

Regulation of oil accumulation in single glands of *Eucalyptus polybractea*

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Summary

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- The accumulation of terpenoid oil was examined in the leaves of *Eucalyptus polybractea* at scales ranging from individual oil glands to the whole plant.
- Variations in oil composition and concentration of oil were measured and related to both morphological and physiological parameters.
- Within a plant, all glands produced oil of broadly similar composition that was not regulated by leaf age or the position of the gland within the leaf. There were, however, distinct differences between plants, suggesting that composition is controlled primarily at the whole-plant level. Oil concentration, too, was regulated primarily at the whole-plant level and was limited by gland capacity. Gland capacity was linked to leaf area and thickness, the final products of leaf expansion.
- Leaf and plant oil composition is determined not by a mosaic of glands specializing in producing a single or a small group of compounds, but rather by glands with remarkably similar capacities for terpenoid biosynthesis, although oil concentration, limited by gland capacity, may be linked to leaf expansion rather than biosynthetic capacity.

Key words: *Eucalyptus*, oil, monoterpene, sesquiterpene, gland, leaf mass per area (LMA).

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Introduction

Some of the most challenging questions arising from the burgeoning fields of genomics, proteomics and metabolomics relate to the regulation of processes within plants. These methods are increasingly being applied at finer and finer scales, probing the regulation of processes at the level of organs, tissues and individual cells (Kehr, 2001, 2003; Brandt, 2005). An ideal candidate for such a micrometabolomics study is the accumulation of essential oils. Essential oils are complex mixtures comprised primarily of the lower classes of terpenes (C₁₀ to C₂₀) (Langenheim, 1994), although their composition and concentration vary widely both within and between species (Gershenzon & Croteau, 1991). Common features of plants that accumulate essential oils are the specialized glands in which the oils are stored (Fahn, 1979). These structures vary widely in architecture, from the glandular trichomes of

the Lamiaceae to the resin ducts of Pinus and the subdermal oil glands of *Eucalyptus* and are typically associated with specialized cells presumed to be the site of oil biosynthesis (Fahn, 1979). Oil glands are both numerous and discretely separated within plants, and thus provide an ideal system in which to examine the regulation of a defined metabolic process at a range of scales, from individual glands to entire plants and populations.

The composition and concentration of sequestered oils vary across genera (Brophy et al, 1991; Dunlop et al, 2003; Khanuja et al, 2005); within and between geographical populations of a species (Homer et al, 2000; Adams et al, 2003; Brophy & Doran, 2004); between cultivars (Labra et al, 2004); and within plants (Gershenzon et al, 2000; Russell & Southwell, 2003; Grassi et al, 2004; Johnson et al, 2004). Diel (Leach & Whiffen, 1989) and seasonal variations have also been observed (Hall & Langenheim, 1986; Leach &

Whiffen, 1989; Silvestre et al, 1997; Johnson et al, 2004). One shortcoming of these studies, however, is that they rely on bulk samples, and are therefore only informative of average oil quality and quantity across a variety of scales (across entire leaves or plants). Given that oil is stored at a much smaller scale, in individual glands, it is unclear whether bulk samples mask much underlying variation.

The composition of oil in any given bulk sample could reflect one of three conditions: (1) all glands produce oil that is identical in composition and therefore is also identical in composition to the bulk sample; (2) glands are specialized, each producing a single compound, therefore the composition in the bulk sample reflects the relative diversity of these individual glands; (3) each gland produces a range of compounds (with composition sometimes varying between glands). This latter condition could involve numerous compositional states of glands.

These ideas have not yet been appropriately tested for any of the major essential oil-containing structures. Some preliminary studies of members of the Lamiaceae have been undertaken and have suggested that, while oil composition does vary somewhat between individual glandular trichomes, the contents of individual glands are broadly similar (Maffei et al, 1989; Voirin & Bayet, 1996; Grassi et al, 2004; Johnson et al, 2004). This is consistent with condition (3) above. Nevertheless, none of these studies was adequately replicated – the recent studies by Grassi et al (2004) and Johnson et al (2004), for example, made use of just a single plant – and some appraised just a subset of components of the oil (Maffei et al, 1989; Voirin & Bayet, 1996; Johnson et al, 2004). Moreover, the focus of all of these studies was on variation in developing rather than mature leaves, which potentially adds an additional source of variation to the experiments.

In the present study, our aim was to conduct a relatively detailed and replicated analysis of the composition of oil in individual glands of fully expanded leaves, and to relate this composition to that of whole-leaf and plant extracts. We chose *Eucalyptus polybracteata* (the mallee) for the research because its leaves contain relatively high concentrations of oil, which is highly variable in composition between individual plants (King et al, 2004). The oil glands are entirely subdermal, embedded within the mesophyll of the leaves, and surrounded by a single layer of nonpigmented secretory cells that are the site of oil biosynthesis and thus determine the composition of the oil (Doran, 1991).

Materials and Methods

Plant material

Leaves were collected from five trees of *Eucalyptus polybracteata* (R.T. Baker) grown in a plantation near Inglewood, Victoria, Australia in October 2004. The plants, grown from mixed seed collected from a single site, were 3 yr old at the time of

sampling. Whole branches (approx. 1 m long) were collected from the east side of each plant. Following transport to the laboratory, they were recut, placed in water and stored at 4°C under lights until sampled. Leaves were removed and analysed within 2 d of sampling. Previous tests have shown that this sampling method affects neither oil composition nor concentration (data not shown). Four to six leaves were selected in sequence along a branch, starting from the first fully expanded leaf on a branch. Individual leaves were removed from the branch just before analysis, whereupon they were measured for area, thickness and weight. Leaves were then photographed on a light box with an appropriate scale. From the resulting photograph, individual oil glands could easily be distinguished from the surrounding leaf lamina, and gland densities could therefore be calculated. Gland densities were determined in 5-mm (of original leaf size) strips across the entire leaf width at three equidistant points along the leaf length in order to determine the mean gland density for each leaf.

