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Differential patterns of mono- and sesquiterpenes with leaf ontogeny influence pharmaceutical oil yield in *Eucalyptus polybractea* R.T. Baker

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1 **Title**

2 Differential patterns of mono- and sesquiterpenes with leaf ontogeny influence pharmaceutical oil yield in
3 *Eucalyptus polybractea* R.T. Baker

4

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15

1 Abstract

2 Pharmaceutical 1,8-cineole oil is commercially harvested from *Eucalyptus polybractea* R.T. Baker grown
3 in short rotation coppice cultivation. Harvests occur throughout the year, with no consideration given to
4 the proportion of leaves present at different ontogenetic stages and the effect leaf ontogeny may have on
5 harvested oil quality and quantity. To determine the optimal leaf stage to harvest pharmaceutical-grade
6 oil, we quantified the relationship between leaf ontogeny and foliar oil quantity and quality, and secretory
7 cavity size and abundance along branches of two *E. polybractea* clones. Our results show that the
8 desirable attributes of oil yield and 1,8-cineole proportion both reach their maximum in fully expanded
9 leaves and that this coincides with maximum secretory cavity size. Across all leaf ages the amount of
10 various monoterpenes were highly correlated with 1,8-cineole, suggesting a single, multi-product
11 monoterpene synthase may be responsible. In the very young leaves, sesquiterpenes such as β -elemene
12 were the most abundant constituents of the essential oil. We suggest there may be differential
13 transcriptional regulation of mono- and sesquiterpene synthases with leaf ontogeny such that
14 sesquiterpene biosynthesis is at a maximum only in young leaves, and that monoterpene biosynthesis
15 dominates during leaf and concomitant cavity expansion. Given *E. polybractea* leaves are produced in
16 major seasonal flushes, there are times throughout the year when the numbers of young leaves with
17 inferior oil quantity and quality are likely to be proportionally decreased. Therefore harvesting times
18 could be manipulated to take advantage of such times and ensure the greatest amount of high quality oil is
19 harvested.

20

21 Keywords

22 Cineole; development; elemene; essential oil; eucalyptol; gland; monoterpene

23

1 **Introduction**

2 *Eucalyptus polybractea* R.T. Baker (Myrtaceae) is the key species used in Australia to produce
3 commercial quantities of pharmaceutical-grade essential oil. Essential oils are a complex mixture of
4 mono- and sesquiterpenes and the largely monoterpenic *E. polybractea* oil is used as an additive in
5 antimicrobial mouthwash, cough suppressants, foods, cosmetics and insect repellents (Doran, 1991).
6 International standards established by various pharmacopœias specify that for eucalyptus oil to be
7 pharmaceutical-grade the monoterpene 1,8-cineole must comprise a minimum of 70% (v/v) of oil,
8 whereas other undesirable constituents must have negligible maximum proportions (Coppen, 2002;
9 Warren, 1991). Commercial production of *E. polybractea* oil is via steam distillation of leaves harvested
10 from stands grown in short-rotation coppice cultivation (Davis, 2002; Wildy and Pate, 2002), with the
11 yield of 1,8-cineole equaling the product of leaf biomass accumulation between harvest rotations, the
12 quantity of oil extracted from leaves, and the proportion of 1,8-cineole in the oil.

13 The frequency and timing of commercial *E. polybractea* harvests (i.e. rotations) is determined by
14 several factors including the rate of biomass accumulation in each rotation, with harvests generally
15 ranging from annual to biannual, and the need to maintain a more or less constant output of oil. Thus,
16 harvesting of coppice plants occurs throughout the year, with no consideration given to environmental or
17 biological factors that may affect oil quality and quantity. One of the most marked changes in coppice
18 plants over the year is variation in the proportions of different aged leaves. For example, in periods of
19 rapid growth, the proportion of young, expanding leaves on coppice plants is much higher than when
20 plant growth is slowed and foliage is largely mature. If leaf ontogeny imparts a strong influence on oil
21 quantity and/or qualitative composition, then it may be possible to time *E. polybractea* harvests when
22 coppice has the greatest proportion of leaves of an age that exhibit maximum 1,8-cineole yields and
23 minimum yields of undesirable constituents.

