CSIRO PUBLISHING
www.publish.csiro.au/journals/fpb *Functional Plant Biology*, 2004, **31**, 451–460

Terpene deployment in *Eucalyptus polybractea*; relationships with leaf structure, environmental stresses, and growth

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Abstract. Terpene deployment was examined in a population of *Eucalyptus polybractea* (R.Baker) trees. *Eucalyptus polybractea* is a terpene-accumulating species, which stores terpenes in oil glands beneath the leaf surface. Using regression analysis, we showed that leaf thickness, measured as leaf mass per area (LMA), influenced terpene content, apparently through regulation of gland dimensions, and thus, gland volume. We also examined how environmental factors affected terpene content through regulation of both LMA, and therefore, storage capacity, and the supply of resources for terpene synthesis. Neither water stress, measured using carbon isotope ratios as an indicator, nor nutrient stress, measured as foliar nitrogen and phosphorus content, accounted for observed variation in either terpene content or LMA. Phenolic content, measured as a possible competing carbon sink, did not account for variation in terpene content, and variation in environmental stresses could not account for differences in growth rate. However, both terpenes and total carbon-based secondary metabolites (terpenes and phenolics) showed positive correlations with growth, suggesting plants gain a growth advantage by deploying greater amounts of secondary metabolites.

Keywords: blue mallee, carbon isotope, defence, $\delta^{13}C$, drought, eucalyptus oil, monoterpene, nitrogen, nutrient availability, phenolic, phosphorus, sesquiterpene.

Introduction

Terpenes are the most diverse group of plant secondary products, with over 15000 known members (Langenheim 1994). However, the roles of these compounds in plant metabolism are not as diverse as their numbers, and there is evidence that most of terpenes are involved in defence against herbivores and pathogens. A relatively small group is involved in growth and development processes, and these can thus be considered to be primary metabolites (Gershenzon and Croteau 1991).

Many of the most abundant terpenes in plants are stored in specialised structures. These structures are diverse in both their form and location within plants, and range from the resin ducts in the leaves and wood of many conifers, through the glandular trichomes of *Mentha* spp. and *Salvia* spp., to the internal foliar oil glands of myrtaceaous species (Fahn 1979). Members of this latter group contain some of the highest mass-based levels of terpenes recorded for any tissue. For example, in several Australian eucalypt mallee species, foliar terpene contents in excess of 7% of fresh weight have been measured (Brophy *et al*. 1991). Production of this quantity of a natural product must come at a considerable cost to the plant, not only in terms of the materials and energy required for biosynthesis, but also in terms of construction of, and allocation of foliar space to, those structures required for storage (Lerdau and Gershenzon 1997).

While considerable progress has been made in research on the mechanisms of terpene biosynthesis (e.g. Mahmoud and Croteau 2002), the factors controlling the overall amount of terpenes stored in glands are not well understood. Theoretically, terpene content may be regulated by two potentially interacting factors: (1) the supply of material to, and capacity for, terpene biosynthesis, and (2) the provision of storage space, i.e. gland number and volume. Both factors are probably under strong genetic control in many species and accordingly, terpene content can vary considerably when plants are grown under uniform environmental conditions (Doran 1991) and both terpene quality and foliar concentration are heritable traits (Barton *et al*. 1991; Doran and Matheson 1994). However, the level of phenotypic control of oil yield is less certain and few if any clear trends have been established (Koricheva *et al*. 1998).

Abbreviations used: CBSM, carbon based secondary metabolites; $\delta^{13}C$, carbon isotope ratio; dw, dry weight; fw, fresh weight; LMA, leaf mass per area; N_A, nitrogen concentration of leaves expressed on an area basis; RGR_{stem}, mean stem relative growth rate; T_A, terpene concentration of leaves expressed on an area basis; T_M, terpene concentration of leaves expressed on a mass basis.

 10.1071/FP03217 1445-4408/04/050451

Studies of the effect of environmental conditions on concentrations of terpenes in leaves have concentrated on water stress, nutrient availability, and high CO_2. For example, under water stress, several species increase foliar terpene concentration (Kainulainen *et al*. 1992; Llusia and Penuelas 1998), while others show no detectable change (Llusia and Penuelas 1998). Similarly variable results were found for a range of species when nutrient (especially nitrogen) supply was varied (Ross and Sombrero 1991; List *et al*. 1995; Lerdau *et al*. 1997; Heyworth *et al*. 1998; Litvak *et al*. 2002). Unfortunately, it is difficult to interpret most of these studies in terms of the regulation of terpene accumulation because the possible effects on biosynthesis were not separated from effects on gland size. For example, water stress can cause an increase in leaf thickness (or allied measures) and a decrease in photosynthetic rate (Li 2000). These may be counteracting effects because decreasing photosynthesis could diminish the capacity to supply carbon and energy for terpene biosynthesis, whereas thicker leaves could allow for a larger final gland volume. One study of terpene accumulation in balsam fir [*Abies balsamea* (L.) P.Mill.] provides information to help separate these two factors. Lamontagne *et al*. (2000, 2002) found that when nitrogen supply was varied, terpene content per unit leaf area increased with leaf mass per unit leaf area (LMA, which is generally proportional to leaf thickness). This result suggests that leaf thickness and gland size do interact, but the result was confounded to a degree by the fact that leaf nitrogen per unit leaf area also increased with LMA. Area based leaf nitrogen is correlated with photosynthetic capacity in many species (Field and Mooney 1986).

