

Intraspecific Variation in Oil Components of *Boronia megastigma* Nees. (Rutaceae) Flowers

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Intraspecific variation in the oil composition of *Boronia megastigma* Nees. (Rutaceae) was examined. *Boronia* absolute is extracted from blossom primarily for use as a food additive. A major component is β -ionone and *B. megastigma* is one of the commercial, natural sources of this compound. Genotypes superior in production of β -ionone and low in monoterpene hydrocarbons were sought from natural populations in the south west of Western Australia as part of a breeding programme. Flowers were collected from 25 plants in each of 29 different populations. Blossom was extracted with ethanol and analysed using a gas liquid chromatograph fitted with ionisation detectors. The contents of β -ionone, dodecyl acetate, α -pinene, β -pinene and limonene in the oil extract were compared. Intra-population variation was as great as inter-population variation and no distinct chemotypes were found. Considerable variation existed in the content of components. The highest β -ionone content was 1787 mg g⁻¹ f. wt. Some genotypes contained all five components analysed, others lacked one or more of the monoterpenes: α -pinene, β -pinene or limonene. Principle components analysis indicated that contents of β -ionone and dodecyl acetate were associated and that they were distinct from the content of the monoterpenes, which were associated with each other. Natural shading was associated with lower levels of monoterpenes but other oils were unaffected. Young plants contained less pinenes than older plants and old plants contained the most dodecyl acetate. Vigorous plants produced more pinenes. Red flowers contained the least β -ionone and dodecyl acetate. © 1999 Annals of Botany Company

Key words: *Boronia megastigma*, boronia, Rutaceae, oil, α -pinene, β -pinene, limonene, β -ionone, dodecyl acetate, monoterpenes, chemotypes.

INTRODUCTION

Boronia megastigma Nees. (Brown boronia) flowers are the source of the highly valued oil, boronia absolute. The species is endemic to south-western Western Australia where it is found in wet or seasonally wet low-lying sites, usually associated with jarrah (*Eucalyptus marginata* Sm.) forests, paperbark (*Melaleuca parviflora* Lindl.) flats, and creeks (Christensen and Skinner, 1978). Volatile oils from the flowers are extracted with hexane or petroleum ether to yield the concrete. *Boronia* absolute can be purified from the concrete by alcohol washing or distillation. Flowers yield about 0.4–0.8% concrete, and the concrete yields about 60% absolute (Guenther, 1949; Penfold and Willis, 1954). The absolute is used primarily in food flavouring to impart a unique richness to many fruit essences (Arctander, 1960; Davies and Menary, 1984; Weyerstahl *et al.*, 1994).

The composition of boronia oil from *B. megastigma* has been documented from plants cultivated in Tasmania (Leggett and Menary, 1982; Davies and Menary, 1984; Roberts and Menary, 1994; Weyerstahl *et al.*, 1994) and from natural populations in Western Australia (Bussell, Considine and Spadek, 1995). More than 150 compounds have been detected, and about half of these have been identified. Many compounds contribute to the aroma and these may be substantial components or present in very small quantities. Davies and Menary (1984) found four

major compounds in the volatile fraction providing the typical boronia fragrance. These were β -ionone, dodecyl acetate, (Z)-heptadec-8-ene and an unidentified compound 'sesquicineole'. The chemical structure of 'sesquicineole' was unknown at the commencement of this experiment and it has only recently been identified (Ghisalberti, 1998). 'Sesquicineole' modified the aroma but it was only found in one clone and (Z)-heptadec-8-ene did not have an important role in the overall aroma impression. The monoterpene hydrocarbons: α -pinene, β -pinene and limonene were present in varying proportions in the three clones examined by Davies and Menary (1984). These compounds do not impart a favourable effect on the aroma.

Chemotaxonomic analysis, using oil constituents of leaves, fruit and flowers within the *Aurantoidea* (Rutaceae), can distinguish between *Citrus* and *Poncirus* genera and species; however varieties are more difficult to separate (Attaway, Pieringer and Barabas, 1966; Scora and Torrisi, 1966; Sawamura *et al.*, 1994). Recently oil analysis has been used to indicate differences in fruit quality in progeny from the *Citrus* breeding programme (e.g. Ruberto *et al.*, 1997). Intraspecific chemical variation, based on essential oil analysis, is found in *Eucalyptus*, *Melaleuca* and *Chamelaucium* and some of these forms have been distinguished as chemotypes (Whiffin and Ladiges, 1992; Butcher, Matheson and Slee, 1996; Egerton-Warburton, Ghisalberti and Considine, 1998). Superior chemotypes, if

present in *B. megastigma*, may have important commercial implications. While variation in essential oil components across natural populations of *B. megastigma* has not been examined, a study of three cultivars demonstrated a considerable variability between individual concretes (Davies and Menary, 1984).

Differences in qualitative and quantitative oil traits are known to be genetically determined in both *Eucalyptus* and *Melaleuca* (Whiffin and Ladiges, 1992; Butcher *et al.*, 1996). However, while both geographic and seasonal differences have been observed in *Citrus* (Scora and Torrisi, 1966), variations due to environmental influences are less well understood. The aim of this study was to investigate variation in the content of commercially significant volatile oil components of flowers from individual plants within and between natural populations of *B. megastigma* and over successive years.

MATERIALS AND METHODS

Sampling

In August and September 1993 *B. megastigma* flowers were collected from plants in 13 natural populations. In August and September 1994 flowers were collected from 16 natural populations. Opening, unpollinated flowers, between Stages III and IV (Bussell *et al.*, 1995), were collected from 25 randomly selected plants from the majority of populations, but where numbers of flowering plants were limited, fewer plants were sampled ($n = 24$ for the Walpole-2 and Palgarup-1 populations; $n = 20$ for the Yallingup population).