24 The majority of knowledge on how leaf ontogeny influences monoterpene quantity comes from
25 studies on numerous Lamiaceae species (e.g. Adzet et al., 1992; Brun et al., 1992; Dudai et al., 2001;
26 Hose et al., 1997; McConkey et al., 2000; Turner et al., 2000). In contrast to *Eucalyptus*, which house
27 oils in secretory cavities embedded within the leaf, Lamiaceae plants store oil in glandular secretory
28 trichomes (GSTs) which protrude from leaf surfaces. The epidermal location of GSTs means leaf
29 ontogeny generally regulates their number and size approximately in synchrony with leaf expansion for
30 plants of a given age and size (see Turner et al. 2000), producing greatest total oil yields in fully expanded
31 leaves. Given the larger size, increased cellular complexity and embedded rather than external location of
32 *Eucalyptus* secretory cavities, lessons learnt from GST development may not necessarily apply to these

1 structures. Nonetheless, it is expected that oil quantity in *E. polybractea* will also increase with
2 ontogenetic leaf expansion due to increases in cavity number, size, or both.

3 The manner by which leaf ontogeny may influence qualitative oil composition in *E. polybractea*
4 is more difficult to predict due to contrasting findings in the literature. In particular, the results of studies
5 on Lamiaceae plants suggest variation in oil quality with leaf ontogeny can be both species- and
6 constituent-dependent. For example, in a study on *Melissa officinalis* the proportion of the monoterpene
7 citronellal increased from a low of 1% in young leaves to 52% in older leaves, whereas citral proportion
8 decreased from 37% to <1% as leaves aged (Hose et al., 1997). Furthermore, a number of studies on
9 seasonality in Pinaceae species have shown the monoterpene composition of oleoresin in resin ducts can
10 vary markedly as needles develop in the growing season and mature thereafter (Forrest, 1980; Hrutfiord et
11 al., 1974; Maarse and Kepner, 1970; Schönwitz et al., 1990; Von Rudloff, 1975). As with the Lamiaceae
12 studies, the direction and magnitude of changes in Pinaceae monoterpenes was shown to vary with
13 species and constituent. Nonetheless, the overall amount of oxygenated terpenes generally increased at a
14 constant rate with age during early needle growth. In one of the few studies on leaf ontogeny and
15 essential oils in a Myrtaceous species, the concentration of 1,8-cineole in seedlings of *Melaleuca*
16 *alternifolia* was found to remain constant throughout leaf ontogeny from first leaves to the tenth leaf
17 (Russell and Southwell, 2002). It should be noted, however, that 1,8-cineole was only a minor
18 component of the seedling leaf oil (<5% in all leaves), whereas other monoterpene components such as
19 terpinolene and terpinen-4-ol showed large and directionally opposite changes as leaves aged, ranging
20 from 40 to 3% and 0 to 33%, respectively. Given the common occurrence of qualitative changes in
21 monoterpene constituents in plants as disparate as the Lamiaceae, Pinaceae and Myrtaceae, it appears
22 likely that leaf ontogeny will influence the proportion of monoterpenes in *E. polybractea*. Nonetheless,
23 the direction and magnitude of such changes cannot be predicted, and the effect such changes may have
24 on harvested oil quality is unknown.

25 In this study we quantified the relationship between *E. polybractea* leaf ontogeny and foliar oil
26 quantity and quality in terms of 1,8-cineole proportion, and that of other abundant mono- and
27 sesquiterpene constituents, to determine the stage at which leaves have the greatest total oil and highest
28 pharmaceutical quality. We also investigated how secretory cavity size and abundance change through
29 leaf ontogeny to determine which cavity parameter drives changes in oil quantity and quality. This will
30 provide information on the factors that underlie variation in eucalyptus oil as leaves develop, and may be
31 used to determine the optimal time to harvest pharmaceutical-grade oil from *E. polybractea* coppice
32 stands.

