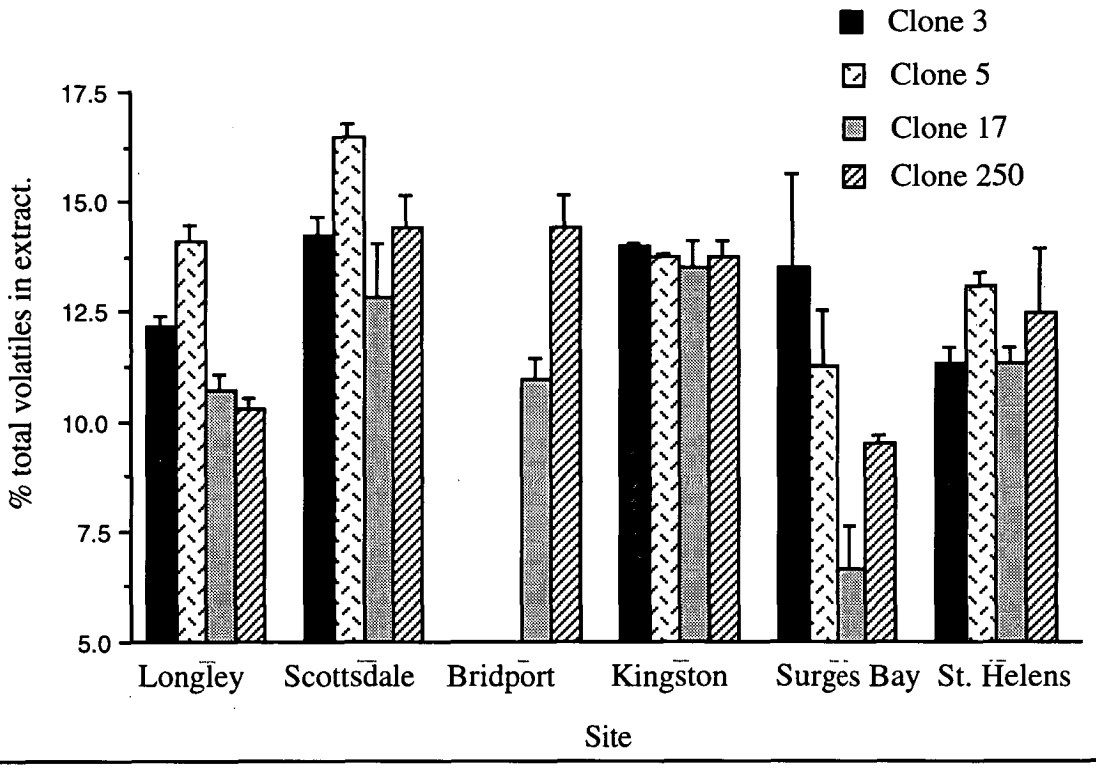
The yields of extract and content of total volatiles and β-ionone as a proportion of the extract for each of the four established clones at each of the six sites sampled are presented in figures 5.3.A, 5.3.B and 5.3.C.

Figure 5.3.A Extract yield from four clones at six sites.



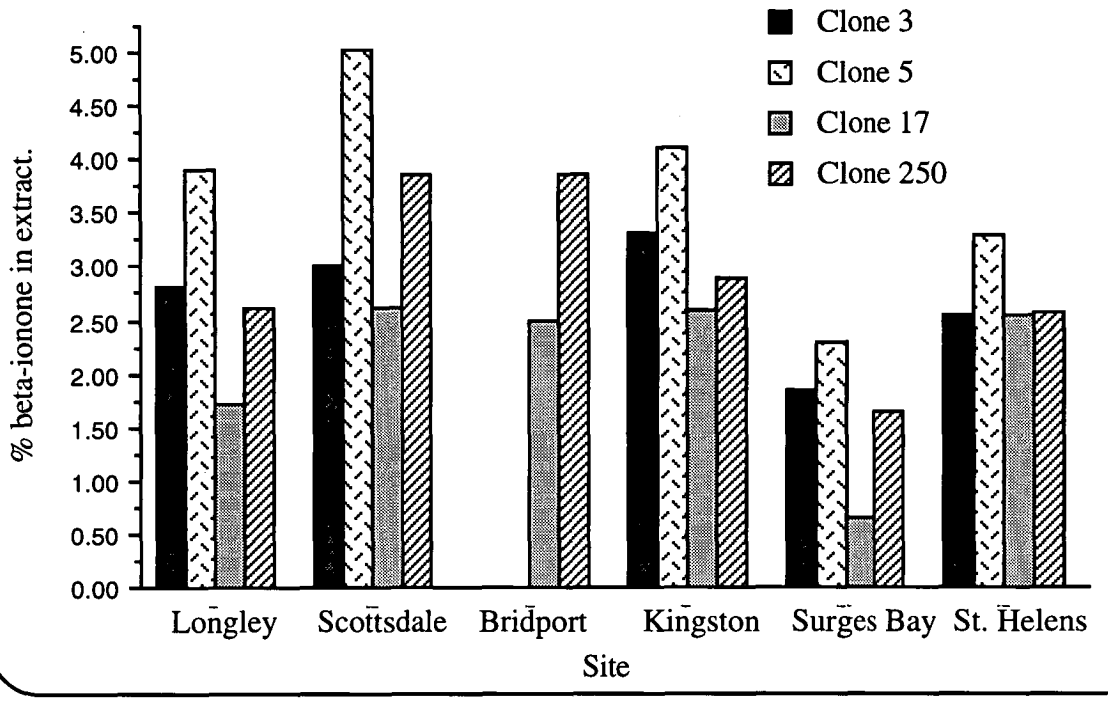
The extract yield from the four different clones is variable at different sites. Clone #5 has a relatively higher extract yield when grown at Kingston, Surges Bay and St. Helens compared with yields at Longley or Scottsdale. Clone #3, which has very high extract yield at Kingston, has a relatively lower yield at St Helens. Clones #17 and #250 tend to have a lower extract yield than clone #3 and #5, especially at Kingston, Surges Bay and Scottsdale. Yields are more similar for all four clones at Longley than at any other site. Clones which do well at one site do not necessarily do so at another site.

Figure 5.3.B Total volatiles in extract from four clones at six sites.



There is as much variation in the percentage of total volatiles in extracts from each clone at each site as there is in the yield of extract. At Longley and Scottsdale, both sites which are marginally wetter and colder than the other three sites where clone #5 is grown, the percentage of total volatiles in extract from clone #5 is higher, but the extract yield lower than at other sites.

Figure 5.3.C beta-Ionone in extract from four clones at six sites.



Clone #5 tends to have the highest proportion of extract comprising β-ionone compared with other clones over all the sites sampled. β-ionone levels are particularly low for all clones at Surges Bay. There are no other consistent trends. A summary of the information presented above is presented in the appendices; the extract yield and composition at each site is averaged for each of the four clones (Appendix 10), and the extract yield and composition of each of the four clones is averaged over each site (Appendix 11).

5.4 Extract production as plants mature: four clones, one environment

Plants generally increase in size over several seasons, and flower production per plant would be expected to increase with plant size. Whether the extract yield per plant, or extract concentration in flowers (% of fresh weight) would also increase was of interest for use in projecting future yields from existing plantations. Two studies were done, study No.1 examined the change in extract yield and composition of one clone in one environment over three successive years; and study No.2 examined the extract yield and composition from plants of different ages, and this study included all

clone in one environment over three successive years; and study No.2 examined the extract yield and composition from plants of different ages, and this study included all four clones in one environment and one season (Section IV.5.1.C). The prevailing temperatures and the total precipitation from April to November during the flowering seasons studied are presented in Appendix 8, and summarised in Section IV.5.3. The results of study #1 may be seen in Table 5.4.A.

Table 5.4.A. Flower and extract yield and extract composition in clone #5 at one site over three successive years.

Year	Flower yield per plant (g)	% extract (fresh wt) +/- std. error	% volatiles in extract +/- std. error	% β–ionone in volatiles +/- std. error	% β – ionone in extract
1992	147 +/- 13.2	0.353 +/- 0.004	11.57+/- 0.363	36.414 +/- 0.39	4.21
1993	148.5	* 0.592 +/- 0.01	* 20.2 +/- 0.64	* 27.70 +/- 1.20	5.60
1994	not assessed	** 0.416 +/- 0.01	** 14.09 +/- 0.40	* 27.76 +/- 0.2	3.91

* means significantly different to 1992 results at the 5% level of significance.

** means significantly different to 1992 and 1993 results at the 5% level of significance.

1993 appears to have been the best season for extract yield, total volatile levels and β–ionone content in the extract for clone #5 plants grown at Longley. There was a depletion of all of these values in 1994. Flower yield per plant did not increase between years 1992 and 1993, and was not assessed in 1994. The results of study No.2 are presented in Table 5.4.B.

Table 5.4.B. Extract yield and composition of four clones of several ages in one environment, in one season.

Clone	Age	% extract +/- std error	% vol in extract +/- std error	% β–ionone in vol +/- std error	% β–ionone in extract
3	2 years	0.489 +/- 0.006	13.55 +/- 0.387	24.977 +/- 0.778	3.38
3	3 years	* 0.435 +/- 0.004	11.327 +/- 0.325	22.503 +/- 0.335	2.55
3	4 years	* ** 0.464 +/- 0.005	9.583 +/- 0.101	24.38 +/- 1.12	2.34
5	3 years	0.487 +/- 0.016	13.07 +/- 0.274	25.123 +/- 0.691	3.28
5	4 years	0.504 +/- 0.018	12.65 +/- 0.407	28.353 +/- 0.517	3.59
17	3 years	0.435 +/- 0.004	11.327 +/- 0.325	22.503 +/- 0.335	2.55
17	4 years	** 0.482 +/- 0.003	13.367 +/- 0.333	20.623 +/- 0.704	2.76
250	3 years	0.446 +/- 0.008	12.45 +/- 1.49	20.59 +/- 1.97	2.56
250	4 years	0.456 +/- 0.005	9.167 +/- 0.179	25.93 +/- 0.105	2.38

* means significantly different to two year old plants at the 5% level of significance.
** means significantly different to three year old plants at the 5% level of significance.

In some cases, for example with clones #3 and #17, four year old plants have a higher extract yield than three year old plants. Two year old clone #3 plants have a higher extract yield than three year old plants. There are no significant differences in the volatile content and β–ionone content in the extract between different aged plants, and there are no consistent trends.

5.5 The effect of flower yield per plant on extract concentration

The effect of flower yield per plant on the extract concentration and yield of extract per plant between clone #5 plants at Longley was studied in the 1992 flowering season (Section IV.5.1.D). This study also allows quantitative analysis of the variation in extract yield between plants of one clone in one environment. The results are summarised in Table 5.5.

Table 5.5 Summary of flower and extract yield per plant, and extract concentration and composition from 15 clone #5 plants at Longley in 1992.

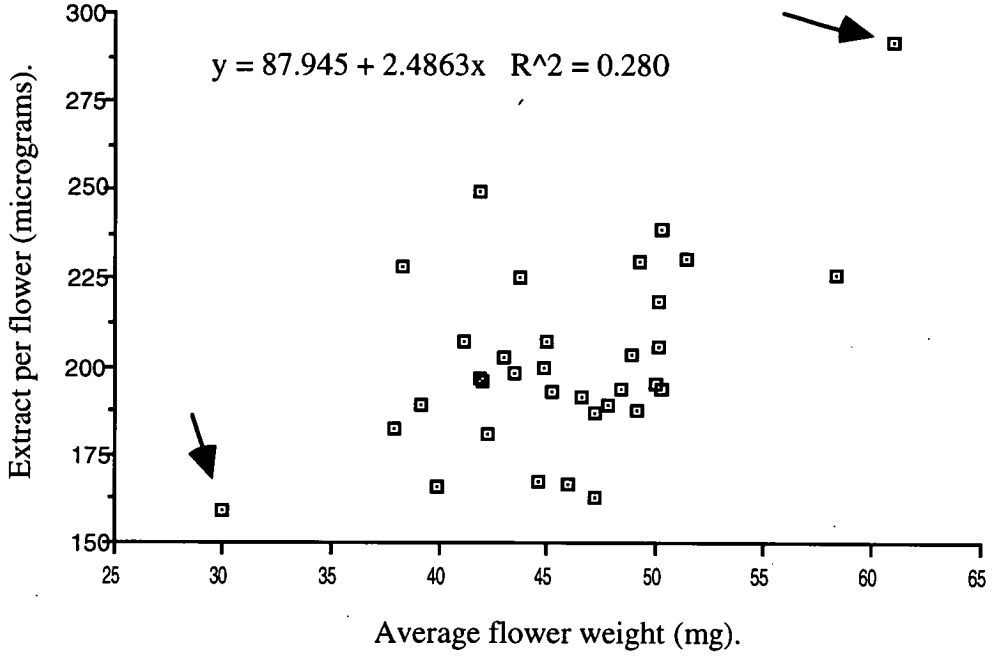
	Flower yield per plant (g)	% extract	Total extract per plant (mg)	% volatiles	% β–ionone in volatiles
Mean	147	.353	520	11.57	36.41
Std. error	13.2	0.004	49.6	0.363	0.39
Min.	58	0.325	186	8.5	33.8
Max	241	0.385	875	13.7	38.7
Coefficient of Variation	34.80	4.729	36.928	12.16	4.141

There is far greater variation in the flower and extract yield per plant, than there is in the concentration of extract in flowers from each plant. There is more variation between plants in the percentage of total volatiles in the extract than there is in the β–ionone levels as a proportion of the total volatiles. There is a significant positive relationship between flower yield per plant and extract yield expressed as milligrams per plant ($R^2 = 0.986$).

5.6 The effect of average flower weight on extract yield

The effect of flower size on the concentration of extract in genetically different plants was studied (Section IV.5.1.E). The results are presented in Figure 5.6.

Figure 5.6 Average flower weight and extract yield per flower. Genetically different plants, two sites.



There is no relationship between flower weight and extract yield per flower. Two values noted in Figure 5.6 (arrows) are outlying from the rest of the values, and these flowers had respectively low flower weight and low extract yield per flower and high flower weight and high extract yield per flower. If these two values are removed, there is no trend seen in the majority of the results.

5.7 The effect of the number of oil glands on the petals and the weight of petals on extract yield

The relationship between extract yield (per flower) and the weight of petals as a proportion of the total flower weight, and the number and density of oil glands on the epidermis of the petals was assessed (Section IV.5.1.F).

There is no significant relationship between the weight of the petal as a proportion of the flower, and extract yield per flower ($R^2 = 0.210$).

The variation in the density of oil glands and the extract yield per flower in genetically different plants is presented in Table 5.7.

Table 5.7. Surface area of petals, oil gland distribution and extract yield in genetically different plants.

Value	Surface area of petals (mm^2)	No. of oil glands per petal	Density of oil glands (No./mm^2)	Extract yield per flower (micrograms)
Mean	181.64	204.29	1.12	194.71
Standard deviation	20.587	39.16	0.158	8.70
Coefficient of variation	11.334	19.169	14.049	14.13

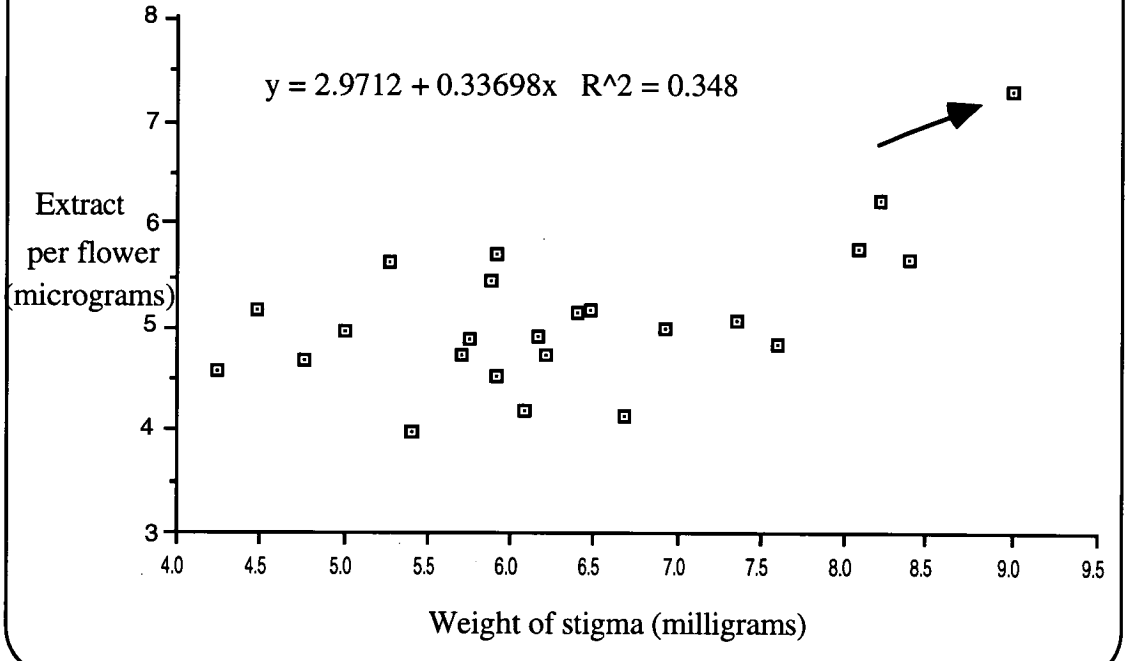
There were no significant relationships between either the number of oil glands, the density of oil glands and the yield of extract when expressed per flower, or as a concentration. There is a non-significant trend for larger petals to have more oil glands than smaller petals (not shown), but the densities of the oil glands between petals of different sizes were similar.

5.8 The effect of weight of the stigma on extract yield

The stigmas of boronia flowers contain large amounts of extract components (Section IV.1.2) and therefore the effect of the weight of the stigma on the yield of extract from genetically different boronia flowers was examined (Section IV.5.1.G). There was a non-significant trend for flowers with a heavier stigma to have more extract per flower (Figure 5.8). There were no other significant trends including when extract yield was expressed as a concentration, or when the weight of the stigma was expressed as a proportion of the total flower.

Figure 5.8

Relationship between stigma weight and extract yield per flower.



5.9 Discussion

The variation in extract concentration and composition in flowers from genetically identical plants grown in one environment is very low, although flower and extract yield per plant may vary significantly between plants (Section IV.5.5). Therefore, any variations which occur between genetically different plants in one environment over a small area are the result of genetic differences alone. Within-site

differences such as soil variations and topographical differences may increase the natural variation.

There is greater natural genetic variation in the composition of boronia extract than in the yield of extract (Section IV.5.2). Generally, the yield of extract is between 0.42% and 0.5%, with some plants having yields as low as 0.33% and as high as 0.63%. The percentage of total volatiles is usually between 10% and 18%, but can be as low as 5% and as high as 25%. Relatively low genetic variability in extract yield compared with extract composition may be a result of low variation in the content of compounds which comprise the bulk of the extract; namely waxes, pigments and other non-volatile compounds. Some of these compounds may be products of primary metabolism and therefore may vary less compared with products of secondary metabolism such as monoterpenes, jasmonates and norisoprenoids. There may be strict regulation of compounds which are the products of primary metabolism or which are involved in primary functions such as compounds which are active in photosynthesis (pigments), in maintenance of turgor pressure (waxes) or cell structure. Some of the varied secondary compounds that are active components of aromatic extracts may arise by one-step processes from more primary compounds (for example degradation of carotenoids into norisoprenoids), or from small modifications to ubiquitous secondary compounds such as geranial. As such, these compounds may vary more between plants as a result of small genetic differences.

The comparison of extracts from four clones in one or several environments is made more difficult by the fact that the proportion of open flowers at any one time affects the yield and quality of extract from that plant (Section IV.4.9), and there is known to be variation in the timing of anthesis between the four clones. It has been observed that flowers on plants in the north of the state generally mature earlier than those in the south of the state (L. Peterson pers. comm.), probably as a result of higher spring temperatures in the north (Appendix 8). For this reason, the harvest dates for each plantation (Section IV.5.1.B) were selected to ensure that clones were at similar stages of anthesis at all sites studied. Surges Bay in the south was harvested early in September, when many flowers had not yet reached anthesis, and this may explain the high variability between clones and the low extract yields from clones #17 and #250 at this site (Appendix 10, Figure 5.3.A). The significance of the extract yields and analyses for these two clones at this site is limited because of this.

