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Article *in* Australian Journal of Botany · May 2017

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Intraspecific diversity of terpenes of *Eucalyptus camaldulensis* (Myrtaceae) at a continental scale

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Abstract. Plants show a high degree of intraspecific variation in several traits including plant secondary metabolites. This variation can be influenced by genetic and environmental factors that result in geographical structure in their distribution. By growing plants from several populations in a controlled environment, we studied variation in foliar terpenes in *Eucalyptus camaldulensis*, which is the widest distributed eucalypt, with a large range both latitudinally and longitudinally. We found that the concentration of terpenes is highly variable among subspecies. We identified four chemotypes dominated by 1,8-cineole, γ-terpinene, α- and β-phellandrene. While the 1,8-cineole chemotype is abundant in all populations, the other three chemotypes are rare in the central area and the north-east of Australia. The γ-terpinene chemotype is mainly restricted to the north and west of Australia, whereas the α- and β-phellandrene chemotypes show an opposite distribution in the north and south of the continent. The annual mean temperature and humidity of the source populations correlate with the abundance of the dominant terpenes. We also tested the effects of elevated CO_2 concentrations on the terpene concentration and found that elevated CO_2 atmosphere reduces the overall accumulation of foliar terpenes. The results suggest that variation in terpene composition in *E. camaldulensis* can be influenced by environmental variables, mainly favouring the 1,8-cineole chemotype in arid locations.

Additional keywords: chemotypes, elevated CO_2, eucalyptus oil, geographical clines, phytochemical diversity, plant secondary metabolites.

Received 15 September 2016, accepted 6 April 2017, published online 30 May 2017

Introduction

Plant secondary metabolites (PSM) are highly diverse both within and among plant species. Most studies of PSMs focus on small-scale variation, but larger, continental-scale studies allow a better understanding of intraspecific diversity, how interactions with abiotic and biotic factors can influence the PSM phenotype among individuals and populations and how populations can respond to future climate changes (De Frenne *et al*. 2013). Several studies have used population differentiation and latitudinal gradients to explain variation in PSMs (Levin 1976; Jaakola and Hohtola 2010; Rasmann and Agrawal 2011; Pratt *et al*. 2014). Traditionally, it has been proposed that concentrations of PSMs, and plant defences more generally, are influenced by latitude, given that populations closer to the tropics would face more intense antagonistic interactions (Dobzhansky 1950; Schemske *et al*. 2009). However, a recent meta-analysis and data from worldwide collections have challenged this idea (Moles *et al*. 2011*a*, 2011*b*). Nevertheless, it is likely that variation in PSM among plant populations is influenced to some degree by differences in climatic conditions and biotic interactions that can correlate with latitude but also with other geographical parameters. Although most previous ecogeographic studies on PSM variation have focussed on its relationship with latitude, few studies have explored how the variation in PSM can be explained at a continental scale, including wide gradients both in latitude and longitude (but see Woods *et al*. 2012). Therefore, continent-wide studies can contribute to our understanding of how geographic and climatic factors influence the intraspecific diversity on PSM by including wide gradients in key abiotic and biotic variables.

Sampling from several natural populations allows the detection of existing clines in the production of PSM, and several studies have investigated the presence of chemotypes in different plant species (e.g. Keszei *et al*. 2010; Taft *et al*. 2015). However, the contribution of genetic and environmental factors to population differences cannot be understood by studying solely

natural populations. Common-garden experiments where seeds from different source sites are grown in a common environment allow genetic-based differences in phenotypic traits to be identified and makes it possible to separate those from the effects of phenotypic plasticity (Woods *et al*. 2012; De Frenne *et al*. 2013; Holeski *et al*. 2013; Pratt *et al*. 2014). As a consequence, these types of studies require an enormous sampling effort and access to collections from many populations of plant species that are widely distributed across a continent.

Terpenes are a highly diverse class of secondary compounds that are produced by a range of organisms from bacteria and fungi to plants and animals. They are abundant across the biosphere and play an important role in regulating environmental processes and ecological interactions between organisms (Lawler *et al*. 1999; Rasmann *et al*. 2005; Ali *et al*. 2010; Kleine and Müller 2011; Moore *et al*. 2014). Terpenes are highly diverse and ~25 000 different terpenes have been identified, with thousands more very likely to exist (Gershenzon and Dudareva 2007). Enzymes that convert prenyl pyrophosphates to terpenes are called terpene synthases and are well characterised in angiosperms and gymnosperms (Tholl 2006; Keszei *et al*. 2010; Külheim *et al*. 2015). Terpenes as well as many other PSMs show intraspecific variation due to ontogenetic effects, somatic mutations or following herbivory, but variation between individuals is also common (Moore *et al*. 2014). Plants producing high concentrations of terpenes in the leaves can present discontinuous variation in the proportion of compounds, which is defined as chemical polymorphisms or chemotypes (Keszei *et al*. 2008). Chemotypes can be the result of only a few genes that are differentially expressed in the same tissues of different plants of a single species. Identification of chemotypes provides a useful way of studying natural genetic variation that can be readily linked to causative genes and the biosynthetic pathways of terpene production (Vernet *et al*. 1986; Keszei *et al*. 2010; Andrew *et al*. 2013). It is still unclear how abiotic factors can affect the accumulation of terpenes. It is expected that predicted changes in atmospheric CO_2 will affect secondary metabolism (Misra and Chen 2015), but whether elevated CO_2 increases, decreases or has minimal effects on terpene accumulation is highly variable (Zvereva and Kozlov 2006; Valkama *et al*. 2007).

Plants from the genus *Eucalyptus* (Myrtaceae) are well known for producing terpene-rich oils in high quantities (Keszei *et al*. 2008; Padovan *et al*. 2014) and these terpenes could be crucial in the success of the trees that dominate the Australian continent. For example, plants emitting isoprene are better at tolerating reactive oxygen species and heating induced by sunlight (Sharkey *et al*. 2008) and some terpenes can protect *Eucalyptus* from herbivores (Stone and Bacon 1994). Although the genus *Eucalyptus* consists of ~800 species, only a few species are distributed across the Australian continent and *Eucalyptus camaldulensis* is the most widespread (Butcher *et al*. 2009). Thus, it provides the opportunity of describing intraspecific trait diversity among different ecosystems at a continental scale. Its mainland distribution ranges along watercourses that can be intermittent and, in many ecosystems, it can be a dominant species that provides habitat for fauna and facilitates river stabilisation (Butcher *et al*. 2009). Recently, seven subspecies have been characterised, on the basis of the variation of 11 microsatellites and morphological traits (Butcher *et al*. 2009; McDonald *et al*. 2009). *Eucalyptus camaldulensis* is also an important plantation tree and is the most widespread eucalypt in the world. It provides wood, shade and shelter as well as pulp and essential oils; thus, several studies have been conducted regarding its terpene profile and concentration. The foliar terpenes of this species show qualitative and quantitative variation in individual compounds (Boland *et al*. 1991; Sadeghi *et al*. 2014); this has been argued to be influenced by water availability with subsequent effects on herbivory (Edwards and Wanjura 1993; Stone and Bacon 1994). Although some studies have found chemical variants in *E. camaldulensis* (Boland *et al*. 1991; Edwards and Wanjura 1993; Moudachirou *et al*. 1999; Padovan *et al*. 2014), no study has thoroughly examined the natural variation in terpenes across its geographic distribution. The geographic distribution of *E. camaldulensis* includes many climatic zones, which, in turn, result in varying degrees of biotic and abiotic stressors. By studying the chemical variation across these zones, we can investigate whether environmental influences have left signatures of adaptation.