Details of flower colour, plant age and plant vigour were recorded for all plants. An approximate plant age was determined using the number of years since the last fire or major soil disturbance. Seedlings were defined as less than 1 year old, young plants 1–2 years, mature plants 2 to 6 years and old plants greater than 6 years. Plant vigour was determined by estimating the density of leaves and flowers on each plant. Weak plants had few flowers and little foliage which was often discoloured, average plants had moderate amounts of foliage and flowers, and vigorous plants had abundant flowers and lush green foliage. The amount of shading by surrounding vegetation was determined for 273 of the plants sampled in 1993 using a Model-A forest densiometer (Lemmon, 1957).

Flowers were collected from seven to ten plants from 12 natural populations in 1993. This included all populations sampled in 1993 except Moodiarup (Fig. 1). These plants were re-sampled in 1994 in order to investigate the quantity of oil components produced by individual plants over successive years.

Oil analysis

Twelve flowers collected from each plant were extracted with 10 ml of absolute ethanol. Collection vials were weighed before and after flower collection and the difference used to determine flower fresh weight. Flowers were extracted for at

least 24 h (Bussell *et al.*, 1995) and sealed vials were stored at 4 °C before oil analysis. Ethyl un-decanoate (1 mg ml⁻¹) was used as an internal standard. An aliquot (4 ml) of the extract was analysed without further purification or concentration using a gas liquid chromatograph (Hewlett Packard 5890A) fitted with dual columns (50 m × 0.25 mm internal diameter BPX70 from SGE International and 50 m × 0.2 mm internal diameter ULTRA1 from Hewlett Packard) connected to a single injection port and twin ionisation detectors. The carrier gas was hydrogen split at ratios of 1:00:1:15:15:2. 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 8 °C min⁻¹ to a final temperature of 260 °C which was held for 10 min. The content ($\mu\text{g g}^{-1}$ f. wt of flowers) of α -pinene, β -pinene, limonene, α -ionone, β -ionone and dodecyl acetate was determined using the areas produced by the ULTRA 1 column. The BPX70 column was used to confirm the presence or absence of α -ionone, β -ionone and dodecyl acetate. Alpha-pinene, β -pinene and limonene co-eluted with the solvent peak on this column.

Statistical analysis

Plant morphology and oil content data were analysed using analysis of variance and means were separated using Fishers PLSD ($P = 0.05$). Comparisons of oil contents between 1993 and 1994 were made using paired two-tailed *t*-tests.

Two principal components analyses were performed on the oil data obtained from each year. The first analysis used the oil data [transformed $\log(x + 1)$] from all individuals sampled to plot the orthogonal factor scores and determine the relationship between the different oil components. The second analysis used the mean data [transformed $\log(x + 1)$] for each oil component from each population to determine the relationship between different populations in terms of the five oil components.

RESULTS

The 29 *B. megastigma* populations sampled over 1993/94 cover most of the now remaining natural range of the species (Fig. 1). Results from the oil analysis on the 718 plants sampled over both years were used to determine a ranking for each oil component (Table 1). All plants contained β -ionone, but α -ionone was either absent or in undetectable amounts and it was therefore excluded from further analysis. β -pinene was absent from 50% of the plants, while α -pinene, limonene and dodecyl acetate were absent from 22, 5 and 1%, respectively.

The mean content of oils varied between populations. Mean β -ionone content was usually greater than dodecyl acetate, which in turn was greater than the monoterpenes (Fig. 2). Alpha-pinene and limonene were often present in similar quantities, and β -pinene was usually the least abundant of the oil components examined. The content of each oil component varied considerably between populations. Although α -pinene, β -pinene and limonene were