Another potentially confounding variable in experiments examining the regulation of terpene concentration is variability in the concentration of other secondary metabolites such as phenolic compounds, which often comprise a sizeable proportion of leaf mass (e.g. Lawler *et al*. 1997). Terpene and phenolic metabolism could effectively compete for resources, and thus variation in the concentration of one class of compounds could affect that of the other. While some studies have examined phenolics and terpenes in a single species (Kainulainen *et al*. 1996; Blodgett and Stanosz 1998; Heyworth *et al*. 1998; Sallas *et al*. 1999; Turtola *et al*. 2002), this type of competitive interaction has not yet been quantified.

The aim of this study is to describe variation in the foliar terpene concentration in a field population of *Eucalyptus polybractea*, one of the oil mallee species of Australia. More specifically, we aimed to measure the effects of water stress, nutrient supply and phenolic level on terpene concentration, and to quantify the degree to which changes in leaf thickness accounts for variation in terpene concentration. We have chosen to study *E. polybractea* because it has relatively large levels of both foliar terpenes, which are stored in glands (Fahn 1979; Doran 1991), and phenolic compounds. Moreover, the population contains even aged trees, allowing us to calculate growth rates and their relationship to the other variables described above.

Materials and methods

Species and site description

Eucalyptus polybractea (R.Baker) is a mallee species restricted to the one region in NSW (West Wyalong) and several disjointed regions in north central Victoria (Chippendale 1988). Plants produce a large subterranean lignotuber that coppices readily following disturbance, typically resulting in a multi-stemmed tree ranging in height from 3–9 m (Costermans 1983). Leaves normally have a high oil content ranging from 1–6% (v/w fw) consisting mainly of monoterpenes together with a few sesquiterpenes (Brophy *et al*. 1991). Existing stands of trees are harvested commercially for eucalyptus oil, which is highly sought after as the oil has a very high 1,8-cineole content. Cineole oils are used for a range of pharmaceutical products (Doran 1991).

The study site was located near Inglewood, approximately 175 km NW of Melbourne in central Victoria, Australia. It consisted of a patch (approximately 3 ha) of *E. polybractea* surrounded by box–ironbark forest, typical of the district (Anonymous 2000). The site was largely mono-specific, with little undergrowth and scattered *E. viridis* R.Baker trees. The site was commercially harvested for eucalyptus oil until 1992. Since then, trees have been allowed to grow without disturbance, resulting in all stems on the site being of even age. Annual average rainfall at Inglewood averaged 487 mm between 1990 and 2000 at Inglewood (data supplied by Bureau of Meteorology, Australia).

Plant material

Sixty-eight trees of *E. polybractea* were sampled along two intersecting transects (east–west: 35 trees and north–south: 33 trees) in June 2002. Transects were restricted to a north-facing slope and positioned to cover a range of altitudes and slopes. In order to estimate tree biomass and growth rate, the number of stems for each tree in the east–west transect was counted and the stem height and stem diameters at various heights recorded. Samples (approximately 10 g) of whole leaves were sampled at random from a pool of > 100 leaves taken from the northern aspect of the tallest stem of each tree frozen immediately in liquid nitrogen. Samples were then stored in liquid nitrogen until analysed (see below). A further 50 leaves from the original collection were retained and measured for area and oven-dried at 55°C for 4 d in order to calculate average leaf size and the relationship between leaf mass and leaf area (leaf mass per area, LMA, $m^2\ kg^{-1}$). An additional 60 leaves from 10 plants were measured for leaf thickness and leaf area, then subsequently oven-dried to determine the relationship between leaf thickness and LMA.

Secondary metabolites

Approximately 4 g of frozen leaves were ground to a fine powder in liquid nitrogen using a blender (Virtis, Gardiner, NY). Three replicate samples (averaging 200 mg fw) were extracted in 3.5 mL hexane containing 100 μg mL^{-1} tridecane as an internal standard in sealed vials for terpene analysis. A further 1 g was freeze-dried for further chemical analyses (see below) and dry weight determination.

Hexane extracts were incubated at 50°C for 2 d with periodic shaking and then stored at –20°C. Samples were dehydrated with anhydrous Na_2SO_4 (75 mg mL^{-1}) and centrifuged before analysis by gas chromatography (GC). Aliquots (1 μL) were analysed using a Perkin Elmer Autosystem XC (Norwalk, CT) fitted with a PE-WAX column [60 m × 0.25 mm id × 0.25 μm film (polyethelene glycol)] and flame ionisation detector with a flow rate of 2 mL min^{-1} and He as the

carrier gas. Separation was achieved by increasing the column temperature from 70 to 110°C at a rate of 2°C min^{-1}, then held at 110°C for 2 min. The temperature was then increased to 230°C at 10°C min^{-1} and held at 230°C for 3 min. Major monoterpenoid components (1,8-cineole, α-pinene, β-pinene, limonene, and *p*-cymene) were identified and quantified by comparison with authentic standards (Sigma, St Louis, MO and Fluka, Buchs, Switzerland). These monoterpenes accounted for 84.46 ± 0.48% (mean ± s.e.) of the total peak area. Unknown peaks were quantified using the average calibration response of the known monoterpenes and identified by subsequent GC-MS analysis, using a 6890 GC coupled to a mass spectrometer (Agilent Technologies, Palo Alto, CA) fitted with a HP5-MS column [(5%-phenyl)-methylpolysiloxane, 30 m × 0.25 mm id × 0.25 μm film]. Data presented are the means of the three replicate samples.