One of the major abiotic impacts on plant growth and chemical composition is the global increase in atmospheric CO_2 (Coley *et al*. 2002; Zvereva and Kozlov 2006; Valkama *et al*. 2007). There are several examples describing modifications in the concentration of terpenes in both gymnosperms (Heyworth *et al*. 1998; Sallas *et al*. 2001) and angiosperms (Johnson and Lincoln 1991; Peñuelas and Llusia 1997; Staudt *et al*. 2001; Rosenstiel *et al*. 2003) grown at elevated CO_2. Nonetheless, the effects are variable (Misra and Chen 2015) and the concentration of foliar terpenes in three species of *Eucalyptus* was not affected by growth under elevated CO_2 (Lawler *et al*. 1997; McKiernan *et al*. 2012). Therefore, the outcome of an increase in CO_2 might depend on each plant species, and it is particularly useful to understand how a widely distributed species such as *E. camaldulensis* will respond to expected changes in atmospheric CO_2. There could be variation at the population (or chemotype) level in response to increases in CO_2, potentially leading to changes in levels of susceptibility to either biotic or abiotic stress, which is important for reforestation and plantation management.

In the present study, we describe the diversity of terpenes among populations of *E. camaldulensis* across the Australian continent; the plants were grown from seed in a controlled environment at ambient and elevated concentrations of CO_2. This allowed the analysis of variation in the foliar terpene composition attributable to genetic-based differences among populations, subspecies and geographic regions. The research questions we addressed in the study are as follows: (1) are there terpene-based chemotypes in *E. camaldulensis*; (2) are these chemotypes equally distributed across the geographic range; and (3) do the concentration and composition of terpene correlate with climatic variables; and (4) does elevated CO_2 affect the concentration of terpenes, which could lead to differences in the response to abiotic and biotic stress?

Materials and methods

Seed collection and growing conditions

Seeds of *E. camaldulensis* obtained from the Australian Tree Seed Centre (Canberra, Australia) were previously collected from

individual mother trees in natural populations. Our samples included six subspecies (49 populations), with between 5 and 12 maternal sources per population. Selected genotypes were distributed over the species' range to capture the breadth of genetic diversity in the species (Fig. 1), broadly representing its range of distribution, including populations from the following six different geographic–climatic regions in Australia: North-west, North-east, West, Central, Central South and South-east.

Seeds were germinated in April 2013 in a germination tray with low phosphorus potting mix and then brought to a nursery room at ambient CO_2 (400 µmol/mol) at a temperature of 28°C on a 16-h day–8-h night light cycle. From sowing until potting, seedlings were watered daily so as to ensure that the soil was damp because small seedlings are prone to desiccation. Four weeks after germination, seedlings were repotted into 10-cm-diameter 0.75-L planter bags with a low-phosphorus potting mix (1/3 river sand, 1/3 peat moss and 1/3 natural compost) suitable for Australian plants, and moved to a glasshouse in similar atmospheric conditions (400 µmol/mol, 24°C) and natural 12-h day–12-night light cycle. Two applications of a systemic fungicide (Fongarid®, AgNova Technologies, Melbourne, Vic., Australia; active constituent: 250 g/kg furalaxyl; dilution: 1 g/L) were applied at ~5 weeks of age and 2 weeks after repotting, to reduce the chance of root fungal diseases. At ~2 months of age, individual healthy seedlings (6–12 plants per population) were transferred to controlled-growth chambers (PGC20 Conviron®, Controlled Environments, Winnipeg, Canada) located at the Australian Plant Phenomics Facility (Canberra, Australia), using a randomised design. At this time Aquasol® (Yates, Clayton, Vic., Australia) soluble plant fertiliser (N : P : K ratio 23 : 3.95 : 14) was provided at half strength, and, subsequently, every month for the duration of the experiment.

Four environmental growth chambers were used to evaluate leaf-level responses. Eucalypt seedlings are prone to abnormalities (intumescences, anthocyanin accumulation) when grown in artificial conditions; thus, special care was taken to minimise the occurrence of those abnormalities and also simulate a realistic natural environment. Temperature was maintained at 18°C during the night and 24°C during the day. These conditions were designed to encapsulate the average maximum and minimum temperatures recorded for each sampled population. Photoperiod was set as a 16-h day–8-night cycle, with conditions approximating natural photoperiod length between 37°S and 14°S, and 115°E and 149°E. Each cabinet was fitted with a backlight (Sylvania FHE 28W/T5/BLB, Sylvania Lighting, Padstow, NSW, Australia) to provide high-frequency light in the ultraviolet-A spectrum for normal growth of eucalypts (Close and Beadle 2003). Light intensity was changed via an hourly step function in the morning and evening to simulate natural light conditions. Plants experienced full light, corresponding to ~1000 µmol m^{-2} s^{-1}, for 7 h each day. Each cabinet was programmed for the same photoperiod, but there was a delay of 2 h between chambers to allow for measurements of plants. Relative humidity (RH) in the cabinet was controlled by a dehumidifier (Belta 601, Australia) connected to each growth

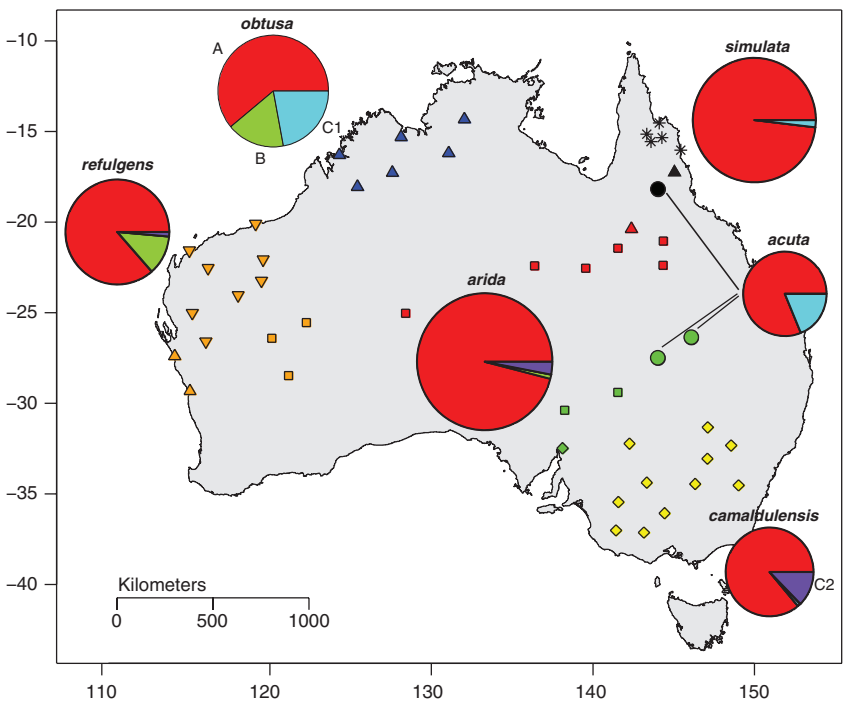

Fig. 1. Map of Australia, showing the sites of *Eucalyptus camaldulensis* seed collection. The pie charts indicate the proportion of plants of each chemotype within a single subspecies. Chemotypes are indicated by the following colours: Chemotype A (red), Chemotype B (green), Chemotype C1 (cyan) and Chemotype C2 (purple). The size of the pie charts is proportional to the mean monoterpene concentration per subspecies, with a range of 15.5–25.2 mg·g^{-1}. Subspecies *acuta* (●), *arida* (■), *camaldulensis* (♦), *obtusa* (▲), *refulgens* (▼) and *simulata* (*), and the six geographic regions of North-west (blue), North-east (black), West (orange), Central (red), South central (green) and South-east (yellow) are indicated.

chamber. This maintained a RH between 50% during the day and 60% at night. Plants were watered to saturation from the base daily for the first 6 weeks, and twice daily from 7 weeks.