It appears from a study of a limited number of sites with quite similar soil types within a relatively small geographical distribution (200 kilometres distance) that there are few environmental factors which can be related to extract yield or quality. Each of the four clones differ in their response to the environment, a good clone in terms of high extract yield at one site may be a poor clone at another site. One could speculate that clone #5 suffers a limit to extract production but an enhancement to the percentage

of total volatiles and β-ionone at comparatively cold, wet sites such as those found at Longley and Scottsdale in comparison with warmer sites, for example St. Helens. Before too many conclusions can be drawn from this study it would be desirable to place all four clones in controlled environments and monitor their responses to changing conditions, and this is a suggested area of future work, perhaps with new clonal selections.

There are distinctive odours to the extracts obtained from each clone (R. C. Menary pers. comm.); the differences in organoleptic assessment of extracts from different clones is probably due to differences in minor compounds (Ohloff 1977) such as jasmonates, norisoprenoids and fatty acid derivatives.

There appear to be few floral characteristics which can be used as criteria for selection of new clones. Extract yield is not correlated with the weight of individual flowers (Section IV.5.6), with the number of oil glands per petal, with the weight of petals as a proportion of the whole flower (Section IV.5.7), or with the weight of the stigma (Section IV.5.8). There may, however, be some flowers with particularly large stigmas that also have high extract yield per flower (Figure 5.8, arrow). This indicates that clonal selection must be based on the performance of plants with regard to extract yield and composition, as well as other factors such as disease resistance and suitability for mechanical harvesting for example; two factors which are currently used to aid selection. It also indicates that activity of enzymes responsible for the production of extract components are more important than the number of oil glands or the weight of floral tissue such as petals and stigmas which are active in non-glandular production of secondary compounds.

There are few, if any, obvious environmental factors which can be linked with extract yield in boronia. The environment obviously has a strong influence given the variability of each clone between sites, however it is not unusual for genetic factors to be more significant than environmental factors (Patra *et al.* 1987). The six sites that were sampled were very similar with regard to soil type, rainfall and temperature regimens. There has been deliberate selection by the company involved in establishment of commercial boronia plantations to utilise sites which mimic the environments where natural stands of boronia occur in Western Australia, it can only be surmised that sites which are not so similar would have an even greater effect on the yield of boronia extract.

There are also no consistent patterns with regard to the effect of the age of the plant on flower and extract yield. Boronia plants are pruned each year after flowering. This removes most of the current seasons growth and keeps the plants short yet bushy. This process itself probably removes any effect that plant maturity may have on the number of flowers initiated each year, because flowers are only initiated on current season's laterals (Roberts 1989).

Increased extract yields per hectare can be obtained by a) selecting plants with the genetic capability of producing high yields of extract with suitable organoleptic properties; and b) by increasing the number of flowers initiated each year, possibly by the use of appropriate fertiliser application (Roberts and Menary 1994b). There are plants present in populations of genetically different plants with up to 50% higher extract yields than any of the clones currently grown. There are also plants present that produce particularly heavy flowers, and flowers with particularly heavy stigmas, and these may be of interest when clonal plants are selected in the future to increase the total weight of flowers on plants.

IV.6 Results

Metabolism of volatiles in flowers after harvest

Continued synthesis of secondary compounds may occur after harvest, and these experiments were designed to identify the effects on yield and composition of extract as a result of storage at room temperature and at -20°C after harvest. The effect of tissue disruption (hand-squeezing flowers), a process which may stimulate oxidative changes as a result of cellular breakdown and bring previously separated compounds into contact with each other, was also studied. The levels of free volatiles, volatiles which exist as glycosidic conjugates, and the activity of *in vivo* glycosidase enzymes after harvest were measured.

6.1 Materials and methods

6.1.A Preliminary investigations

Clone 5 flowers were obtained 2.5 hours after harvest. Duplicate 100g flower samples were 1) extracted immediately (+2.5 hours) (Section III.3.2); 2) kept at 26°C for 20 hours, then extracted (+22.5 hours); 3) hand-squeezed, left at 26°C for 20 hours, then extracted (squeezed +22.5 hours); or 4) frozen for four days, thawed, hand-squeezed, left at 26°C for 20 hours and then extracted (frozen, squeezed +22.5 hours). All extracts were analysed by GC (Section III.4.3).

6.1.B Effect of squeezing flowers prior to storage and extraction

Clone 5 flowers were obtained within one hour of harvest. Flowers were generally more mature than those used in Section IV.6.1.A, showing more signs of senescence (pigments were faded, some petals had abscised). Duplicate 100g flower samples were either 1) extracted immediately (Section III.3.2); 2) hand-squeezed then extracted immediately; 3) left for 21 hours at room temperature, then extracted; or 4) hand-squeezed, left at room temperature for 9, 21 and 31 hours, then extracted. Where indicated, each 100g sample of flowers was squeezed a standard number of times. Extracts were analysed by GC (Section III.4.3). Extracts from unsqueezed flowers at zero time (A), and extracts from squeezed flowers that were left at room

temperature for 9 (B), 21 (C), and 31 (D) hours were assessed for organoleptic quality (Section III.5).

6.1.C Comparison of changes in flowers and flower buds after harvest

Flowers from pot-grown clone 5 plants were used. Samples comprising five buds or flowers were excised from plants and placed into 7ml glass vials. At zero time, and after 4 hours of storage at room temperature, triplicated samples of each bud stage and open flowers were extracted (Section III.3.3). To enhance extraction of buds, two petals were excised from each bud, to ensure solvent came into contact with the stigma, petals so removed were included in the extraction. Samples were analysed by GC (Section III.3.3).

6.1.D Free and glycosidically-bound volatiles and activity of glycosidase enzyme(s) during storage of flowers after harvest

6.1.D.1 Experiment No.1, 8 - 14 September 1994

Flowers from 3 year old clone 5 plants grown at St. Helens were used, harvested at approximately 80% open flowers. Flowers were stored in cardboard apple boxes with plastic liners and cardboard lids; air exchange with the environment was minimal, and light was excluded. The boxes contained about 7kg of flowers each. Two boxes containing open flowers and large buds (stage #4) (Section IV.4.2) were used, designated as 'open flowers' box#1 and box#2). Box #1 was sampled throughout the entire experiment; box #2 was sampled only during the second half of the experiment, after 54 hours of undisturbed storage after-harvest. This was to allow comparison of changes that result from storage in an enclosed environment, with changes resulting from storage in an environment that is continually being disturbed by introduction of fresh air. One box containing buds at stage 3 and smaller (Section IV.4.2), and some leaf material was also examined during post-harvest storage, designated as 'buds and leaf'. This box was sampled throughout the entire experiment. The plant material in the 'buds and leaf' box represents material that has been separated from open flowers and larger buds by sieving, and is not generally retained for commercial extraction.

Into each box was placed a Delphi Temperature Logger (model #861, lithium cell, developed in association with the Meat Industry Research Institute of New Zealand); a so-called 'thermo-logger'. The thermo-loggers monitored the temperature

every 15 minutes. A print-out of the temperature fluctuations experienced by each logger was obtained at the completion of the experiment.

* At regular intervals throughout the experiment, samples of approximately 50g were taken from each box. Half of each 50g sample was immediately made into an acetone powder (Section III.8.1), which was subsequently stored at 4°C ('crude' enzyme). The activity of glycoside-hydrolysing enzymes within the acetone powders was assayed (Section III.9.2.C).

The other half was immediately frozen at -20°C. From frozen samples, 'precursor solutions' were made (Section III.9.2.A) and incubated with β–glucosidase (Section III.9.2.B).

6.1.D.2 Experiment No.2, 12-16 October 1994

The experiment described in Section IV.6.1.D.1 was repeated at a later date during the same flowering season, using clone 5 flowers from 3 year old plants from Longley. These flowers were more mature, and came from plants on which 90% or more of flowers were open; pigments were faded and some petals had abscised. Two boxes of open flowers were used, box #1 was sampled throughout the entire experiment, box #2 was sampled only after 48 hours of undisturbed storage. In this experiment, thermo-loggers were attached to the outside, and placed inside each box to compare external and internal temperatures during the experiment. Samples were taken and analysed as in Section IV.6.1.D.1 from * onwards.

6.1.E The effect of freezer storage on extract yield and composition

Clone 5 flowers from one plantation were used, obtained 2.5 hours after harvest. Flowers were extracted immediately (Section III.3.2), or frozen at -20°C in duplicated 100g lots for specified periods of time, after which they were extracted (Section III.3.2), followed by GC analysis (Section III.4.3).

6.2 Results of preliminary investigations

Preliminary experiments examined the effects of storage of fresh, frozen and squeezed flowers after harvest on the yield and composition of floral extract (Section IV.6.1.A). Flowers in control samples were extracted immediately after harvest and transportation to the laboratory; fresh flowers were stored in polythene bags; other flowers were hand-squeezed a standard number of times to bring about cellular destruction and oxidative changes and then stored in polythene bags; and other flowers were treated in this last manner after freezing and thawing. The results are presented in Table 6.2

Table 6.2. Effect of post-harvest treatments on extract yield and composition.

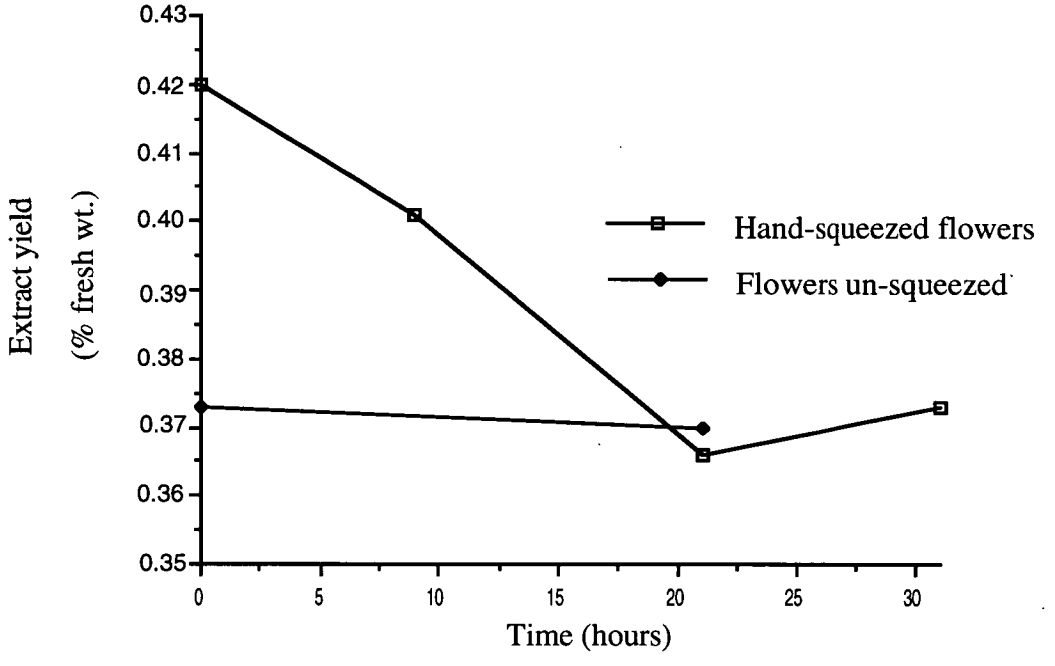
Treatment	% extract	% volatiles	% β–ionone in volatiles	% β–ionone in extract
Control	0.462	18.01	30.25	5.45
Stored 20 hours	0.465	15.97	34.75	5.55
Squeezed and stored 20 hours	0.557	18.66	39.34	7.34
Frozen, squeezed, and stored 20 hours	0.494	14.21	30.97	4.40

The yield of extract is increased when flowers undergo some form of tissue disruption, (i.e. are hand-squeezed), prior to storage at room temperature for 20 hours. There is an increase in β–ionone as a proportion of total volatiles in fresh flowers which were left at room temperature for 20 hours; the concentration of β–ionone in the extract is enhanced if flowers undergo tissue disruption prior to storage. Extract yield was unaffected in flowers which had been frozen prior to squeezing and storage, however a decrease in total volatile content in extract from such flowers occurred. Further investigations into the rate of change of secondary components in fresh, squeezed and un-squeezed flowers after harvest were made.

6.3 Changes in squeezed and un-squeezed flowers after harvest

The effects of squeezing flowers (i.e. tissue disruption), followed by storage at room temperature were studied in more detail (Section IV.6.1.B). All flowers were fresh, and obtained soon after harvest. The yield of extract from flowers treated to various squeezing and storage treatments is presented in Figure 6.3.A; the total volatile contents in the extracts are illustrated in Figure 6.3.B; and the proportion of total volatiles comprising β–ionone is presented in Figure 6.3.C.

Figure 6.3.A Extract yield from flowers stored at room temperature after harvest, with and without tissue disruption (squeezing).



Treating flowers with some form of gentle tissue disruption such as hand-squeezing prior to extraction increases extract yield. Storage of squeezed flowers at room temperature causes a depletion in extract yield, such that after 20 hours, the yield enhancement resulting from squeezing is completely lost. Extract yield from un-squeezed flowers does not alter during 20 hours of post-harvest storage.

Figure 6.3.B
Total volatiles (% in extract) from flowers stored at room temperature after harvest, with and without tissue disruption.

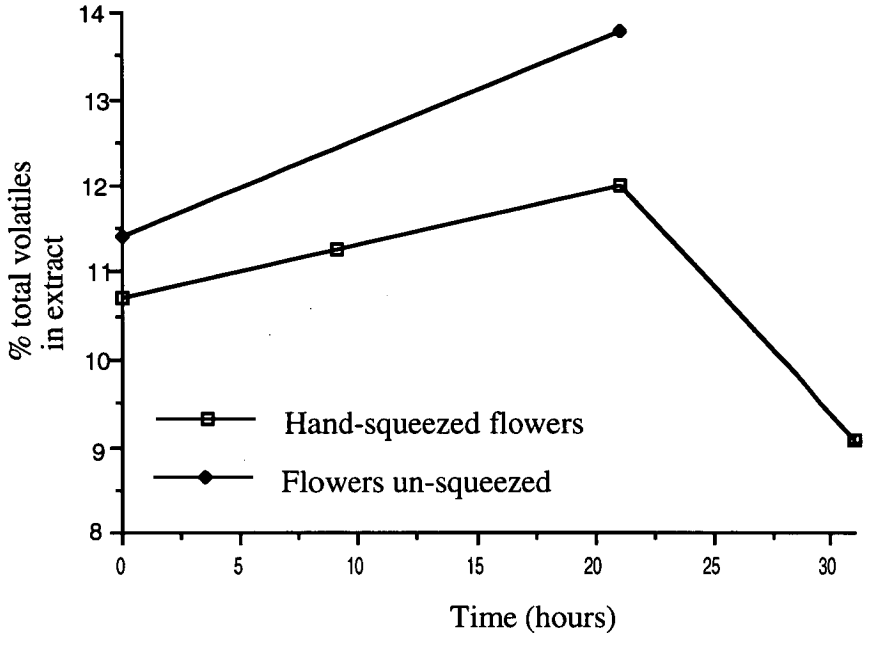
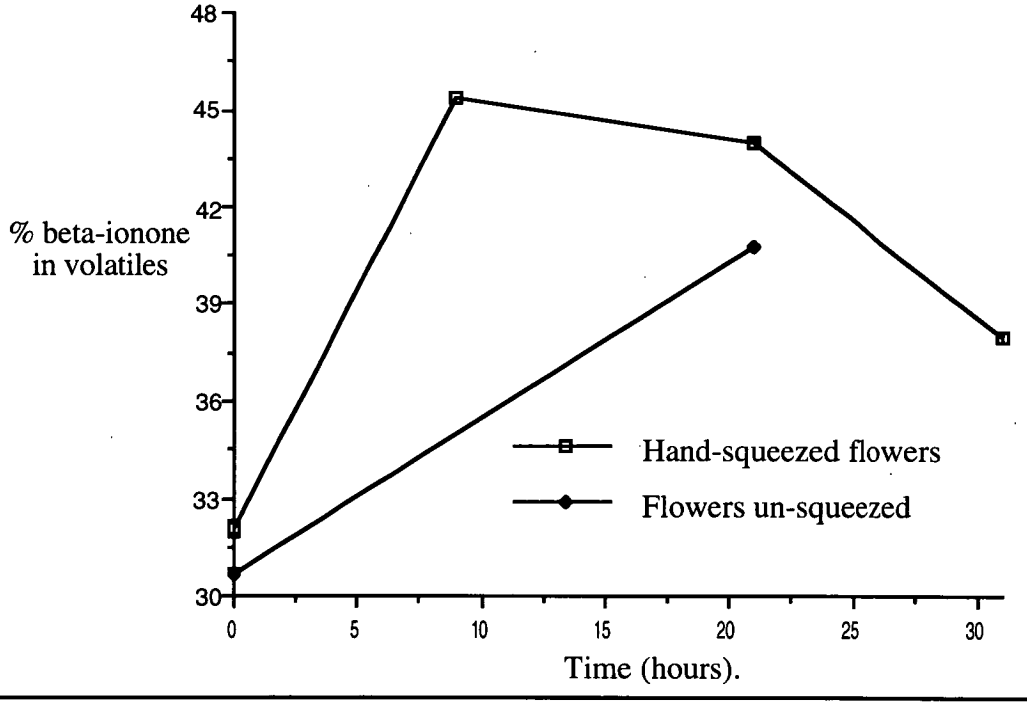


Figure 6.3.C
beta-Ionone(% of total volatiles) in extract from flowers stored at room temperature after harvest, with and without tissue disruption.



Increased levels of total volatiles and β–ionone as a percentage of total volatiles occurred in extract from flowers which were stored at room temperature for up to 20 hours, regardless of the amount of tissue disruption that occurred prior to storage. Storage for longer than 20 hours reduced the percentage of total volatiles in extract from squeezed flowers, however β–ionone (as a percentage of total volatiles) remained increased compared with zero time values.

Organoleptic assessments were made of extracts from fresh flowers (immediately after harvest) (A); squeezed flowers that had been stored for 9 hours (B), 21 hours (C), and 31 (D) hours after harvest. Samples were ranked according to their perceived odour from 'most floral' to 'most citrus'. A and B were described as 'floral', C was described as 'citrus/floral', and D, as 'citrus'. They were initially ranked from most desirable quality to least desirable quality:

$$C > B > A > D.$$

After fifteen minutes the solutions were re-examined to assess their lasting quality, and they were then ranked as:

$$B > A > C > D.$$

Therefore, storage of squeezed flowers for 9 hours after harvest noticeably improves the organoleptic quality of the extract.

6.4 Changes in β–ionone levels after harvest in flowers compared with flower buds

The aim of this experiment was to determine whether flower buds prior to anthesis also have the potential to produce increased levels of volatiles during storage after harvest. The level of β–ionone in fresh, un-squeezed flowers and buds before and after 4 hours of post-harvest storage are presented in Table 6.4.

Table 6.4. β–Ionone in flowers and flower buds before and after post-harvest storage.

Developmental stage	Time (hours)	β–ionone ± std. error (micrograms)	Change after storage (micrograms)
Open flower	0	61 ± 1.7	
(stage #5)	4	* 82 ± 4.39	* + 21
Large bud	0	28.2 ± 2.7	
(stage #4)	4	27.6 ± 1.6	- 0.6
Medium-size bud	0	nil	
(stage #3)	4	nil	nil

* means significantly different from zero time value at 5% level of significance.

Open flowers are the only developmental stage that is capable of producing increased levels of β–ionone during post-harvest storage.

6.5 Free and glycosidically-bound volatiles, and activity of glycosidase enzyme(s) in flowers after harvest

There were two experiments performed to examine the possibility that increased levels of volatiles in flowers after harvest was the result of cleavage of volatile-glycoside conjugates. The first experiment (Section IV.6.1.D.1) used flowers harvested earlier in the flowering season (80% open flowers), and the second (Section IV.6.1.D.2), used flowers harvested when 90% or more of flowers on most plants had reached anthesis. These experiments were intended to supply information regarding the effect of harvest date (i.e. % open flowers) on the potential for post-harvest changes to secondary compounds.

6.5.A Experiment No.1, 8-14 September 1994

The temperature profiles inside the three boxes containing floral material undergoing post-harvest storage (Section IV.6.1.D.1) are presented in Appendix 12. The two boxes which were sampled throughout the entire experiment reached 35°C (open flowers, box #1) and 30°C ('buds and leaf') in the first 50 hours of storage after harvest. After 50 hours, the temperature within both boxes which were regularly sampled decreased to 23-25°C. The box of open flowers which was sampled only after the first 50 hours of storage, reached a temperature of only 23-25°C during the first 50 hours, and subsequently maintained this temperature (23°C) for a further 48 hours. After 100 hours of storage, the temperature increased to almost 30°C until the completion of the experiment.