33

1 **Materials and methods**

2 *Plant material*

3 In excess of 20 ramets from each of 20 genetically different micropropagated clones of *Eucalyptus*
4 *polybractea* R.T. Baker (Goodger et al., 2008) were planted in a plantation near Glenalbyn, Victoria,
5 Australia (36.5335° S, 143.7346° E) at 2×2 m spacing in October 2008. An equal-aged and similar-sized
6 sapling ramet from each of two different clones was randomly selected for this study. The chosen ramets
7 are designated Clone 1 and Clone 2 hereafter. The ortet from which Clone 1 was micropropagated was a
8 seedling grown from seed collected from West Wyalong, NSW, Australia (33.9497° S, 146.9999° E;
9 purchased from CSIRO Australian Tree Seed Centre, Seedlot #19649, Tree # T319). The ortet from
10 which Clone 2 was micropropagated was a coppiced adult tree growing in Kooyoora State Park, Victoria,
11 Australia (36.5350° S, 143.7269° E). Thus the two clones are from distinct provenances that are
12 geographically well separated. The fact that the ortets of each clone were of different ages when
13 micropropagated has been shown to have no influence on essential oil traits in subsequent clones of the
14 species (Goodger and Woodrow, 2009). Two adjacent branches were harvested from the selected Clone 1
15 sapling and from the Clone 2 sapling in January 2011. Branches had developed from the main trunk over
16 the previous three months. All leaves were removed from each branch and scanned for leaf area using a
17 flatbed scanner. Leaves from branch 1 were cleared for microscopic secretory cavity determinations
18 whereas oil was extracted from leaves of branch 2. Ontogenetic examinations were therefore based on
19 independent leaves per branch, as non-destructively analysing individual leaves at different ontogenetic
20 stages was not possible.

21 *Microscopy*

22 Fresh leaves from branch 1 were cleared using a method modified from (Fuchs, 1963) as described in
23 Goodger and Woodrow, (2012), with mean secretory cavity size and number determined in a five mm²
24 region of lamina from the middle of each leaf lengthwise. Cavities were assumed to be spherical and
25 were measured as the external volume of each complex. It should be noted that cavity volumes estimated
26 by this technique will be greater than the amount of oil housed in each cavity for two reasons. First,
27 external cavity volumes include secretory cells bounding the oil-containing lumen. Second, non-volatile
28 glucose esters are known to be co-housed with oil in cavity lumina of *E. polybractea* (Goodger et al.,
29 2009) and can comprise over 50% of cavity lumen volume (Goodger et al., 2010).

30 *Oil extraction and analysis*

31 Fresh leaves from branch 2 were frozen in liquid nitrogen and ground to a fine powder in a mortar. Total
32 oil was extracted in hexane containing the internal standard tri-decane (Goodger et al., 2008). Total oil

1 and constituents in extracts were quantified by gas chromatography with flame ionization detection as
2 described in Goodger et al., (2007). It should be noted that the column used was unable to differentiate
3 between possible co-eluting isomers.

4 *Statistical analyses*

5 Linear and non-linear regressions and parallel line analyses were performed using Sigmaplot 12 (Systat
6 Software, San Jose, USA).

7

8

9 **Results**

10 *Leaf expansion*

11 The two branches removed from each Clone for respective microscopic (branch 1) and oil (branch 2)
12 analyses had a wide range of leaf sizes from newly emerged leaf tips through to large leaves at or
13 approaching full area expansion. The leaf area distribution of Clone 1 branches ranged from 7-783 mm²
14 for branch 1 (16 leaves) and 7-686 mm² for branch 2 (17 leaves; Fig. 1A), and Clone 2 had similar ranges
15 of 13-601 mm² for branch 1 (16 leaves) and 6-795 mm² for branch 2 (19 leaves; Fig. 1B). Leaf size
16 generally increased with leaf ontogeny on all branches, with the largest leaves found in the oldest cohort
17 of basal leaves, although the largest leaf was not necessarily the oldest. Significant linear relationships
18 were observed between leaf area and leaf dry mass for branch 2 from each clone. Regression analyses
19 performed on the data gave equations of $g_{dw} \text{ leaf}^1 = -0.005 + 1.2 \times 10^{-4} \text{ mm}^2 \text{ leaf}^1$ for Clone 1 ($F = 607$, P
20 <0.0001 ; $r^2 = 0.98$) and $g_{dw} \text{ leaf}^1 = -0.003 + 9.3 \times 10^{-5} \text{ mm}^2 \text{ leaf}^1$ for Clone 2 ($F = 460$, $P <0.0001$; $r^2 =$
21 0.97). The regression slopes were significantly different between the clones (Parallel line analysis $F = 13$,
22 $P <0.01$).