CO_2 conditions

At 2 months after germination, plants from 43 populations were exposed to ambient [CO_2] (aCO_2, 400 µmol/mol) over 10 weeks followed by elevated [CO_2] (eCO_2, 800 µmol/mol) for a further 8 weeks (±20 µmol CO_2). This included a period of 2 weeks to allow plants to acclimatise to the cabinets before commencing the ambient CO_2 exposure. The rationale for choosing the conditions for our study was based on current, and projected (Year 2100) atmospheric CO_2 concentrations in an extreme scenario (if anthropogenic emissions keep on increasing and peak after Year 2080; IPCC 2013). To maintain [CO_2] at the desired levels, a non-dispersive CO_2 analyser (GMT220 Vaisala Carbocap® CO_2, Vaisala, Vantaa, Finland) continuously measured [CO_2] in each chamber and directly controlled a solenoid valve that released CO_2 into the chambers as necessary from a cylinder of industrial-grade compressed CO_2. Every second week over the course of the experiment, trays within each cabinet were rotated to avoid potentially confounding position effects. Additionally, between treatment phases, plants were re-randomised within and between cabinets. One or two individuals from 31 populations were maintained at 400 µmol/mol for the duration of the experiment as a control set, whereas 5–10 plants from the 43 populations were subjected to both aCO_2 and eCO_2 phases. This was necessary because of the sequential design of the cabinet experiment, to account for possible developmental effects over the two experimental time points. The number of individuals and populations were reduced as a result of space limitations in the cabinets.

Terpene analysis

At Week 18 after germination, we collected ~1 g of mature leaves in Ziploc bags on dry ice from 414 plants from 49 populations growing at aCO_2 and immediately transferred the samples to a −80°C freezer. After the plants were kept in the elevated CO_2 treatment (Week 26 after germination), we again collected ~1 g of mature leaves for 43 control and 327 test plants (only from 43 populations), making sure that these leaves were developed after the start of the elevated CO_2 treatment with the help of a colour mark that was painted on the youngest leaf's petiole of each plant immediately before commencing the elevated CO_2 treatment. The number of plants sampled was lower at the second time point, because of mortality or unavailability of enough mature tissue. A quantity of ~0.5 g of tissue was immediately transferred to pre-weighted vials with 5 mL of ethanol and tetradecane (0.25 $g.L^{-1}$) as an internal standard. The remainder of the leaf material was weighed and transferred into paper envelopes and oven dried (50°C for 3 days), then reweighed again to determine fresh to dry mass ratio. The frozen leaf tissue from the first leaf collection was treated similarly. After 7 days in ethanol, we injected 1 µL of the extract onto an Agilent 6890 gas chromatograph (Agilent Technologies, Deerfield, IL, USA), at 250°C and 1 : 25 split ratio. Ethanol extracts were separated on a SGE BPX-35 column (SGE Analytical Science, Ringwood, Vic., Australia). The column was 60 m long (internal diameter 0.25 mm), with a stationary-phase film thickness of 0.25 µm, and helium was used as the carrier gas. The temperature ramp was as follows: 100°C for 4 min, ramping to 180°C at 20°C·min^{-1}, held at 180°C for 10 min, followed by a ramp at 20°C·min^{-1}, and held at 250°C for 4.5 min. The total elution time was 26 min. Mass spectra were obtained through a mass selective detector (HP 5073; Agilent Technologies, Deerfield, IL, USA) at a temperature of 250°C (source) and 150°C (quad), with transfer line at 280°C and ion source-filament voltage of 70 eV.

The gas chromatography–mass spectrometry files were processed with the software package PyMS (O'Callaghan *et al*. 2012) to extract the mass spectral peaks, quantify the peak areas and align the peaks across the files. Peak areas were estimated relative to the area of the internal standard, to obtain concentrations as milligrams per gram dry matter. Individual terpenes were identified by comparison of the elution order and mass spectra with published data (NIST database) and previous literature using a similar 35% phenyl polysilphenylene–siloxane column (Southwell and Russell 2002). In addition, we compared the peaks with authentic standards for α-pinene, β-pinene, myrcene, β- phellandrene, terpinen-4-ol, 1,8-cineole, α-terpineol, α-terpinyl acetate and β- caryophyllene (99% purity, made available by Mike Lacey and Thomas Wallenius, CSIRO, Canberra; Keszei *et al*. 2010).

Chemotyping

We performed a hierarchical cluster analysis on the correlation-based dissimilarity matrix of monoterpene and sesquiterpene proportions, using the Ward agglomeration method (*pvclust* package in R; Suzuki and Shimodaira 2006). For this analysis, we included the plants from 49 populations that were sampled for terpenes at ambient CO_2 concentrations at the first sampling time (see Terpene analysis section earlier). A multiscale bootstrap resampling was used for calculating the *P*-values of each cluster (10 000 pseudoreplications). In a preliminary correlation analysis, α-thujene was correlated with several groups and, because it is known that it can arise from different biosynthetic pathways (as can other compounds) via γ-terpinene synthase (Alonso and Croteau 1991; Külheim *et al*. 2015) and sabinene synthase (Chang and Chu 2011), it was excluded from the present analysis. We selected the main clusters of compounds and identified discontinuous distributions in these groups. We then made scatterplots of the proportional contribution of each group of monoterpenes to the total monoterpene concentration and classified each discrete group of samples as chemotypes.

Environmental variables

Environmental variables of each source population were sourced from the Atlas of Living Australia (2015). We calculated Pearson's correlation between the latitude, longitude and several environmental variables of each population that were likely to interact with terpene production in *E. camaldulensis*. Consequently, we selected three independent environmental variables for the selected locations (mean annual temperature,

mean relative humidity and soil nutrient index) to analyse the relationship with terpene traits across populations.

Statistical analysis

We performed linear mixed models within the R environment (ver. 3.2.2; package *lme4* ver. 1.1-10, Bates *et al*. 2015) to identify the contribution of geographic region, subspecies and population to variance in terpenes under ambient CO_2 (the first sampling time). The models included the random factors region, subspecies within region and population within subspecies. The amount of variance explained by the random factors indicates the level of genetic differentiation among them. Significance of variance estimates for random factors was tested by a likelihood test between models with and without a given random factor. The response variables were the total concentration of terpenes at the initial sampling (before the elevated CO_2 treatment) for monoterpenes and sesquiterpenes, or concentrations of the main individual terpenes (1,8-cineole, γ-terpinene, α-pinene, β-pinene, α-phellandrene and β-phellandrene) with total concentration of terpenes as a covariate. The analysis of the effects of elevated CO_2 on terpene concentration was performed using similar mixed models, but with the concentrations from the second sampling (after the CO_2 treatment) as the response variable. We added the fixed factors 'treatment' (control or test plants) to evaluate the differences between plants under ambient and elevated CO_2, 'chemotype' to control for the effect of differences in the proportion of chemotypes between control and test plants, and the interaction between both factors to test whether the response to CO_2 treatment depended on the plant chemotype. We performed an ANOVA based on Type II sum of squares on these analyses to interpret independently the main effects and interaction effects (see Langsrud 2003).