To determine foliar phenolic concentrations, finely ground freeze-dried leaf samples (50 mg) were extracted four times in 50% acetone (v/v) and measured with Folin–Ciocalteau's reagent (Cork and Krockenberger 1991) as modified by Gleadow and Woodrow (2002). Gallic acid was used as a standard and measurements were expressed as gallic acid equivalents.

Element analysis

Carbon isotope ratios were used as an indicator of water stress. Carbon isotope ratios of each freeze-dried leaf sample were measured using an on-line VG Isochrom mass spectrometer (VG Microtech, Uckfield, East Sussex, UK; precision 0.1‰) after combustion in a Carlo Erba 1110 elemental analyser (Thermoquest, Rydalmere, Australia). Carbon isotope ratios were expressed as $\delta^{13}C$ using the PeeDee Belemnite standard (Farquhar *et al*. 1989).

Foliar levels of the plant nutrients nitrogen and phosphorus, were determined as possible sources of nutrient stress. Total foliar nitrogen content was measured on freeze-dried leaf samples (7 mg) using a Perkin Elmer 2400 Series 2 CHNS/O analyser (Norwalk, CT), calibrated with high purity acetanilide (Perkin Elmer).

To determine total phosphorus content, 25 mg of freeze-dried tissue was ashed at 550°C in a muffle furnace and extracted with 5 mL of 1% (v/v) perchloric acid ($HClO_4$) at room temperature for 2 d. The supernatant was then analysed following Murphy and Riley (1962). Analytical grade KH_2PO_4 was used as a standard.

Biomass and growth

As *E. polybractea* is not commercially harvested for its wood, there have been no studies relating its above ground biomass to stem dimensions, nor on any other mallee species, to our knowledge. In this study the above ground biomass of each stem was estimated for 35 plants (east–west transect) by describing the bole of the tree as sequential conoid cylinders topped by a cone using Newton's formula (Philip 1994). Because the crowns of the trees were sparse and did not display an extensive branching habit, it is unlikely that they contributed greatly to biomass. The biomass for each stem of an individual was calculated in this way and summed to give an estimate of the above ground biomass per plant.

Since coppicing mallee species draws significantly from resources stored in swollen subterranean lignotubers (Wildy and Pate 2002), variation in lignotuber size, and thus resources, could introduce a confounding variable when examining the growth of even aged stems. In order to minimise the impact of lignotuber size on growth when calculating relative growth rates of individuals, stem number was integrated as a surrogate measure of lignotuber size. The number of stems per plant was dependant on the amount of the lignotuber that was exposed at the soil surface, which was assumed to be indicative of total lignotuber size. In order to compare the growth rate of trees with different lignotuber sizes we calculated the mean stem relative growth rate as follows:

$$RGR_{stem} = \frac{\ln\sum(\text{stem biomass/stem number})}{\text{Age of stem}} \times 10^{-3} \text{ mg g}^{-1} \text{ d}^{-1}. \quad (1)$$

In this equation RGR_{stem} is effectively the relative growth rate corrected for lignotuber size, stem biomass is the estimated total biomass per plant in grams, stem number is the number of stems per plant, and age of stem is the time since the site was last harvested in days.

Statistics

Data were analysed (regressions, multiple regressions) using Minitab Release 13 (Minitab, State College, PA). All data were found to be normally distributed using Anderson–Darling tests.

Results

Secondary metabolites

The average concentration of total foliar terpenes (i.e. mono- and sesquiterpenes) of this population of *E. polybractea* was (± 1 s.e.) 2.08 ± 0.09% (w/fw). In order to minimise any difference caused by instantaneous difference in water content between individuals, we also calculated the concentration of terpenes on a dry weight basis. Trees varied more than 4-fold in foliar terpene concentration on both a weight (range: 1.44–6.46% (w/dw); mean ± 1 s.e. = 3.64 ± 0.16%; Fig. 1*A*; Table 1) and area basis (range: 4.53–23.18 g m^{-2}; mean ± 1 s.e. = 11.42 ± 0.53 g m^{-2}). The most abundant terpene was 1,8-cineole, a monoterpene, accounting on average for over three quarters of the total foliar terpene concentration (77.79 ± 1.15%), although this was quite variable (Table 1). Other significant components were the monoterpenes *p*-cymene (3.72 ± 0.34%), α-pinene (1.68 ± 0.28%), limonene (0.69 ± 0.06%), and β-pinene (0.66 ± 0.13%), and the sesquiterpenes globulol (2.45 ± 0.19%) and spathulenol (1.09 ± 0.11%).

Total foliar phenolic concentration was less variable, ranging from 8.96 to 18.07% (w/dw) (Fig. 1*B*; Table 1). Overall, the average concentration of phenolics was 13.80 ± 0.25% (w/dw) (Fig. 1*B*; Table 1). This equated on an area basis to a range from 25.75 to 60.40 g m^{-2} with a mean of 43.10 ± 7.54 g m^{-2}. A positive regression was evident between phenolic and terpene content on an area basis (r^2=0.095, P= 0.011); however, there was no significant corresponding mass based correlation. Moreover, additional regression analysis using the various components of the terpene pool did not show any significant relationships with leaf phenolic concentration (data not shown).