Figures 6.5.A and 6.5.B illustrate the changes observed in flowers during post-harvest storage in the levels of free volatiles and glycosidically-bound volatiles, and in the activity of enzyme(s) capable of glycosidase cleavage (boxes #1 and #2). The level of free volatiles is the concentration extracted from flowers by an aqueous buffer, and is therefore not comparable with the concentration obtained by organic solvent extraction.

Figure 6.5.A Changes in free volatiles, glycosidically-bound volatiles and activity of glycosidase enzymes during post-harvest storage. Experiment No.1: 8-14 September 1994, Open flowers box #1.

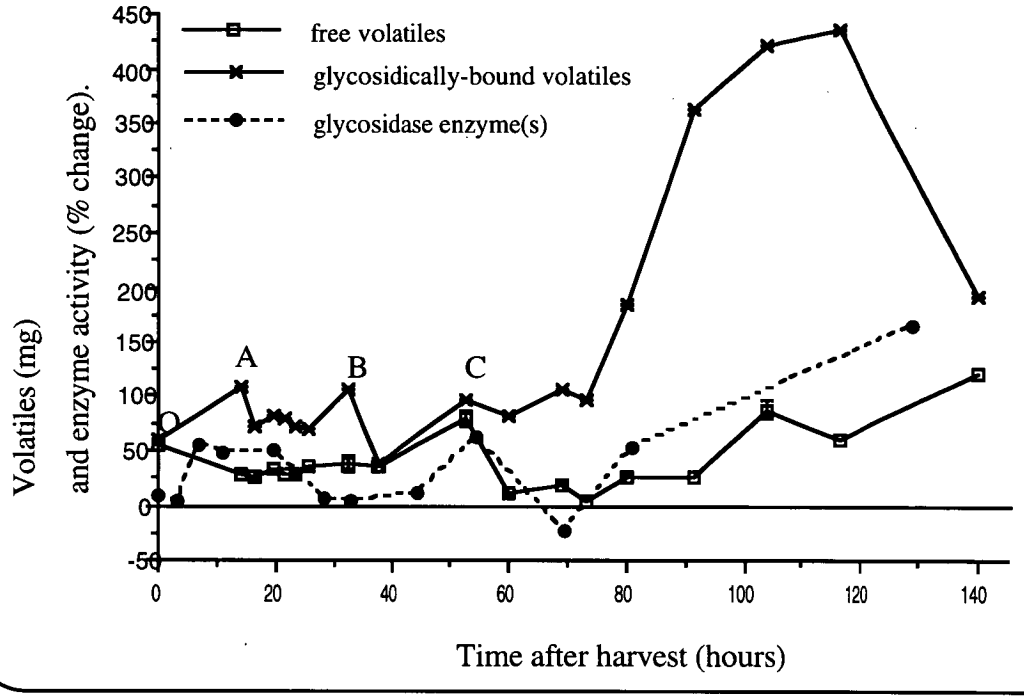
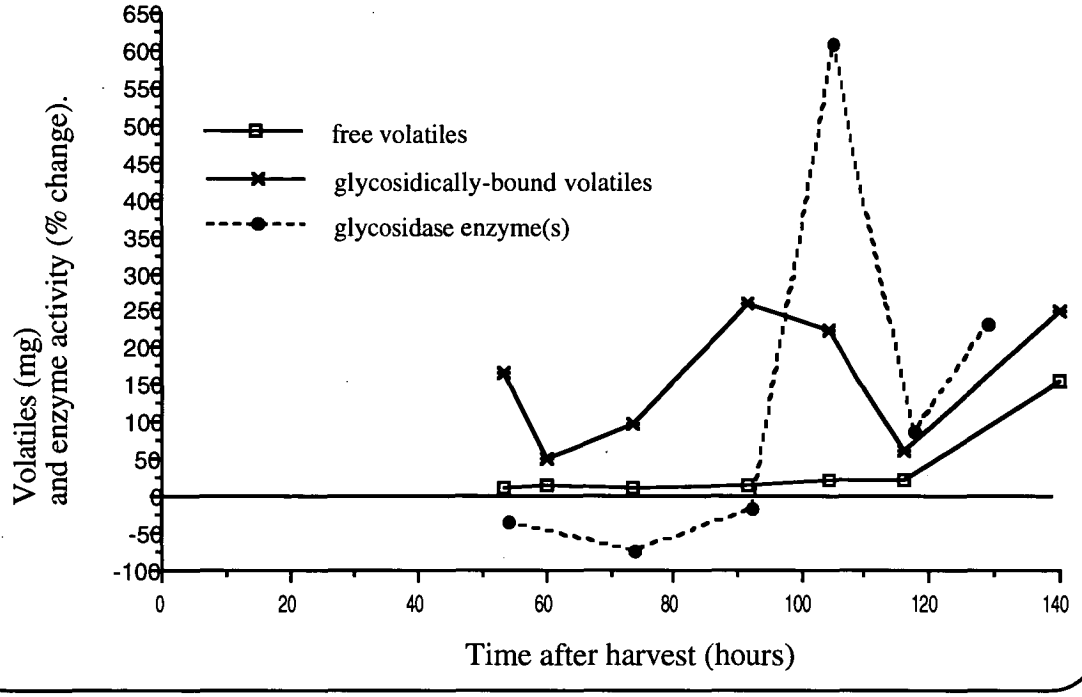


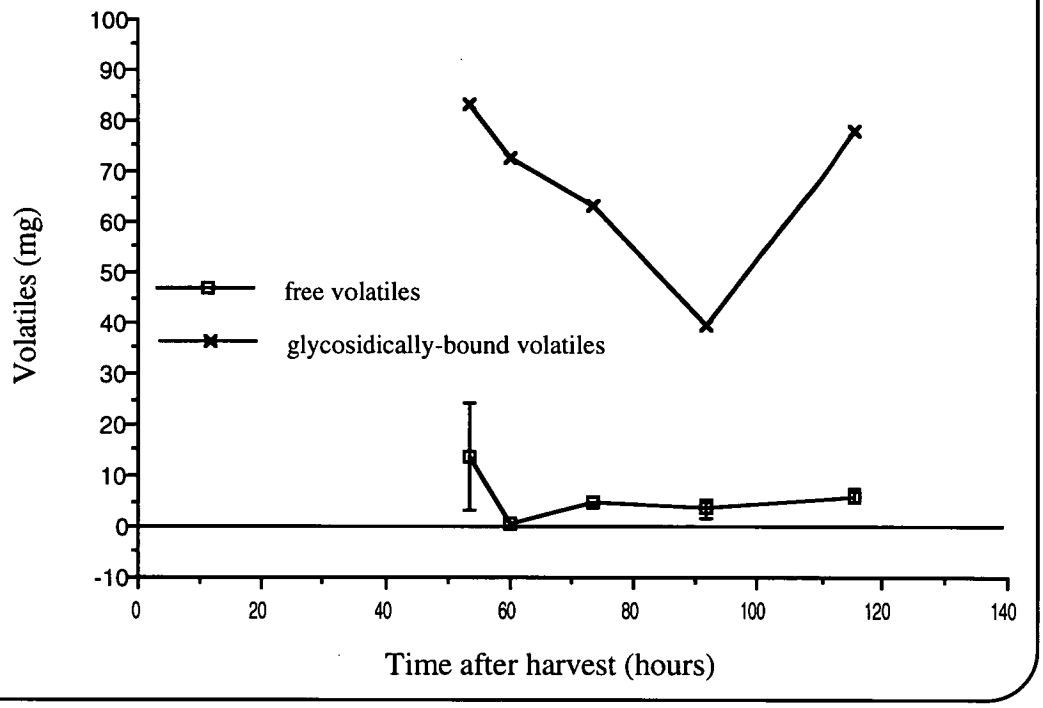
Figure 6.5.B Changes in free volatiles, glycosidically-bound volatiles and activity of glycosidase enzymes during post-harvest storage. Experiment No.1: 8-14 September 1994, Open flowers box #2.



A general pattern may be observed, most obvious where an increase in the level of glycosidically-bound volatiles occurs after about 70 hours of storage (in both Figures 6.5.A and 6.5.B). Subsequent to an increase in the levels of glycosidically-bound compounds, the activity of glycosidase enzyme(s) increases. Concomitantly, an increase in the level of free volatiles occurs, followed by a decline in the level of glycosidically-bound volatiles. There are short (20 hour) cycles similar to the one described above in the levels of all assayed compounds and enzyme activity during the first 60 hours of storage (Figure 6.5.A). The main difference between the regularly sampled box #1 (Figure 6.5.A) and box #2 which was sampled only after 55 hours of storage (Figure 6.5.B) is that the level of free volatiles declines (compared with zero time) between zero-45 hours and between 55-100 hours in box #1, and in box #2 it remains fairly constant between 55 and 115 hours, after which it increases.

Figure 6.5.C illustrates the levels of free and glycosidically-bound volatiles in a small number of samples of 'buds and leaf' during post-harvest storage. Activity of glycosidase enzyme(s) was not assessed in 'buds and leaf' samples.

Figure 6.5.C. Changes in free and glycosidically-bound volatiles in 'buds and leaf' during post-harvest storage. Experiment No.1: 8-14 September 1994.



While the level of free volatiles remains fairly constant between 50 and 100 hours of storage, the level of glycosidically-bound volatiles decreases until after 90 hours of storage, after which it increases again.

6.5.B Experiment No.2 12-16 October 1994

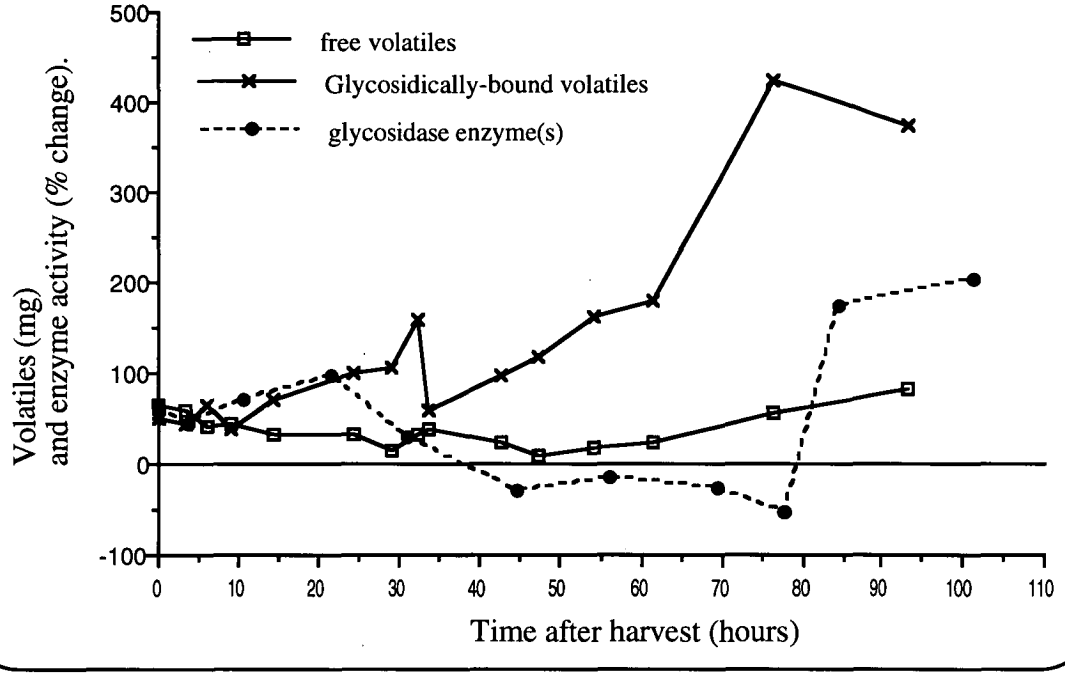
In this experiment, harvested when more than 90% of the flowers were open (Section IV.6.1.D.2), only two boxes of flowers were used. One box (box #1) was again sampled throughout the entire experiment, the other box (box #2) was sampled only during the second half of the experiment. The other difference compared with the previous experiment (Section IV.6.1.D.1) was that in this experiment, thermo-loggers were placed both within the boxes and attached to the external surface of the boxes to assess the effect of the external environment on the flowers within.

The temperatures within boxes of flowers stored after harvest for more than five days may be seen in Appendix 13. In box #1, the temperature within the box increased to 30°C in the first 24 hours, and was maintained at this level except for a small decrease after 50 hours, probably in response to a decreased external temperature; the temperature increased to 30°C again after this time. In the box of flowers which were not sampled until 50 hours of storage time had elapsed (box #2), the temperature within the box increased more slowly, reaching 25°C after 30 hours of

storage, and maintained this temperature for the next 60 hours, after which it began to decline.

Figure 6.5.D illustrates the changes in the levels of free volatiles, glycosidically-bound volatiles and activity of glycosidase enzyme(s) during the storage of open flowers after harvest.

Figure 6.5.D Changes in free volatiles, glycosidically-bound volatiles and activity of glycosidase enzymes during post-harvest storage. Experiment No.2: 12-16 October 1994, Open flowers box #1.



An increase in glycosidically-bound volatiles begins after 45 hours of storage, and continues until 80 hours of storage, after which levels decline. A rapid increase in activity of glycosidase enzyme(s) occurs between 78 and 85 hours of storage and then plateaus. The level of free volatiles is reduced during storage of up to 85 hours, compared with the level at harvest.

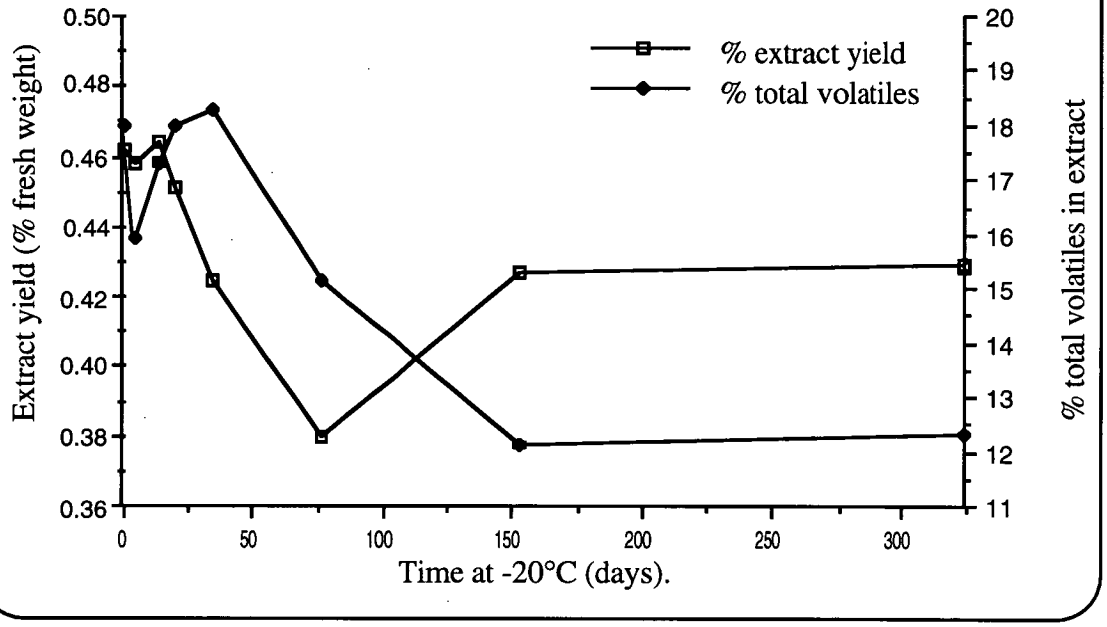
The levels of free and bound volatiles in box #2 (sampled only during the second half of the experiment) were not assessed, however the activity of glycosidase enzyme(s) in flowers from box #2 (not shown) mirrored that seen in box #1 (Figure 6.5.D).

Generally, the levels of free volatiles are less changeable than the levels of glycosides and glycosidases. Large changes in the levels of glycosides are not always reflected in the levels of free volatiles.

6.6 The effect of freezer storage

It is known that the effects of oxidative reactions are enhanced upon prolonged storage. The effect of such reactions on secondary compounds in boronia flowers was deemed worthy of study. The yield of extract and some aspects of extract composition during 324 days of freezer storage at -20°C were studied (Section IV.6.1.E). The effect of freezer storage on extract yield and the proportion of extract comprising volatile compounds is presented in Figure 6.6.A.

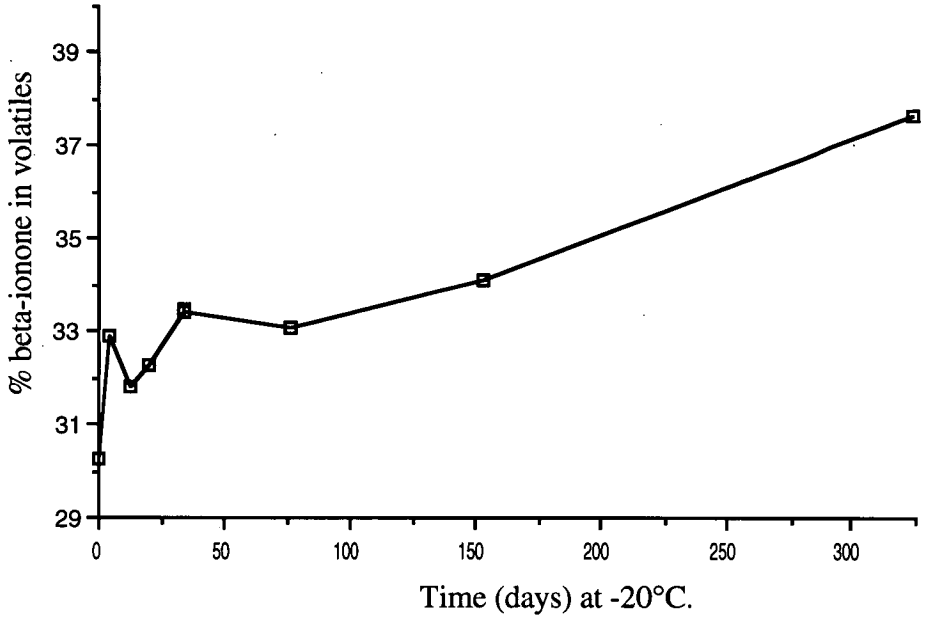
Figure 6.6.A Change in extract yield and % volatiles in extract during freezer storage.



The yield of extract is reduced after 34 days of freezer storage, and is maintained at a reduced level over the next 300 days of freezer storage. The proportion of volatile compounds decreased after 75 days of freezer storage; levels continue to decline for the next 75 days, after which volatile levels remain constant until 324 days of storage. The effect of freezer storage on β–ionone expressed as a proportion of total volatiles in the extract is presented in Figure 6.6.B.

Figure 6.6.B

Effect of freezer storage on beta-ionone (% of total volatiles in extract).



The relative concentration of β–ionone in volatiles from flowers frozen for between one and 150 days is increased compared with unfrozen flowers. A further increase occurs over the next 150 days.

6.7 Discussion

The process of gentle tissue disruption by hand-squeezing flowers initially increases the yield of extract (Section IV.6.3; also Section IV.7.3), presumably by allowing better access of the solvent to thick tissues such as the stigma. As a result of prolonged storage (20 hours) after squeezing, the yield enhancement is lost, presumably through the action of oxidative and degradative processes acting on extract components other than volatile compounds. There is evidence for continued production of volatile compounds during storage of flowers at room temperatures immediately after harvest (Section IV.6.3); the action of tissue disruption may alter the nature of the post-harvest changes. This result is akin to the findings of other researchers (Tyutyunnik and Ponomaryova 1977). The effect of post-squeezing storage on flowers varies between experiments, particularly between Sections IV.6.2 and IV.6.3. In Section IV.6.2 all factors related to extract yield and quality were increased by squeezing followed by storage for 20 hours; in the Section IV.6.3 extract yield was initially enhanced and subsequently declined to similar levels as those from unsqueezed flowers while extract composition mirrored changes seen in unsqueezed

flowers during storage. This is most likely caused by the predominance of older, more mature flowers in the second experiment compared with the first (Section IV.6.1.B). Flowers which are more mature and have begun senescence appear to have less ability to undergo increased extract yield and quality during post-harvest storage than younger flowers. Presumably loss of active enzymes and retrieval of substrates from the flower prior to abscission have affected the biosynthetic ability of the flower.