Sampling of oil glands

To sample oil from individual glands, leaves were viewed under a dissecting microscope with the light source located beneath the leaf, enabling glands to be easily distinguished. Glands were then punctured with glass capillaries pulled to a fine point (approx. 50 µm OD), and a portion of oil was removed from the gland by a combination of positive gland pressure and suction applied to the capillary by a small-volume syringe connected via narrow-bore tubing to the capillary. This oil was then immediately injected into 40 µl hexane containing 100 µg ml⁻¹ tridecane⁻¹ as an internal standard, and the sample was kept at -20°C until analysis. Ten glands were sampled from each leaf according to a predetermined pattern. Glands were sampled across the leaf, from the leaf lamina and also from near the leaf margin and next to the leaf midrib. The sampling of glands also encompassed the entire leaf length and the precise location of each sampled gland was recorded. Following sampling of glands (if performed), leaves were immediately frozen and stored in liquid nitrogen until analysis.

Oil extraction from leaves

To extract oil from whole leaves, leaves were ground to a fine powder under liquid nitrogen using a mortar and pestle. Two amounts of approx. 100 mg were weighed into separate vials and 2 ml hexane containing 100 µg ml⁻¹ tridecane as an internal standard was added. Samples were extracted at room temperature for 25 d with periodic shaking. Vials were weighed before and after this period to account for any possible solvent loss, although no appreciable loss (> 0.1%) was detected. An aliquot of 1 ml of each extract was dried with anhydrous Na₂SO₄, then stored at -20°C until analysis. The

remaining solvent was evaporated, then each sample was dried at 65°C and reweighed to determine the dry weight of each sample. The ratios of fresh weight to dry weight for samples from each leaf were averaged and subsequently used to estimate whole-leaf dry weight and to calculate leaf mass per area (LMA).

Oil analysis

Oil samples were analysed by a method modified from King et al (2004). All samples were analysed twice by gas chromatography-flame ionization detection using a Perkin Elmer Autosystem XC (Melbourne, Australia) fitted with a Sol-Gel WAX column (30 m × 0.25 mm id × 0.25 µm film, SGE, Melbourne, Australia) using helium as a carrier gas at a flow rate of 1 ml min⁻¹. The column temperature was held at 50°C for 5 min following injection, then ramped at 10°C min⁻¹ to 190°C and held for a further 4 min. Analysis of samples extracted from single glands differed from that of whole-leaf extracts in that an increased volume was injected and the split ratio decreased. Compounds were identified by retention time by comparison with known standards (1,8-cineole, p-cymene, limonene, aromadendrene, terpinen-4-ol, myrcene, β-pinene, α-pinene; Sigma, St Louis, MO, USA/Fluka, Buchs, Switzerland), and quantified by comparison with the same standards. For compounds for which a standard was not used, quantities were determined by using an average response ratio of all compounds. Compound identification was confirmed by GC–mass spectrometry analysis on a subset of samples, conducted using a 6890 GC coupled to a mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) operating with the same column and conditions identical to those above. Data presented are mean values from all analyses of a sample.

Microscopy

Two leaves from each plant were removed approx. 4 h after collection from trees. These leaves were cut into strips across the width of the leaf, then fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 5.5. All leaf sections were then dehydrated in a progressive ethanol series (25, 50, 70%) over a period of 1 wk, then processed on an LX120 Tissue Processor (Innovative Medical Systems Corp., Ivyland, PA, USA), which further dehydrated the samples (70–100% ethanol), then cleared the tissue with Histo-lene solvent (1-methyl-4(methylthenyl)cyclohexane, Gale Scientific, Ringwood, Australia). Dehydrated tissue was then embedded in a matrix of paraffin and plastic polymers (Paraplast tissue embedding medium, Oxford Labware, St Louis, MO, USA) using the methods of Gordon (1990), and serial sections were cut at 10 µm with disposable blades on a Microm HM350 rotary microtome (Zeiss, Walldorf, Germany). The resulting sections were cleared of wax and stained with 0.5% toluidine

blue, and examined and photographed using an Olympus BH2 microscope (Olympus, Tokyo, Japan) fitted with a Leica DC300F camera (Leica, Vienna, Austria). For each plant, a total of eight transverse sections (four from each leaf), from various positions along the leaf and covering the entire leaf width, were analysed using graphics software (CORELDRAW 10, Corel, Ottawa, Canada) and the percentage of the area of the transverse section present as oil glands was determined for each section. The mean percentage of area as glands of sections from a single plant was taken as an estimate of the percentage of leaf volume present as oil glands. In addition, the cuticle thickness was determined on each section.

Ordinations and statistics

In all, 41 compounds were detected at >0.01% of total oil in at least one sample (Table 1). However, as the amounts extracted from glands were small, and to maintain accuracy and precision, only compounds that existed at levels above 0.1% in samples were included in analyses. Across all samples this reduced to 34 the number of compounds included in analyses. All oil compositional data are presented as a percentage of total oil, standardized so that included components in any sample sum to 100%. Dissimilarity matrices were constructed using both the Manhattan metric and the Gower metric distance measures using the PATN software package (CSIRO, Canberra, Australia). The Manhattan metric measures were used for within-plant analyses, as this measure places a greater emphasis on differences in the concentration of the more abundant compounds (which are more informative with regard to oil chemometric analyses in certain situations (Dunlop et al, 2000) vs the range-standardized (each component of equal weight) Gower measure that was used for between-plant analyses. PATN was also used to produce ordinations of the data set using the nonmetric multidimensional scaling (NMDS) technique. Five ordination analyses were performed on each data set using random starting configurations and 40 iterations within each analysis, after which the ordination with the lowest stress value (best fit) was used. The relationships of the individual oil components within each of the ordinations were examined by constructing loading plots. The line (or direction) of best fit for each compound within the ordination was calculated, and the relevant correlation coefficient was used as an estimate of the fit of the component with the observed pattern of the ordinations. All ordinations were rotated such that the direction of the slope of best fit for 1,8-cineole (the most abundant compound in all samples) was equivalent to the x axis of the plotted ordinations. Principal components analyses were also conducted on the same data matrices using SPSS software (ver. 14; SPSS, Chicago, IL, USA). All other statistics (regressions, ANOVAs, t-tests) were calculated using MINITAB software (ver. 14.1; Minitab, State College, PA, USA).