Leaf morphology

Leaf mass per unit leaf area (LMA) was used in this study as a surrogate for leaf thickness. Measurements of leaves from *E. polybractea* showed that leaf thickness was highly correlated with LMA (r^2=0.710, P< 0.001; y = 16.00 + 663.61x). LMA varied from 267.2 to 358.5 g m^{-2}, with an overall population mean of 312.1 ± 2.6 g m^{-2} (Fig. 1*C*). Terpene

concentration was significantly positively correlated with LMA on both a weight (r^2=0.068, P=0.032; Fig. 2*A*) and an area basis (r^2=0.187, P<0.001; Fig. 2*B*). In contrast LMA and leaf phenolic concentration were not correlated when phenolics were considered on a weight basis, though they were correlated when phenolics were considered by area (r^2=0.290, P<0.001). Using multiple regression analysis, it was found that the relationship between terpenes and LMA was independent of variation in phenolic concentration (r^2=0.005, P= 0.547). However, variation in LMA did account in part for the relationship between phenolics and terpenes (r^2=0.156, P< 0.001).

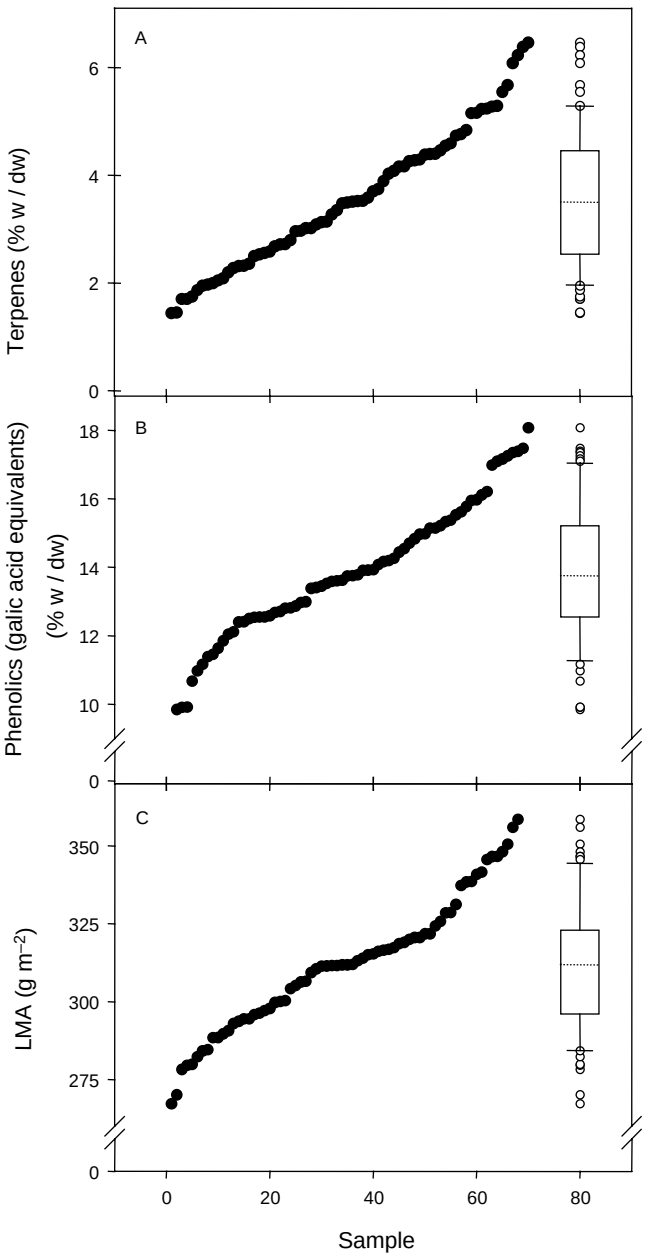

Fig. 1. Foliar terpene (*A*) and foliar phenolic (*B*) concentrations, and leaf mass per area (LMA). (*C*) values from a population of *Eucalyptus polybractea* trees. Plots represent 68 individual plants sampled in two transects. Box plots represent entire sample population and are delimited by the 25th and 75th percentiles with error bars extending to the 10th and 90th percentiles. Values outside these ranges are represented by open circles. Mean (dotted lines) and median (solid lines) values were 3.67 and 3.49% dw, respectively, for leaf terpenes, 13.80 and 13.68% dw, respectively, for leaf phenolics and 312.1 and 311.8 g m^{-2} for LMA.

Leaf elemental composition

Various aspects of leaf elemental composition were examined to quantify the environmental stresses of both limited nutrient and water availability. In order to quantify the degree of water stress, foliar samples were analysed for stable carbon isotope content. In general, higher $\delta^{13}C$ (i.e. less negative) indicates a greater degree of water stress (Farquhar *et al*. 1989). Leaf $\delta^{13}C$ values varied considerably, from –26.8 to –23.3‰, with an overall mean (± 1 s.e.) of –25.1 ± 0.1‰ (Table 1).

Nitrogen and phosphorus are known to be limiting in many Australian soils, and were thus examined in this study as possible sources of nutrient stress. The total concentration of foliar nitrogen did not vary widely, ranging from 0.93 to 1.47% (w/dw) [mean ± s.e = 1.19 ± 0.02% (w/dw)] (Table 1). This corresponds with an area based concentration range from 2.91 to 4.85 g m^{-2} (mean = 3.71 ± 0.06 g m^{-2}). Likewise total leaf phosphorus concentration, ranging on a mass basis between 0.43 to 0.94 mg g^{-1} dw (mean = 0.64 ± 0.01 mg g^{-1} dw) (Table 1). On an area basis, this equated to a range of 0.144 to 0.316 g m^{-2} (mean = 0.199 ± 0.004 mg g^{-1} dw). Plants with higher leaf nitrogen concentrations also tended to have higher leaf phosphorus on both an area (r^2=0.133, P=0.002) and a mass (r^2=0.098, P=0.009) basis.

