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Flower and Volatile Oil Ontogeny in *Boronia megastigma*

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The volatile oils of flowers of *Boronia megastigma* Nees. are important commercially but little has been published regarding their localization or the dynamics of their synthesis. In this study we examined the histochemistry, anatomy and volatile oil composition of the floral organs of plants from a native population by SEM.

Lysigenous glands occurred in all organs except the stigma and androecium and were associated with the presence of α- and β-pinene and limonene in tissue extracts. These compounds increased in concentration throughout fruit development. Petals, stamens, staminodes and stigmas were glandular and contained phenolic deposits, a dense cytoplasm and prominent intercellular spaces. Extracts of these tissues contained dodecanol, β-ionone and dodecyl acetate, the concentrations of which were greatest at the time of stigma receptivity. The location and timing of their synthesis together with their biological activity suggests that these volatile oils may have a role in pollination.

Key words: *Boronia megastigma* Nees., Rutaceae, essential oils, volatile oils, reproductive biology, α-pinene, β-pinene, limonene, β-ionone, dodecyl acetate, heptadecene.

INTRODUCTION

Flowers of *Boronia megastigma* Nees. (Rutaceae) are the source of a volatile oil that is used in fragrances and flavours. The composition of the petroleum ether extracts used in commerce has been reported to include β-ionone and related epoxides and dihydro compounds; α- and β-pinene and limonene; fatty acid methyl and ethyl esters; acetates of decyl- and tetra-decyl alcohols; dihydroactinidiolide; isomers of methyl jasmonate and wax hydrocarbons from heneicosane to tritriacontane (Davies and Menary, 1984).

Plant volatile oils are associated usually with secretory structures; resin ducts, lysigenous glands and glandular trichomes (Schnepf, 1974; Dell and McComb, 1978*a*, *b*; Fahn, 1979). Haberlandt (1928) described lysigenous glands in leaves of *B. megastigma* while Porsch (1906) had earlier suggested that the fragrance of flowers was produced in an apparatus (gland) similar to that which occurred in leaves. While no modern account exists of the ultrastructure of these glands, they are probably similar to those of *Citrus* (Bosabalidis and Tsekos, 1982*a*, *b*). The distribution of glands and the development of volatile oil components in individual organs during the reproductive cycle have not been reported. The aim of this study was, therefore, to identify the distribution of glands in flowers and fruits of *B. megastigma* and to relate these to intrafloral and ontogenetic variation in volatile oil composition.

MATERIALS AND METHODS

Six plants of *B. megastigma* were selected at random from within a natural population at Nannup, Western Australia (Latitude 33° 59′ S, longitude 115° 46′ E). Fresh flowers and fruits were collected from these for anatomical studies and volatile oil analyses at particular flower stages of development (Fig. 1).

The distribution of glands and organ histochemistry was determined for each stage. Fresh material was sectioned and stained directly or fixed in 3% gluteraldehyde, 0·05 M phosphate buffer pH 7·2 and prepared for either scanning electron microscopy by critical point drying (Phillips 505 SEM) or for light microscopy by dehydration with methyl cellosolve and embedding in glycol methacrylate (O'Brien and McCully, 1981). Toluidine blue was used as a general stain for morphology (O'Brien, Feder and McCully, 1964). Phenolic compounds were identified by colour with toluidine blue staining and by autofluorescence of unstained material (O'Brien and McCully, 1981) and lipophilic material by fluorescence following staining with aqueous 0·01% Auramine O (Considine and Knox, 1979).

Ontogenetic variation in volatile oil was determined from a combined sample of 0·35 to 0·75 g plant material harvested at each of the above stages and extracted with 10 ml of absolute AR ethanol (used in all extractions). A further ten fresh, open flowers from each plant were dissected into petals, stigma, stamens, staminodes and combined re-