Table 1 Components found in the terpenoid oil of leaf extracts and isolated oil glands from five individual plants of *Eucalyptus polybractea*

Component	Plant 1		Plant 2		Plant 3		Plant 4		Plant 5	
	Leaf (n = 5)	Glands (n = 26)	Leaf (n = 5)	Glands (n = 28)	Leaf (n = 4)	Glands (n = 23)	Leaf (n = 6)	Glands (n = 39)	Leaf (n = 5)	Glands (n = 26)
1 α -pinene	5.36	3.17**	18.20	8.40**	1.38	1.10 ns	13.20	8.70**	2.10	1.87 ns
2 β -phellandrene		trace		nd		nd	0.99	0.10**	0.30	0.26 ns
3 β -pinene	3.81	1.49**	0.72	0.73 ns	2.05	2.21 ns	0.74	1.12 ns	0.72	0.34**
4 γ -terpinene		trace		nd		nd		trace		nd
5 limonene	0.79	0.59 ns	1.76	0.93**	0.99	0.77*	2.58	1.05**	1.08	0.70 ns
6 myrcene		nd		trace		nd		trace		nd
7 <i>p</i> -cymene	6.43	5.23 ns	3.16	2.78 ns	1.45	1.42 ns	7.52	7.37 ns	2.65	2.48 ns
8 sabinene		trace		nd		trace		trace	0.24	0.26 ns
9 verbenene		trace		trace		trace		trace		nd
Total hydrocarbon monoterpenes	16.34	10.48**	23.90	12.84**	5.87	5.50 ns	25.03	18.32**	7.09	5.91*
10 α -terpenyl acetate		nd	0.39	0.37 ns	0.56	0.72 ns	2.36	1.74 ns		nd
11 α -terpineol		nd		trace		trace	0.38	0.27 ns	0.11	0.10 ns
12 carveol	1.20	0.74*	0.91	1.15 ns	0.33	0.55 ns	1.04	0.67*	0.50	0.40 ns
13 carvone	0.41	0.72 ns	0.39	0.88*	0.33	0.60 ns	0.31	0.29 ns	0.38	0.49 ns
14 1,8-cineole	58.21	62.62 ns	55.88	61.71*	87.82	83.89 ns	41.03	47.10*	86.10	87.69 ns
15 cryptone	4.12	5.14 ns	1.29	1.73 ns	0.39	0.77 ns	4.87	6.47 ns	0.62	0.59 ns
16 cuminal	2.59	1.92 ns	0.77	0.49**	0.94	0.82 ns	1.22	1.25 ns	0.51	0.28**
17 δ -terpineol		trace		trace	0.19	0.32 ns	0.28	0.27 ns	0.20	0.20 ns
18 myrtenal	1.31	1.57 ns	0.36	0.53 ns	0.60	1.42 ns	1.13	0.75*		trace
19 pinocarvone	1.31	1.79 ns	0.56	0.50 ns		trace	0.34	0.49*	0.18	0.22 ns
20 <i>p</i> -cymen-8-ol	0.80	1.06 ns	0.53	1.04 ns	0.21	0.66 ns	0.46	0.90*		trace
21 <i>p</i> -menthen-2-ol		trace		trace		trace	0.20	0.15 ns	0.12	0.08*
22 terpinen-4-ol	1.35	1.15 ns	0.99	0.93 ns	0.58	0.65 ns	1.81	0.89**	0.40	0.17**
23 <i>trans</i> -pinocarveol	2.14	2.02 ns	1.21	1.05 ns	0.74	0.74 ns	0.48	0.78*	0.19	0.20 ns
24 verbenol	1.54	1.77 ns	2.22	3.26*	0.31	0.46 ns	0.71	1.17 ns	0.33	0.31 ns
25 verbenone	1.10	2.38**	1.66	4.10**	0.25	0.66 ns	0.33	0.77**	0.40	0.59 ns
Total oxygenated monoterpenes	76.08	82.89**	67.10	77.72**	92.64	90.87 ns	56.95	63.97**	90.40	91.97 ns
Total monoterpenes	92.43	93.37 ns	91.00	90.56 ns	98.51	96.37 ns	81.98	82.29 ns	97.49	97.68 ns
26 α -copaene		trace		nd		nd	0.50	0.35 ns		nd
27 α -cubebene		trace		trace		nd	0.17	0.10*		nd
28 α -muurolene		trace		trace		trace		trace		trace
29 alloaromadendrene	0.34	0.50 ns	2.14	2.88 ns	0.27	0.47 ns	2.67	3.03 ns	0.61	0.78 ns
30 aromadendrene		trace	0.46	0.34 ns	0.60	1.42 ns		trace	0.22	0.16 ns
31 β -elemene		nd		trace		nd		trace		nd
32 calamenene		trace		trace		trace	1.04	0.77**		trace
33 δ -cadinene		trace		trace		trace	0.47	0.54 ns		trace
Total hydrocarbon sesquiterpenes	0.34	0.50 ns	2.60	3.21**	0.87	1.89 ns	4.85	4.80 ns	0.83	0.94 ns
34 C ₁₅ H ₂₆ O (a)	0.70	0.65 ns		trace		trace	1.27	1.26 ns		trace
35 C ₁₅ H ₂₆ O (b)	1.34	1.16 ns		trace		trace	1.14	1.32 ns		trace
36 caryophellene oxide	2.37	1.83 ns		trace		trace	0.99	0.93 ns		trace
37 epi-globulol		trace		trace		trace		trace		trace
38 globulol	0.82	0.98 ns	2.66	2.53 ns	0.62	1.74 ns	3.12	3.45 ns	1.69	1.38 ns
39 ledol		trace		trace		trace	0.45	0.34 ns		trace
40 spatheulenol	1.96	1.51 ns	3.75	3.70 ns		trace	6.20	5.62 ns		trace
41 viridiflorol		nd		trace		nd		trace		trace
Total oxygenated sesquiterpenes	7.24	6.13 ns	6.41	6.23 ns	0.62	1.74 ns	13.17	12.91 ns	1.69	1.38 ns
Total sesquiterpenes	7.57	6.63 ns	9.00	9.44 ns	1.49	3.63 ns	18.02	17.71 ns	2.51	2.32 ns