Neither nitrogen nor phosphorus correlated significantly with $\delta^{13}C$ on either a weight or an area basis, suggesting that nutrient uptake was not inhibited by variation in water availability. Water stress, as measured by $\delta^{13}C$, did not significantly correlate with either terpenes or phenolics when considered on either an area or weight basis, nor was it correlated with LMA. The only measured variable that was significantly related to water stress was average leaf size (r^2=0.064, P=0.036), with smaller leaves occurring under higher water stress (i.e. less negative $\delta^{13}C$).

Nitrogen concentration did not account for any of the variation observed in either terpene or phenolics concentration when these were considered on a weight basis. When all were considered on an area basis, however, a slight though significant positive correlation was evident with terpenes (r^2=0.070, P=0.028; Fig. 3*A*), although not with phenolics. Phosphorus concentration was not correlated with either terpenes or phenolics, regardless of whether the components were considered on an area or weight basis.

LMA was not significantly related with either nitrogen or phosphorus concentration as calculated by weight. However, when considered on an area basis, both nitrogen and phosphorus concentrations were positively correlated with LMA (r^2=0.176, P<0.001; Fig. 3*B*; r^2=0.088, P=0.014, respectively). Further analysis using multiple regression analysis revealed that the relationship between phosphorus per area and LMA was determined in part by variation in nitrogen concentration per area (r^2=0.070, P=0.029). This analysis also revealed that the relationship between nitrogen and phosphorus is independent of LMA (r^2=0.024, P=0.211).

Multiple regression analysis was also used to test whether the relationship between LMA and terpenes was affected by variation in nitrogen. This analysis demonstrated that the relationship between terpenes and LMA was independent of variation in nitrogen (r^2=0.006, P=0.505). The analysis also revealed that the relationship between terpenes and nitrogen was dependant upon variation in LMA (r^2=0.096, P=0.010).

Plant growth

Eucalyptus polybractea trees at the site were last harvested for oil (i.e. cut back to ground level) in 1992. Despite this, there was considerable variation in stem number (range: 1–13, mean = 4.05 ± 0.47) and plant height (range: 1.95–5.90 m, mean = 4.03 ± 0.09 m) between trees. Using biomass estimation techniques, the average above ground biomass was highly variable, with a 30-fold difference between the smallest and largest individuals (1.35–30.5 kg). However, when relative growth rates were corrected for lignotuber size (RGR_{stem}, eqn 1), variation was less than a tenth of that amount (approximately 30%, 1.69–2.46 mg g^{-1} d^{-1}), with an overall mean (± 1 s.e.) of 2.08 ± 0.03 mg g^{-1} d^{-1} (Table 1). RGR_{stem} effectively treats each stem as an individual plant and this index was used in all subsequent regression analyses.

Variation in RGR is often attributed to the availability of nutrients and water. To test this we compared RGR_{stem} with the degree of water stress ($\delta^{13}C$) and foliar nutrient composition. None of these environmental variables could account for the variation observed in RGR_{stem}. A positive, though weak, correlation was detected between growth rate and total mass based terpene concentration (r^2=0.111, P=0.051; Fig. 4*A*) but not between growth rate and phenolic concentration. When both pools of secondary metabolites in each plant were summed per plant, to give total carbon based secondary metabolites (CBSM, % w/dw), and compared with RGR_{stem}, there was a significant correlation which accounted for more of the variation in RGR_{stem}, compared with either phenolics or terpenes alone (r^2=0.125, P=0.038; Fig. 4*B*).

Discussion

This study confirms that the terpene concentration in *E. polybractea* foliage is comparable with the highest values recorded for the genus, and indeed for terpene-storing plants in general (Brophy *et al*. 1991). However, there was considerable variation in total terpene concentration between individual plants (Fig. 1*A*), which is typical of *Eucalyptus* species (Brophy *et al*. 1991). There were also significant differences in terpene composition, with all of the main components of the terpene pool varying considerably, although the monoterpene 1,8-cineole was the main component of the terpene pool in all individuals. A physiological variable that could account for either the variation in terpene composition or a sizeable part of the variation in terpene concentration was not identified and it is likely that both types of variation are largely under genetic control. This conclusion is consistent with previous studies that have determined high heritability values for terpene content and composition in *E. camaldulensis* (Barton *et al*. 1991; Doran and Matheson 1994) and the related species *Melaleuca alternifolia* (Butcher *et al*. 1994).

Table 1. Leaf characteristics and growth parameters of *E. polybractea* trees in a natural population

Total terpene, phenolic, nitrogen and phosphorus concentrations are given as a proportion of dry leaf mass (dw), % 1,8-cineole refers to the proportion of total terpenes as 1,8-cineole, and LMA is the leaf mass to area ratio. RGR_{stem} is the mean stem relative growth rate, a measure that controls for lignotuber size. Means and standard errors (s.e.) are of 68 plants (n = 68) for all parameters except RGR_{stem} (n = 35)