We performed multiple regressions to detect effects of the environmental variables on the total mono- and sesquiterpene concentrations of the plants at the first sampling point. Then, we performed similar regressions for the main individual terpenes, but included the total terpene concentration as a covariate.

Results

Terpene composition

We investigated the terpene composition among 49 populations of *E. camaldulensis* from across the Australian mainland (Fig. 1), using plants growing in a controlled environment. In most of the leaf extracts among plant samples, 1,8-cineole was the dominant terpene; however, other compounds were dominant in some samples (γ-terpinene, α-phellandrene, β-phellandrene and *p*-cymene). Monoterpenes were more abundant than sesquiterpenes relative to the total concentration of terpenes (68–100% and 0–32% respectively). Monoterpenes formed four highly supported clusters ($P > 0.95$; Fig. 2) classified as Group *mono1* (α-phellandrene, piperitone, *p*-cymene and monoterpene 4), Group *mono2* (sabinene, trans-sabinene hydrate, cis-sabinene hydrate, β-phellandrene and linalool), Group *mono3* (limonene, α-pinene, β-pinene, 1,8-cineole, β-trans-ocimene, α-terpineol, α-terpinyl acetate and monoterpene 1) and Group *mono4* (γ-terpinene, terpinen-4-ol, α-terpinene and terpinolene). Sesquiterpenes formed two highly supported clusters which were *sesq1* (α-caryophyllene, β-caryophyllene)

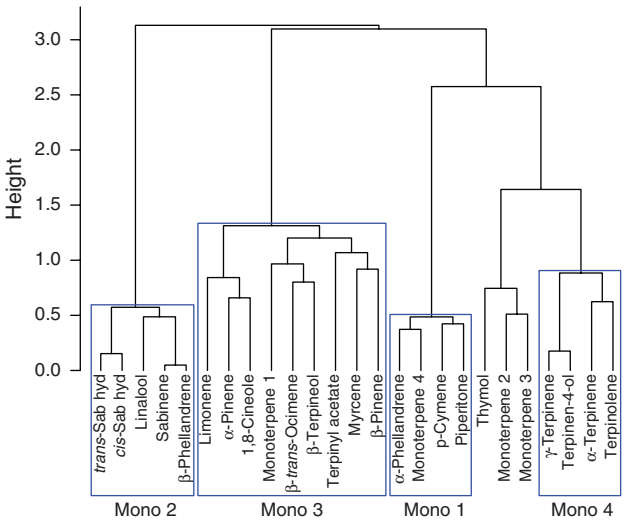

Fig. 2. Cluster analysis of the correlations of monoterpene proportions in all *Eucalyptus camaldulensis* plants. Unbiased approximate test was used to calculate the *P*-values by multiscale bootstrap resampling (10 000 pseudo replications). Clusters highly supported by the data are enclosed in a blue box ($P > 0.95$).

and *sesq2* (α-gurjunene, β-gurjunene, aromadendrene and alloaromadendrene; data not shown). Monoterpene groups showed evidence of discontinuous distribution, whereas sesquiterpene groups did not. The relationships among monoterpene groups showed a pattern with four main profiles that were identified as chemotypes (Tables 1 and 2), including Chemotype A (more than 30% of Group *mono3*, and low percentage of the other terpene groups, dominated mainly by 1,8-cineole), Chemotype B (more than 60% of Group *mono4*, dominated by γ-terpinene), Chemotype C1 (more than 40% of Group *mono1*, dominated mainly by α-phellandrene) and Chemotype C2 (more than 20% of Group *mono2*, dominated mainly by β-phellandrene).

Distribution of chemotypes

Across all subspecies, Chemotype A plants were dominant, with more than 95% of individuals in subspecies *E. c. arida* and *E. c. simulata* being Chemotype A (Fig. 1). *Eucalyptus c. refulgens* was dominated by Chemotype A plants but 12% of the plants were Chemotype B. Subspecies *E. c. obtusa* had the greatest diversity of chemotypes, including plants of Chemotype A (61%), B (17%) and C1 (high in α-phellandrene, 22%). Chemotype C2 (high in β-phellandrene) was found in populations of subspecies *E. c. arida* (3%) and *E. c. camaldulensis* (13%, Fig. 1).

Sources of variation

To explore to which extent spatial and biological factors could explain the variation in terpenes, we performed linear mixed models. Variation in concentration of total monoterpenes was explained mostly by 'subspecies' (11.2%, Fig. S1, available as Supplementary Material to this paper), with 'population' explaining 6.5% of the variance, whereas 'region' explained only little variation. However, only the 'population' factor was detected as significant. Patterns in the concentration of total sesquiterpenes were similar (Table 3, Fig. S1). When the total

Table 1. Mean concentration (mg·g DM^{-1}) per compound among the four identified chemotypes (A, B, C1 and C2)
Standard errors of the mean are shown in parentheses. The three most abundant terpenes per chemotype are in bold and shaded