Components are listed at mean standardized relative concentration unless listed as trace (component present at average levels < 0.10%) or nd (component was not detected). Leaf and gland means were compared using Tukey pairwise comparisons: significantly different at *, $\alpha = 0.05$; **, $\alpha = 0.01$; ns, not significantly different.

Results

Variation in oil composition

To analyse variations in the content of individual oil glands on leaves of an individual plant, oil was extracted from glands on three to four leaves along an age gradient (typically the first, third and fifth fully expanded leaves) from each plant. Overall, oil was extracted from some 142 oil glands from 15 leaves from a total of five plants, in quantities sufficient for analysis. The average amount of oil extracted from glands was 867 ± 48 (SE) ng, although it varied from 10 ng to 3.29 μg . Glands that yielded > 100 ng were used in the analysis to maintain precision and accuracy. We thus excluded 18 of the 160 glands originally sampled.

Across all gland samples a total of 41 compounds were detected, comprising both oxygenated and hydrocarbon mono- and sesquiterpenes. There was considerable variation in the complement of oils in the glands, both qualitative and quantitative (Table 1). Overall, the oils were dominated by monoterpenes, both in concentration (between 70.39 and 99.17%) and in the number of compounds detected (between 21 and 25 compounds). 1,8-cineole [14] was the major compound in every sample, with a mean of 67.28% (± 1.27). This compound varied in concentration across samples from 31.6 to 91.2%, and was the only compound to comprise $> 1\%$ of total oil in every sample. While oxygenated monoterpenes dominated the oil profile, other consistently abundant compounds detected were the hydrocarbon monoterpenes α - [1] and β -pinene [3] (5.77 ± 0.37 and $1.22 \pm 0.09\%$, respectively) and *p*-cymene [7] ($4.33 \pm 2.1\%$), and the modified monoterpene cryptone [15] ($3.30 \pm 0.23\%$). All four compounds were present in appreciable quantities in most gland samples. Although a number of sesquiterpenes were detected in each sample (between 12 and 16 compounds), they rarely comprised more than 10% of the total oil. The most common and abundant sesquiterpenes were the hydrocarbons alloaromadendrene [29] ($1.68 \pm 0.11\%$) and aromadendrene [30] ($0.62 \pm 0.05\%$), and the alcohols globulol [38] ($2.18 \pm 0.10\%$) and spathulenol [40] ($4.02 \pm 0.25\%$).

We found that all gland samples from a single plant contained the same characteristic suite of compounds: neither individual glands nor leaves could be distinguished by their qualitative composition. We did, however, find considerable variation between plants in the number of compounds present in significant amounts (defined as a relative concentration $> 0.1\%$ of total oil), varying from 20 compounds in plant 3 to 32 compounds in plant 4 (Table 1). Plants could thus be differentiated by the presence or absence of certain compounds in significant amounts. For example, β -phellandrene [2] was present in significant amounts in plants 4 and 5, and in trace ($< 0.1\%$) amounts in plant 1, but was absent in samples from plants 2 and 3. Similarly, samples from plant 4 could easily be differentiated by the large amounts of several

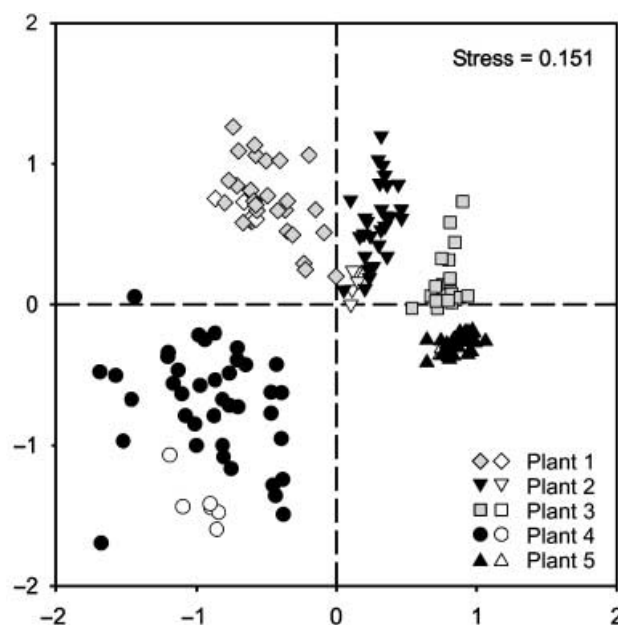


Fig. 1 Nonmetric multidimensional scaling (NMDS) ordination of oil-composition data from individual oil glands (closed symbols) and leaf extracts (open symbols) of five plants of *Eucalyptus polybractea* corresponding to those in Table 1. The stress of the ordination is 0.151.

sesquiterpenes that were absent in other plants, or present only in trace quantities.

In contrast to the qualitative data, the relative concentrations of compounds varied considerably between gland samples, within each leaf and plant, and between plants. To analyse this variation, we applied the data-reduction technique of NMDS, in which points that are located more closely within the ordination space are more similar. In the resulting two-dimensional ordination (stress = 0.151), samples from individual plants formed clear, discrete groups (Fig. 1). Within the plant groupings a degree of scattering between gland samples was evident, indicating that there was quantitative variation in oil composition within plants. The degree of this scatter varied considerably between plants; the data from plant 5 clustered very tightly in comparison with the other plants, while the data from plant 4 were the most scattered. The degree of clustering was largely related to the average concentration of 1,8-cineole [14], given that this compound was the most abundant in every gland. Thus plants 3 and 5 (which had high average levels of 1,8-cineole of 84.47 and 87.43%, respectively) had relatively tightly clustered points, while plant 4 (which had the lowest 1,8-cineole concentration of 46.27%) had relatively widely spread points. Plants 1 and 2 had intermediate levels of 1,8-cineole and clustering. Results obtained using principal components analysis were identical in terms of the separation of plants, and thus have not been included here.