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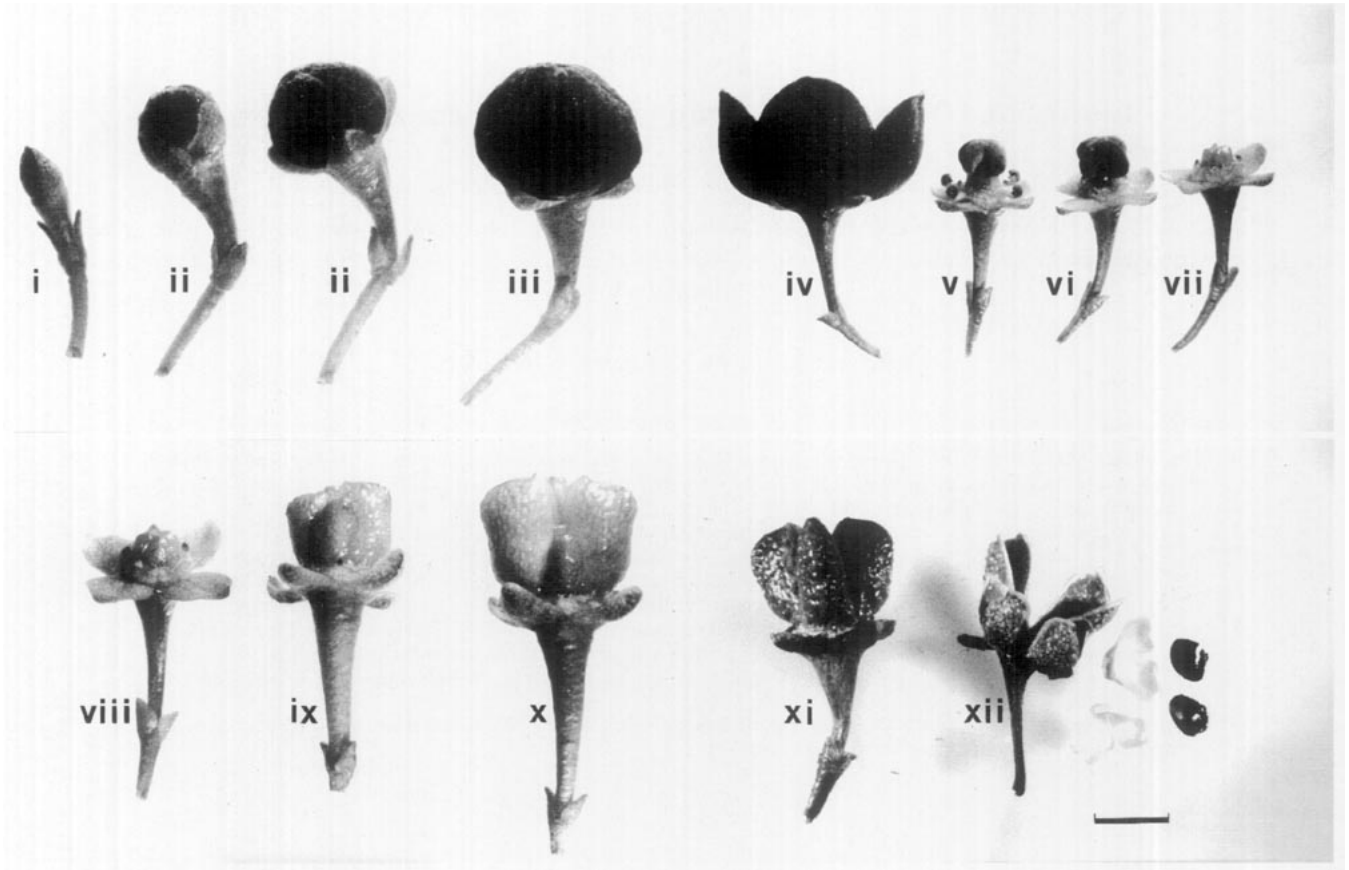


FIG. 1. Stages of reproductive development in *B. megastigma*. (i) Petals fully enclosed by the sepals; (ii) petals emergent from between sepals of immature flower bud and bud 2–4 mm diameter; (iii) balloon stage, bud 4–6 mm diameter; (iv) flower open; (v) flower pollinated and stigma discoloured; (vi) petals, stamens, staminodes and stigma abscised; (vii) fruit 2 mm diameter; (viii) fruit 5 mm diameter, seed testa transparent; (ix) seed testa brown; (x) fruit mature, seed complete with developed endosperm and embryo; and (xi) seed dispersed. (Bar = 3 mm)

ceptacle, ovary and nectary organs and the material placed immediately in ethanol and placed in an insulated box. All material for volatile oil analysis was extracted for at least 24 h before analysis.