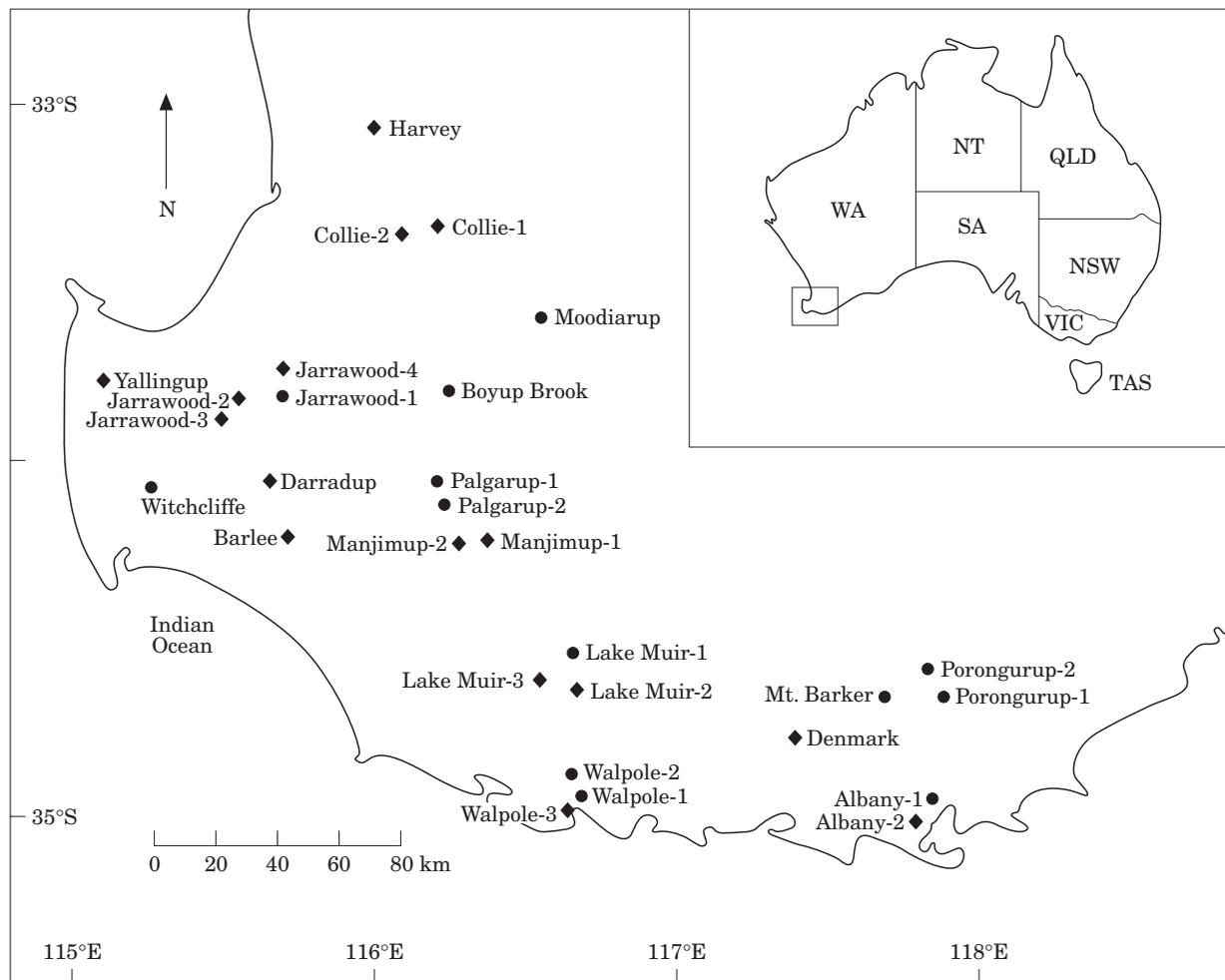


FIG. 1. *Boronia megastigma* populations in the south-west of Western Australia. ●, Populations initially sampled in 1993 and re-sampled for comparison in 1994 (except Moodiarup which was not re-sampled in 1994); ◆, populations only sampled in 1994.

TABLE 1. Ranking of *Boronia megastigma* plants by the content of oil components

Rank	% of total plants	Oil content ($\mu\text{g g}^{-1}$ f. wt)				
		α -pinene	β -pinene	limonene	β -ionone	dodecyl acetate
Extremely high	5	420–1001	298–940	264–893	803–1787	577–1291
Very high	5	303–419	207–297	184–263	646–802	518–576
High	10	193–302	140–206	118–183	548–645	427–517
Medium	60	0–192	0–139	33–117	260–547	178–426
Low	10	0	0	25–32	199–259	115–177
Very low	5	0	0	15–24	162–198	84–114
Extremely low	5	0	0	0–14	70–161	0–83

Oils were extracted from flowers collected from plants growing in their natural habitat which is restricted to Western Australia.

commonly absent from individual plants (Table 1), each of these oil components was found in the flowers from at least one plant within all populations sampled. However, location had some effect on the content of these monoterpene hydrocarbons. South-eastern populations, such as Albany-1, Albany-2, Mt Barker and Denmark had quite high mean monoterpene contents, while northern populations such as Boyup Brook, Moodiarup, Collie-1, Collie-2 and Harvey

were all low. Location was not always a good indicator of the content of oil components. For example, of the Jarrowood populations sampled in 1994, Jarrowood-3 produced more than twice the amount of α -pinene and β -pinene as Jarrowood-2 and Jarrowood-4, even though the three populations were only a short distance apart. Location trends were not evident for β -ionone and dodecyl acetate. Of the populations sampled in 1993, plants from the Boyup