	Mean	s.e.	Minimum	Maximum
Foliar terpenes (% w/dw)	3.64	0.16	1.44	6.46
% 1,8-cineole (% w/w)	77.79	1.15	49.14	93.42
Foliar phenolics (% w/dw)	13.8	0.25	8.96	18.07
$\delta^{13}C$ (‰)	–25.1	0.1	–26.8	–23.3
Foliar nitrogen (% w/dw)	1.19	0.02	0.93	1.47
Foliar phosphorus (mg g^{-1} dw)	0.64	0.01	0.43	0.94
LMA (g m^{-2})	312.1	2.6	267.2	358.5
RGR_{stem} (mg g^{-1} d^{-1})	2.08	0.03	1.69	2.47

Leaf thickness

The variation in terpene concentration could be accounted for by variation in leaf mass per area (LMA), which was directly proportional to leaf thickness. More specifically, terpene concentration was positively correlated with LMA when expressed on both an area (T_A) and a mass (T_M) basis (Fig. 2). Similar area-based increases in terpene concentration were measured for balsam fir (*Abies balsamea*) needles by Lamontagne *et al*. (2000, 2002). However, these authors measured a decrease in mass-based terpene concentration with increasing LMA. Assuming that gland number was not a variable, the positive relationship between LMA and T_A is likely the result of gland dimension in the plane perpendicular to the leaf surface scaling with leaf thickness. Thus, as leaf thickness increases, so too does the depth, or height of the gland. The positive relationship between LMA and T_M measured here indicates that the overall volume-based concentration of terpenes also increases with LMA. Thus, with increasing LMA, a greater proportion of leaf volume is occupied by glands. This is probably the consequence of gland dimension also varying in the plane parallel with the leaf surface, suggesting that gland height and width co-vary. Therefore, given that eucalypt oil glands are spherical to ovoid in shape (Fahn 1979), any increase in leaf thickness should result in a non-linear increase in gland volume and consequently terpene content. This hypothesis could be tested by measuring average gland size in leaves with varying LMAs. The reason why the volume-based concentration of terpenes did not increase with LMA in balsam fir is unclear, but it may relate to the structure of the resin ducts in which the terpenes are stored in this species.

Our leaf phenolic data contrasted with the terpene data. We found that total phenolic concentration increased with LMA when expressed on an area basis, but not on a mass basis. Given that phenolics are not stored in specialised structures, but rather are likely stored in vacuoles of cells, the overall volume-based concentration of phenolics should remain roughly constant with varying LMA, as long as the vacuolar phenolic concentration does not change. Thus, the

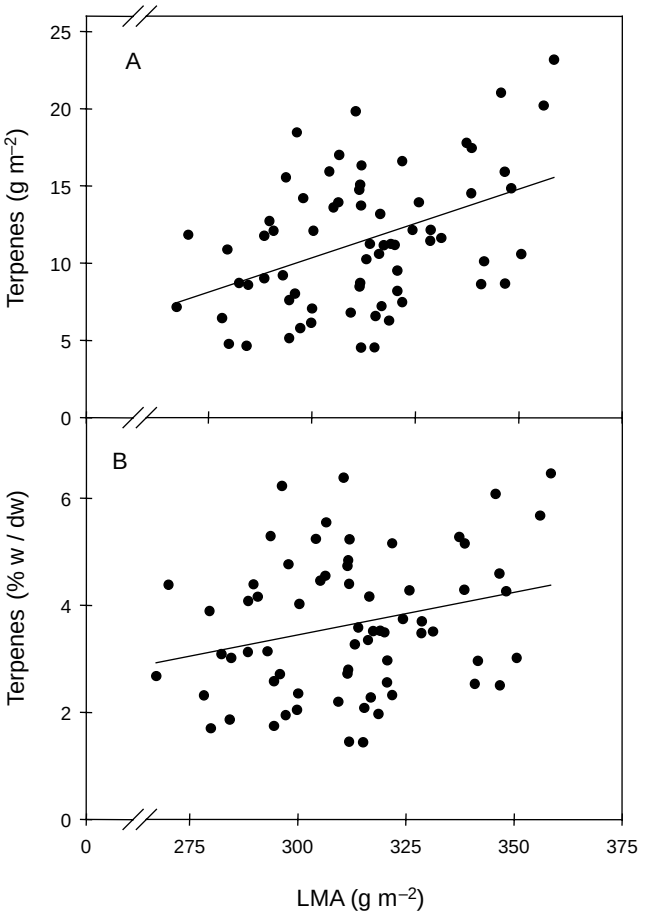

Fig. 2. Relationships between leaf mass per area (LMA) and foliar terpene concentrations of 68 *Eucalyptus polybractea* trees. Terpene concentration was significantly correlated with LMA when calculated on an (*A*) area (r^2=0.187, P<0.001) and (*B*) weight (r^2=0.068, P=0.032) basis.