An aliquot of the ethanolic extract (3 μl) was analysed without further purification or concentration using a Hewlett Packard 5890A GLC fitted with dual columns connected to a single injection port (50 m × 0·22 mm i.d. BPX70 and 50 m × 0·2 mm i.d. BP1) and twin flame ionization detectors. The carrier gas was hydrogen split at ratios of 2·2:2·5:64. The injector and detector temperatures were set at 275 °C. An initial oven temperature of 60 °C was held for 5 min and then increased at a rate of 4 °C min^{-1} to a final temperature of 275 °C which was held for 10 min.

The identity of particular eluates was determined from the retention times of authentic standards run concurrently and by co-chromatography (Jennings and Shibamoto, 1980). Ethyl un-decanoate (1 mg ml^{-1}) was used as the internal standard for α-pinene, β-pinene, limonene, β-ionone, dodecyl acetate and heptadecene. Values for β-ionone, dodecyl acetate, and heptadecene concentrations are the mean value obtained from both columns while those for α-pinene, β-pinene and limonene concentrations were calculated from the BP1 column only because they co-eluted with the solvent peak on the polar column (BPX70). Heptadecene refers to 8-heptadecene, the response factor for which was assumed to equal that of 1-heptadecene.

RESULTS

Flower and fruit development

Figure 1 represents the stages of flower and fruit development distinguished for the purposes of this study. Stages (i) to (iv) represent maturation of the flower bud and

FIG. 2. Light micrographs of glycol methacrylate embedded tissue stained with toluidine blue (A,B,C,E) and scanning electron micrographs (D,F) of gland types in *Boronia megastigma*. A, Multicellular trichomes in axil of vegetative shoot. Bar = 0·15 mm. B, Lysigenous glands (arrows), in nectary, petal and sepal of immature flower bud. Bar = 0·25 mm. C, Lysigenous glands and vasculature in nectary and ovary of immature fruit. Bar = 0·15 mm. D, Papilli on stigma surface. Bar = 0·25 mm. E, Stigma papilli and underlying glandular tissue. Bar = 0·22 mm. F, Surface of lysigenous gland and hair trichomes on receptacle. Bar = 0·02 mm. T, Multicellular trichome; Ax, axillary meristem; A, apical meristem; St, stigma; Sa, staminode; O, ovary; Pe, petal; S, sepal; H, hair trichomes; Ly, lysigenous glands; Ph, phenolic deposits; P, papilli.