The amount of β–ionone as a proportion of total volatiles increases to maximal levels after between nine and 21 hours of post-harvest storage (Section IV.6.2), and the concentration in the extract can be increased by 50% by squeezing flowers prior to storage (Section IV.6.2). This may be the result of oxidation of compounds such as β–carotene, or hydrolysis of stored glycosides. Or, it may represent preferential volatilisation of more volatile compounds such as monoterpenes, causing an apparent increase in the concentration of less volatile compounds such as β–ionone. When frozen flowers are thawed, squeezed and stored, there is no subsequent increase in β–ionone levels, but there is degradation of other volatiles. Enzymatic and non-enzymatic oxidation, hydrolysis or volatilisation of volatile compounds occurs after harvest and upon tissue disruption. This suggests that the enzyme(s) responsible for the production of β–ionone after harvest are sensitive to freezing (Section IV.6.2); they are also enhanced by tissue disruption (Section IV.6.3) and active only in open flowers (Section IV.6.4). The enzyme(s) responsible may occur in organelles or cells not normally in contact with substrates such as β-carotene, which is plastid-localised. Tissue disruption may improve enzyme/substrate contact, increase the availability of cofactors, or oxidise cofactors such as linoleate, which may be necessary for the reaction (Firn and Friend 1972). Destruction of carotenoids has been shown to occur by enzymes isolated from organelles including plastids (Friend and Mayer 1960) and mitochondria (Dicks and Friend 1967). The enzymes could be either lipoxygenases (Blain *et al.* 1968) or haemoprotein-type enzymes (Blain and Styles 1959), both of which are aerobic.

The temperatures in stored boxes of flowers which were regularly opened and disturbed during the first 50 hours of storage after harvest were generally at least 5°C higher than in undisturbed boxes during this time. This is indicative of higher respiratory activity in disturbed boxes as a result of the replacement of oxygen and removal of ethylene and carbon dioxide during sampling, high levels of which in closed systems will inhibit respiration. Higher maximum levels of glycosidically-bound volatiles and higher maximum activity of glycosidase enzyme(s) occurred in boxes which were continually disturbed, compared with those which were undisturbed during the first 50 hours of storage (Figure 6.5.A compared with Figure 6.5.B). This indicates that most enzymes may have been stimulated by higher respiratory activity and higher temperatures in disturbed boxes. There is a direct relationship between the

rate of respiration (aerobic) and changes in essential oil yield after harvest (Tyutyunnik and Ponomaryova 1977). Enzymic activity after harvest may degrade larger metabolites, catalyse synthesis of secondary compounds *de novo*, or continue with pre-determined processes which are characteristic of senescence, the rate of which may be increased as a result of excision from the plant.

During the first 35 hours of storage of open flowers there is more variability in the levels of glycosidically-bound volatiles and glycosidase enzyme(s) than in the levels of free volatiles. Loss of free volatiles by volatilisation occurs prior to harvest (Section IV.1.3), and presumably continues in open flowers after harvest. In the first 60 hours after harvest there are certain patterns in the levels of free and bound volatiles and hydrolytic enzymes, particularly evident in samples in which most flowers were newly opened (Section IV.6.5.A, Figure 6.5A) compared with those in which the majority of flowers were older and more senescent (Section IV.6.5.B, Figure 6.5.D). In less senescent flowers, there is a series of peaks in the levels of glycosidically-bound volatiles (Figure 6.5A, zero-60 hours), the maxima of which (O, A, B, C) are approximately 20 hours apart. Between 'O' and 'A', the levels of free volatiles declines, and the activity of hydrolytic enzyme(s) increases and remains constantly high. Between 'A' and 'B', the level of glycosidically-bound volatiles declines, after which the activity of hydrolytic enzymes also decreases (free volatiles are constant). Between 'B' and 'C', the same pattern in glycosidically-bound volatiles occurs, and as the activity of hydrolytic enzymes increases, the levels of free volatiles also increase. After 60 hours there is less control of equilibrium levels, i.e. the concentration of glycosidically-bound volatiles increases to far higher levels, followed by increased activity of hydrolytic enzymes and an increase in free volatiles. Flowers during the first 60 hours after harvest appear to maintain equilibrium levels of free and bound volatiles by activity of hydrolytic enzymes, and presumably also by activity of enzymes responsible for forming free volatiles *de novo* and glycosidic linkages (not assayed). This indicates *in vivo* regulation of formation of free and bound volatiles. The length of time between maxima of glycosidically-bound volatiles (20 hours) during the first 60 hours suggests the occurrence of circadian rhythms in accumulation and degradation of such compounds. The lack of similar rhythms in free volatiles may be caused by increased rates of volatilisation of free volatiles as a result of the high temperatures reached in the boxes (Section IV.6.5). The 20-hour rhythms in glycosidically-bound volatiles are less obvious after 60 hours, and in Experiment No.2, in which flowers were at a later stage of senescence. This may indicate that senescence removes the control of regulation of such compounds, and that the act of storage increases the rate of senescence and associated reactions.

The loss of volatiles from stored flowers occurs even at -20°C (Section IV.6.6), a factor of commercial significance. The storage of flowers at -20°C has a

finite limit after which depletion in yield and composition of product will ensue. Storage at lower temperatures (-70°C for example) may improve the otherwise deleterious effects of freezer storage for longer than one month. The depletion of volatiles at freezing temperatures is probably caused by autoxidative changes occurring, reducing the amount of volatile material present; the activity of enzymes would be negligible at such temperatures.

The increased level of free volatiles in flowers during the first 20 hours after harvest (Sections IV.6.2 and IV.6.3), is not only caused by enhanced cleavage of volatile-glycoside moieties, although enzymes capable of this are active during this time. It is likely that most enzyme types are enhanced as a result of increased respiratory activity at increased temperatures reached during storage in an insulated, but not an anaerobic environment. The synthesis of glycoside moieties is increased after 60 hours of post-harvest storage, and a small amount of free volatiles may be released after this time by activity of *in vivo* glycosidase enzymes.

IV.7 Results
The process of extraction

A rapid, precise and complete extraction method was required for laboratory use for the studies presented in this thesis, and for the routine analysis of boronia flowers. Commercially, the solvent used for the extraction of boronia flowers incurs one of the major costs for production. Solvent can be recovered, redistilled and re-used, however these processes do not recover 100% of the solvent, and are costly and time-consuming. Freezer storage for longer than one month results in depletion of volatiles, therefore time is of the essence in the processing of the large amounts of boronia flowers harvested each year. To minimise capital investment in extractors, a maximum throughput of flowers in this equipment is also necessary. The effect that extraction constraints such as time and the washing regimen have on the final yield needed consideration. The potential for producing different products by different extraction strategies was also investigated.

7.1 Materials and methods

7.1.A Solvent

The solvent used was commercial grade petroleum ether (boiling point 40-60°C). Other solvents used were AR grade, re-distilled. Solvent was not re-used in experimental treatments.

7.1.B Pilot-scale extraction method

A 200L drum which had been modified so that it revolved and tipped back and forth when placed on a motor-driven belt was used. This was placed on an electric balance of capacity 250kg (+/- 0.5kg). 10kg of frozen flowers which had been left at room temperature for 24 hours were placed into the extractor along with an appropriate volume of solvent (assessed by weight using the density of the solvent). The flower weight:solvent volumes examined were 1:6, 1:3 and 1:1.5; solvent volume was divided evenly between three washes.

After one hour of constant movement, the extractor was stopped. Solvent was decanted and the weight change of the extractor was noted as an assessment of the volume of solvent removed. Some solvent was lost as a result of evaporation and this was recorded. Solvent was replenished and the extraction continued for * 30 minutes for the second wash, and 15 minutes for the third wash. External temperature conditions were similar throughout the experiment.

At the completion of the extraction the flowers were discarded. Solvent from the three washes was mixed together, and a 500ml sub–sample was taken. The solvent/extract was dried down at 60°C for five minutes after the last traces of solvent were removed. The yield of extract was calculated using final (recovered) solvent volumes. Samples were analysed by GC (Section III.4.3) and by organoleptic assessment (Section III.5)

* For the time trial, all three washes were of 60 minutes duration, and total flower weight: solvent volume ratio was 1:6.

7.1.C Commercial-scale extraction method

Verification of lab-scale and pilot scale experiments were done on a commercial scale by P. Coulson of Essential Oils of Tasmania. The experiments used 535kg of thawed flowers for each batch, and each protocol was repeated twice. The protocols examined were:

Protocol/Wash number	Flower weight:solvent volume ratio	Time per wash
Protocol #1	1 : 7.3 (535kg:3900L total)	9 hours total
wash #1	1 : 3	3 hours
wash #2	1 : 3	4 hours
wash #3	1 : 1.5	1.5 hours
wash #4	1 : 1	0.5 hours
Protocol #2	1 : 5.8 (535kg:3100L total)	8 hours total
wash #1	1 : 2.5	2 hours
wash #2	1 : 2	4 hours
wash #3	1 : 1	1.5 hours
wash #4	1 : 1	0.5 hours
Protocol #3	1 : 4.7 (535kg:2500L total)	7 hours total
wash #1	1 : 2	1 hour
wash #2	1 : 1	4 hours
wash #3	1 : 1	1 hour
wash #4	1 : 1	1 hour

7.2 The effect of solvent and time on extraction of pigments and volatiles

Petals were extracted for 20 hours in hexane. During the extraction process, samples were taken and observed under a dissecting microscope (for epidermal discolouration) and a light microscope (to observe hand cut sections). The pigment distribution was observed and recorded.

Pigments: Anthocyanins rapidly become dull brown coloured and are depleted unevenly. Carotenoids in cells surrounding the oil gland and in the palisade layer below the epidermis on the adaxial surface remain after twenty hours of extraction. The contents of the oil glands were easily identified as grey, oily droplets. The contents were absent in samples that had been extracted for three and a half hours or more. None of the samples observed had ruptures in the epidermal caps of oil glands.

Pigments and volatiles: The absorption spectra and volatile levels from extracts of petals produced by extraction with dichloromethane (DCM), chloroform, acetone, ethanol and hexane, were analysed several times during a twenty hour extraction.

The carotenoid absorbance in hexane extracts was the same after fifteen minutes extraction as it was after twenty hours of extraction. β–ionone levels doubled between 15 minute and 3.5 hour hexane extractions, and remained the same between 3.5 and 20 hour extractions. DCM and chloroform extracted more carotenoids than hexane, by almost two-fold. Even larger amounts of carotenoid were obtained with acetone and ethanol, however these two solvents also extracted chlorophyll, flavonoids and water soluble components; the petals after extraction were completely devoid of pigments. In a separate experiment it was found that homogenisation of flower material greatly enhances carotenoid extraction.

DCM and hexane extracted similar amounts of β–ionone from petals; chloroform and ethanol extracted similar but reduced amounts compared with DCM and hexane. Acetone extracted twice the amount of β–ionone compared with hexane and DCM.

After extraction, whole petals were examined under the SEM; on none of the petals examined were oil glands with ruptured epidermal caps seen. Measurements of the thickness of the remaining cuticle were not made.

7.3 The effect of scale on laboratory extractions

Occasionally, only small sample sizes are available for extraction. A range of small sample sizes were examined for the extract yield produced. In all the (duplicated) extractions, flower weight : solvent volume ratio, time, and the washing regimen were kept constant. The results are presented in Table 7.3.

Table 7.3 Extract yield and composition from small samples.

Sample size	extract yield ± Std. error	% volatiles ± Std. error
100 g (control)	0.349 ± 0.008	16.75 ± 0.145
50g	0.360 ± 0.009	16.94 ± 0.0
25g	0.349 ± 0.032	18.02 ± 1.62
10g	0.392 ± 0.029	15.78 ± 1.04
5g	* 0.520 ± 0.024	* 11.28 ± 0.63

* means significantly different to control and all other samples at the 5% level of significance.

Extract yield and composition remain essentially the same in samples of between 10g and 100g. The variability increases in samples of 25g and smaller. Samples smaller than 10g have an increased extract yield, and depleted volatile levels.

7.4 The effect of flower treatment prior to, and during extraction

The effect of treatments supposedly causing tissue disruption, such as sonication and chopping up flowers prior to and during extraction, were examined using laboratory-scale extraction methods. The results may be seen in Table 7.4.

Table 7.4. Effect of flower treatment prior to and during extraction.

Treatment	% oil ± std. error	% volatiles	% β-ionone in extract
1. Control (no treatment)	0.343 ± 0.005	18.57	6.59
2. Sonication during extraction	0.347 ± 0.001	18.57	6.59
3. Hand-squeezing prior to each wash	* 0.381 ± 0.001	18.13	6.68
4. Agitation during extraction	0.349 ± 0.002	18.43	6.80
5. Chopping (each flower chopped into <10 pieces)	* 0.502 ± 0.011	18.0	6.33

* means significantly different to control at the 5% level of significance.

Treatments which included mechanical disruption of the flower tissue by squeezing and partial homogenisation (chopping flowers into 5 to 10 pieces) significantly increased the yield of extract obtained. The composition of major volatile components of the extract were not altered.

7.5 The effect of flower weight : solvent volume (g/ml)

The effects on extract yield of two solvent regimens was studied. The total volume of solvent over four washes, each of 60 minutes duration, was changed to test the flower weight : solvent volume ratios 1:4.3, 1:8.7 and 1:17.4. Duplicate extractions were made for each treatment. The results may be seen in Table 7.5.A.

Table 7.5.A Extract yield from extractions using different flower weight : solvent volume ratios.

Flower weight : solvent volume ratio (solvent volume = total from four washes)	% extract ± standard error
1 : 17.4	0.44 ± 0.01
1 : 8.7	0.460 ± 0.04
1 : 4.3	0.383 ± 0.02

There were no differences. Because this result may be of significance to the commercial method, a larger scale experiment, using the pilot-scale extractor was done. Solvent volumes were kept to a minimum to find the lowest possible level for addition before depletion of extract yield ensued. In this experiment, triplicate extractions were made for each treatment. The results may be seen in Table 7.5.B.

Table 7.5.B Results of pilot-scale flower weight : solvent volume experiment.

Flower weight: solvent volume (total of three washes)	% extract ± std. error	% volatiles ± std. error	% β−ionone in volatiles ± std. error
1 : 6 (control) Sample A	0.4138 ± 0.03	10.11 ± 1.34	36.87 ± 0.70
1 : 3 Sample B	* 0.3431 ± 0.013	10.99 ± 0.60	* 41.68 ± 0.79
1 : 1.5 Sample C	* 0.3325 ± 0.004	10.54 ± 0.34	* 45.22 ± 0.25

* means significantly different to control at the 5% level of significance.

A 50% reduction in the solvent volume used significantly reduced extract yield. A further 50% reduction had no further effect. The total volatiles in the extract did not vary with different flower weight:solvent volume ratios, however significantly more β–ionone (as a percentage of the total volatiles) was extracted at reduced solvent volumes.

Organoleptic assessment of the extracts

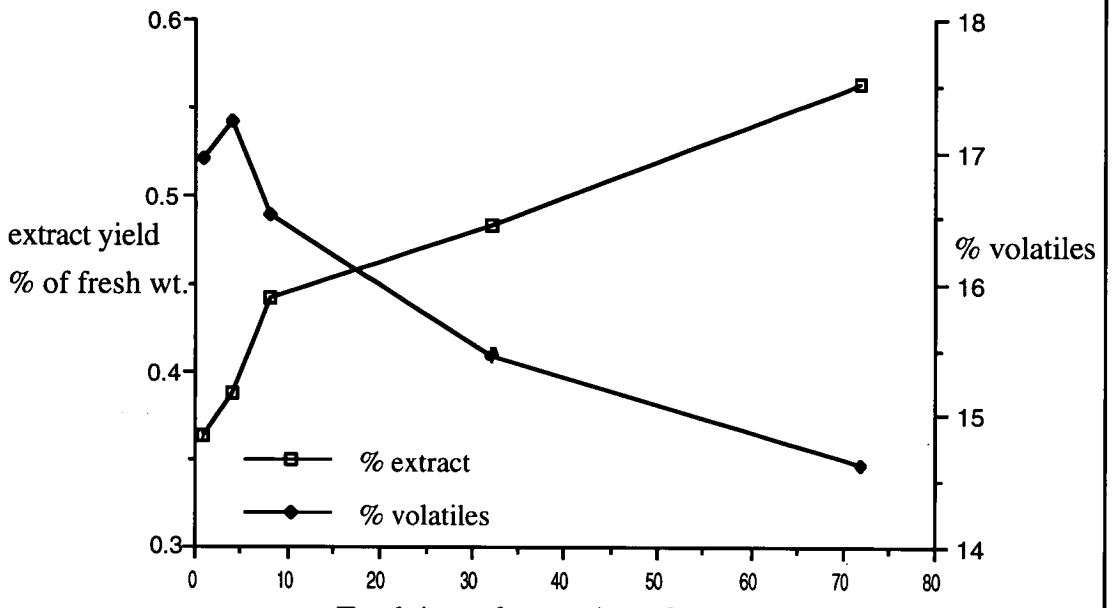
The samples were ranked according to their perceived quality from 'most floral' to 'most citrus' (Section III.5). Sample C (lowest solvent volume) was ranked highest in terms of quality compared with all others. Sample B was ranked after sample C, and Sample A (highest solvent volume) was ranked lowest, it had an oily smell and was quite unpleasant.

7.6 The effect of the duration of extraction

The total length of time boronia flowers are in contact with solvent during extraction is spread over a number of washes. The time of each wash and its affect on extraction yield may be different at different stages of the extraction (i.e. first compared with third washes).

The length of time of a complete extraction was increased evenly throughout four washes. The total yield of extract after complete extraction was assessed in duplicate extractions, and the extract was analysed by GC (Section III.4.3). The results are presented in Figure 7.6.A.

Figure 7.6.A. The effect of total extraction time on % extract yield and % volatiles.



There is a positive relationship between extraction time and extract yield up to 72 hours of extraction time. However, in samples which were extracted for longer than four hours, there is a depletion of volatiles.

The extract obtained from <u>each</u> wash of four sequential 15 minute washes, and four sequential eight hour washes was analysed. The same flower weights and solvent volumes were used in both extractions. The extract obtained from each wash may be seen in Figure 7.6.B. The analysis of the extract obtained from each wash is summarised in Figure 7.6.C.

Figure 7.6.B

Comparison of extract yield from 15 minute and 8 hour washes.

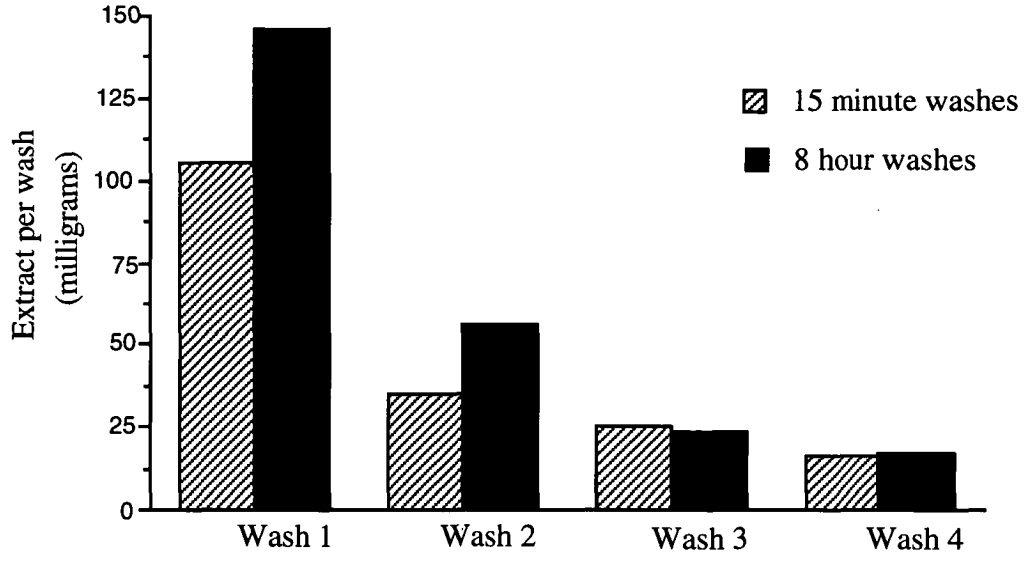
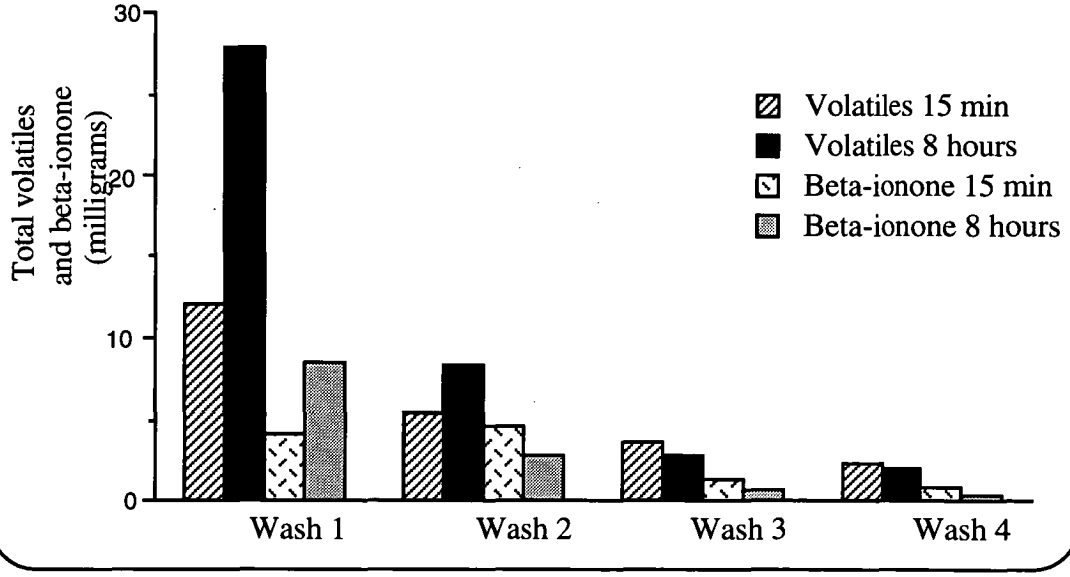


Figure 7.6.C Comparison of total volatiles and beta-ionone from 15 minute and 8 hour washes.