Parameter	A		B		C1		C2	
N	353		23		22		16	
α-Thujene	0.04	(0.00)	0.06	(0.02)	0.37	(0.03)	0.63	(0.12)
α-Pinene	**3.67**	(0.15)	0.19	(0.02)	0.74	(0.15)	1.32	(0.26)
Sabinene	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)	0.42	(0.09)
Myrcene	0.18	(0.01)	0.03	(0.01)	0.10	(0.01)	0.30	(0.06)
β-Pinene	0.42	(0.07)	0.00	(0.00)	0.01	(0.01)	1.29	(0.53)
α-Phellandrene	0.08	(0.02)	0.05	(0.02)	**6.60**	(0.84)	1.23	(0.33)
α-Terpinene	0.00	(0.00)	0.09	(0.02)	0.05	(0.02)	0.00	(0.00)
trans-β-Ocimene	0.08	(0.02)	0.00	(0.00)	0.00	(0.00)	0.07	(0.07)
Limonene	**1.87**	(0.08)	0.27	(0.04)	0.78	(0.11)	0.63	(0.10)
β-Phellandrene	0.09	(0.01)	0.00	(0.00)	0.30	(0.03)	**7.13**	(1.38)
p-Cymene	0.15	(0.02)	**1.31**	(0.18)	**2.97**	(0.37)	0.03083	(0.23)
1,8-Cineole	**12.09**	(0.32)	1.07	(0.37)	**1.41**	(0.34)	**3.75**	(0.70)
γ-Terpinene	1.12	(0.13)	**14.67**	(1.41)	0.65	(0.15)	0.87	(0.28)
trans-Sabinene hydrate	0.00	(0.00)	0.02	(0.01)	0.00	(0.00)	0.17	(0.04)
Terpinolene	0.21	(0.02)	0.45	(0.08)	0.13	(0.02)	0.04	(0.02)
Linalool	0.01	(0.00)	0.02	(0.01)	0.01	(0.01)	0.19	(0.05)
cis-Sabinene hydrate	0.01	(0.00)	0.08	(0.04)	0.01	(0.00)	0.27	(0.06)
Terpinen-4-ol	0.16	(0.02)	**1.23**	(0.14)	0.15	(0.03)	0.11	(0.04)
Monoterpene 1	0.22	(0.01)	0.09	(0.07)	0.23	(0.03)	0.27	(0.04)
Terpenoid 2	0.10	(0.01)	0.20	(0.03)	0.01	(0.01)	0.00	(0.00)
α-Terpineol	0.35	(0.02)	0.06	(0.02)	0.10	(0.02)	0.24	(0.06)
Monoterpene 3	0.01	(0.00)	0.02	(0.01)	0.00	(0.00)	0.00	(0.00)
Monoterpene 4	0.00	(0.00)	0.00	(0.00)	0.08	(0.01)	0.04	(0.02)
Monoterpene 5	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)	0.04	(0.02)
Piperitone	0.01	(0.00)	0.01	(0.01)	0.20	(0.02)	0.02	(0.01)
Sesquiterpene 1	0.02	(0.00)	0.01	(0.00)	0.00	(0.00)	0.00	(0.00)
Sesquiterpene 2	0.00	(0.00)	0.00	(0.00)	0.10	(0.02)	0.04	(0.02)
Terpinyl acetate	0.12	(0.02)	0.00	(0.00)	0.00	(0.00)	0.20	(0.10)
α-Gurjuene	0.33	(0.02)	0.26	(0.07)	0.02	(0.01)	0.13	(0.04)
β-Caryophyllene	0.06	(0.01)	0.00	(0.00)	0.04	(0.04)	0.25	(0.11)
β-Gurjuene	0.04	(0.00)	0.01	(0.01)	0.00	(0.00)	0.00	(0.00)
Aromadendrene	0.46	(0.02)	0.46	(0.13)	0.06	(0.03)	0.37	(0.11)
α-Caryophyllene	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)	0.01	(0.01)
Alloaromadendrene	0.20	(0.01)	0.11	(0.04)	0.02	(0.01)	0.38	(0.09)
Viridiflorene	0.02	(0.00)	0.03	(0.02)	0.00	(0.00)	0.03	(0.02)
Bicyclogermacrene	0.07	(0.01)	0.00	(0.00)	0.29	(0.13)	**1.53**	(0.29)
Sesquiterpene 3	0.01	(0.00)	0.01	(0.01)	0.00	(0.00)	0.01	(0.01)
Sesquiterpene 4	0.02	(0.01)	0.00	(0.00)	0.00	(0.00)	0.01	(0.01)
Total terpenes	22.75	(0.55)	21.35	(1.67)	15.77	(1.10)	23.38	(3.25)

Table 2. Differences in the relative abundance of the four proposed monoterpene groups (Fig. 2) among chemotypes, shown as the percentage of the total monoterpene concentrations per plant
Values in parentheses express the standard error of the mean

Group	Chemotype							
	A		B		C1		C2	
mono1	1.16	(0.13)	6.97	(0.64)	65.94	(2.63)	11.88	(1.16)
mono2	1.36	(0.07)	0.66	(0.20)	2.73	(0.19)	38.43	(3.88)
mono3	6.78	(0.59)	82.12	(1.97)	6.49	(0.91)	6.20	(1.84)
mono4	89.42	(0.66)	8.77	(2.16)	22.34	(3.14)	39.72	(4.79)

terpene concentration was used as a covariate, variation in individual terpenes was explained to a large extent by 'population' and 'region'. Concentration of 1,8-cineole was explained by 'region' (15.8%) and among-population variance (29.9%) with a negligible contribution from 'subspecies'. The variation in γ-terpinene was mainly explained by 'population' (44.5%). Most of the variation in other individual terpenes followed a pattern similar to that observed in 1,8-cineole (Table 3); however, the estimated deviation of the variance components was higher for these terpenes and the 'region' factor was not detected as significant.

Environmental variables

Variation in terpene production across the geographic scale was partly related to environmental variables. Total monoterpene concentration was positively associated with the temperature of the source site ($t_{(410)} = 3.46$, $P = 0.0006$), but it was not related to humidity or the soil-nutrient index ($t_{(410)} = -1.3$, $P = 0.2$ and $t_{(410)} = -1.45$, $P = 0.15$ respectively). The concentrations of sesquiterpenes were positively correlated with temperature and humidity ($t_{(410)} = 3.58$, $P = 0.0004$ and

$t_{(410)} = 4.71$, $P < 0.0001$ respectively), but not with the soil nutrient index ($t_{(410)} = -1.24$, $P = 0.22$). In general, individual terpenes were positively correlated with either temperature or humidity (Table 4).

Elevated CO_2 treatment

The treatment with elevated CO_2 had a small but significant effect on the concentration of terpenes (Fig. 3, Table S1, available as Supplementary Material to this paper). Plants exposed to elevated CO_2 had a lower concentration of total monoterpenes ($\chi^2_{(1)} = 4.7$, $P = 0.029$, Fig. 3*a*) than did control plants. Sesquiterpene concentrations were similar between control plants and those exposed to elevated CO_2 (Table S1). There was no significant effect of the interaction between CO_2 treatment and chemotype (Table S1). When the comparison was made among the same groups of plants, but with the data obtained before the elevated CO_2 treatment (Fig. 3*a*), neither monoterpenes ($\chi^2_{(1)} = 1.33$, $P = 0.25$) nor sesquiterpenes ($\chi^2_{(1)} = 0.23$, $P = 0.63$) showed significant differences among treatment groups. Thus, plants under ambient CO_2 conditions showed an increased concentration of monoterpenes at the second sampling point, but this increase was lower when plants were moved to elevated CO_2 (Fig. 3*a*) When taking total terpenes as a covariate, α-phellandrene and β-pinene were less abundant in plants under elevated CO_2 ($\chi^2_{(1)} = 6.3$, $P = 0.012$ and $\chi^2_{(1)} = 15.7$, $P < 0.0001$ respectively), whereas other measured terpenes showed no difference between the CO_2 treatments (Fig. 3*b*). The

Table 3. Variance partition between spatial and subspecies random factors for terpenes in *Eucalyptus camaldulensis*

The percentage of the total variance explained is also indicated. The variance components were obtained from the linear mixed models analysing the variance of the total concentration of monoterpenes and sesquiterpenes, and the abundance of the most dominant terpenes with total terpenes as a covariate. Values in parentheses indicate the standard deviation of each component. Significant estimates are shown in bold. *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$

Parameter	Population	Subspecies	Region	Residual variance	Population (%)	Subspecies (%)	Region (%)	Total variance
Monoterpenes	**5.81***	10.00	0.12	73.27	6.51	11.21	0.14	89.20
	(2.41)	(3.16)	(0.35)	(8.56)				
Sesquiterpenes	**0.13****	**0.27***	0.02	1.12	8.34	17.62	1.40	1.54
	(0.36)	(0.52)	(0.15)	(1.06)				
1,8-Cineole	**6.29*****	0.00	3.32	11.44	29.88	0.00	15.76	21.04
	(2.51)	(0.00)	(1.82)	(3.38)				
γ-Terpinene	**7.07*****	0.00	0.00	8.80	44.52	0.00	0.00	15.87
	(2.66)	(0.00)	(0.00)	(2.97)				
α-Phellandrene	**0.26****	0.00	0.82	2.20	7.93	0.00	24.94	3.27
	(0.51)	(0.00)	(0.90)	(1.48)				
β-Phellandrene	**0.32****	0.00	0.35	2.38	10.66	0.00	11.38	3.05
	(0.57)	(0.00)	(0.59)	(1.54)				
α-Pinene	**2.01*****	0.00	0.14	3.35	36.49	0.00	2.60	5.51
	(1.42)	(0.00)	(0.38)	(1.83)				
β-Pinene	**0.11****	0.00	0.20	1.40	6.42	0.00	11.87	1.71
	(0.33)	(0.00)	(0.45)	(1.18)				
p-Cymene	**0.17*****	0.28	0.00	0.44	18.52	31.80	0.00	0.89
	(0.41)	(0.53)	(0.00)	(0.67)				
Bicyclogermacrene	**0.02*****	0.00	0.02	0.18	11.20	0.00	7.23	0.22
	(0.16)	(0.00)	(0.13)	(0.42)				