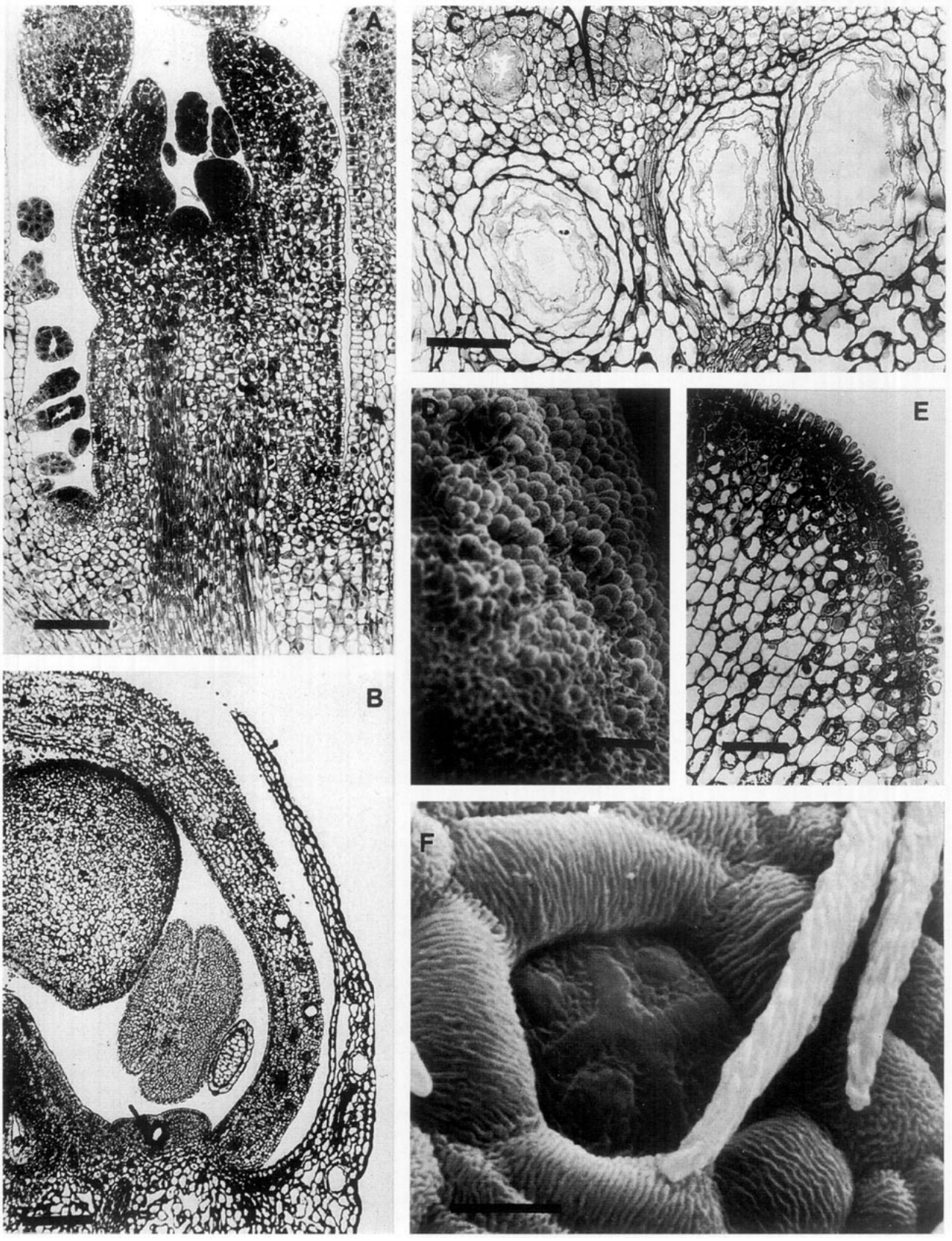
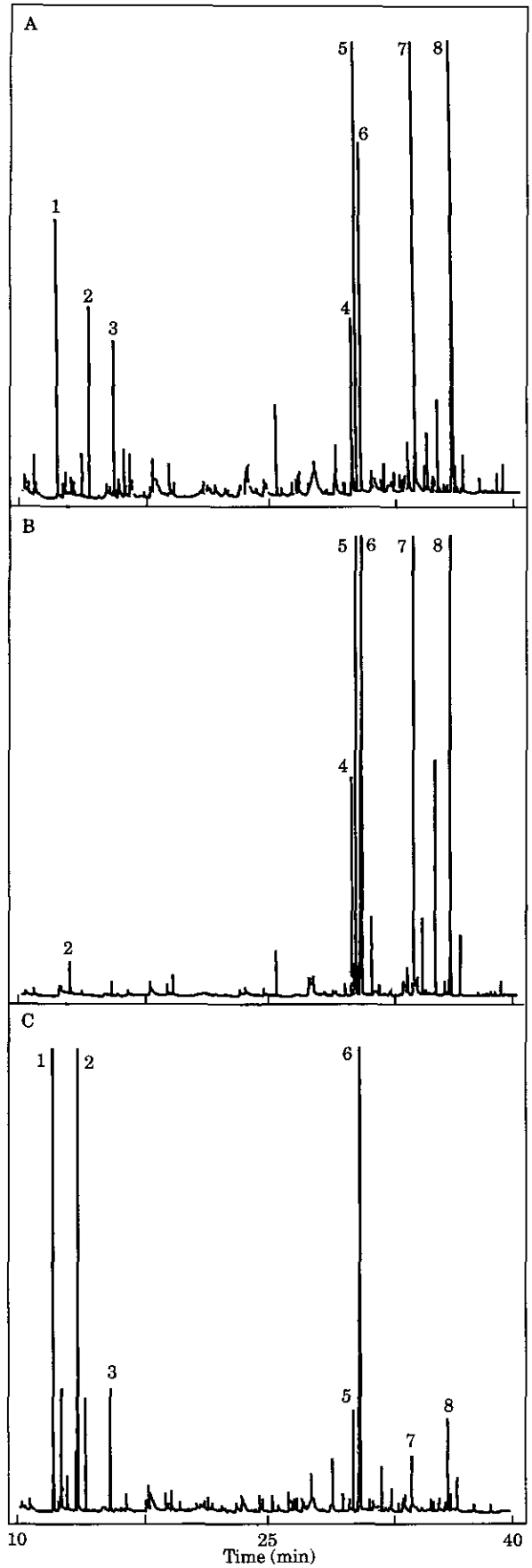