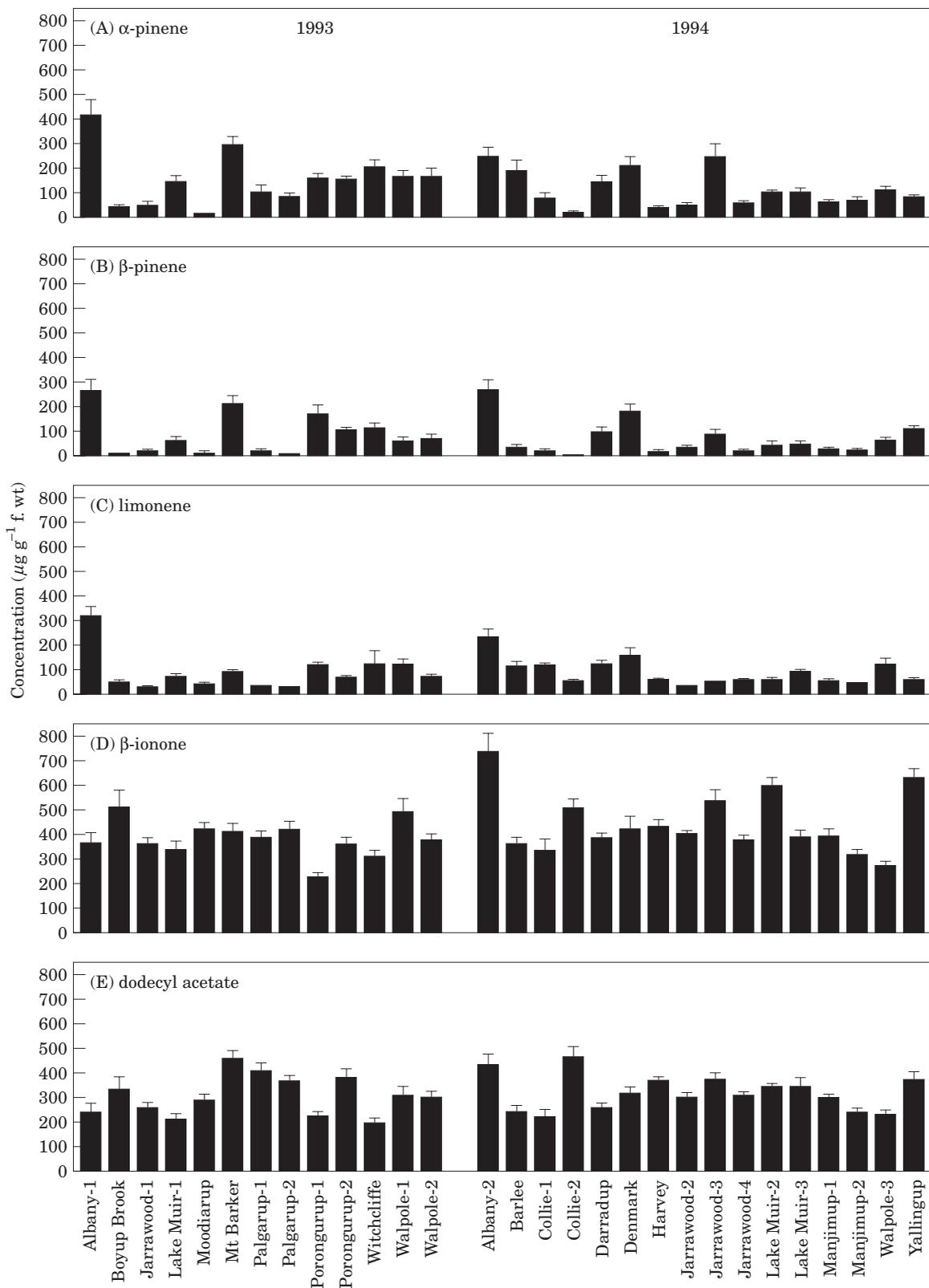


FIG. 2. Contents of oil components: α -pinene (A), β -pinene (B), limonene (C), β -ionone (D) and dodecyl acetate (E) in *Boronia megastigma* flowers collected in 1993 and 1994 from plants growing in their natural habitat. The 29 populations are ordered alphabetically within each year. Bars indicate s.e.

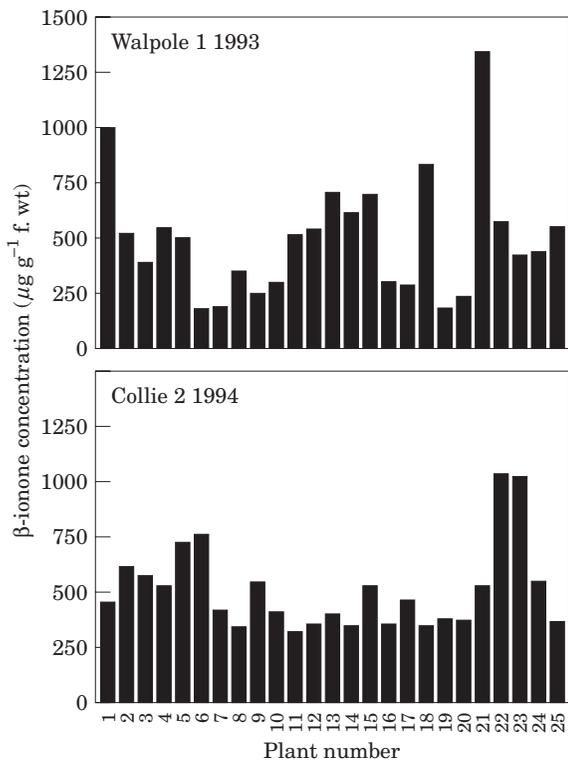


FIG. 3. Variation in β -ionone content within two populations of *Boronia megastigma*.

Brook population produced the highest mean content of β -ionone (511 mg g^{-1}) and plants from the Porongurup-1 population produced the lowest (227 mg g^{-1}). In 1994, mean β -ionone content ranged from 736 mg g^{-1} at Albany-1 to 274 mg g^{-1} at Walpole-3.

The content of oil components within populations also varied considerably (Fig. 3). The Walpole-1 population and Collie-2 population were typical of the variation in β -ionone content that existed within populations. Beta-ionone concentration ranged from very low (175 mg g^{-1}) to extremely high (1342 mg g^{-1}) in plants from Walpole-1, and from medium (314 mg g^{-1}) to extremely high (1028 mg g^{-1}) in plants from Collie-2.

Separate principal component analyses on the 1993 and 1994 data are shown in Fig. 4. In 1993, the first principle axis accounted for 43.1% of the variance and the second principle axis 29.1%, whilst these values were 39.5 and 33.6%, respectively, in 1994. The distance between any two oil components is an indication of the relationship between them according to their content in the plants sampled. Beta-ionone and dodecyl acetate were closely related, especially within the plants sampled in 1993, as were α -pinene and β -pinene, and to a lesser extent, limonene.

Plotted scores from principal components analyses on the mean oil content of each population did not show any distinct sets or groupings of related populations (Fig. 5), though certain populations were adjacent and therefore similar in the mean content of the five oil components. Some of the populations were located in close proximity geographically but others were distinct. As an illustration, Boyup Brook, Palgarup-1 and Palgarup-2 are within 33 km of each other and are closely related, as are Jarrawood-2 and Jarrawood-4. Jarrawood-3, however, is quite distinct. Jarrawood-2 and Jarrawood-4 are located within the same catchment, while Jarrawood-3, although only 8 km from Jarrawood-2, is in a different catchment area. Similarly, Collie-1 and Collie-2 are located in adjoining water catchment areas, and differ markedly. Thus distance is not a reliable indicator of relatedness but catchment area may be.