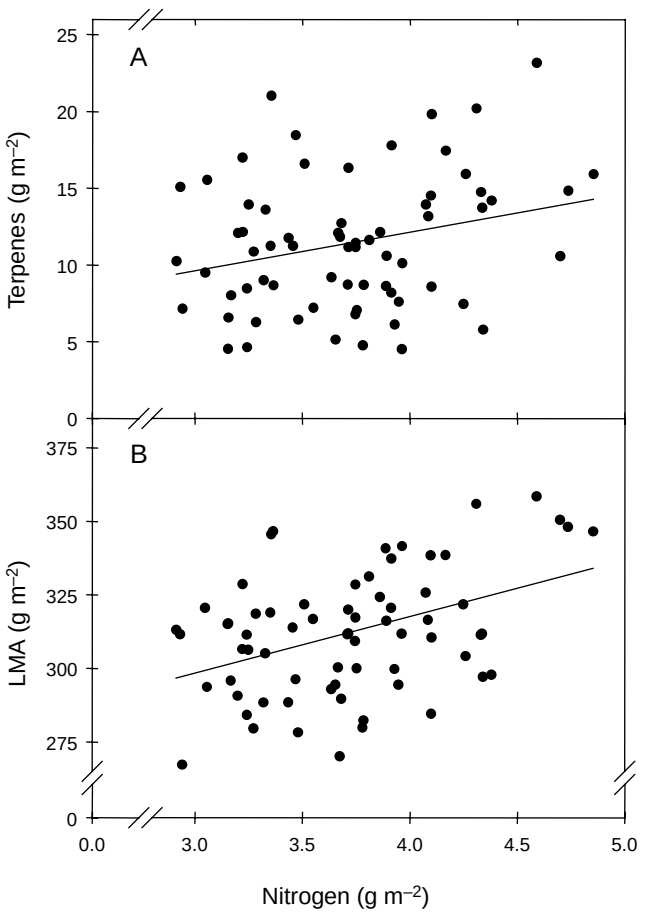

Fig. 3. Relationships between foliar terpene concentration by area (*A*, r^2=0.071, P= 0.028) and leaf mass per area (LMA) (*B*, r^2=0.176, P<0.001) and nitrogen concentration by area in leaves of 68 *Eucalyptus polybractea* trees.

positive relationship measured between phenolic concentration per area and LMA is probably the result of variation in leaf thickness and the associated change in total vacuolar volume.

Given the highly significant relationship between LMA and T_A and T_M, it follows that one way in which environmental factors may influence terpene concentration is through their effects on LMA. Although LMA is known to be a heritable trait within *Eucalyptus* (Li *et al*. 2000), studies have demonstrated that LMA also varies with factors such as water stress (Li *et al*. 2000; Pita and Pardos 2001; Gleadow and Woodrow 2002), phosphorus availability and nitrogen availability (Anderson *et al*. 2000; Burns *et al*. 2002; Gleadow and Woodrow 2002). Nevertheless, these factors can also affect primary metabolism, and may, therefore, affect terpene content not only through regulation of gland volume (via effects on LMA), but also through regulation of the supply of carbon for biosynthesis. Phenolic biosynthesis too may impact on terpene content by acting as a competing carbon sink.

Water stress

We found that trees at the study site varied considerably in their foliar carbon isotope ratio ($\delta^{13}C$), and it is likely that this reflects varying degrees of water stress. In support of this suggestion, we did not find a relationship between $\delta^{13}C$ and nitrogen per unit leaf area, which is correlated with photosynthetic capacity in a range of plants (Field and Mooney 1986) including eucalypts (Anderson *et al*. 2000). This indicates that variation in $\delta^{13}C$ was due primarily to variation in stomatal conductance and presumably water availability. It should be noted that the average leaf $\delta^{13}C$ value obtained for *E. polybractea* is amongst the highest (i.e. least negative) ever recorded for a *Eucalyptus* spp. (Schulze *et al*. 1998; Miller *et al*. 2001; Woodrow *et al*. 2002), indicating that overall water stress at the study site was considerable.

In contrast with our hypothesis, we did not detect a significant relationship between either $\delta^{13}C$ and LMA or $\delta^{13}C$ and terpene concentration (T_A and T_M). This indicates that water stress did not regulate terpene content either directly or indirectly through effects on LMA. These findings are consistent with other studies of *Eucalyptus* spp. that showed no relationship between water availability and terpene content. However, the data presented here contradict anecdotal evidence from commercial oil producers, who experience a decrease in terpenoid oil yield from *E. polybractea* following conditions of extreme drought (P. Abbott, personal communication). These observations, however, have been based on bulk oil distillation (i.e. oil per hectare). Taking into account the results found in this study, these observations may reflect alterations in biomass allocation as a result of water stress, particularly allocation to leaves, rather than an increase in foliar terpene concentration.

Other species of *Eucalyptus* have been shown to have a higher LMAs under water stress (Li *et al*. 2000; Pita and Pardos 2001; Gleadow and Woodrow 2002). According to our results this, on its own, would result in an increased gland volume and foliar terpene concentration. Water stress, however, is also known to result in a reduced photosynthetic capacity in eucalypts and other species (Li 2000), which may decrease the supply of carbon to terpene synthesis, offsetting, to a degree, the LMA effect. We were not able to resolve these counteracting effects in this study, but they may be a reason why a meta-analysis of numerous studies could not distinguish any consistent relationship between terpene content and water stress across a range of species (Koricheva *et al*. 1998).