23 **FIGURE 1**

24 *Oil analyses*

25 The amount of total oil extracted per leaf increased slowly with increasing leaf age in the youngest cohort
26 of leaves on branch 2 (*ca.* leaves 1-7) from each clone before increasing rapidly up until the fourteenth
27 leaf, after which oil per leaf was relatively constant in the basal leaves (Fig. 2A). On a concentration
28 basis (mg g_{dw}⁻¹), oil increases with leaf ontogeny occurred at a more constant rate in young and
29 intermediate leaves, but appeared to reach a maximum concentration in leaves older than approximately
30 the twelfth oldest leaf. Oil concentration in leaves ranged from 15–94 mg g_{dw}⁻¹ for Clone 1 and from 22–
31 133 mg g_{dw}⁻¹ for Clone 2.

1 FIGURE 2

2 The extracted oil from the leaves of each branch had proportions of 1,8-cineole that increased
 3 rapidly with leaf ontogeny in the youngest leaves before the rate of increase was reduced in older leaves.
 4 The proportion of 1,8-cineole ranged from a low of 31% in the youngest leaf to a high of 79% in the
 5 oldest leaves of Clone 1 and from a low of 26% in the youngest leaves to a high of 76% in the oldest
 6 leaves of Clone 2 (Fig. 3A). On a concentration basis, 1,8-cineole appeared to increase in a more
 7 consistent manner with leaf ontogeny to reach maxima of 74 and 101 mg g_{dw}⁻¹ in branches from Clones 1
 8 and 2, respectively (Fig. 3B). Interestingly, the proportion of the sesquiterpene β-elemene - the most
 9 abundant essential oil constituent in the youngest leaves from branches of both clones - decreased with
 10 increasing leaf age from a high of 49% in young leaves to a low of 1% in old leaves of Clone 1, and from
 11 a high of 46% to a low of 2% in Clone 2 (Fig. 3C), corresponding to respective concentration minima of
 12 1.1 and 2.3 mg g_{dw}⁻¹ in the oldest leaves of both clones (Fig. 3D).

13 FIGURE 3

14 Given such dramatic and directionally opposite changes in 1,8-cineole and β-elemene proportion
 15 and concentration with leaf ontogeny, we next examined the relationship between 1,8-cineole and other
 16 key mono- and sesquiterpene constituents in the harvested leaves from each clone, irrespective of age.
 17 Because leaf age and leaf mass were positively associated, we related the concentrations of each
 18 constituent per volume of hexane extract (μg ml⁻¹) to each other to avoid the potentially confounding
 19 influence of tissue mass variation. The concentration of the monoterpenes sabinene, limonene and
 20 myrcene increased in a positive, and tightly linear fashion with 1,8-cineole concentration (Fig. 4).
 21 Regression analyses performed on the data were all highly significant, although intercept coefficients
 22 were non-significant (Table 1). Different slopes were observed for each monoterpene constituent relative
 23 to 1,8-cineole (Table 1), and parallel line analyses detected significant differences in the slopes between
 24 clones for each monoterpene relative to 1,8-cineole (sabinene $F = 54$, $P < 0.0001$; limonene $F = 4$, $P =$
 25 0.05 ; myrcene $F = 19$, $P < 0.001$). In contrast, the concentration of the sesquiterpenes β-elemene (Fig.
 26 4D), δ-cadinene (Fig. 4E) and β-caryophyllene (Fig. 4F) increased initially with increasing 1,8-cineole
 27 concentration, but then appeared to reach an approximate maximum. Despite variability in the data
 28 around these maxima, non-linear (exponential rise to maximum) regressions fitted to the data were all
 29 highly significant (Table 1), suggesting the biosynthesis of each of these sesquiterpenes ceases whilst 1,8-
 30 cineole biosynthesis continues as leaves age.