The loading plot (Fig. 2) associated with the ordination (Fig. 1) shows the influence of the individual compounds on

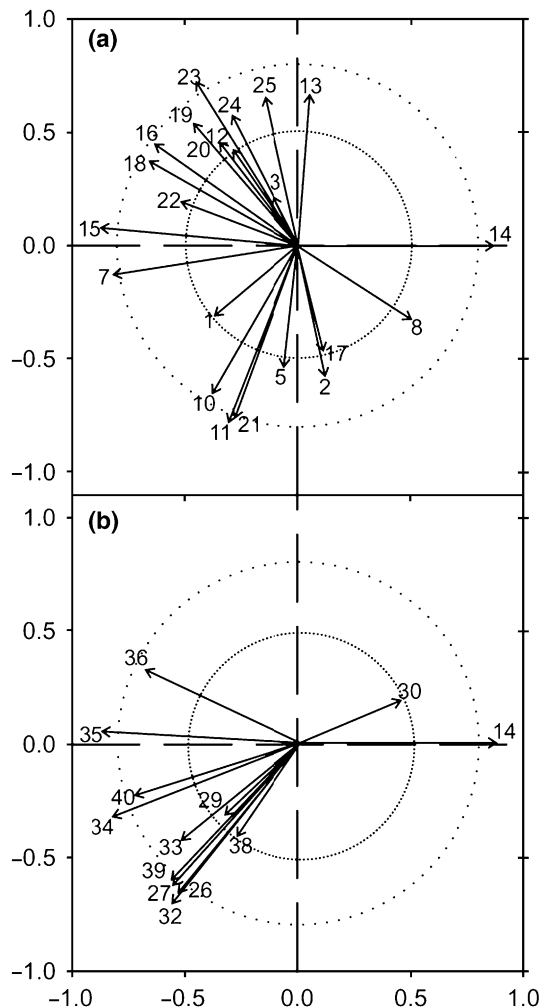


Fig. 2 Loading plots associated with the nonmetric multidimensional scaling (NMDS) ordination in Fig. 1 for (a) monoterpene; (b) sesquiterpene components of oil from *Eucalyptus polybractea*. Each line represents the direction of the line of best fit for the relevant component of the oil (see Table 1 for component names) within the ordination. The length of the line is equal to the regression coefficient (r) of the component data along that line, and is indicative of how strongly that component influenced the ordination. Dotted circles, r values of 0.5 and 0.8.

the spatial distribution of the samples. Within the loading plot, the compounds did not form discrete groups based on whether they were either monoterpenes or sesquiterpenes, or whether or not they contained oxygen (Fig. 2). Nevertheless, it is notable that the direction of influence of all but five compounds was over 90° away from that of 1,8-cineole [14]. Accordingly, the concentration of 1,8-cineole [14] was negatively correlated with that of 26 of the remaining 33 compounds ($P < 0.05$). Among these 33 compounds, positive correlations were often detected between structurally similar compounds. This corresponded with compounds being closely allied within the loading plot (Fig. 2). For example,

the concentrations of verbenol [24] and verbenone [25] ($r^2 = 0.78$, $P < 0.001$); transpinocarveol [23] and pinocarvone [19] ($r^2 = 0.58$, $P < 0.001$); α -terpineol [11] and α -terpinyl acetate [11] ($r^2 = 0.63$, $P < 0.001$); and spathulenol [40] and globulol [38] ($r^2 = 0.64$, $P < 0.001$) were all strongly correlated. There were, however, exceptions to this rule. For example, aromadendrene [30] and allo-aromadendrene [29] were not significantly correlated; and α -pinene [1] and β -pinene [3] were weakly negatively correlated ($r^2 = 0.03$, $P = 0.027$). In some cases, correlations were detected between oxygenated compounds and their hydrocarbon equivalents; for example p -cymene [7] was correlated with p -menthen-2-ol [21] ($r^2 = 0.34$, $P < 0.001$) and cuminal [16] ($r^2 = 0.33$, $P < 0.001$). Nevertheless, no correlation was detected between p -cymene [7] and p -cymen-8-ol [20], for example.

The loading plot also showed that 14 compounds had lines of best fit with a corresponding r value of > 0.80 (Fig. 2). This indicates that they had a significant influence on the spatial distribution of data within the ordination. Of the 14 compounds, seven were monoterpenes and seven were sesquiterpenes. As noted above, the sesquiterpenes differentiated plant 4, which had the largest amount and most diverse range of sesquiterpenes and, to a lesser degree, plant 2. The levels of the monoterpenes 1,8-cineole [14], cryptone [15], cuminal [16], transpinocarveol [23] and p -cymene [7], all major components ($> 1\%$) of the extracted oils (Table 1), defined the majority of the variation between the remaining plants. All compounds contributed to the variation within the plant groupings (Figs 1, 2).

It is noteworthy that oil samples extracted from whole leaves clustered relatively tightly within the grouping of each plant (Fig. 1, open symbols; Fig. 3). This tight clustering indicates minimal variation in oil composition between whole-leaf oil samples compared with the glands sampled from the same leaves. We used ANOVA to examine further the relationship between the oil composition of the glands and that of their parent leaves. We did not detect a difference in the overall levels of monoterpenes or sesquiterpenes, but we did detect differences in the relative concentrations of a number of individual compounds in glands vs leaves in all plants (Table 1). There were, however, few consistent trends across all plants. For example, carveol [12] was found in significantly higher amounts in the whole-leaf extracts than in the glands in plants 1 and 4, although no difference was detected in the other three plants (Table 1).