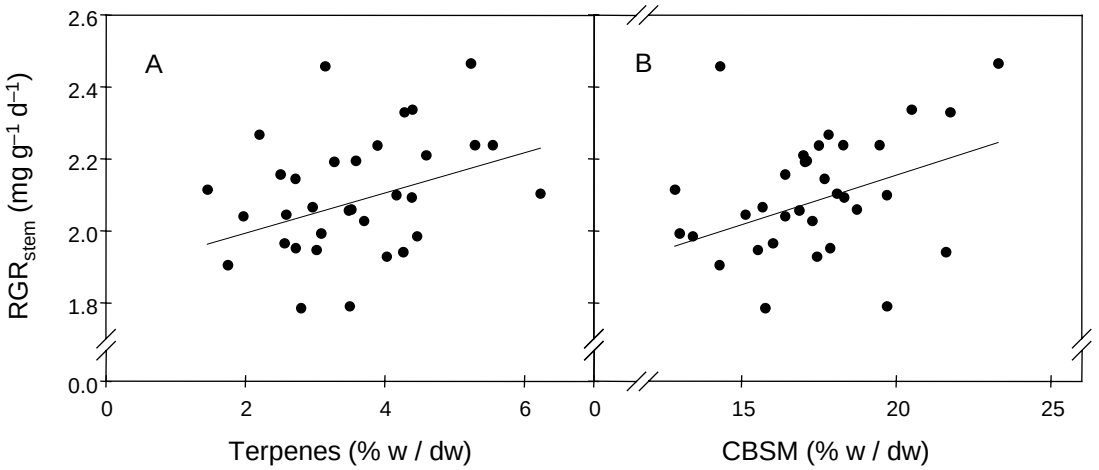

Fig. 4. Relationships between mean stem relative growth rate (RGR_{stem}) and (*A*) foliar terpenes (r^2=0.111, P=0.051) and (*B*) total carbon based secondary metabolites (CBSM, r^2=0.125, P=0.037) of 35 *Eucalyptus polybractea* trees. RGR_{stem} effectively treats each stem as an individual tree, which controls for lignotuber size. CBSM is the sum of total foliar terpenes and phenolics.

Nutrients and phenolics

Previous studies of the effects of nitrogen and phosphorous supply to eucalypts found a decrease in LMA with increasing mass based nutrient concentrations (Anderson *et al*. 2000; Burns *et al*. 2002; Gleadow and Woodrow 2002). However, we did not detect a significant relationship between LMA and mass-based nutrient concentrations. The positive relationships found on an area basis are therefore likely to be representative of increased leaf volume per unit leaf area. Significant relationships between the concentration of either nutrient and T_M were not detected in this study. The only significant relationship identified was a positive correlation between area based nitrogen concentration (N_A) and T_A (Fig. 3*A*). Lamontagne *et al*. (2000, 2002) identified a similar correlation in balsam fir. In both cases it is likely that the relationship is a consequence of the fact that both T_A and N_A increase with LMA. Multiple regression analysis of our data showed that under constant LMA, N_A was not correlated with T_A. If we assume that N_A is proportional to photosynthetic capacity, then at least under the conditions of this study, the capacity to supply carbon does not have a measurable affect on terpene concentration.

It appears that competition for carbon from the phenolic biosynthetic pathways does not affect the concentration of terpenes. Multiple regression analysis showed that the relationship between LMA and T_A was not significantly affected by area or mass based phenolic concentration. Consistent with this finding, mass based terpene and phenolic concentrations were not significantly correlated.

Impacts on growth

Plant growth rate of *Eucalyptus* and other species is sensitive to water supply (Li *et al*. 2000; Gleadow and Woodrow 2002), especially in environments where water is relatively scarce. Similarly, there is usually a positive relationship between nutrient supply and growth (Anderson *et al*. 2000; Burns *et al*. 2002; Gleadow and Woodrow 2002). It is surprising then, that we failed to detect a significant relationship between growth rate and any environmental variable in this study. This may be explained by the particular adaptations of *E. polybractea* to its environment. Although nutrient levels and average rainfall were relatively low (approximately 480 mm per annum), and the degree of water stress was high at this site, there was a marked lack of phenotypic plasticity observed in almost all characters measured. We suggest that this may be a result of selection pressures selecting for plants with high intrinsic WUE, together with high constitutive levels of defence. Such features would enhance survival through rare periods of catastrophic drought and herbivory stress. Alternativley, maintenance of adaptations to such extreme events may constrain the ability of the plant to respond to periods of relatively high nutrient and water availability, and thereby account for the lack of plasticity noted in growth, secondary metabolism, and leaf morphology.

Given that mallee roots can be extremely long lived, the prevalence of *E. polybractea* in many small disjointed monospecific stands suggests that these areas previously experienced a catastrophic event, selecting for species and individuals exhibiting such survival features. Moreover, the relative paucity of the species in the box-ironbark forest surrounding the experimental site suggests that in such areas, *E. polybractea* is out-competed by other species able to exploit higher resource availability. It is noteworthy though, that growth of above-ground biomass was positively related to concentration of secondary metabolites (Fig. 4*B*). The fact that terpene concentration is the major contributing factor to this result (Fig. 4*A*) is particularly interesting, as the costs of deploying monoterpenes are generally considered high. Estimated costs of terpene biosynthesis are high (approximately 3.4 g glucose g^{-1}; Gershenzon 1994) when compared with those estimated for ordinary leaf biomass of evergreen species (1.55 g glucose g^{-1}; Villar and Merino 2001), and glands required for terpene storage also have high construction costs. Moreover, continuing biosynthetic rates may be relatively high, as *Eucalyptus* spp. are known to emit high quantities of terpenes (Guenther *et al*. 1991; He *et al*. 2000). It appears, then, that *E. polybractea* may gain a significant growth advantage due to increased deployment of secondary metabolites, possibly due to their role in herbivore defence, although the correlation is small and growth is still primarily determined by other factors not measured in this study.

Acknowledgments

The authors would like to especially thank Peter Abbott, Kerin Lee and Des Lamprell of Felton, Grimwade and Bickford Pty Ltd for their considerable assistance with all aspects of this research. This research was funded by a Linkage grant from the Australian Research Council to IEW. DJK was a recipient of a Melbourne Research Scholarship.

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Manuscript received 5 November 2003, accepted 5 February 2004