An increase in the length of each wash from 15 minutes to 8 hours increases the yield of extract and the percentage of total volatiles extracted per wash during the first two washes. The extraction of β–ionone per wash is increased only by an increase in the duration of the first wash. Subsequent washes extract the same amount of extract and volatiles regardless of the duration.

An experiment using the pilot-scale extraction facility was done to examine the effect of extraction time on the yield of extract obtained. A total flower volume of 1:6 was used, the first treatment getting a 60 minute, a 30 minute and a 15 minute washing regimen, and the second getting three washes of 60 minutes each. Each treatment was repeated in triplicate. The results may be seen in Table 7.6.

Table 7.6 The effect of extraction time in pilot-scale extractions

Washing regimen	% extract ± std error	% volatiles ± std. error	% β–ionone in volatiles ± std. error
60 + 30 + 15 minutes (control) Sample D	0.4138 ± 0.0295	10.11 ± 1.34	36.87 ± 0.70
60 + 60 + 60 minutes Sample E	0.4206 ± 0.0038	10.85 ± 0.26	* 38.89 ± 0.20

* means significantly different to control at the 5% level of significance.

In samples given a similar extraction time during the first wash, a reduction in the time of subsequent washes does not decrease extract yield significantly. However, a longer extraction time in the second and third washes may increase the amount of volatiles extracted, such as β–ionone.

Each of the extracts from the pilot-scale extraction experiment were organoleptically assessed (Section III.5). Sample D (less time) had a 'citrus' odour and an unpleasant oily odour, and was ranked lower than Sample E (most time) which had a typical odour: 'floral, rose'.

7.7 The effect of different extraction regimens

Using the same total extraction time (60 minutes) and total solvent volume, five different washing regimes were studied in duplicate for their effect on the total yield and composition of extract produced. The results are summarised in Table 7.7.

Table 7.7 Results of different washing regimens on extract yield and % total volatiles.

Washing regimen	Extract yield (%)	% volatiles
15 + 15 + 15 + 15 minutes **(control)**	0.363	16.94
30 + 15 + 15 minutes	0.329	16.02
30 + 30 minutes	0.329	15.08
45 + 15 minutes	0.322	15.47
60 minutes	0.308	15.89

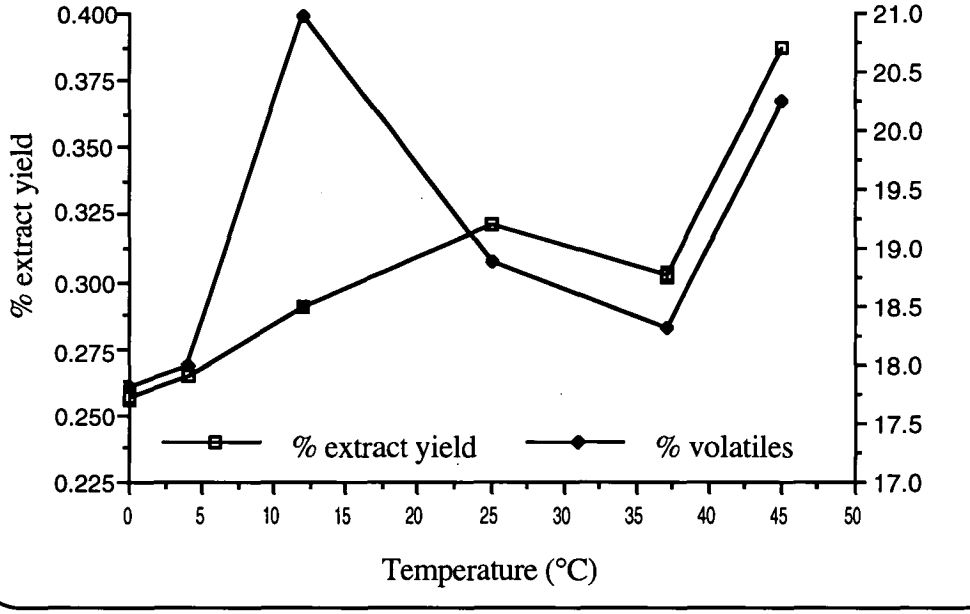
The extract yield and composition from extractions using two or three washes is similar. When four washes are used, extract yield and the percentage of total volatiles increases. When only one wash is used, low yield of extract is produced, however volatile levels are similar to those produced from three washes.

7.8 The effect of extraction temperature

The effect of increasing the temperature during an extraction involving one wash of two hours was studied in duplicate. It was necessary to add extra solvent to the extractions set at 45°C to counteract the effects of evaporation of solvent. The results are displayed in Figure 7.8.

Figure 7.8.

Extract yield and % volatiles at different extraction temperatures.



Increasing the extraction temperature increases oil yield and percentage of total volatiles. The maximum amount of volatiles over the temperature range studied are extracted at 12°C. Temperatures greater than 12°C deplete volatiles, with an exception at 45°C. At 45°C, evaporation of lower boiling point constituents of the solvent may change the extraction efficiency of the solvent, and this should be kept in mind.

7.9 Commercial scale verifications

Some of the findings described in this section were examined on a larger scale using the commercial-scale extractor (Section IV.7.1.C). Protocol #1 involved the highest solvent usage and longest time, and protocol #3 the lowest solvent usage and lowest time. The results are presented in Table 7.9*.

Table 7.9* Effect of solvent volume and time on extractions involving four washes in the commercial extractor.

Protocol Number	Description	% extract yield	% volatiles in extract	% β-ionone in extract
#1	1 : 7.3 kg:L 9 hours	0.47	10.51	2.75
#2	1 : 5.8 kg:L 8 hours	0.485	10.73	2.87
#3	1 : 4.67 kg:L 7 hours	0.41	11.72	3.0

*courtesy of P. Coulson, Essential Oils of Tasmania.

There is a slightly lower extract yield produced by protocol #3 compared with the other two methods, however the yield of volatile compounds including β-ionone is highest when the solvent volume used, and the duration of the extraction time are minimised (Protocol #3).

7.10 The effect of time and temperature of dry-down

The temperature and time of dry-down of the extract after removal of solvent on a rotary vacuum evaporator may influence the yield and composition of the final extract. These factors were studied by making a single extraction of 200g flowers and using triplicated sub–samples of the final extract-containing solvent for each dry-down condition. The results of this experiment are presented in Table 7.10.

Table 7.10 The effect of temperature and duration of dry-down on the yield and composition of extract.

Trt. No.	Treatment	Yield of extract (mg) +/- std error	% volatiles in extract +/- std error	% β−ionone in volatiles +/- std error
1	10min @ 30°C	47.87 +/- 0.61	8.44 +/- 0.27	40.77 +/- 0.34
2	10min @ 30°C + 5 min @ 60°C	* 33.23 +/- 0.83	* 13.53 +/- 0.17	40.90 +/- 0.18
3	10min @ 30°C + 15 min @ 60°C	* 31.07 +/- 0.98	* 13.40 +/- 0.22	*1 39.35 +/- 0.43
4	10min @ 30°C + 15 min @ 60°C + 15 min @ 80°C	* 31.13 +/- 0.75	*2 10.12 +/- 0.36	*2 32.87 +/- 0.38

* means significantly different to treatment No. 1 at the 5% level of significance
1 means significantly different to treatment No. 2 at the 5% level of significance
2 means significantly different to treatments No. 2 & 3 at the 5% level of significance

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Dry-down at 30°C does not remove all residual solvent present. A subsequent dry-down for five minutes at 60°C produces consistent estimates of yield and maximises volatile content. Continued dry-down at 60°C for longer than five minutes depletes volatiles. The point at which all visible traces of solvent were removed could be identified by a deepening in the colour of the extract, that appears to 'solidify' at the base of the round bottom flask; the time at 60°C was measured precisely after this point.

7.11 Discussion

The process of release of secondary compounds from boronia flowers is a passive diffusive one, after dissolution of compounds in the solvent. Different compounds diffuse out at different rates, particularly from organs of different density (Section IV.7.2). The pigments in the petals and the contents of the oil glands on the epidermis of the petal are rapidly extracted, however the pigments and volatiles within

the firm, thick stigma are dissolved more slowly (Section IV.7.2) and extraction may be aided by tissue disruption (Sections IV.7.2 and IV.7.4). Removal of the contents of the epidermal oil glands does not require rupture of the epidermal cap (Section IV.7.2), however dissolution of waxes in the cuticle may aid solvent penetration into the central cavity.

The effect of the volume of solvent used during the extraction is not critical in the estimation of extract yield, however it appears that extract yield can be maximised by use of a total flower weight : solvent volume ratio of 1 : 6 during the complete extraction, distributed over several washes (Sections IV.7.5 and IV.7.9). Additional solvent may cause increased extraction of waxes and other non-volatile compounds (Section IV.7.9). Minimal solvent volumes may produce products with different organoleptic properties (Section IV.7.5), as a result of higher proportions of floral compounds such as β–ionone compared with more vegetative/citrus compounds such as monoterpenes (Sections IV.7.5 and IV.7.9). A similar result is found by increasing the extraction time. Prolonged extractions produce a higher extract yield, however the additional extract obtained comprises non volatile compounds and green-smelling monoterpenes, typical of the leaf and calyx (Sections IV.7.6 and IV.7.9). The first two washes are where maximum benefits from increasing the extraction time are gained (Sections IV.7.6 and IV.7.9). Replenishment of solvent a number of times is desirable (7.7), and it is likely that careful experimentation with the number and duration of each wash and the solvent volume used may make the extraction process more efficient commercially, and may produce 'new' products.

The temperature of extraction has a large influence on the extract yield, a maximum temperature of 12°C can be recommended not to cause depletion of volatiles (Section IV.7.8). Once again, use of different thermal conditions may influence the nature of the product extracted; there appear to be a range of volatiles that become more soluble at temperatures of 45°C (Section IV.7.8), possibly as a result of changing solvent content due to evaporation of lower boiling point fractions such as pentane.

In the laboratory, samples as small as 10g can be reliably extracted, however to reduce variability between samples, a sample size of 25g or more is recommended (Section IV.7.3). Even after extraction, removal of the solvent can influence the yield and composition of product obtained (Section IV.7.10). Excessive use of high temperatures during solvent removal may cause depletion of monoterpenes and more volatile compounds, and therefore may decrease extract yield.

V. General discussion

In this thesis, each results chapter concludes with a detailed discussion. In this section, the relevant findings are brought together, presenting the development of the project from the initial acquisition of biological and biochemical information to the implications of these findings on the ecological niche of native boronia plants. Other research focussed on the effects of management practices on the extract yield and composition of accumulated compounds, and the commercial implications of this work will be discussed. Recommendations will be made on ways to ensure maximisation of available product(s) from existing crops of boronia.

The sexual organs contribute significantly to the amount and range of compounds emitted from the boronia flower. This may be indicative of the activity of particular volatile compounds in the attraction of pollinators, or the repellence of herbivorous arthropods, however it may also indicate adsorption of compounds from other organs (Dobson *et al.* 1990). Higher levels of β-ionone being emitted from large buds compared with open flowers, and the uneven flowering pattern in boronia may be of significance both ecologically and commercially. If β-ionone is active in attraction of Lepidoptera pollinators, as it is likely to be (Buttery *et al.* 1982), then high levels of emission from large buds may ensure that moths are present to receive pollen immediately upon anthesis, which would be necessary if the flowers are in fact protandrous. Other compounds, such as dodecyl acetate, which are emitted from the stigma and anthers in larger amounts after anthesis, may be significant for the attraction of moths which have already obtained pollen from other boronia plants. The uneven flowering pattern in boronia may help achieve cross-pollination and species survival (Wyatt 1982). It would be interesting to study in more detail the effect of anthesis and senescence on the headspace emitted from boronia flowers, and the effect of pollination on these emissions. The commercial significance of buds emitting higher levels of β-ionone than open flowers is that this emission may represent lost product(s) before harvest. It is recommended that the harvest is done as close as possible to anthesis to reduce the loss of β-ionone and other compounds.

Maximum levels of emission of volatiles may not coincide with the highest levels of extract yield, since the extract is made up of many compounds, most of which are not volatile but may confer other vital properties to the final product, such as keeping quality (Gopalakrishnan 1994). Commercially, the extended flowering pattern in boronia necessitates careful selection of the optimum harvest time to maximise flower and extract yield and other desirable aspects of extract composition.

The distribution of pigments throughout boronia flowers results in regions of contrast, such as between the functional and non-functional anthers, the functional anthers and the stigma, and the stigma and the epidermis on the adaxial surface of the petals. These colour contrast boundaries may be detected by insects in search of food rewards such as nectar or pollen, and may aid in detection of target organs for pollination (Penny 1983). It is likely that the green coloured disc beneath the brown coloured stigma of boronia flowers is active in nectar secretion as is typical of Rutaceae (Armstrong 1975; Fahn 1979b). The rewards for pollination could be nectar or pollen, or both.

There is evidence for diurnal or circadian rhythms in the levels of free and glycosidically bound volatiles. Rhythms in emission may concur with periods of activity of pollinators, but it is not possible to conclude whether emission of volatiles from boronia flowers is controlled by circadian or diurnal rhythms. There is a trend toward decreased tissue levels of volatiles, and perhaps concomitant increases in the emission of volatiles as a result of increased temperatures. Increased temperatures may increase the rates of evaporation of droplets of oil present in the cytoplasm of epidermal and epithelial cells surrounding the oil glands on the petals, which then diffuse through the cell walls and cuticle, thus depleting tissue levels. The release of volatiles is via diffusion because ruptures in the epidermal cap cell of oil glands on the petals are not a prerequisite for emission of volatiles, as has been suggested (Haberlandt 1928). Ruptures in the caps on petals that are in late stages of senescence are seen, probably caused by the depletion of moisture and volatiles with senescence.

Cells of most floral organs have the potential to be active in the synthesis of secondary products. The cells of tissues from immature buds contain numerous mitochondria and vesicles. In mature tissues, cells are largely devoid of distinct organelles except for vesicles, some ER and disintegrated plastids filled with droplets which are variably osmiophilic. The plastids are most often associated with droplets of what could be oily material. Carotenoids may be stored within droplets in the plastids (Davies 1977). The presence of β-ionone in isolated plastids suggests that this compound may also be formed within plastids, although the small amount found may have resulted from incomplete extraction, or may be the result of contamination of plastids during isolation. The role of the ER in the production of vesicles which are filled with material of the same matrix as plastidial droplets suggests the involvement of ER in the synthesis or conversion of secondary compounds of interest. The presence of ER and dictyosomes can be related to the synthesis of polysaccharides or to the glucosylation of secretory compounds (Venkaiah 1992), and ER-mediated synthesis of mono- and sesquiterpenes has been documented (Gleizes *et al.* 1980; Kleinig 1989). There is evidence for accumulation of secondary compounds not present in the extract,

such as vacuolar tannins and phenolics, and some of these may also be produced by the ER.

The chromoplasts within cells of boronia flowers are pleomorphic in form. Chromoplasts in the normal mesophyll cells are elongated and densely osmiophilic with flattened parallel membranes and few lipid droplets (Section IV.2, Plate 10). In cells surrounding the central cavity of oil glands the chromoplasts have a more rounded structure and fit the description of globulous chromoplasts (Whatley and Whatley 1987), containing more and larger droplets. Occasionally membranous whorls which resemble typical membranous chromoplasts are seen (Stubbs and Francis 1971), they have a spherical or deformed shape and contain concentric membranes and no oil droplets (Section IV.2, Plates 11, dashed arrow, 13A and 15A). In some chromoplasts, osmiophilic droplets were occasionally seen within the plastidial membranes (not shown), suggesting biosynthesis at this site, a possibility raised by other researchers (Heinrich *et al.* 1983).

The activity of other cell organelles, especially the lysosomal cell compartment, which comprises the ER, golgi, extracellular space and numerous vesicles, also appears to increase in mature flowers, which is not surprising since this cell compartment is active in senescent processes (Matile 1974). The many vesicles in cells of most organs in boronia flowers may contain digestive enzymes such as hydrolases and glycosidases. They may also contain enzymes responsible for formation of glycoside conjugates if such conjugation is a prerequisite for catabolism of compounds. The separation of glycosidases from the vacuolar contents prevents random hydrolysis of glycosides, and suggests that the cleavage of glycosides is a regulated process.

The site(s) of subcellular synthesis of secondary products such as monoterpenes, ionones and jasmonates may be more easily identified by microscopical studies using techniques such as immunogold labelling with antibodies raised to specific moieties. Without such studies, and with poor fixation of lipoidal compounds due to standard dehydration techniques, the elucidation of biosynthetic sites for different compounds is difficult and evidence is largely circumstantial.

There appears to be a pool of glycosides present in most tissues of the boronia plant examined. In flowers, glycosidic conjugates between most floral volatiles are found. Generally, the level of glycosidically bound volatiles are lower than the levels of free volatiles in flowers, which is the opposite of the situation described for other species (Francis and Allcock 1969; Gunata *et al.* 1988; Williams *et al.* 1992). Watanabe *et al.* (1993) showed that isolated enzymes from open flowers were able to cleave glycosides from mature buds of the same species, and concluded that glycosides are produced in buds and are precursors for free volatiles which are produced by the action of glycosidases prior to or after anthesis. This strict precursor/product role

delineated by phenological changes seems unlikely in boronia because glycosidase activity and emission of volatiles occurs in buds prior to anthesis. In fact, there is evidence for high levels of activity of glycosidase enzymes in buds prior to anthesis, indicating that conjugation and cleavage of glycosides are continual processes, probably existing in equilibrium, and genetically regulated.

Diurnal changes in levels of free volatiles in flowers may be caused by changes in equilibrium positions as a result of changing external conditions or changes in rates of primary reactions such as photosynthesis. The activity of glycoside-conjugating and hydrolytic enzymes may decrease during senescence, followed by a decline in the level of bound volatiles. It was the rise and fall of glycosides prior to similar behaviour in free volatiles that caused Francis and Allcock (1969) to suggest that glycosides are precursors to free volatiles.

It has been suggested that the process of harvesting flowers stimulates an acceleration of the controlled or unregulated processes of senescence (Mookherjee *et al.* 1986, 1989). The decline in glycosides that occurs in mature flowers is absent in flowers which are harvested and then stored in boxes at room temperature for 80 hours. In fact, there appear to be 20 hour cycles in the levels of glycosides during the first 60 hours, and over the following 60 hours, a large increase in the level of glycosides occurs, after which levels decline. Also, levels of glycosidically-bound volatiles are much higher compared with free volatiles after even short periods of post-harvest storage. Increased temperatures as a result of high respiratory activity in sealed boxes may increase the rate of evaporation of compounds from flowers, thus reducing tissue levels of free volatiles.

Croteau and Martinkus (1979) raised the possibility that transport of glycosides throughout the entire plant could occur via the phloem cells. It is known that waste compounds may be accumulated in organs which are to be abscised from the plant, or energy-rich compounds may be salvaged from those same organs (Zimmermann 1969). As such, transport of volatile conjugates into or out of flowers prior to abscission may occur. Bugorskii and Zaprometov (1983) proved that glycosides could be transported into flowers through the xylem vessels, however this does not necessarily indicate that this in fact occurs in plants. Loughrin *et al.* (1992) suggested that monoterpenes in rose flowers were synthesised in the leaf, glycosylated and transported via the phloem to the flower buds. The synthesis of typical floral compounds does not appear to occur in boronia leaves, and therefore it is unlikely that volatiles found in the flowers are synthesised elsewhere and transported into the flower. It is possible that some of the monoterpenes found both in the flower and leaves may enter flowers as glycosides transported from synthetic sites in the leaves. Glycosidase activity in the leaves declines steadily during flowering, and this may

cause an accumulation of glycosides in the leaves available for transport to other organs, however glycosides of typical floral volatiles were not found in boronia leaves.