We also found that in four of the five plants the levels of hydrocarbon monoterpenes were significantly higher in leaf extracts than in oil glands (Table 1). This was largely caused by the significantly lower levels of α -pinene, the most abundant of the hydrocarbon monoterpenes, detected in the glands compared with the whole-leaf extracts (on average 32% lower) in four of the plants (Table 1). Conversely, the average level of oxygenated monoterpenes in leaves was significantly lower in three of the five plants when

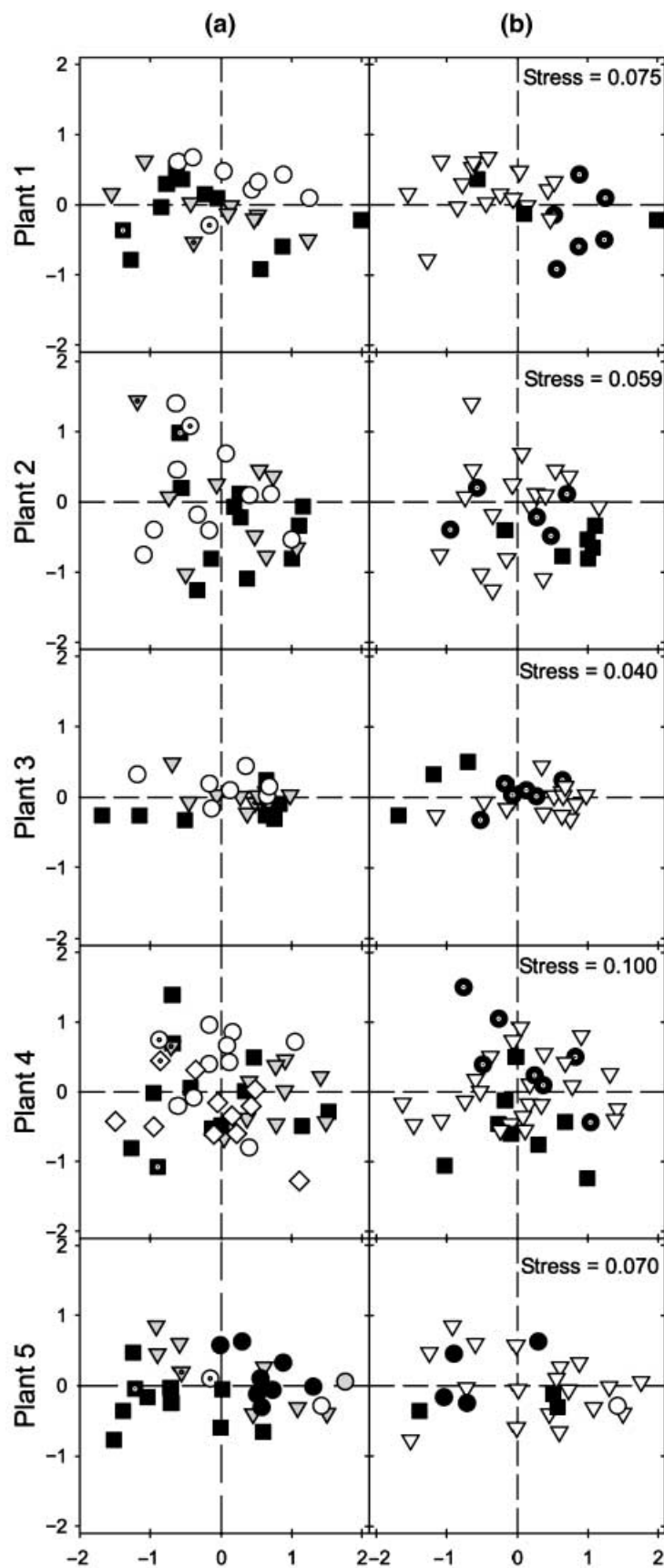


Fig. 3 Nonmetric multidimensional scaling (NMDS) ordinations of oil-composition data from individual oil glands and leaf extracts from five individual plants of *Eucalyptus polybractea*, as listed in Table 1. Data are grouped (a) by leaf, with leaves differentiated by symbol; (b) by location within the leaf, with glands being located either in the leaf lamina (open triangles), next to the midrib (closed circles), or on the leaf margin (closed squares).

Table 2 Leaf oil content, oil glands and characteristics from five individual plants of *Eucalyptus polybractea*

Parameter	Plant 1 (n = 5)	Plant 2 (n = 5)	Plant 3 (n = 4)	Plant 4 (n = 6)	Plant 5 (n = 5)	P
Oil						
Percentage by weight (% wt/DW)	3.86 ± 0.14	5.65 ± 0.22	4.65 ± 0.27	5.94 ± 0.26	4.71 ± 0.16	0.000
by area (g m ⁻²)	9.94 ± 0.50	18.67 ± 0.78	13.24 ± 0.81	17.19 ± 0.94	14.57 ± 0.47	0.000
Oil glands						
Density (glands cm ⁻²)	306 ± 18	475 ± 20	324 ± 27	381 ± 19	434 ± 32	0.000
Glands per leaf	3071 ± 273	3174 ± 169	3201 ± 462	3260 ± 235	3090 ± 278	0.916
Gland volume (% leaf volume)	10.81 ± 0.36	15.24 ± 0.37	9.96 ± 0.34	17.76 ± 0.39	12.5 ± 0.49	0.000
Leaf characters						
Area (cm ²)	10.01 ± 0.58	6.7 ± 0.34	9.75 ± 0.66	8.58 ± 0.54	7.15 ± 0.58	0.000
Thickness (µm)	508 ± 15	635 ± 8	557 ± 7	521 ± 17	564 ± 11	0.000
Leaf mass per area (g m ⁻²)	257.5 ± 3.5	330.8 ± 7.5	284.9 ± 2.5	288.6 ± 4.7	309.5 ± 3.8	0.000

All values are the mean of each plant ± SE. Values of *n* apply to all measures except gland volume, where *n* = 8 for all plants. *P* values are the results of one-way ANOVAS.

compared with glands (Table 1). No differences were detected in average levels of either hydrocarbon or oxygenated sesquiterpenes.