Table 4. Estimates of the multiple linear regressions testing the effects of environmental variables on total concentration of monoterpenes and sesquiterpenes and abundances of the main terpenes, with total terpenes as a covariate

Standard errors of the estimates are shown in parentheses. Significant values are shown in bold. *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$

Parameter	Temperature		Humidity		Nutrient index		Total terpenes	
Monoterpenes	**0.46*****	**(0.13)**	–0.08	(0.06)	–1.66	(1.15)		
Sesquiterpenes	**0.06*****	**(0.02)**	**0.04*****	**(0.01)**	–0.18	(0.15)		
1,8-Cineole	**–0.28*****	**(0.07)**	**–0.07***	**(0.03)**	0.69	(0.56)	**0.5*****	**(0.02)**
γ-Terpinene	**0.28*****	**(0.06)**	**0.08****	**(0.03)**	**–1.23***	**(0.50)**	**0.07*****	**(0.02)**
α-Phellandrene	**0.06***	**(0.03)**	–0.01	(0.01)	0.32	(0.22)	–0.01	(0.01)
β-Phellandrene	**–0.12*****	**(0.02)**	0	(0.01)	0.22	(0.21)	**0.04*****	**(0.01)**
α-Pinene	**–0.08***	**(0.03)**	**–0.1*****	**(0.01)**	–0.41	(0.28)	**0.15*****	**(0.01)**
β-Pinene	0.04	(0.02)	**0.04*****	**(0.01)**	0.12	(0.16)	**0.03*****	**(0.01)**
p-Cymene	**0.05*****	**(0.01)**	0.01	(0.01)	0.06	(0.11)	0	(0.00)
Bicyclogermacrene	–0.01	(0.01)	0	(0.00)	0.02	(0.06)	0	(0.00)

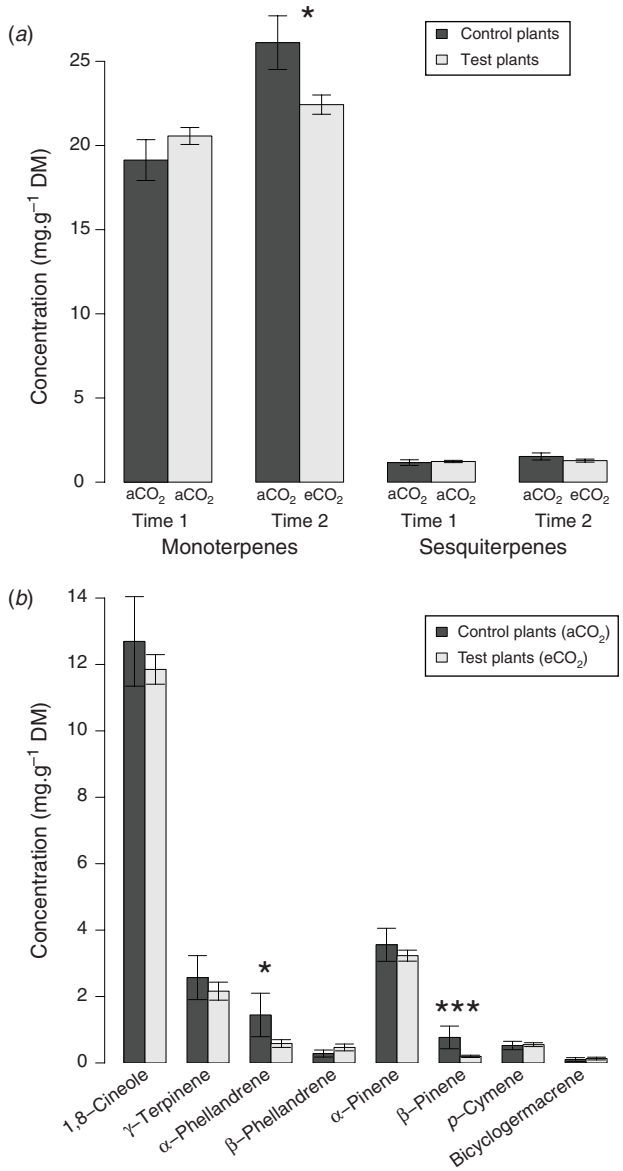

Fig. 3. Differences in mean terpene concentration between control and test plants under ambient (aCO_2) and elevated CO_2 (eCO_2) conditions. (*a*) Mean values for total monoterpene and sesquiterpene concentrations at Time points 1 and 2 for control and test plants. (*b*) Mean values for the most abundant terpenes at Time point 2. Thin vertical bars indicate ± s.e.m. Significance values are derived from the linear mixed models described in the Materials and methods section in text; $*P < 0.05$, and $***P < 0.0001$.

interaction between the CO_2 treatment and chemotype had a significant effect on α-phellandrene ($\chi^2_{(3)} = 103$, $P < 0.0001$), with plants of the α-phellandrene chemotype (C1) showing a larger difference between the CO_2 treatments. This interaction was significant also for β-phellandrene and *p*-cymene ($\chi^2_{(3)} = 10.6$, $P = 0.014$ and $\chi^2_{(3)} = 11.0$, $P < 0.012$ respectively); these compounds were more abundant in plants of Chemotype C2 (β-phellandrene) under elevated CO_2 conditions; however, these results must be taken with caution because only one plant of Chemotype C2 was under ambient CO_2. Other compounds were not affected by the interaction between the CO_2 treatment and chemotype (Table S1).

Discussion

We described the variation of the abundance of foliar terpenes of *E. camaldulensis* across a very wide continental distribution. These data captured both quantitative and qualitative variation and allowed us to identify chemical polymorphisms or chemotypes. We identified four main chemotypes on the basis of variation in monoterpenes. Variation in terpene concentration was explained mainly by the subspecies identity, whereas environmental variables were correlated with the abundance of individual terpenes. We also detected a reduction in monoterpene concentration when plants were subjected to elevated CO_2 conditions in a controlled environment.

Quantitative and qualitative variation in terpenes

Concentrations of total monoterpenes and sesquiterpenes were partially explained by the subspecies group (11%); therefore, the genetic differences among subspecies must correlate with differences of expression in genes that determine the quantitative variation in terpene production. These are likely to be the genes regulating the methylerythritol phosphate pathway (MEP) in the chloroplast or the mevalonate pathway (MVA) in the cytosol, as shown in *Eucalyptus globulus* and *Melaleuca alternifolia* (Külheim *et al*. 2011; Webb *et al*. 2013).