It is also possible that biosynthetic or ecological relationships between glycosides and free volatiles do not in fact exist; free volatiles may be formed for the purpose of volatilisation and subsequent pollinator activity. At times when external temperatures prevent or reduce evaporation, excess volatiles may be conjugated with sugars as a prerequisite for transport to other parts of the plant. Alternatively they may be solubilised to enable digestive enzymes, initially including glycosidase(s), to act on the volatile compound bringing about catabolism and reutilisation of energy and carbon. This would also explain the 20 hour cycles in levels of glycosides after harvest, resulting from diurnally changing levels of volatiles.

Future work could focus on methods of purification of glycosides, sugar moieties and hydrolytic enzymes; evidence may be found for the failure of various published methods for the preparation of glycoside extracts from boronia flowers. One of the complications that arose during attempts to repeat published methods for purification of aqueous glycoside extracts through an Amberlite column (Gunata *et al.* 1985) was the immiscibility of the different mobile phases suggested: aqueous followed by organic with no gradients in between. Adding an internal standard to an aqueous extract prior to purification resulted in high levels of variability. The most efficient and reliable method found was a simple method based on a number of published reports. It may also be useful to trace labelled compounds through the pool of free and bound volatiles and observe the phenological changes, the diurnal patterns, and the changes after harvest.

The commercial significance of volatile-glycoside conjugates is the unavailability of these water-soluble compounds for extraction into organic solvents. To ensure maximum levels of free volatiles, a high level of hydrolysis of glycosides is desirable prior to extraction, perhaps via careful use of various post-harvest storage conditions. Apart from immediate post-harvest treatments of fresh flowers, the potential for treatment of thawed, frozen flowers to enable hydrolysis of glycosides could be examined for use prior to extraction. This would allow for greater control over experimental conditions than could be achieved at the many plantation sites immediately after harvest.

One of the major aspects of biochemical interest in this study of boronia flowers and their secondary compounds is the potential for elucidating, or at least providing some evidence for the theorised biosynthetic pathway for β-ionone, suggested to be via carotenoid degradation (Enzell 1985). Floral organs which emit high levels of compounds such as β-ionone also contain high tissue levels of carotenoids. The predominant carotenoids are β-carotene and lutein, both capable of supplying suitable end-groups for β-ionone production, and a loosely identified apocarotenoid: a partially

degraded carotenoid. There is evidence that enzymes capable of cleaving β-carotene exist in boronia flowers, which is to be expected since this process is a universal part of normal plant senescence (Booth 1960). However, production of β-ionone levels do not increase during senescence; quite the opposite occurs. Exposing flowers to conditions that would be expected to bring about enhanced oxidation of labile compounds, such as freezing and thawing, does not increase the rate of production of β-ionone after harvest. However, storage of newly opened flowers after harvest increases both carotenoids and β-ionone, and after a certain time, levels decline again. Increased accumulation of β-carotene and a reduction in levels of β-ionone occurs in boronia flowers which are treated with DCPE, a lipoxygenase inhibitor at low levels of concentration and an inhibitor of MVA incorporation at higher levels (Berry *et al.* 1974). Clearly, production of β-ionone is an enzyme-regulated process, the stimulation of which is lost as a result of increasing senescence, after freezing, and after long periods of post-harvest storage. It is likely that β-ionone is in fact produced by cleavage of carotenoids, however *de novo* synthesis cannot be ruled out. Glycosidic conjugation of β-ionone also suggests that directed production of this compound occurs, especially if the processes of formation and glycosidic bonding are heavily regulated.

Norisoprenoid-producing carotenoid degradation is a genetically-controlled process, and one that is distinct from senescence-related carotenoid degradation via autoxidation or random enzyme hydrolysis. If β-ionone is produced in the plastid, then both synthetic and degradative carotenogenic enzymes exist in one compartment. This may be brought about by selective importation of necessary cofactors at various times. It is possible to speculate on the changing activities of organelles, cells and tissues during development and senescence of flowers, providing an hypothesis for the control of norisoprenoid production. Thus, within flower buds of early developmental stages, the main activities of plastidial enzymes are in the synthesis of carotenoids and chlorophylls, the latter being active in photosynthesis possibly until after anthesis. The plastids may undergo structural changes, and other modifications as a result of the development of chloroplasts into chromoplasts in later bud stages. Close to anthesis, the rate of photosynthesis in the flower bud may decline, and the predominant enzymes may be active in the synthesis of terpenoids such as carotenoids. Subsequently, enzymes which bring about increased rates of destruction of carotenoids are synthesised. These may include enzymes which cleave β-carotene (for example) into molecules lacking one cyclic end group and subsequently shorten the hydrocarbon skeletons, or it may include enzymes which cleave the molecule centrally and then hydrolyse the halves into smaller molecules, or both. Carotenoid synthesis may also continue at this time, so that there is an equilibrium point between synthesis and catabolism of carotenoids. During senescence the rate of carotenoid synthesis may

decline, as a result of decreased enzyme activity (Borochov and Woodson 1989). The stimulation of carotenogenesis by compounds such as β-ionone is known to occur in some species (Yokoyama *et al.* 1984), and this may prolong the duration of carotenogenesis, until the point when activity of β-ionone-producing enzyme(s) also declines with senescence. In later stages of senescence, increased activity of oxygenases and hydrolases may occur. These may be capable of degrading both carotenoids, norisoprenoids and many other compounds more randomly and in a less regulated fashion. This would be expected to occur especially after plastidial disintegration which releases droplets containing carotenoids and other terpenoids into the vacuole.

It is likely that the genetic control of β-ionone is a complex one, probably controlled by more than one gene, because no mutants which lacked the ability to produce β-ionone were found in genetically different populations and amongst diverse colour mutants. As in other species (Jackson *et al.* 1992), mutants of boronia which accumulate different anthocyanins were found. These often result from mutations in only one gene, however the complement of carotenoids and volatiles were similar to 'normal' flowers. This is consistent with published information that control of carotenoid accumulation is more complex than anthocyanin accumulation (Spurgeon and Porter 1980).

Lipoxygenases which produce jasmonic acid and related compounds from linolenic acid also would be activated at this time, possibly stimulating the processes of senescence by their presence (Puchalski *et al.* 1989; Saniewski *et al.* 1987a, 1987b). Methyl jasmonate may also stimulate accumulation of carotenoids such as β-carotene and therefore the whole process may be more complicated (Hamberg and Gardner 1992). The only evidence that may be interpreted as being indicative of *de novo* synthesis of β-ionone is the specific enhancement of β-ionone in extracts of boronia flowers after nitrogen application (Roberts and Menary 1994b), although improved nutrition would probably promote most biochemical pathways, and it is not possible to distinguish the exact cause. Labelling studies, once again, would be advantageous, although such studies are made difficult by the labile nature of the substrates and their predilection for conversion into the products of interest without enzymic involvement, during isolation (Isoe *et al.* 1969).

The regulation of secondary metabolism in boronia flowers has already been mentioned. The external temperature around flower-bearing plants has little effect on the level of volatiles in the flower, although the emission of volatiles may change in response to temperature, causing depletion of volatiles via evaporation which may be replaced by glycoside hydrolysis. The effect of different external temperature conditions may be more pronounced when plants are grown under such conditions for longer periods of time. Plants flower earlier in warmer conditions, such as in the north

of Tasmania compared with the south, however there were no obvious effects on the yield and composition of extract as a result of being grown in these different environments.

There is no detailed information on the length of the flowering period in different regions of Tasmania. Long periods of warm, dry weather in the flowering season occur more frequently in the north-east, and this type of weather increases the rate at which flowers reach anthesis and senesce. There is conflicting evidence as to whether a reduced flowering period adversely affects extract yield (Hornok 1988; Franz *et al.* 1986; Patra *et al.* 1987). During shorter flowering seasons, a careful assessment of the percentage of open flowers will enable selection of the optimum harvest date. Reduced extract yield from shortened flowering periods is more likely to be caused by harvesting too late, when most flowers are in late stages of senescence, as found in this study (Section IV.4.9).

Light levels can alter the number of flowers reaching anthesis (Roberts 1984). In tissue culture, unusual lighting and temperature regimens including continuous light and dark, and short, cold days, reduced the yield of volatiles produced in comparison with cultures grown under mild, long days. This is reminiscent of the results seen in cultures of *Ruta* grown under different conditions (Corduan and Reinhard 1972) where dark-grown cultures produced compounds similar to root oil, and light grown cultures produced compounds found in leaf oil. It was expected that high light would stimulate the production of β-carotene (Demmig-Adams and Adams 1992), and perhaps also β-ionone, however supportive results were not found in the tissue culture experiment documented in this thesis (Section IV.3.9).

Preliminary investigations suggest that callus cultures are unlikely to be useful in studies of the biosynthesis or conversion of typical floral compounds, unless suspension cultures could be made from floral tissue, an idea which has been trialed without success (Norman 1989). The potential for *in vitro* flowering (Roberts *et al.* 1993) suggests that future work could be directed toward identifying regulatory mechanisms for secondary metabolism in boronia flowers by the use of hormones and inhibitors of specific enzymes.

Genetic control of secondary metabolism and therefore extract yield and composition from boronia flowers is more significant than the environmental conditions, as has been found in other floral extracts and oils (Franz *et al.* 1986). There is large variation in the potential for genetically different plants to accumulate extract with desirable characteristics. However, selection of plants should only be based on the yield and composition of extract produced in the flowers, and cannot successfully be based on floral characteristics such as the number of oil glands on the petals, or the size (or weight) of the floral organs, or the whole flowers. This is similar to the findings of Patra *et al.* (1987) and Sharma and Farooqi (1990) on the

relationship between the weight of individual rose flowers and the yield of essential oil. Usually there is a relationship between the yield of extract produced by oil-gland bearing tissues and the number or density of oil glands (Henderson *et al.* 1970; Tanaka *et al.* 1989). Lack of a relationship of this kind in boronia flowers bearing oil glands on the petals suggests that non-glandular production of secondary products by floral organs may contribute more extract per flower than the amount of extract produced and accumulated in glands on the petals. It also appears that the level of activity of enzymes responsible for biosynthesis of secondary products is more significant than the number of active cells and the available cellular space for accumulation of product. One of the most significant factors that affects extract yield and composition from boronia flowers is the timing of harvest to coincide with particular proportions of open flowers on the whole plant. As with most oils and extracts (Burbott and Loomis 1961; Putievsky *et al.* 1984), the yield of extract increases and the composition develops in complexity during development of the organ (in this case the flower bud), and declines during senescence of the tissue(s), i.e. after anthesis. This is the result of increased activity of catabolic enzymes in mature cells (Croteau 1987, 1991; Croteau *et al.* 1981). The uneven flowering pattern in boronia indicates that the balance between the rates of anabolism and catabolism of secondary compounds which has to be considered when harvesting essential oil crops is even more complex in boronia flowers. During the flowering season, the number of immature buds with high levels of biosynthetically active enzymes decreases, and the number of open flowers with progressively less biosynthetic enzymes and increasing catabolic activities increases. Another factor causing depletion of volatiles in mature flowers are the effects of oxidation and polymerisation which accumulate in plant tissue over time, and the cumulative loss of product(s) via evaporation (Guenther 1972; Haagen-Smit 1972).

One of the main recommendations to come out of the studies presented in this thesis is to begin the harvest when approximately 55-60% of flowers are open, instead of when 90% or more flowers have reached anthesis, which is the current practice. Higher yields will be obtained by harvesting when there are high levels of volatiles in flowers prior to and shortly after anthesis even when only 50% of flowers on a plant have reached anthesis and the rest of the buds are mostly medium-sized or larger, compared with later stages of flowering when many of the flowers have lost petals, volatiles and moisture.

Various post-harvest treatments have been considered and found to have the potential for increasing the level of free volatiles in the extract, most notably the increase of β-ionone as a proportion of total volatiles by 40% in flowers that were crushed by hand, and then left at room temperature for nine hours. This increase could be a relative one, caused by depletion of other volatiles such as monoterpenes, however this is unlikely to be the case because the levels of total volatiles either

remained constant or also increased. When the level of total volatiles also increases, this causes an increase in the content of β-ionone as a proportion of the extract also. It is not necessary to crush or squeeze the flowers prior to storage; this process actually stimulates catabolic processes which deplete extract yield during storage.

Post-harvest storage at room temperature for 10-20 hours after harvest may yield a product of different organoleptic qualities as a result of enhanced volatiles, particularly β-ionone, and this is an important finding for commercial application. Freezing flowers prior to storage inhibits the post-harvest changes. Changes in volatile levels after harvest do not occur in buds prior to anthesis, and therefore there may be different optimum harvest dates for a) maximisation of post-harvest activity and production of different products than for b) high yield of standard extract, best obtained from early harvests when 50-60% of flowers are open.

High levels of respiration raised the temperature within enclosed boxes of flowers to a greater extent when boxes were opened for sampling regularly during storage than when they were undisturbed. Biosynthetic enzymes were most likely very active after harvest, and increased rates of respiration probably increased the rates of enzyme activity responsible for secondary metabolism. This also occurred in rose flowers after harvest (Serrano *et al.* 1992). It does not appear that hydrolysis of volatile-glycosides are responsible for the increased rates of volatile production after harvest.

The levels of glycosidically-bound volatiles increase dramatically after 80 hours of post-harvest storage, which is probably indicative of their function in solubilisation of volatiles in preparation for catabolism into smaller compounds, or as a means of transportation from the flower. The increase in glycosides may also be a result of the loss of control of biosynthetic pathways and relationships which normally exist in equilibrium, caused by a disruption in transport mechanisms into and out of the flower. There is evidence for 20 hour cycles in the levels of glycosides during the first 60 hours of post-harvest storage; cycles that are more obvious in flowers harvested earlier rather than later in the flowering season. This may be because regulatory mechanisms falter as a result of senescence.

There is large potential for the control of secondary metabolism even after harvest and clearly there is a fine line between desirable and undesirable changes. Increased levels of glycosides after extended storage will further reduce the level of free volatiles available to the solvent during extraction. Areas where future work would be useful include the effects on post-harvest changes of temperature, the concentration of particular gases such as oxygen and carbon dioxide during storage, and the possibility of exposure of frozen/thawed flowers to exogenous enzymes such as glycosidases. Observations on the changes in cellular structure during storage may shed light on the effects on normal physiological processes of accelerated senescence.

Storage at -20°C does not prevent loss of compounds of relatively high volatility, and may not inhibit other reactions which deplete extract yield, such as polymerisation and oxidation of compounds (Burbott and Loomis 1969). Therefore, if flowers are to be frozen and subsequently exposed to conditions that may bring about the same changes as would normally occur in fresh flowers after harvest, then storage at reduced temperatures such as -40°C or -70°C may be necessary.

The extraction of products from boronia flowers is clearly one of the main areas where small changes can affect the extract yield and composition, and can produce extracts of different organoleptic properties. Two of the greatest factors are the duration of extraction and the washing regimen: the number and duration of separate washes during a complete extraction. It has been demonstrated that it is difficult to completely extract boronia flowers; continued extraction of waxes and pigments occurs even after seven washes, each of fifteen hours duration. This is to be expected with the use of pet. ether, which does not penetrate well through moist tissue. For this and other reasons, the colour of the solvent after a wash is not an accurate indication of the content of extract in the solvent, particularly of volatile compounds. The first and second replenishments of solvent are the most critical, and it is suggested that the bulk of the solvent and the longest extraction times be incorporated into these washes, with subsequent washes (one or two) using restricted solvent and being of short duration. Later washes act mainly as a rinse for the flowers, removing extract that has been released from the cellular matrix, but which adheres to the epidermis of the floral organs, and removing waxes from epidermal cells. The commercial significance of these findings may be great, particularly because reduced solvent volumes tend to selectively extract compounds such as β-ionone in preference to more volatile compounds such as monoterpenes, and this produces extracts with different and perhaps enhanced organoleptic properties. The opportunity cost of reducing the solvent volume and perhaps also the duration of extraction must be assessed by taking into consideration the reduced cost of extraction, the increased rate at which large amounts of flowers can be processed, the slightly reduced extract yield that may ensue in some situations, and the increased levels of desirable extract components.

Another important finding for the commercial production of boronia extract is the enhanced yield of extract that has high levels of volatiles from flowers that were crushed or partially chopped up prior to extraction. This process probably causes disruption of floral tissues such as the stigma and the non-functional anthers, both organs which contain floral volatiles that may not be accessible to the solvent because of the firm nature of the tissue, particularly the stigma. It was thought that crushing flowers in this way immediately after harvest may enhance the post-harvest increases in volatile levels in flowers, because of the increased cellular destruction and decreased regulation that ensues. However, crushing and storing flowers reduced yield

compared with that obtained by crushing alone, probably as a result of increased oxidative effects. Therefore, the use of these two procedures, crushing and storage, should be done in reverse order: harvest the flowers and if possible expose them to aerated conditions for at least ten hours, but not more than 20 hours, subsequently freeze the flowers (preferably at less than -20°C) or crush them and then freeze them. It is probably more advisable to freeze the flowers first, and then when the time for extraction approaches, thaw the flowers and crush them, and subsequently extract in the suggested way. Controlled use of these practices may yield a product of both increased yield, and enhanced quality.

In summary, high levels of emission of volatiles from buds and newly opened flowers indicates that rapid loss of product occurs after anthesis. Most of the typical floral volatiles are probably synthesised in cells of the glandular epidermis of floral tissues, the activity of oil glands on the petals is not exclusive. The involvement of plastids in the production of secondary compounds, particularly β-ionone, is suggested. The involvement of pigments in the production of some volatile compounds is likely. As a result of a study of phenology and extract accumulation and catabolism, recommendations have been made for an early harvest, when approximately 50 to 60% of flowers on a plant have reached anthesis. This maximises extract yield and the concentration of desirable components. It is suggested that glycosidic conjugates with volatiles probably act mainly as prerequisites for catabolism of volatiles or as a means of removal from the flower. Levels of glycosides are regulated and kept in equilibrium with free volatiles until late senescence or after long periods of post-harvest storage. The genetic predisposition of a particular boronia plant or clone to produce extract is more important in determining subsequent extract yield than environmental or floral characteristics other than the developmental stage. This indicates that levels of enzyme activity are significant, and that future work should be directed toward studying this form of regulation. The potential for post-harvest treatments and variations in the extraction protocol to improve the composition, yield and quality of extract indicate that these are powerful tools for commercial consideration.

In the future it is envisaged that a range of products could be produced from existing plantations by utilising the high yield of extract in newly opened flowers, and the ability of such flowers to continue synthesis of secondary products after harvest. There is scope both for commercial experimentation to produce improved yield and product diversity, and detailed biochemical and perhaps molecular studies to produce information about biochemical pathways which are at best speculative at present.

VI. Bibliography

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VII. Appendices

APPENDIX 1. CAROTENOID ANALYSIS BY HPLC

This method used a Waters Associates liquid chromatograph comprising M6000A, M45 and M501 pumps, a Valco injector, a Hewlett Packard 8450A diode array spectrophotometer, and a Waters 440 absorbance detector, connected via a Waters System Interface Module to a microcomputer running Waters Maxima software. The column used was a stainless steel 30cm x 4.6mm ID, 5micron particle size C18 column. Pigment detection was at 436nm, the solvent system was as follows:

Solvent A: 80:20 methanol : 0.5M ammonium acetate (aq.; pH 7.2 v/v)
Solvent B: 90:10 acetonitrile (210nm UV cut-off grade) : water (v/v)
Solvent C: ethyl acetate (HPLC grade).