Finally, we tested whether leaf position on the plant, or gland position within the leaf, correlated with oil composition (Fig. 3). Various classifications of position were used, including distance to the gland from the base of the leaf, the side of the midrib from which the gland was sampled, and also whether the gland sampled was from the leaf margin, lamina, or next to the leaf midrib. Although differences were found in the relative concentrations of individual compounds in all plants according to these classifications (data not shown), no consistent pattern was observed across all plants. When data from each plant were subjected to NMDS analysis, no clearly defined groups were observed in any plant when samples were defined by parent leaf (Fig. 3a) or by gland position (Fig. 3b).

Variation in leaf oil concentration

The amount of oils detected in the leaves of the plants ranged from 3.45 to 6.62% (wt/DW), which is equivalent to a leaf area-based range of 8.81–20.50 g m⁻². As with oil composition, the amount of oil in the leaves varied primarily between plants, rather than within plants, with differences detected between plants on both a mass and area basis (Tables 2, *F* = 16.15 and 21.16, respectively, *P* = 0.001 in both cases). The variation detected within plants could not be explained by leaf age, as defined by position on the branch, nor could it be explained by variation in any of the individual oil components (data not shown).

In order to characterize further the relationship between the oil content of glands and whole plants, we next examined whether gland number and size could account for oil concentration. Gland density and number of glands per leaf were measured for all leaves used in oil-concentration measurements. Gland density ranged from 262 to 509 glands cm⁻²,

although this variation was again detected primarily between plants (Tables 2, *F* = 9.08, *P* < 0.001). Gland density was significantly and positively correlated with oil concentration when the latter was calculated by mass (*r*² = 0.399, *P* = 0.001) or by area (*r*² = 0.548, *P* < 0.001). In contrast to the density data, no difference was detected between plants in the total number of glands per leaf (Table 2, *F* = 0.08, *P* = 0.916). Significant negative correlations were accordingly detected between leaf area and gland density (Fig. 4a, *r*² = 0.335, *P* = 0.002); and between leaf area and oil concentration per area (Fig. 4b, *r*² = 0.295, *P* = 0.005). A weak and negative correlation was detected between leaf area and oil concentration per mass, although this was not significant (*r*² = 0.147, *P* = 0.059).

Gland volume (calculated as a percentage of leaf volume) was determined for leaves that were similar to those from which oil was extracted. Thus comparisons with oil content were made on a plant basis rather than a leaf basis. Despite a corresponding drop in statistical power, a significant positive correlation was detected between mean gland volume and mean mass-based oil concentration (Fig. 4c, *r*² = 0.805, *P* = 0.039). When oil concentration was expressed on an area basis, a strong positive correlation with gland volume was detected, although this was not significant (*r*² = 0.653, *P* = 0.098).

We found that leaf characters such as leaf size, average leaf thickness and LMA were relatively uniform within individual plants, but were significantly different between plants in all cases (Table 2, ANOVA, *P* < 0.001). The LMA, the product of leaf thickness and density, was governed primarily by leaf thickness across all the leaves tested (*r*² = 0.731, *P* < 0.001). All leaves examined had between seven and nine layers of palisade mesophyll and lacked any conspicuous spongy mesophyll (Fig. 5). The degree of plasticity in density is therefore likely to be minimal simply because of the paucity of air spaces within leaves. In addition, larger leaves tended to be thinner, with negative correlations detected between leaf

Discussion

Oil composition

Our results can be interpreted within two 'boundary' hypotheses: either all oil glands produce an oil of identical composition; or glands are specialized, each producing a single compound. Our results are intermediate between these two hypotheses. We found that all oil glands within a plant contained an identical range of compounds (they were qualitatively identical), yet there was significant variation between glands in the relative concentrations of these compounds (they were quantitatively different). We also found that most of the variation was attributable to glands; there was little variability between leaves within a plant (Fig. 1).

This quantitative variation in the oil profile of glands is consistent with previous work on the glandular trichomes of the Lamiaceae family (Maffei et al, 1989; Voirin & Bayet, 1996; Grassi et al, 2004; Johnson et al, 2004). For example, in examining the oil composition in the trichomes of a single developing sage (*Salvia officinalis* leaf, Grassi et al (2004) found discrete groupings of glands according to the position of the gland on the leaf. This finding was partly explained by a relationship between position and the age of the trichomes caused by the initiation of glands being linked to leaf expansion. The production of the subdermal oil glands of *Eucalyptus* leaves is not, however, governed by the same factors that govern the peltate trichomes of the Lamiaceae. Carr & Carr (1976) described the formation of oil glands in *Eucalyptus* occurring in conjunction with the development of venation within the leaf. The first, and therefore the oldest, glands are thus initiated next to the midrib and the marginal vein, which are the first veins to appear during leaf development. New glands are then initiated throughout the leaf lamina during leaf expansion as the leaf venation lattice develops. In this study, the position of the oil gland did not account for the compositional variation detected between glands within a single plant. Moreover, the variation detected could not be attributed to differences between the parent leaves from which the glands were sampled (Fig. 3). These results suggest that the age of the gland has little to do with determining the oil composition within *E. polybractea*.

The variation in oil composition between plants and between glands probably reflects the biosynthetic machinery in the secretory cells that surround the individual oil glands, although differential turnover and vaporization cannot be ignored. Although no functional terpene synthase has been isolated from a eucalypt (or any member of the Myrtaceae), enzymes have been isolated from a number of plant families in both native and recombinant forms. These enzymes have individually been shown to produce a range of terpene products (e.g. *Abies grandis* (Sijzen et al, 1991; *Salvia officinalis* Wise et al, 1998; *Vitis vinifera* Martin & Bohlmann, 2004; *Arabidopsis thaliana* Eildt et al, 2003), which are typically

similar in structure. The complexity of essential oils observed here and elsewhere is consistent with this lack of product specificity of the relevant enzymes, and with the many positive correlations detected between structurally similar compounds. It is likely that the variation we have noted in oil composition, particularly between the glands sampled within a single plant, represents a small shift in the activity of several terpene synthases in the surrounding secretory cells of the individual glands.