Also, the distribution of chemotypes across Australia is not homogeneous, because some of the chemotypes are restricted to a few areas; for example, the α-phellandrene chemotype (Chemotype C1) is present only in the North-west region and β-phellandrene chemotype (C2) is just found in the south regions (Fig. 1). The distribution of chemotypes must be spatially restricted due to a low genetic flow between geographic regions or, alternatively, because some chemotypes might be at a selective disadvantage in some populations (Thompson *et al*. 2013). Populations from the North-west region showed a higher chemical diversity on the basis of the chemotypes, so this could be related to the higher genetic diversity in those populations, probably reflecting the tropical origin of *E. camaldulensis* (Butcher *et al*. 2009). Because terpenes can function as plant defences (Stone and Bacon 1994; Gershenzon and Dudareva 2007; Oates *et al*. 2015), it is also possible that variation in the distribution of chemotypes is maintained by interactions with natural enemies such as herbivores or pathogens. Additional studies on the local adaptation of plant chemotypes to natural enemies would be needed to explore this possibility.

Consistent with the distribution of chemotypes, the relative abundance of the individual terpenes was more influenced by the population and geographic area than by subspecies; thus, variation in the proportion of the main terpenes was more important at the spatial scale. The variation in individual terpenes was also associated with environmental variables such as mean temperature and mean humidity. This suggests a link between the spatial structure of the plant populations and the environmental variables that influence the terpene composition. Chemotype B (dominated by γ-terpinene) was found in the North-west and West regions. Accordingly, we

expected that γ-terpinene would be more abundant in plants from locations with higher annual mean temperature but also with higher humidity, whereas 1,8-cineole-rich plants should be more common in locations with lower mean temperatures. Both of these expectations were supported by the data. The compound β-phellandrene was more abundant in populations at lower temperatures, which are located in the Central South and South-east regions. Whether this relationship between the environment and distribution of genetically variable chemotypes is a result of adaptation to abiotic conditions is unknown and, to find out, it would require specific experiments at different locations that evaluate the selective advantage of a set of chemotypes under different climatic conditions.

The dataset presented here highlights the variation that exists in foliar terpene abundance in *E. camaldulensis* at a large scale. The 1,8-cineole chemotypes are common in most populations, but those in the North-west and South have phellandrene chemotypes. These regions experience medium to high relative humidity; it is, therefore, possible that these chemotypes are at a disadvantage in very dry locations, such as in the West or Central regions. Also, most of the plants with the γ-terpinene chemotype are located in the west of the continent where they face high temperatures (West region) or high temperatures combined with high humidity (North-west region). Therefore, the environmental patterns along the latitudinal scale, but also at the longitudinal scale, help explain the distribution of chemotypes.

Dispersion patterns

The distribution of *E. camaldulensis* across the Australian continent is likely to be a result of its ability to disperse through rivers and drainage basins. Seeds can float more than 10 days before sinking (Pettit and Froend 2001), so they can travel many kilometres along a river and disperse to downstream locations. However, genetic similarity is higher among closer geographic locations independent of the river system and it has been proposed that gene flow is driven more by pollen transfer than by seed dispersal (Butcher *et al*. 2002, 2009). Therefore, gene flow among populations within the same region could maintain chemical traits that are relatively similar within a region. Despite the sampled populations experiencing very different environments, ranging from desert to rainforest ecosystems, the terpene diversity over the species distribution is not greater than that observed in other *Eucalyptus* species with much narrower distributions (e.g. Wallis *et al*. 2011; Andrew *et al*. 2013). This could be a result of a recently expanded range of *E. camaldulensis* across the river systems, or it could indicate low selection pressures of abiotic variables on terpene traits. Butcher *et al*. (2009) found evidence that historical processes have a significant influence on the genetic structure of *E. camaldulensis*, which suggests that selection has played only a small role in the distribution of genetic diversity.

Biosynthetic inference

The groups of monoterpenes that were identified in *E. camaldulensis* are consistent with their expected biosynthetic relationships and, thus, the chemotypes obtained through our analysis can help identify the enzymatic differences among chemotypes. Monoterpene synthases can produce different terpenes from the same precursor, geranyl diphosphate (GPP; Tholl 2006) and some terpene synthases have been reported to be multi-substrate (Pazouki and Niinemets 2016). In Fig. 4 we propose the steps involved in carbocation transition and stabilisation that can lead to the diversity of monoterpenes found in *E. camaldulensis*. Some of these steps have been confirmed by functional characterisation of terpene synthases (Degenhardt *et al*. 2009; Falara *et al*. 2011), whereas others can currently be inferred only through the systematic co-occurrence of metabolites (Keszei *et al*. 2008).

The groups observed in the cluster analysis of monoterpenes (Fig. 2) are consistent with their proposed carbocation origins (Keszei *et al*. 2010). In Fig. 4, the compounds on the lowest level (α-terpinyl acetate, *p*-cymene and piperitone) are derived from modifications of the original product of the terpene synthase modification. In some cases, such as with α-terpinyl acetate, formed by the addition of an acyl group to α-terpineol (Liaw and Liu 2010), only one possible terpene can contribute to the formation of a particular product. Therefore, the distribution of this compound will correlate strongly with the group of terpenes containing its precursor (α-terpineol). Others, such as *p*-cymene, piperitone and thymol, may arise by spontaneous oxidation and non-specific de-phosphorylation (or alternatively the action of a specific P450 reaction), and may be associated with multiple terpene synthases from any of the major groups (Poulose and Croteau 1978).

All cyclic terpenes in the *mono3* group come from the α-terpinyl carbocation, and the pinyl carbocation, which is formed from it via a hydride shift (Croteau *et al*. 1989). The two acyclic monoterpenes are both derived from the linalyl carbocation, which is the last intermediate before cyclisation of the terpene backbone, so common catalytic origins can be assumed (Degenhardt et al. 2009). This group includes 1,8-cineole and is characteristic of the genus *Eucalyptus* (Padovan *et al*. 2014). The terpenes of the Group *mono4* can all be derived from the γ-terpinyl carbocation, and may in fact all be products of a single terpene synthase (Keszei *et al*. 2008).

The terpenes of the Group *mono2* come from different origins. As with other acyclic monoterpenes being synthesised from GPP, linalool is a possible non-specific product of previous steps of the terpinyl carbocation (Alonso and Croteau 1991; van Schie *et al*. 2007; Schilmiller *et al*. 2009; Külheim *et al*. 2015). Although this grouping is not as biosynthetically intuitive as are *mono3* and *mono4*, the catalysis of the formation of both sabinene and β-phellandrene by the same terpene synthase has already shown to be possible in *Solanum lycopersicum* by van Schie *et al*. (2007).