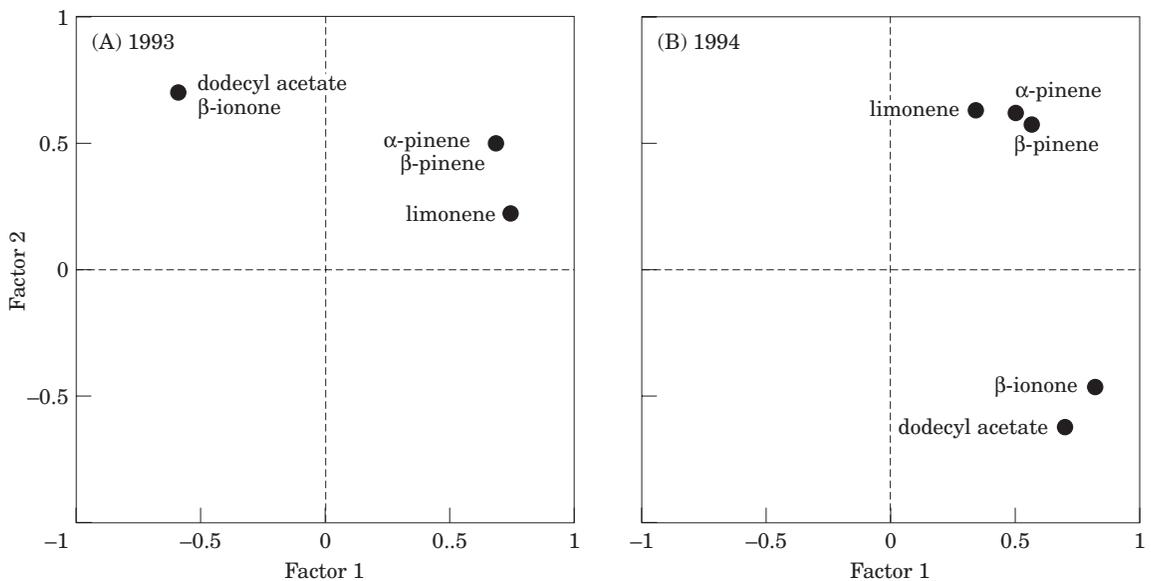


FIG. 4. Unrotated orthogonal plot of factor 1 and factor 2 resulting from principal components analysis of oil components from individual *Boronia megastigma* plants sampled from their natural habitats in 1993 (A) and 1994 (B).

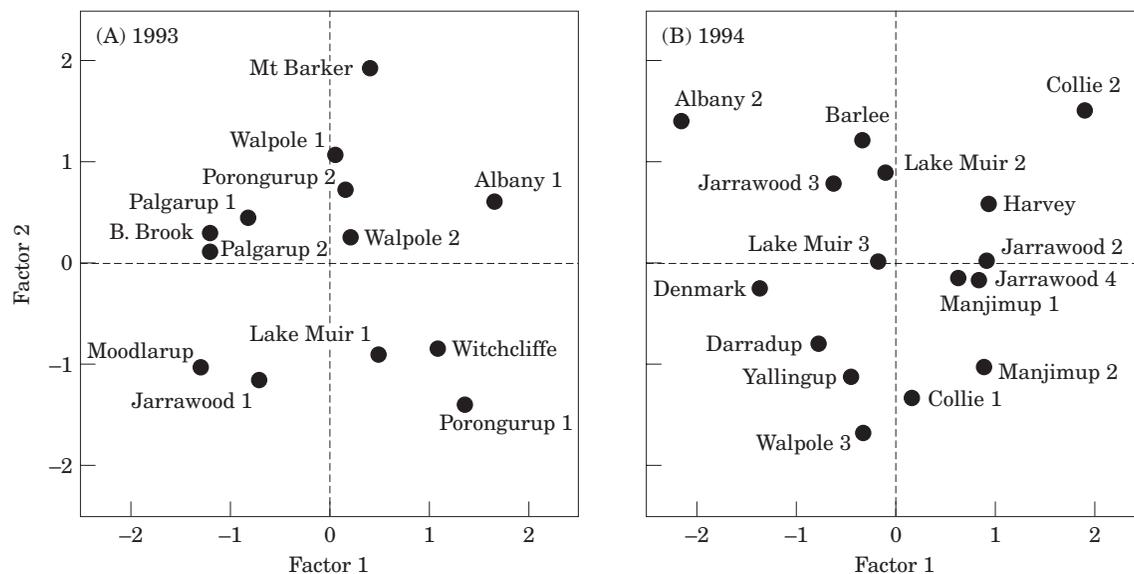


FIG. 5. Plotted factor scores from principal components analyses of the mean content of oil components from natural *Boronia megastigma* populations sampled in 1993 (A) and 1994 (B).

Effect of shading

The amount of canopy cover above *B. megastigma* plants was found to affect the content of the terpenoids, α - and β -pinene and limonene extracted from flowers (Fig. 6A–C), but no simple relationship was apparent. Plants shaded by a high level of canopy cover produced a smaller range of monoterpene hydrocarbon values than plants in areas with little canopy cover. Beta-ionone and dodecyl acetate contents were unaffected by the surrounding canopy cover (Fig. 6D and E).

Plant age, plant vigour and flower colour

Plant age, plant vigour and flower colour each affected the content of at least one of the five essential oil components investigated. Similar trends occurred in both years and so only 1993 data are presented (Fig. 7). Age affected four essential oil components. Only two plants of seedling age were present in the 13 populations sampled in both 1993 and 1994. These contained high levels of β -pinene (230 mg g^{-1}) and β -limonene (233 mg g^{-1}) but were removed from the statistical analysis. Young plants contained less α -pinene and limonene than mature plants and less β -pinene than mature and old plants. Old plants had the most dodecyl acetate. Plant vigour, as distinct from age, also affected the quantity of oil extracted per flower. Vigorous plants tended to produce more β -pinene and limonene than less vigorous plants, with average plants being intermediate in content.

Most plants sampled in 1993 ($n = 338$) were brown (56%), followed by red/brown (35%) and only a small proportion were red (8%) or yellow (1%). Colour appeared to be associated with oil content; red flowers contained the least β -ionone and dodecyl acetate. There was a tendency for red/brown flowers to have less β -ionone and dodecyl

acetate than brown flowers, while the yellow flowers assessed contained no detectable amounts of β -pinene.

Comparison of 1993 and 1994 oil samples

When the oil content of the 109 plants sampled in both 1993 and 1994 was compared using paired *t*-tests, β -ionone and dodecyl acetate content was found to differ significantly between the two years. The mean content of β -ionone and dodecyl acetate was higher in 1994 than 1993, while α -pinene, β -pinene and limonene content did not differ significantly (Fig. 8).