Methanol, acetonitrile and ethyl acetate were HPLC grade reagents from Waters, used without further purification other than filtration and degassing by sonication. Water was purified using a Millipore Milli-Q system. Ammonium acetate was A.R. grade.
Flow rate was 1 ml/min. The gradient system used was :

Time (min)	Flow rate ml/min	%A	%B	%C	Conditions
0	1	100	0	0	Injection
4	1	0	100	0	linear grad.
18	1	0	20	80	linear grad.
21	1	0	100	0	linear grad
24	1	100	0	0	linear grad.
29	1	100	0	0	equilibration

APPENDIX 2. MEDIA FOR TISSUE CULTURE

Chemical	mg/L
NH_4NO_3	1000
KH_2PO_4	170
$MgSO_4$	370
NaFeEDTA	40.7
KCl	114
$CaCl_2.2H_2O$	440
$MnSO_4.H_2O$	22.4
$ZnSO_4.7H_2O$	8.6
KI	0.85
H_3BO_3	6.2
$NaMoO_4.2H_2O$	0.25
$CoCl_2.5H_2O$	0.025
$CuSO_4.5H_2O$	0.025
Glycine	2.0
Thiamine	0.1
Pyridoxine	0.5
Nicotinic acid	0.5
MyoInositol	100
Adenine sulphate	80
Sucrose	3% (30 g/L)
IAA	4.0
BAP	1.5
Agar	0.8%

N.B. This media was developed by G. Luckman (1989), Univeristy of Tasmania, specifically for culturing proliferating *B. megastigma* Nees. callus cultures.

APPENDIX 3. DESCRIPTIONS OF SITES SAMPLED DURING VARIOUS STUDIES.

REFER TO MAP, OVER PAGE, FOR LOCATIONS.

Bridport. 40,000 plants, 2 ha, planted in December 1991. Northerly aspect, moderate slope. Well sheltered from prevailing wind. Soil pH 4.8.

Kingston - I. South-easterly aspect, flat site. Sheltered from westerly and sou-westerly prevailing winds. Soil: a sandy podzol, pH 3.7.

Kingston - M. South-easterly aspect, flat site. Sheltered from westerly and sou-westerly prevailing winds. Soil: a sandy podzol, pH 4.2. Drip irrigated as needed.

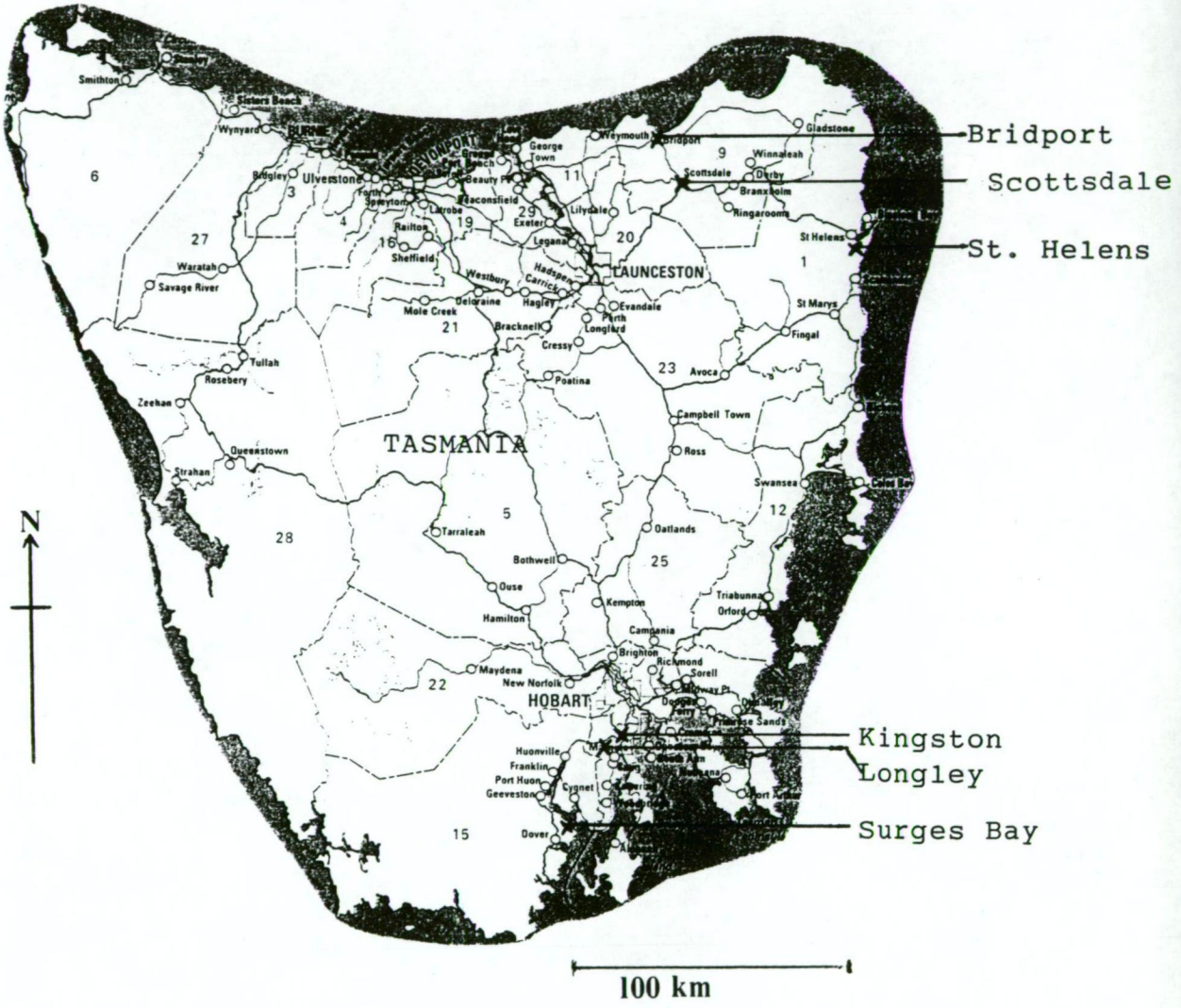
Longley. Approximately 1 ha, planted in July 1990. Easterly aspect, extreme slope (relatively). Sheltered from sou-easterly wind. Drip irrigated. Sandy soil, pH 4.6.

Scottsdale. 74,000 plants, 3 ha., various planting's. Moderate slope, North to North-westerly aspect. Exposed to westerly and nor-westerly winds. Soil pH 5.0.

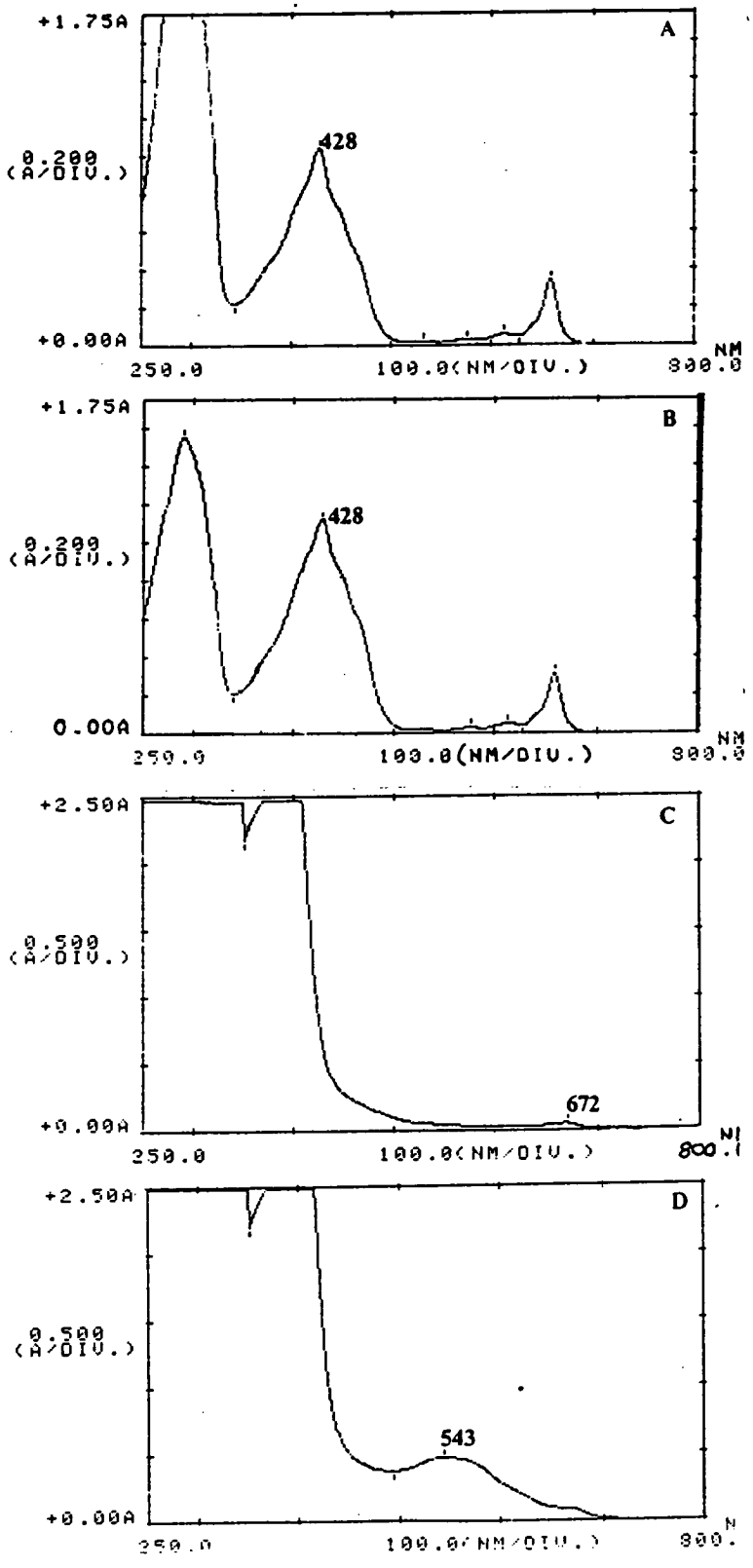
Surges Bay. 1 ha. planted December 1991. Moderate slope, some areas flat. Aspect, northerly. Sheltered from northerly wind. Soil pH 4.7.

St. Helens. 306,000 plants, more than 8 ha. Plantings in May 1991 (4 years old), August 1992 (3 years) and May 1993 (2 years). Flat site, exposed to easterly and westerly winds.

APPENDIX 3. CONTINUED. MAP OF TASMANIA SHOWING LOCATION OF SAMPLING SITES.

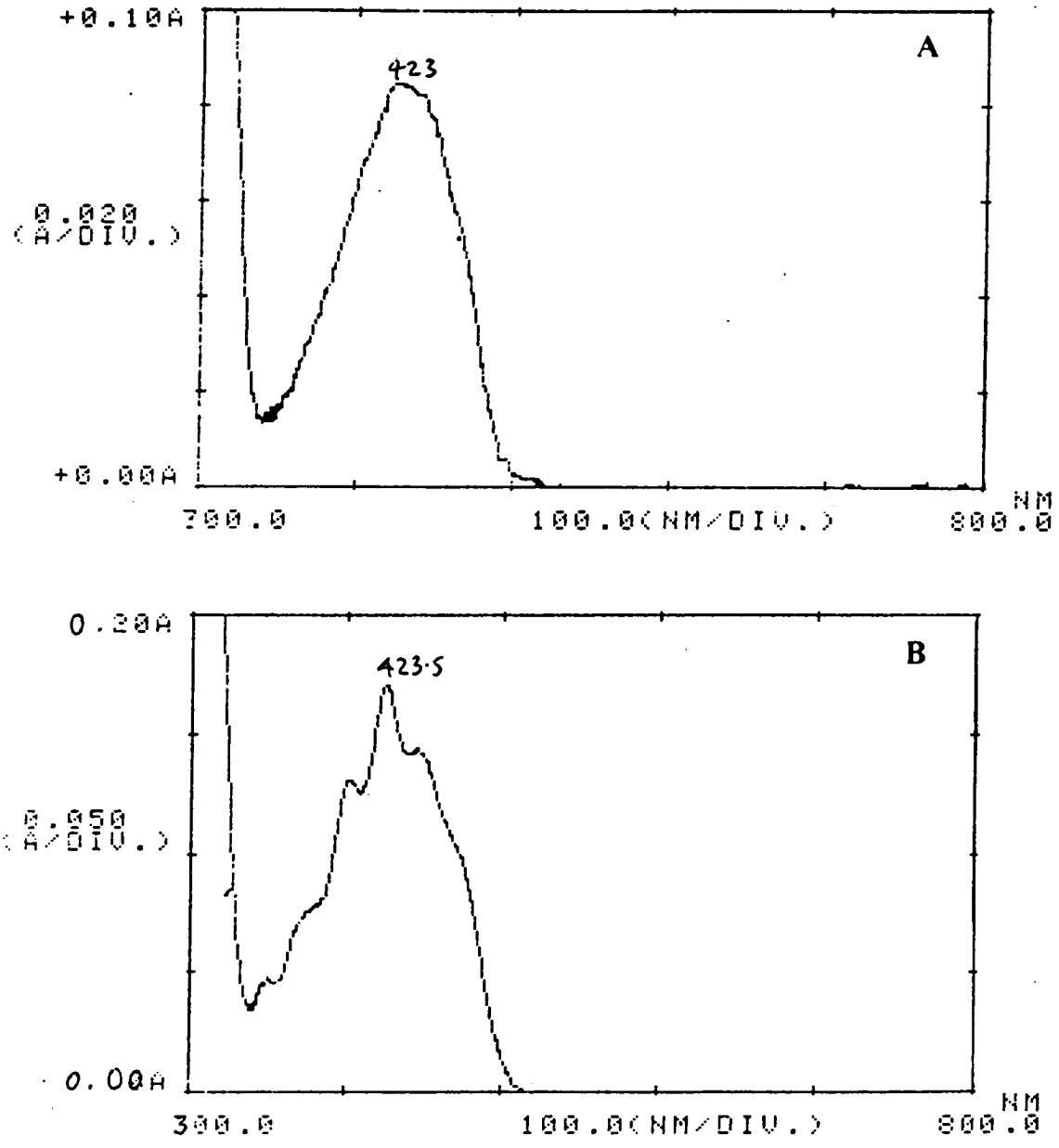


APPENDIX 4.



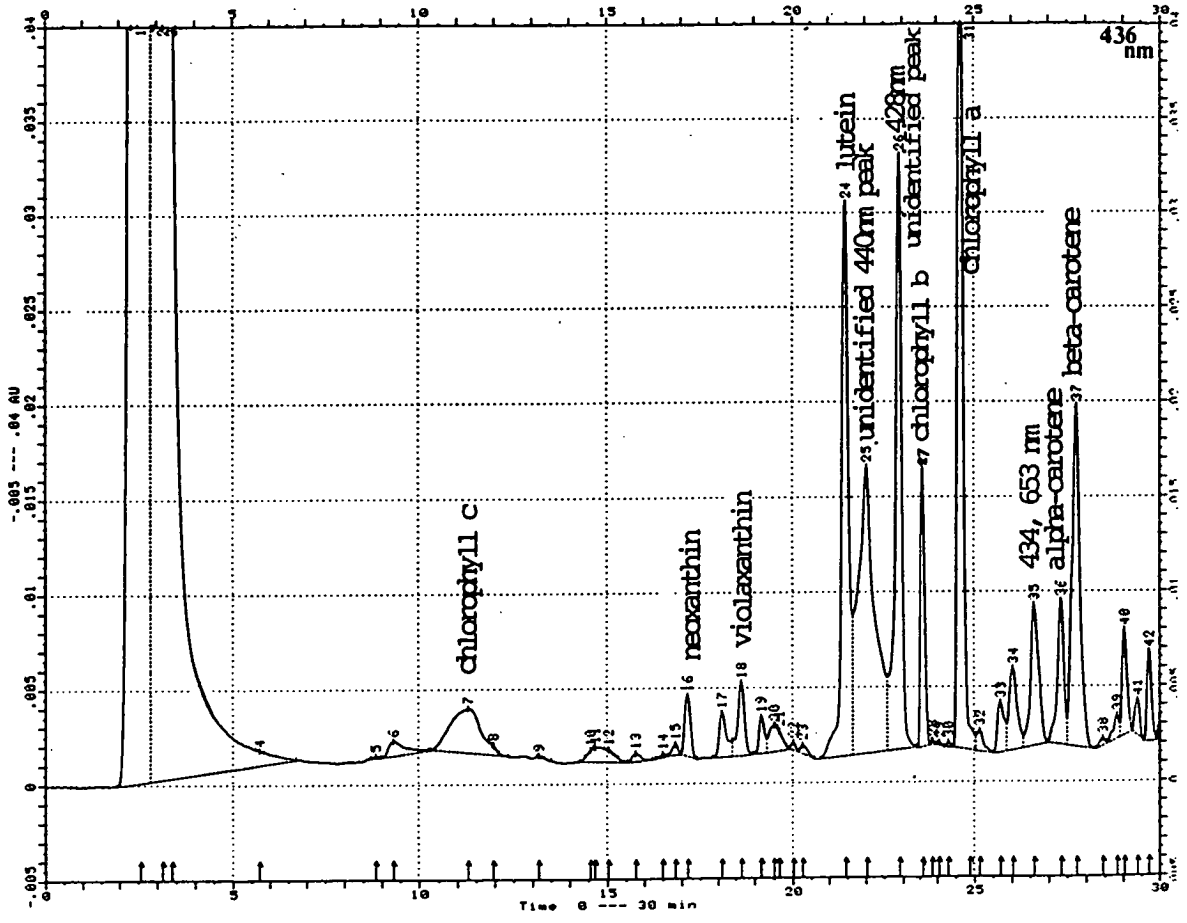
A-D: Spectra of pigment extracts. A = Pet.ether extract of 'Lutea', B = Pet. ether extract of normal petals, C = Aqueous solution from 'Lutea', D = aqueous solution from normal petals. All extracts are from petals.

APPENDIX 5.



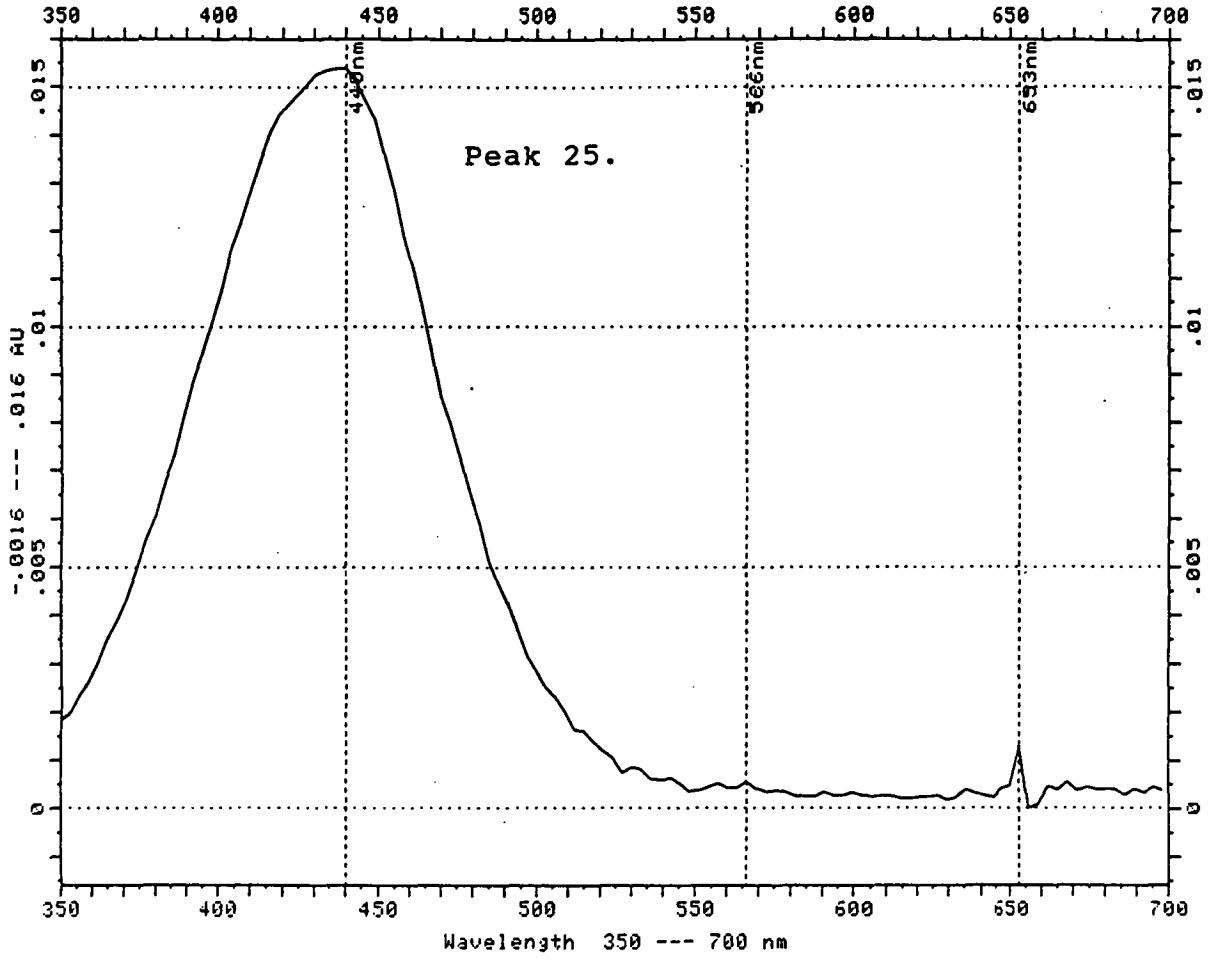
Spectra of pet. ether extracts of A (petals), and B (whole flowers), with homogenisation.