Effect of CO_2 on terpenes

We detected a small but significant negative effect of elevated CO_2 on the accumulation of foliar terpenes, which was similar across the different chemotypes. The effect was particularly higher for the proportions of α-phellandrene and β-pinene. This result is opposite to the response seen in some other plant systems (Peñuelas and Llusia 1997; Staudt *et al*. 2001) and with the overall effect of elevated CO_2 in combination with elevated O_3

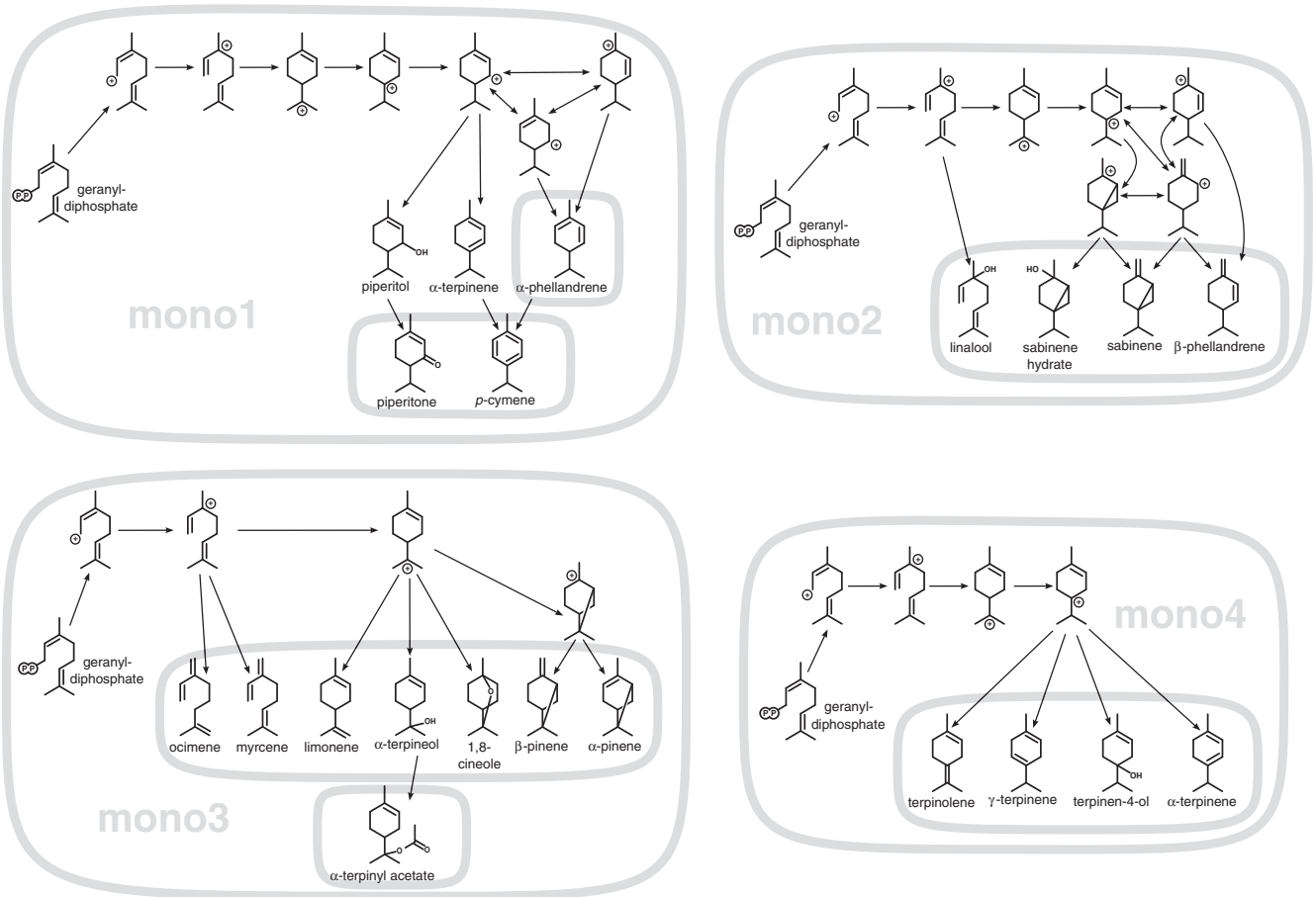

Fig. 4. Biosynthetic relationships among monoterpenes grouped in the hierarchical cluster analysis found across the populations of *Eucalyptus camaldulensis* (Fig. 2). The proposed carbocation transitions are linked to the final products of terpene synthase in the four groups of monoterpenes.

(Valkama *et al*. 2007). However, green tissue from woody plants tends to contain a lower abundance of terpenoid compounds under elevated CO_2 conditions (Zvereva and Kozlov 2006). Other *Eucalyptus* species that have been tested under elevated CO_2 conditions have not shown any significant changes in the accumulation of terpenes (Lawler *et al*. 1997; McKiernan *et al*. 2012). Therefore, the response of terpene accumulation to different concentrations of CO_2 would very likely depend on the genetic background of the species. The *Eucalyptus* genome contains more than 100 terpene genes involved in terpene expression, which is many more than for any other genome studied (Külheim *et al*. 2015); so, eucalypts could react to changes in CO_2 very differently from other genera. The increase in the concentration of monoterpenes from the first to the second sampling was smaller in plants under elevated CO_2 conditions, indicating that CO_2 can regulate the ontogenetic changes in terpene accumulation. In contrast, sesquiterpenes were not affected by the change in CO_2, suggesting that elevated CO_2 is mainly affecting the MEP or other plastid biosynthetic steps that are responsible for the synthesis of monoterpenes.

Little is known about the biotic interactions of the two monoterpenes that showed the strongest reduction under elevated CO_2, namely α-phellandrene and β-pinene. The monoterpene α-phellandrene has been shown to attract insects (Kant *et al*. 2009), which can affect herbivores in two ways, namely, either as insect herbivore attractant or as insect herbivore-predator attractant. Oates *et al.* (2015) showed that α-phellandrene is present only in *Eucalyptus grandis* genotypes that are resistant to a gall wasp *Leptocybe invasa*, whereas β-pinene was present in both resistant and susceptible *Eucalyptus* genotypes, but was induced in susceptible *E. grandis* genotypes after infestation with *L. invasa* (Oates *et al*. 2015). Without further study, it is difficult to predict whether the small quantitative changes of these two monoterpenes would affect plant–herbivore interactions.

Plants having lower concentrations of terpenes might be regarded as less well defended against plant herbivores, because they can offer protection (Stone and Bacon 1994); however, experiments testing the herbivore performance on other *Eucalytpus* species have shown that *Paropsis atomaria*, a common specialist on *Eucalyptus*, performs worse under elevated CO_2 (Gherlenda *et al*. 2015). Nevertheless, it is likely that a reduction in terpene production will affect terpene emissions of *E. camaldulensis* under higher temperatures and compromise the ability of this species to tolerate heat stress in the future (Sharkey and Monson 2014).

Conclusions

The current study has presented the patterns of terpene variation in populations of *E. camaldulensis*, and, although the variation is substantial, it is similar to the variation found in other *Eucalyptus* species with a much narrower distribution. It is likely that the observed structure is a result of the dispersion processes from the centre of origin of the species, as well as the suitability of chemotypes to contrasting environments. Experiments that assess the performance of the different monoterpene chemotypes found in *E. camaldulensis* to their local biotic and abiotic conditions are needed to establish whether the present distribution of chemotypes is a reflection of adaptive processes or whether it is solely a product of neutral variation and the dispersion patterns along the river systems. The reduced accumulation of terpenes in plants under elevated CO_2 conditions implies that physiological responses to global changes will have an impact on the health of natural populations and plantations of the most common eucalypt species around the world.

Acknowledgements

We thank David Kainer for developing the python pipeline for GC–MS data processing using PyMS. Thanks go to Amanda Padovan for commenting on an earlier version of the manuscript. CBS was supported by CONCYT and ANU scholarships. Experimental part was supported by funding from the CSIRO Transformational Biology Catalytic Platform. Analyses and the writing of the paper were funded by an ARC Discovery Project DP140101755 to WJF.

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Handling Editor: Linda Broadhurst