The mean content of the majority of oil components for each population sampled in 1994 was the same as for 1993. Differences could be attributed to either large differences in an individual plant, or numerous small differences in several plants within the population. Twenty-six of the 109 plants were found to have no detectable α -pinene in at least one of the years sampled. Of the plants found to have no detectable α -pinene present in either the 1993 or 1994 analysis, 69% also had no detectable α -pinene in both years. No detectable β -pinene was found in 53 of the 109 plants in at least one of the years sampled and of these plants 85% had no β -pinene in both years. Of the plants lacking limonene, only one in five had no detectable limonene in both years. Many plants consistently had two or more of the hydrocarbon monoterpenes absent, and several were lacking all three.

DISCUSSION

Whilst substantial variation was present in the floral oils of *Boronia megastigma* the qualitative and quantitative variation was continuous and not partitioned into chemotypes. The lack of chemotypes was clearly supported by principle components analysis which consistently failed to separate populations. The content of the five oil components, β -

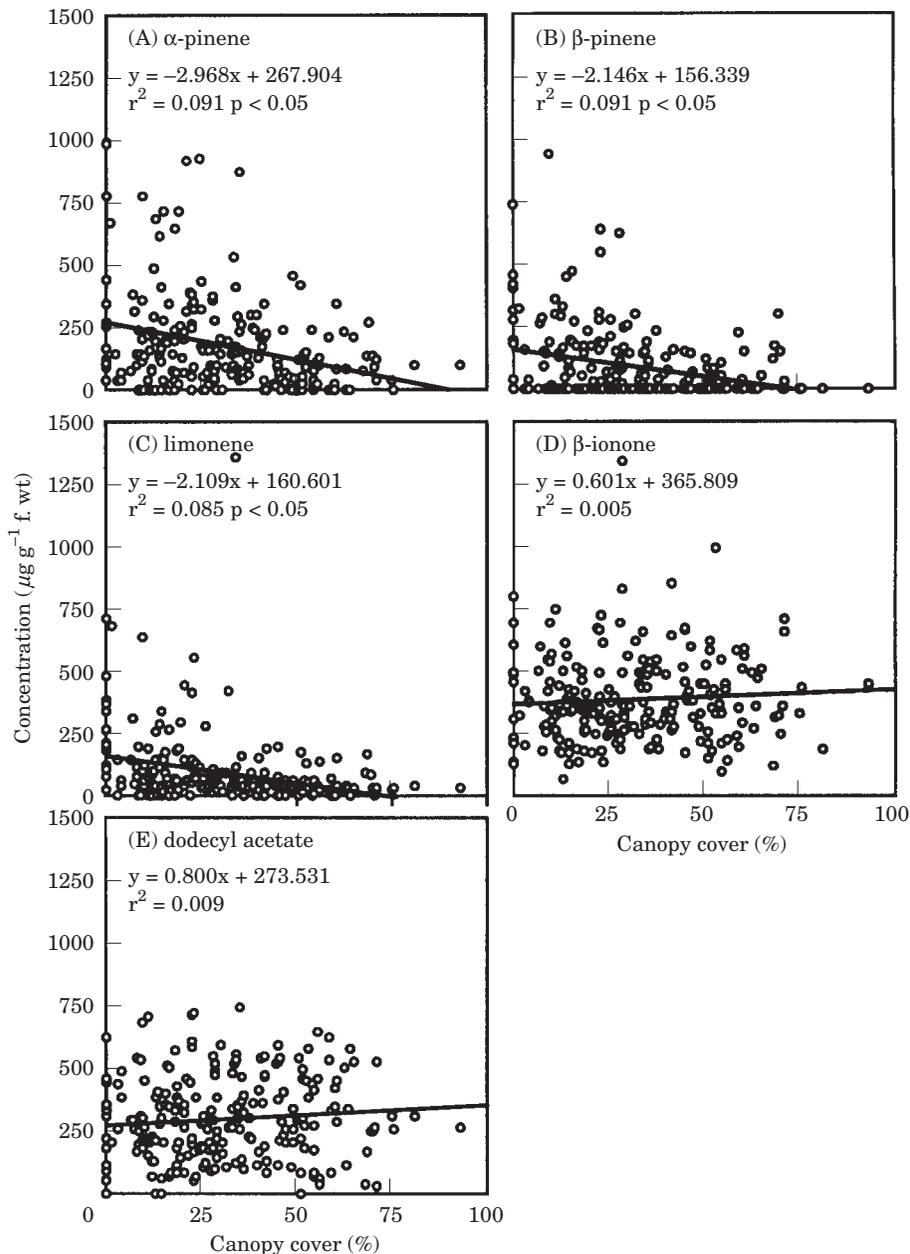


FIG. 6. Effect of canopy cover on the content of the oil components: α -pinene (A), β -pinene (B), limonene (C), β -ionone (D) and dodecyl acetate (E) in *Boronia megastigma* flowers collected from plants growing in natural habitats in 1993 and 1994.

ionone, dodecyl acetate, α -pinene, β -pinene and limonene varied considerably both within and between populations. Some plants lacked at least one of the four compounds (dodecyl acetate, α -pinene, β -pinene or limonene) and some lacked more. However, all populations had at least one plant which contained each of the monoterpenes. On average, plants within south-eastern populations had high concentrations of monoterpenes, while northern populations all had low concentrations and contained many plants without α -pinene, β -pinene or limonene. Variations in concentrations were also continuous. No consistent trends were observed in either β -ionone or dodecyl acetate content. The populations at Boyup Brook and Albany-1 had the highest mean concentrations of β -ionone but they

did not contain plants with the highest β -ionone concentration. Indeed populations which contained plants with the highest β -ionone concentration also contained plants with some of the lowest concentrations.