FIG. 2. For legend see facing page.



the succeeding stages fruit set and maturation. Pollination of the flower led to abscission of petals (v), then stamens and staminodes (vi), and finally the stigma (vii). The carpels then expanded (viii to x) and changed from being translucent (ix and x) to a dark brown (xi) as testa hardening began following the cessation of fruit expansion (x). The endosperm of the seed filled during the penultimate stage of development after which the embryo and seed matured (xi). Seed dissemination occurred after fruit senescence, browning and drying of the pericarp (xii).

Gland types and distribution

Multicellular trichomes, lysigenous glands, unicellular papillae and hair trichomes were identified during flower and fruit development of *B. megastigma* (Fig. 2). Multicellular trichomes with a single basal cell, two to five stem cells and a cap of 5–20 cells occurred on the adaxial surface of flower bracts and the leaf bases of axillary meristems (Fig. 2A) and were not examined for any glandular function.

Lysigenous glands were present in the mesophyll tissue of mature leaves, flower bracts, sepals, petals, the receptacle, ovary and nectary (Fig. 2B, C). The glands were isodiametric and varied in size from 0·06–0·3 mm. The lysigenous cavity was capped by four dorso-ventrally flattened cells on the epidermis (Fig. 2F). Unicellular papillae were abundant on the upper surfaces of the stigma (Fig. 2D, E) and occurred on the tip of staminodes and on the adaxial surface of petals. Extracellular, lipophilic compounds were identified as a well defined layer above the stigma, the toral nectary and on the cuticle of the receptacle and other vegetative tissues.

Phenolic compounds were associated with the cell walls of epidermal and subepidermal stigmatic cells (Fig. 2E), the cortex of the stamen filament and within the testa of the developing seed. Phenolic deposits were also present in the distal tissue of the staminode, the adaxial epidermis of petals, and in the walls of the mature fruit. These tissues were characterized by a densely stained cytoplasm and intercellular spaces.

Volatile oils of floral organs

Three classes of tissue were identified from the volatile oil profiles of individual flower organs (Fig. 3). The first class (Fig. 3A) was characteristic of petal tissue and contained relatively low concentrations of all of the volatile compounds observed (Table 1). The second class was characteristic of stigma, stamen and staminode tissue and possessed dodecanol, β-ionone, dodecyl acetate and heptadecene amongst the principal components (Fig. 3B and Table 1) but the monoterpenes associated with class 3 (vegetative) tissue

FIG. 3. Distribution of volatile oil components in unprocessed ethanolic extracts of petals (A), stigmas (B) and ovaries (C) of *B. megastigma*. Chromatographs from 50 m × 0·2 mm BP1 column with a split ratio of 32:1 otherwise as per Materials and Methods. (1) α-pinene; (2) β-pinene; (3) limonene; (4) dodecanol; (5) β-ionone; (6) internal standard; (7) dodecyl acetate; (8) heptadecene.