APPENDIX 6.



HPLC Chromatogram of 90% acetone extract of Boronia petals.

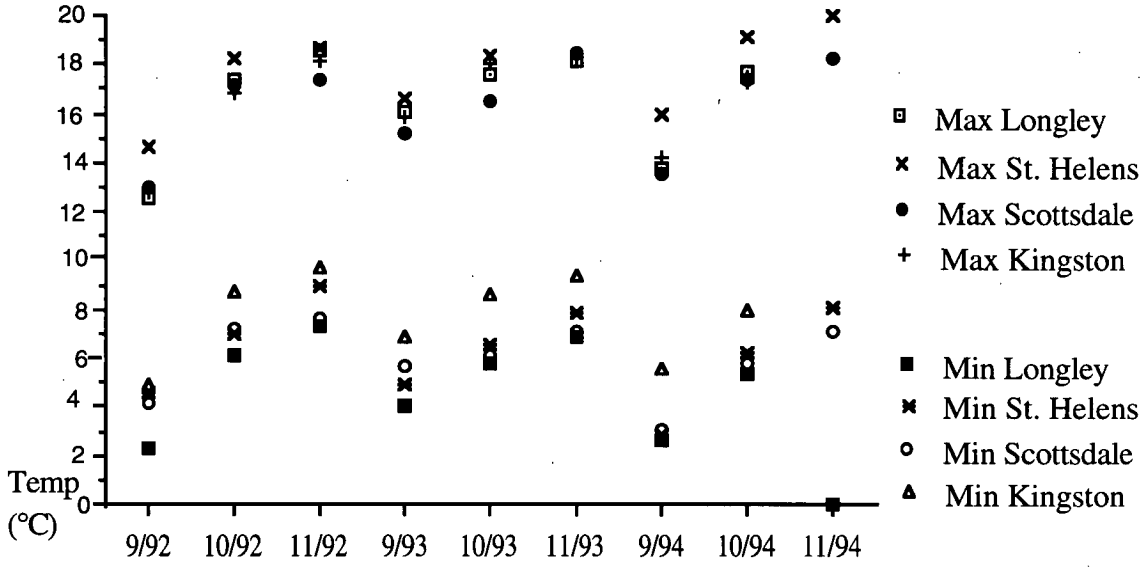
APPENDIX 7.



Absorbance of Peak number 25, from HPLC of 90% acetone extract of Boronia petals.

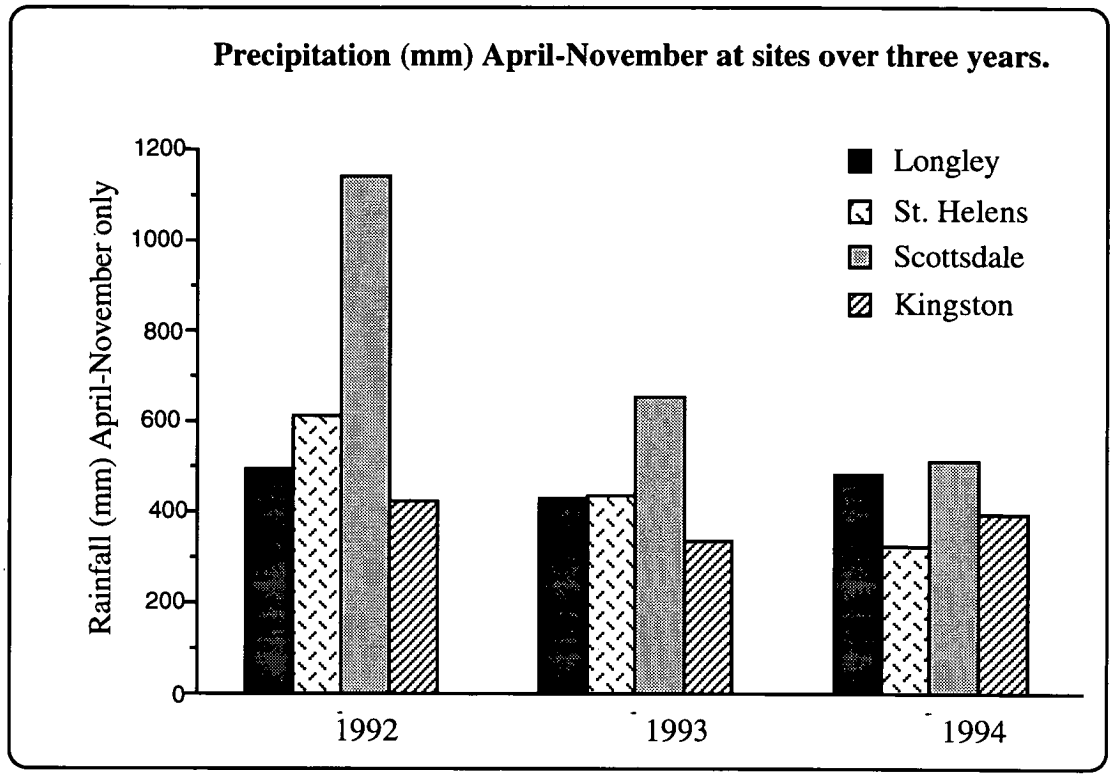
APPENDIX 8. TEMPERATURE AND PRECIPITATION OVER THREE YEARS AT FOUR SITES.

Temperatures (°C) during September, October and November at sites over three years.



September, October and November, 1992-1994.

APPENDIX 8. CONTINUED.



APPENDIX 9. ANALYSES OF VARIANCE FOR EXTRACT YIELD AND COMPOSITION FROM GENETICALLY DIFFERENT PLANTS IN THREE ENVIRONMENTS (SECTION IV.5.2).

ANOVA for yield of extract

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
Between subjects	15	.1	.0067	1.7868	.0825
Within subjects	32	.1194	.0037		
treatments	2	.0375	.0188	6.8753	.0035
residual	30	.0819	.0027		
Total	47	.2195			

Reliability Estimates for- All treatments: .4403 Single Treatment: .2078

ANOVA for percentage of total volatiles in extract

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
Between subjects	15	199.2641	13.2843	.8903	.5809
Within subjects	32	477.4883	14.9215		
treatments	2	134.8638	67.4319	5.9043	.0069
residual	30	342.6245	11.4208		
Total	47	676.7524			

Reliability Estimates for- All treatments: -.1232 Single Treatment: -.038

ANOVA for beta-ionone as a proportion of total volatiles

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
Between subjects	15	150.243	10.0162	.2614	.996
Within subjects	32	1225.9475	38.3109		
treatments	2	251.1292	125.5646	3.8642	.0321
residual	30	974.8183	32.4939		
Total	47	1376.1905			

Reliability Estimates for- All treatments: -2.825 Single Treatment: -.3266

APPENDIX 9. CONTINUED.

ANOVA for beta-ionone in extract

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
Between subjects	15	16.7148	1.1143	.4366	.9547
Within subjects	32	81.6727	2.5523		
treatments	2	21.0972	10.5486	5.2242	.0113
residual	30	60.5756	2.0192		
Total	47	98.3875			

Reliability Estimates for- All treatments: -1.29 Single Treatment: -.2312

APPENDIX 10. EXTRACT YIELD AND COMPOSITION OF FOUR CLONES AVERAGED OVER SIX SITES. (SECTION IV.5.3).

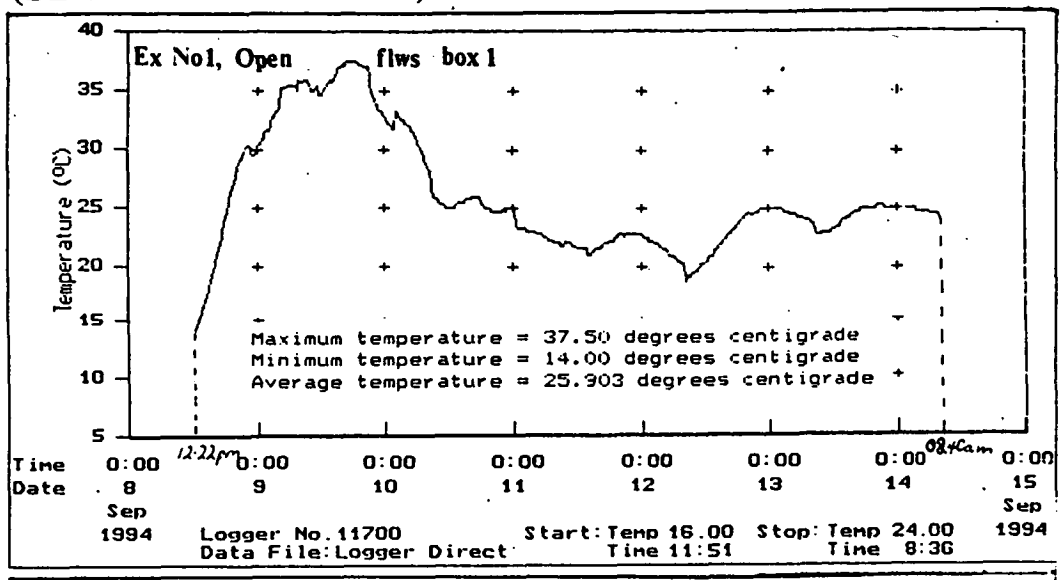
	Clone #3	Clone #5	Clone #17	Clone #250
% extract yield				
Mean	0.473	0.460	0.411	0.413
Standard deviation	0.045	0.035	0.042	0.030
Range	0.12	0.071	0.111	0.076
Coefficient of variation	9.515	7.520	10.339	7.354
% total volatiles in extract				
Mean	13.356	13.72	10.99	12.47
Standard deviation	0.840	1.88	2.384	2.14
Range	2.09	5.2	6.814	4.92
Coefficient of variation	6.287	13.70	21.70	17.12
% β–ionone in extract				
Mean	2.76	3.72	2.10	2.90
Standard deviation	0.549	1.018	0.788	0.845
Range	1.47	2.74	1.97	2.20
Coefficient of variation	19.905	27.37	37.49	29.11

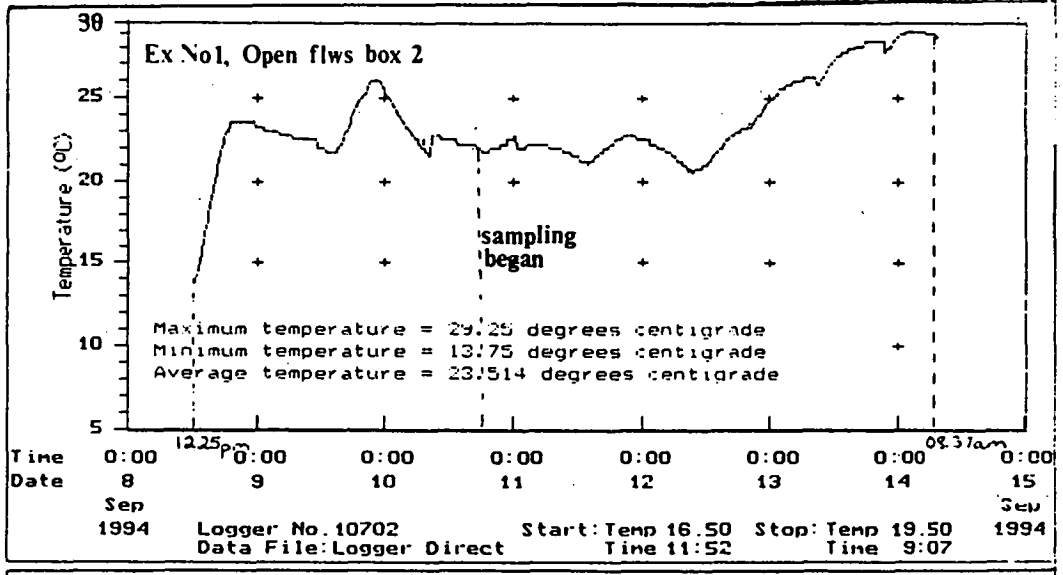
APPENDIX 11. EXTRACT YIELD AND COMPOSITION AT SIX SITES, AVERAGE OF FOUR CLONES. (SECTION IV.5.3).

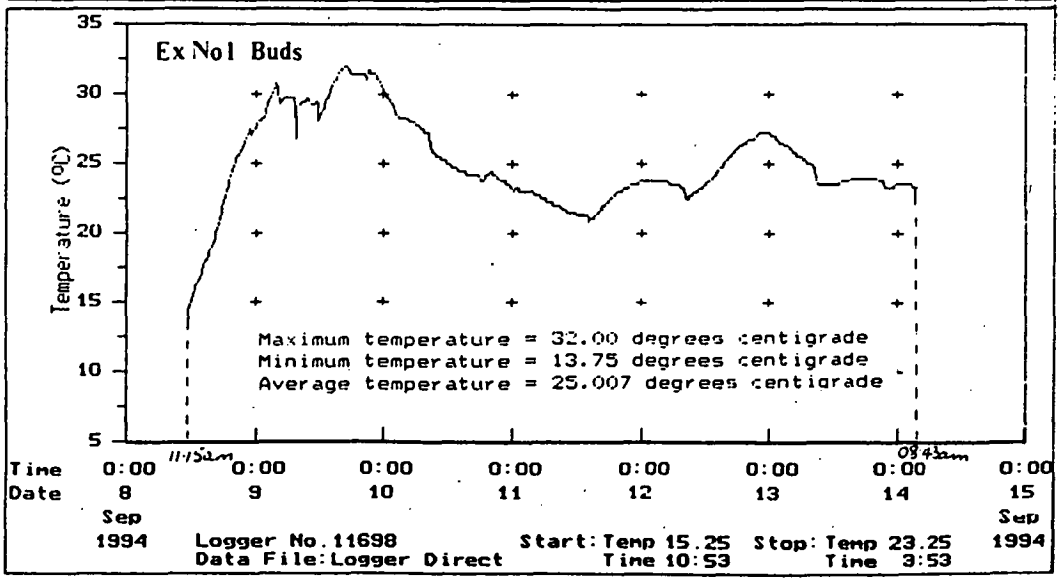
	Longley	Scottsdale	Bridport *	Kingston	Surges Bay	St. Helens
% extract yield						
Mean	0.433	0.428	0.403	0.477	0.417	0.445
Standard deviation	0.017	0.046	0.01	0.041	0.076	0.031
Range	0.035	0.098	0.01	0.087	0.152	0.075
Coefficient of variation	3.95	10.70	2.46	8.49	18.19	7.05
% total volatiles in extract						
Mean	11.81	14.49	12.70	13.72	10.23	12.44
Standard deviation	1.72	1.51	2.45	0.20	2.89	0.79
Range	3.81	3.67	3.46	0.50	6.84	1.74
Coefficient of variation	14.53	10.42	19.27	1.49	28.24	6.32
% β–ionone in extract						
Mean	2.77	3.62	3.17	3.23	1.61	2.62
Standard deviation	0.90	1.06	0.97	0.66	0.69	0.60
Range	2.19	2.4	1.37	1.52	1.63	1.43
Coefficient of variation	32.55	29.38	30.61	20.46	42.95	22.70

* Clones #17 and #250 only.

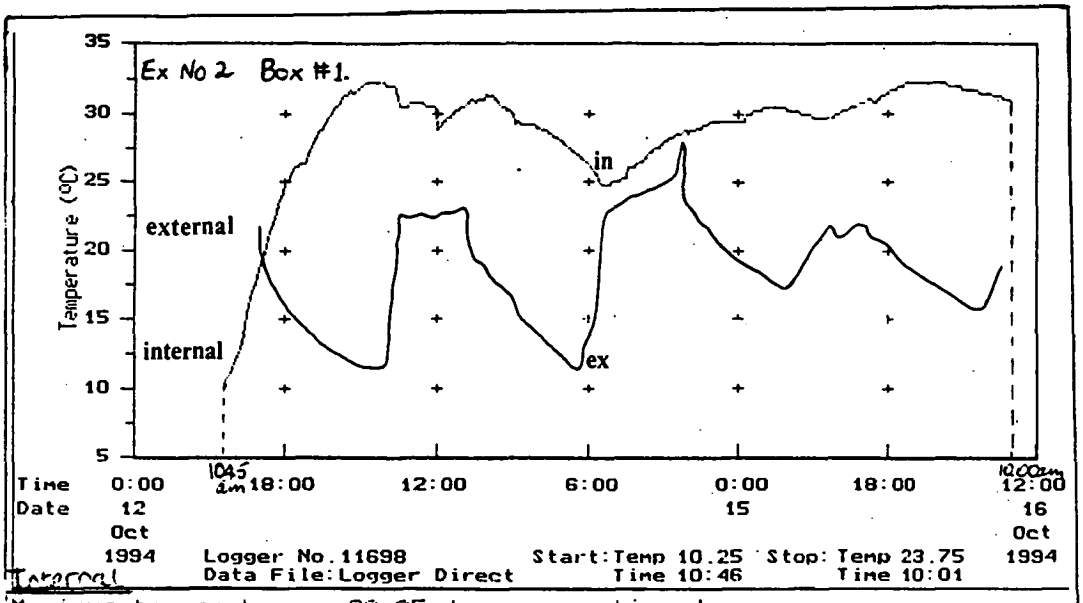
APPENDIX 12. TEMPERATURE PROFILES IN BOXES OF FLOWERS AND BUDS AFTER HARVEST. (SECTION IV.6.1.D.1).







APPENDIX 13. TEMPERATURE PROFILES IN BOXES OF FLOWERS AFTER HARVEST. (SECTION IV.6.1.D.2).

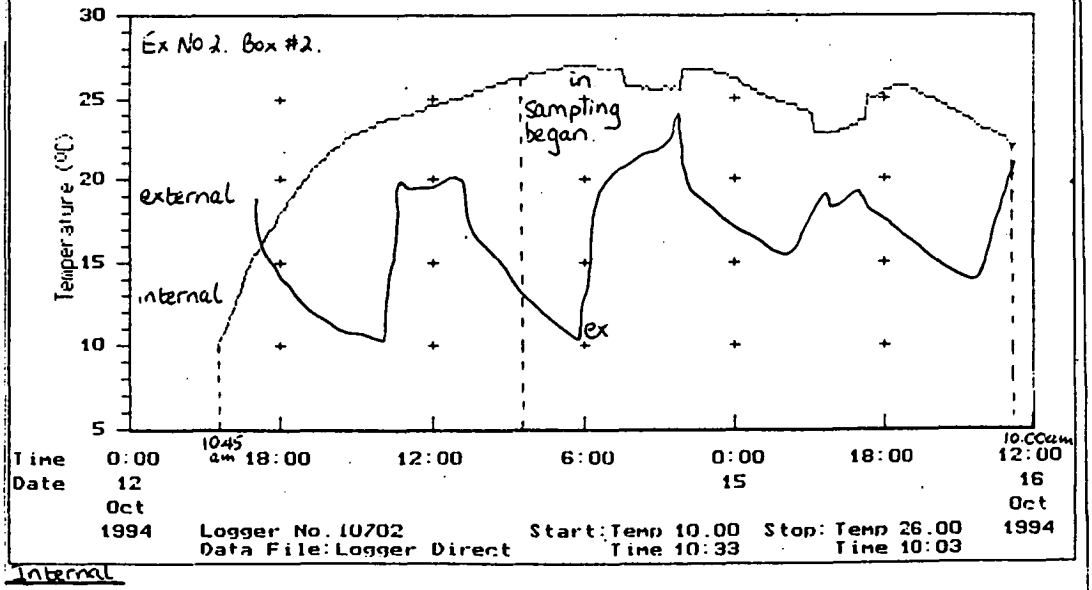


Internal

Maximum temperature = 32.25 degrees centigrade
Minimum temperature = 10.25 degrees centigrade
Average temperature = 28.640 degrees centigrade

External

Maximum temperature = 29.50 degrees centigrade
Minimum temperature = 11.00 degrees centigrade
Average temperature = 18.379 degrees centigrade



Internal

Maximum temperature = 27.00 degrees centigrade
Minimum temperature = 10.00 degrees centigrade
Average temperature = 23.807 degrees centigrade

External

Maximum temperature = 25.75 degrees centigrade
Minimum temperature = 15.25 degrees centigrade
Average temperature = 20.561 degrees centigrade