In many genera, qualitative and quantitative variation in oil is genetically determined (Scora and Torrisi, 1966; Leach and Whiffin, 1989; Sawamura *et al.*, 1994; Butcher *et al.*, 1996). Variation in essential oil composition is therefore an indicator of genetic variation. Although some individual populations are now quite isolated from one another similar variation exists in oil composition in populations at the centre and extremes of the range. Generally gene flow is required to maintain genetic variation. The isolation of *B. megastigma* populations may be relatively recent and has

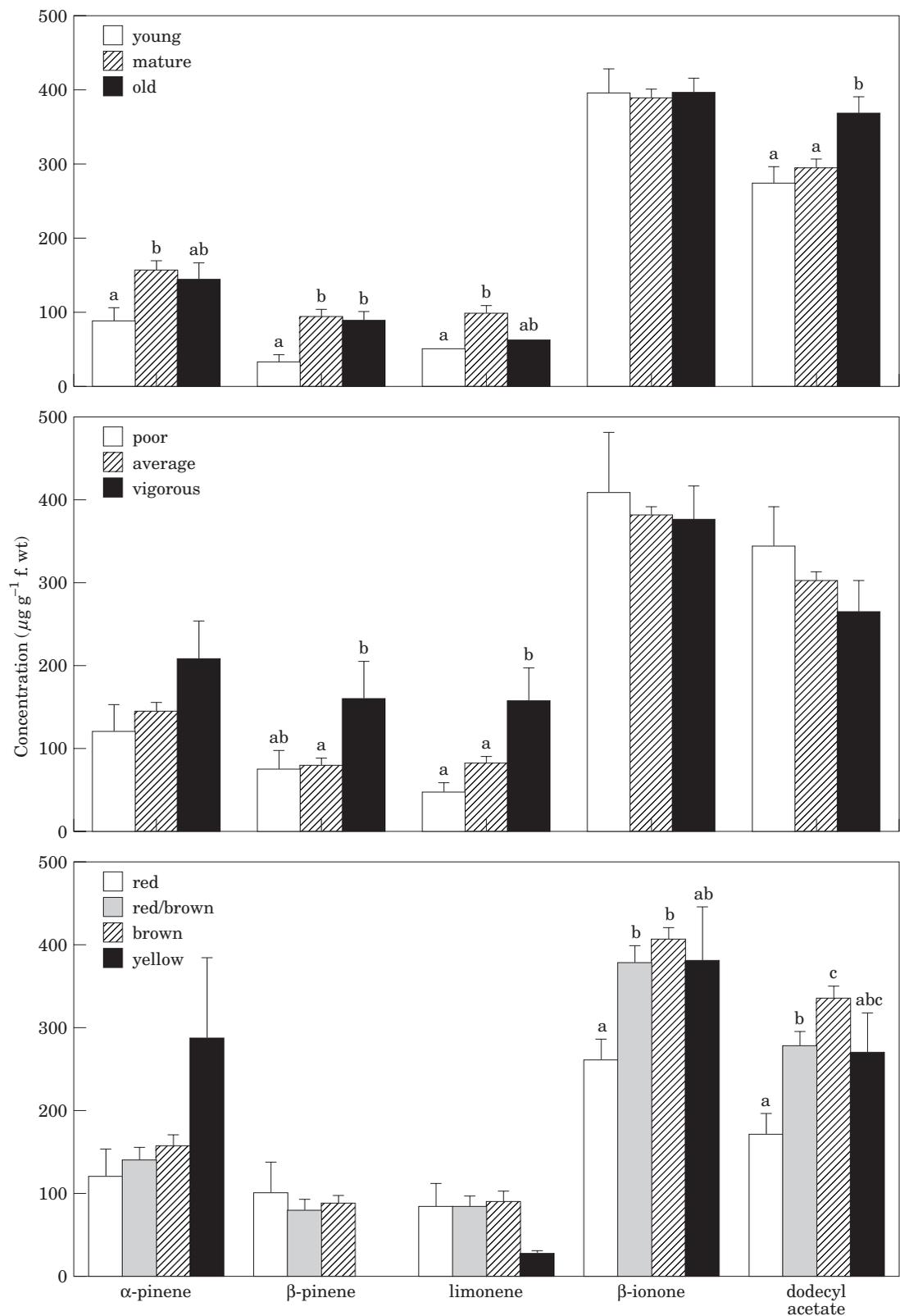


FIG. 7. Effect of plant age (A), plant vigour (B) and flower colour (C) on the concentration of oil components in *B. megastigma*. Bars indicate s.e. Letters indicate significant differences between means within each oil component. Replication within categories: plant age—[seedlings (2)—not presented], young (46), mature (246), old (44); plant vigour—poor (11), average (300), vigorous (27); flower colour—brown (188), red/brown (119), red (28), yellow (3).

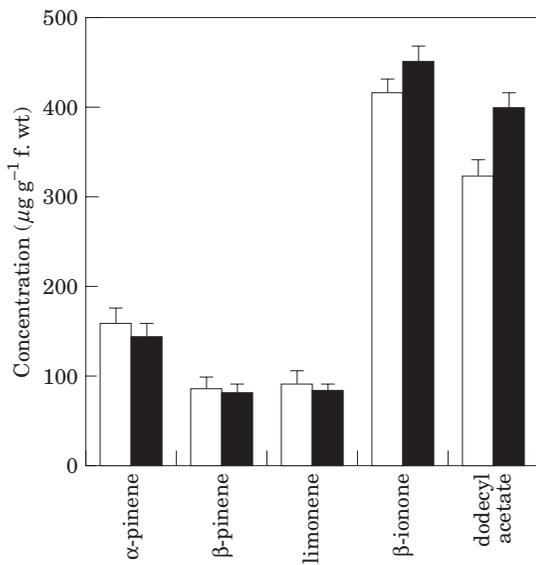


FIG. 8. Mean quantity of oil components from *Boronia megastigma* flowers collected from plants growing in their natural habitats in 1993 (□) and 1994 (■). Bars indicate s.e. $n = 109$.