TABLE 1. *Variation in the concentration (μg. g⁻¹f. wt) of essential oil compounds detected in flowers from 6 individuals of* Boronia megastigma

	α-Pinene	β-Pinene	Limonene	β-Ionone	Dodecyl acetate
Freshly open flower					
Mean	52	56	37	408	284
Range	0–138	0–109	8–88	115–1092	112–631
Mature fruit					
Mean	418	165	17679	14	5
Range	142–877	39–240	16996–26691	0–58	0–24

TABLE 2. *Correlation matrix for component content in all samples at all stages of flower development. (The bold values are statistically significant at* $P \leqslant 0{\cdot}05$, $n = 58$)

	α-Pinene	β-Pinene	Limonene	β-Ionone
β-Pinene	0·741			
Limonene	0·616	0·360		
β-Ionone	−0·264	−0·068	−0·393	
Dodecylacetate	−0·266	−0·089	−0·342	0·889

were not detectable. Staminode tissue contained the highest proportion of heptadecene, while dodecyl acetate and β-ionone concentrations were highest in stigma extracts. Extracts of combined ovary, nectary and receptacle tissue contained primarily the volatile compounds α-pinene, β-pinene and limonene (Fig. 3C).

Correlation analysis showed a highly statistically significant relationship between α and β-pinene and between α-pinene and limonene but and to a lesser degree between β-pinene and limonene (Table 2). β-Ionone and dodecyl acetate were also highly correlated with one another and were negatively correlated with α-pinene and limonene content, but independent of β-pinene.

Ontogenetic change in volatile oil content

Developing whole flowers displayed large changes in volatile oil content during maturation and further development into mature fruits (Fig. 4). The content of β-ionone and heptadecene rose sharply at flower opening and declined rapidly as petals, anthers, staminodes and stigma were shed (stages v to vii). Heptadecene was only present in appreciable amounts at flower opening while β-ionone was present at the balloon stage of flower development but was much reduced after petal, androecium and stigma abscission. Limonene was present at a low level only at flowering and increased almost linearly as the fruit matured. α-Pinene and β-pinene concentration also increased with fruit maturation although a transitory peak was observed immediately following the abscission of floral parts and at the beginning of fruit development.

Considerable differences occurred in the content of particular volatiles between individual plants (Table 1). Thus the content of β-ionone in freshly open flowers varied nearly ten-fold, dodecylacetate six-fold, while the vegetative volatiles, α- and β-pinene and limonene were not detectable or present in moderate levels only. Extracts from mature fruit also varied considerably (30- to 60- to 200-fold for β- and α-pinene and limonene, respectively).

DISCUSSION

Lysigenous glands are the main volatile oil secreting organs in *B. megastigma* and are distributed throughout all plant tissues except those of the stigma and androecium. These

TABLE 3. *Relationship between floral histology and volatile oil composition*

Organ	Anatomical features: Trichomes†	Lysigenous glands	Surface papillae	Oil composition*
Leaves	✓	✓	—	B
Flower bracts	✓	✓	—	NT
Sepals	—	✓	—	NT
Receptacle	—	✓	—	B
Nectary	—	✓	—	B
Ovary	—	✓	—	B
Petals	—	✓	✓	A+B
Stamens	—	—	—	A
Stigma	—	—	✓	A
Staminodes	—	—	✓	A

* A, Dodecanol, β-ionone, dodedyl acetate and heptadecene; B, α- and β-pinene and limonene; NT, not tested.
† ✓, indicates presence; —, indicates abscence.