The oil extracted from glands, when averaged across the entire plant, broadly reflected that of whole leaves, but differences were detected in the mean relative concentrations of certain compounds between leaves and the population of glands tested. This, again, is consistent with previous work on other oil glands (Grassi et al, 2004). It was surprising, however, that the variation in oil profiles that we described among the glands was not reflected in variation among their parent leaves. This suggests that plants exert a tighter control of oil composition across entire plants than is implicit in an analysis of the glands alone. Oil composition is assumed to be primarily genetically regulated, as demonstrated in previous work on *Eucalyptus camaldulensis* (Doran & Matheson, 1994) and the related *Melaleuca alternifolia* (Barton et al, 1991), in which high levels of heritability were found for certain oil components.

It is noteworthy that hydrocarbon monoterpenes were commonly present in lower relative concentrations in glands than in leaves. This suggests that, in addition to the glands, there is another sink of hydrocarbon monoterpenes in the leaves of *E. polybractea*. Such a sink would not have to be very large to bring about the differences seen, although it would need to be lipophilic, as hydrocarbon monoterpenes are more volatile and readily soluble in lipophilic domains than their oxygenated counterparts (Fichan et al, 1999). One possible sink is the leaf cuticle (Schmid et al, 1992), which, in the leaves studied, was on average 0.57 ± 0.03 mm thick. Supporting this idea is research showing that emissions of (particularly) hydrocarbon monoterpenes are not regulated by stomata (Guenther et al, 1991; Niinemets et al, 2002). Thus a significant portion of monoterpenes is volatilized via the cuticle.

Variation in oil concentration

We found considerable variation in total oil concentration between plants, but this was not correlated with variation in the concentration of any one compound or group of compounds. This is consistent with the finding of Litvak et al (2002) that, in *Pseudotsuga menziesii*, biosynthetic capacity for monoterpenes was not related to overall monoterpene concentration. Presumably, the factors controlling the development and final size of oil-storing structures has a strong influence on the oil concentration of plants. Indeed, we found a strong positive correlation between oil concentration and the volume of the leaf occupied by the oil glands (Fig. 4c).

The total volume of oil glands within leaves is determined by two factors: the size of the individual glands; and the number or density of glands across the leaf. Accordingly, we also detected positive correlations between gland density and oil content, and total gland volume and oil content. Surprisingly, this correlation has not commonly been detected for eucalypts (Doran, 1991) or other oil-storing species.

Gland densities were quite consistent among the leaves of individual plants, yet they differed between plants, suggesting that density is tightly controlled within plants. Further, we detected a negative correlation between gland density and leaf area, and between leaf area and oil concentration per area. Given that leaf area is the result of leaf expansion, it follows therefore that the distance between glands, and therefore gland density, is dictated by the amount of leaf expansion that occurs during and after gland initiation. Implicitly, the control of the timing of gland initiation, a process known to occur through various stages of leaf expansion (Carr & Carr, 1976), plays an important role in determining gland density and oil content, and would provide an interesting focus for future research. Larger leaves would thus be more likely to have a lower gland density than smaller leaves and, in turn, would have a lower oil content. Consistent with this, we found a negative correlation between leaf area and oil concentration per area.

We also found positive correlations between both mass- and area-based oil content and LMA (Fig. 4d,e). Variation in the latter was driven primarily by leaf thickness. King et al (2004), in a study of a natural population of *E. polybractea* reported similar findings and concluded that the relationship between oil content per area and LMA is indicative of glands being larger in transverse section in thicker leaves: for a given unit of leaf area in thicker leaves, glands are larger through the leaf. We accounted for variation in leaf thickness by measuring oil concentration on a mass basis; however, a positive relationship between oil concentration per mass and LMA was still detected, although with a reduced r^2 of 0.29 (Fig. 4d). This indicates that thicker leaves have wider glands in a plane parallel to the leaf surface. In this scenario, with increasing leaf thickness, glands are larger and occupy a greater proportion of the leaf volume than in thinner leaves. The fact that glands expand in both transverse and parallel planes is unsurprising, as glands tended to be spherical unless impeded by leaf venation or other glands (Fig. 5).

The finding that gland dimensions are linked to leaf thickness may also be explained by a link with leaf expansion. Once initiated, a gland may expand within the expanding leaf; thus thicker leaves, those that have undergone a greater amount of expansion in that plane, will have larger glands on average. It is interesting to note that thicker leaves were more likely to be smaller in area than thinner leaves, suggesting that the expansion of leaves in one plane is, to a degree, mutually exclusive of expansion in the other. Within *E. polybractea* the measurements that are the direct results of leaf expansion – leaf area,

thickness and LMA – were conservative features within the plants studied, suggesting that the controls on leaf expansion within plants are tight.

Concluding remarks

Here we describe significant variation in both the composition and concentration of oil within a single species. This variation was detected primarily between plants. Within plants, oil composition varied more between individual glands than between leaves. We could find no positional or temporal reason for this discrepancy, raising interesting questions concerning the spatial regulation of oil biosynthesis within plants. We also found no discernible link between oil composition and concentration. We suggest, rather, that it is gland capacity that restricts the accumulation of oils. We present data suggesting that the gland capacity of *E. polybractea* is linked to three-dimensional leaf expansion. This raises the question as to whether or not the controls on oil accumulation are related to those on oil biosynthesis. Rather, the timing of gland initiation and leaf expansion may govern oil content. The question remains as to what drives gland expansion: is it driven by oil biosynthesis, or by a more stochastic link with leaf expansion?

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