certainly increased since settlement. *Boronia megastigma* flowers are hermaphroditic and pollination is by an unknown species of moth. Certain oil components, such as dodecyl acetate, act as sex pheromones for other female moth species (Nesbitt *et al.*, 1975). The boronia moth copulates and then oviposits in the ovary of the flower and in doing so leaves pollen on the stigmatic surface. Essential oil composition is closely linked with flower development and so components are probably acting as attractants to the boronia moth (Bussell *et al.*, 1995; MacTavish and Menary, 1997). *Boronia megastigma* is not self-pollinating and is self-incompatible (Weston, Carolyn and Armstrong, 1984) and movement of the moth from flower to flower would also promote outbreeding. However, it is possible that as populations have become more isolated, the movement of the moth could be restricted to within an individual population or a few close populations. As a result, distant populations may become more genetically isolated over time. Populations in the south-eastern area of the species' range had comparatively high concentrations of α -pinene, β -pinene and limonene compared with northern populations but it is unlikely that this is attributable to genetic isolation of these two areas.

Populations in close proximity to each other may remain genetically similar not only due to the movements of pollinating moths, but also water movements that may wash seeds from one population to another. Populations within the same water catchment appeared more closely related than nearby populations located within a different catchment area. However, location did not always explain differences between populations in terms of their oil content, as the Porongurup populations sampled in 1993 show. Porongurup-1 and Porongurup-2 are located in the same water catchment but Porongurup-1 had higher mean β -pinene and limonene and lower β -ionone and dodecyl acetate concentrations than Porongurup-2.

Volatile oils of *B. megastigma* flowers appear to act as pheromones and vary with floral development (Bussell *et al.*, 1995; Mactavish and Menary, 1997). In particular, β -ionone content increases ten-fold and dodecyl acetate six-fold during flower opening. Presumably this assists in their role of attracting moths following anthesis and during stigma receptivity. This substantial change in oil content during the period when flowers were harvested would increase the variation observed between genotypes and in years of collection.

Oil yield can be substantially modified by environmental factors (Scora and Torrisi, 1966). Variation in *B. megastigma* oil was observed in extracts from the same plants between different years, and some differences could be correlated with aspects of the environment. Small but consistent quantitative differences in essential oils due to environmental factors have also been recorded in other Rutaceous species. The terpenic, alcoholic and aldehydic oil fractions of *Citrus sinensis* 'Valencia' fruit, are higher from coastal areas of California, whereas linalool and geraniol are higher inland (Scora and Torrisi, 1966). More southern populations of *B. megastigma* would tend to receive more rainfall and are exposed to cooler temperatures which may affect oil composition.

Light also influenced oil quantity. Although not an accurate measure of light intensity, higher levels of canopy cover above plants generally result in higher levels of shading. This, however, was not a controlled experiment, as shade levels would change throughout the day with the movement of the sun. Nevertheless, it was demonstrated that in open conditions with little canopy cover and shade, *B. megastigma* produced high amounts of α -pinene, β -pinene and limonene, while in denser forest areas where the canopy cover and shade levels were high, these compounds were produced in low amounts. In peppermint, *Mentha piperita*, highest oil yields, including the production of limonene, result from high photon flux density (Clark and Menary, 1980). Quantitative differences in the production of particular oil components are also seen in *Pinus monticola* where southern facing branches (northern hemisphere) have higher α - and β -pinene and limonene content than the more shaded northern facing branches (Hanover, 1966). Shading effects would also be compounded by differences in leaf temperature but this was not explored.

Chemotaxa are not necessarily associated with particular morphological characteristics. However, in *B. megastigma*, flower colour was correlated with particular essential oils. Although yellow flowered specimens were rare, no β -pinene was detected. Red flowers contained less β -ionone and dodecyl acetate than brown flowers suggesting a link between these chemical and morphological traits. Carotenes are pigments which affect flower colour and β -ionone is synthesized from β -carotene (Sanderson and Gonzalez, 1971). Flowers of vigorous plants tended to have higher concentrations of monoterpenes as did seedling plants. Both vigorous plants and seedlings would have a predominance of new growth and this may be causal. The production of abundant new growth may be genetically controlled or due to their juvenile status. It may also be due to greater access to water and nutrients, especially in seedlings which emerge

after fire in the nutrient rich ashbed when competition is reduced (Christensen and Skinner, 1978).

Principal components analysis indicated that β -ionone and dodecyl acetate were more closely associated with each other than with α -pinene, β -pinene and limonene. This may be advantageous in selecting plants with high β -ionone and dodecyl acetate and low monoterpene concentrations. The relationships between these components are supported by their location in *B. megastigma* flowers. The stigma and petals contain most of the β -ionone and dodecyl acetate, whereas the receptacle and ovary contain α -pinene, β -pinene and limonene (Bussell *et al.*, 1995; MacTavish and Menary, 1997). Beta-ionone, dodecyl acetate, α -pinene, β -pinene and limonene are all derived from acetyl SCoA (Mann, 1994). Dodecyl acetate is derived from the fatty acid pathway. The other compounds have a similar precursor of mevalonic acid but limonene, α -pinene and β -pinene follow the terpenoid pathway, whereas β -ionone is a diterpene derivative. Thus the biochemical pathways to β -ionone and dodecyl acetate are unrelated and their close association following principle components analysis is probably random. The monoterpenes are tightly linked on the same pathway and this is reflected in their close association following principle components analysis.

To conclude, chemotypes associated with β -ionone, dodecyl acetate, α -pinene, β -pinene or limonene were not identified within *B. megastigma*. However, plants with extremely high β -ionone and dodecyl acetate and low monoterpene content were identified. These characteristics enhance oil quality and may provide a basis for selection of superior genotypes for essential oil production. The performance of these superior genotypes needs to be examined under cultivated conditions to determine their vigour, disease resistance and overall productivity.

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