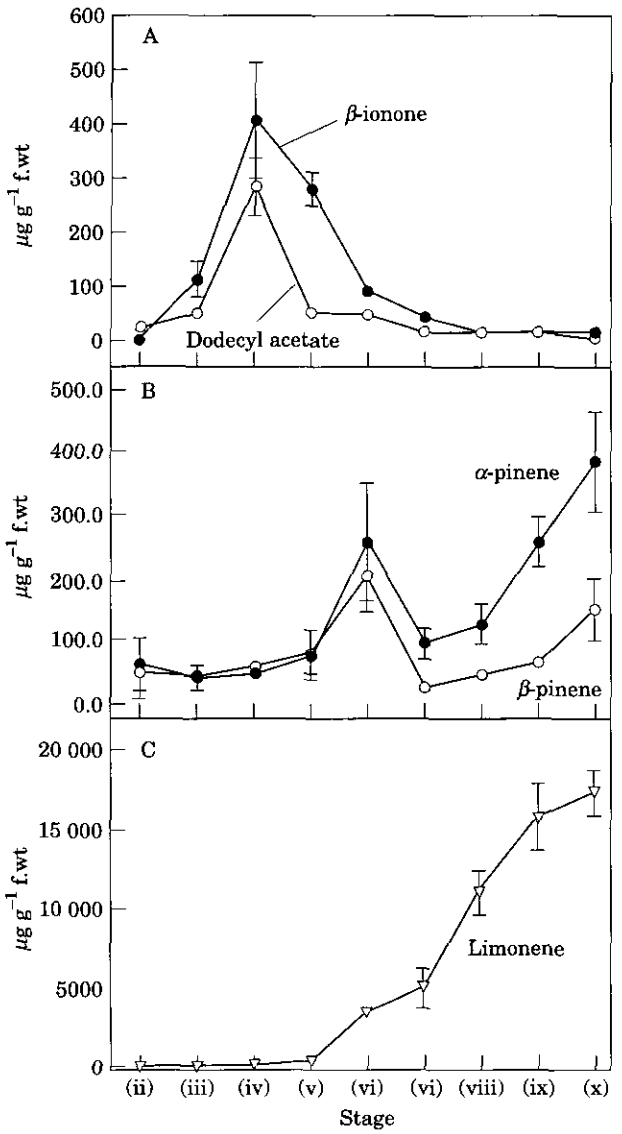


FIG. 4. Changes in volatile oil content ($\mu g\ g^{-1}$ f.wt) and composition during reproductive development of *B. megastigma*. The stages are as per Fig. 1 except that ('ix') is an intermediate stage of development of maturity of the seed characterized by a tan colour which subsequently becomes black as the seed matured (x). The time from stage (ii) to (iv) was 26 d and from (iv) to (x) another 78 d. A, β-Ionone (●) and dodeoyl acetate (○). B, α- (●) and β- (○) pinene. C, Limonene (▽). Bars represent ± s.e..

glands are the principal source of the monoterpene hydrocarbons α- and β-pinene and limonene, in a base of higher boiling point compounds identified previously by Davies and Menary (1984). During flowering, individual concentrations of these compounds rarely exceed 0·02% of the fresh weight of the flower. However, the volatile oil extracted from the ovary wall and nectary of the developing fruit was shown here to contain up to 2% limonene. This proportion is consistent with the limonene values obtained in the developing fruit of *Citrus* (Johnson and Peterson, 1974; Kekelidze, Lomidze and Janikashvili, 1989).

Stigma, staminodes and stamens are the primary sites of accumulation of the volatile oil components, dodecanol, β-ionone and heptadecene although petal tissue and possibly maturing fruit also contained these compounds. Unicellular papillae were evident on stigma and staminode organs, although, no distinct glandular structures were observed on or in the stamens. All tissues of the stigma and androecium were characterized by a dense cytoplasm, intercellular spaces and the accumulation therein of phenolic compounds. Hence, we suggest that β-ionone, dodecyl acetate and heptadecene are synthesized in glandular tissues associated with floral organs rather than in the discrete structures usually associated with volatile oil production (Table 3). The presence of the acetate derivatives in developing fruit is uncertain due to the very high levels of terpenes and requires confirmation by GC-MS or other appropriate methods.

Vogel (1990) suggested that the term osmophore be used to describe tissues that produce volatile secretions as attractants for pollinators. Structurally, the epithelium, or 'emission layer', of osmophores overlays a 'production layer' with a developed system of intercellular spaces, starch reserves and phenolic secretions. Structural similarities between the osmophores described by Vogel (1990) and the glandular tissues described in this study are apparent. Identification of an ecological role for volatile oil compounds, particularly with regard to their pheromone qualities would establish whether the term osmophore is an appropriate description for the stigma and androecial organs of *B. megastigma*.

The large variation in oil content observed between plants within a single population and in particular, the presence of plants with undetectable levels of the undesirable monoterpenes, α- and β-pinene and limonene, indicates that clonal selection may be a promising option for improving the quality and yield of boronia flower extracts for the fragrance and flavour trades.

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