31 FIGURE 4 TABLE 1

32 *Secretory cavity determinations*

1 The majority of foliar mono- and sesquiterpenes in *E. polybractea* are housed within secretory cavities,
 2 although a small proportion can partition into other lipophilic structures such as the leaf cuticle and
 3 epicuticular waxes (King et al., 2006; Schmid et al., 1992). Therefore increases in oil concentration with
 4 leaf ontogeny are predicted to be largely facilitated by increases in secretory cavity size or number as
 5 leaves expand, or a combination of both parameters. In the cleared leaves of branch 1 from each clone we
 6 observed average cavity volume to increase with increasing leaf age, ranging from 0.01-1.6 nl per cavity
 7 from the youngest to oldest leaves in Clone 1 and from 0.02-2.0 nl per cavity in Clone 2 (Fig. 5A).

8 FIGURE 5

9 We also found that the smallest and youngest expanding leaves of each clone had relatively high
 10 secretory cavity densities, which initially increased with increasing leaf age in the first few leaves to reach
 11 density maxima of 152 cavities mm⁻² in leaf 3 of Clone 1 and 108 cavities mm⁻² in leaf 5 of Clone 2 (Fig.
 12 5B). Cavity density then decreased with increasing leaf age and size to reach minima of 6.3 and 9.6
 13 cavities mm⁻² in the oldest leaves of Clones 1 and 2, respectively. An exponential decay regression
 14 performed on the combined data sets from the clones was significant giving an equation of *cavity density*
 15 = $9 + 117 \times e^{(-3 \times \text{cavity volume})}$ ($r^2 = 0.93$, $F = 194$, $P < 0.0001$). Images of representative young, intermediate
 16 and fully expanded leaves from Clone 2 are presented in Fig. 6 and illustrate well the observed increase in
 17 cavity size and concomitant decrease in cavity density with leaf ontogeny. Interestingly, cells bounding
 18 the larger cavities in older leaves were darkly stained with toluidine blue, whereas smaller cavities in
 19 those leaves were only lightly stained and no staining was detected in any cavities of younger leaves (Fig.
 20 6).

21 FIGURE 6

22 In the cohort of young leaves on each branch (*ca.* leaves 1-8), total cavity number per leaf
 23 increased with increasing leaf age (Fig. 5C). These leaves had not yet reached half of the leaf area of the
 24 basal cohorts of leaves (Fig. 1) and cavity number would be expected to increase further if these leaves
 25 had been left to develop to their full size. Ostensibly in contrast to this, cavity number per leaf was lower
 26 in the oldest cohort of leaves (*ca.* leaves 11-16) compared to the middle cohort (*ca.* leaves 6-10; Fig. 5C).
 27 The lower number of cavities in the oldest leaves may be a result of these leaves attaining a smaller final
 28 leaf size. We have commonly observed that each subsequent leaf after the basal leaf attains a greater final
 29 size than the previous leaf, at least for the first 6-8 leaves on *E. polybractea* branches (unpublished
 30 observations). The constant cavity density observed in the five oldest leaves (Fig. 5B) supports this
 31 possibility. Alternatively, given that each leaf developed in sequence from the basal leaf, rather than
 32 concurrently, the observed differences in cavity number between the oldest and middle leaf cohorts may
 33 result from the different environmental conditions each leaf was exposed to as it formed and expanded.

1 Total cavity volume per leaf, the product of average cavity volume and cavity number,
2 consistently increased with leaf ontogeny in the youngest seven leaves per branch, but then remained
3 relatively constant in the older cohort of leaves from both clones (Fig. 5D). This relative consistency in
4 total cavity volume of mature leaves is a consequence of the negative relationship observed between
5 average cavity volume and cavity density across all leaves from both clones (Fig. 6D). Moreover, the
6 increase in total cavity volume with leaf ontogeny up to an apparent maximum is consistent with the oil
7 data from branch 2 (Fig. 2) and suggests that total cavity volume is a good estimator of leaf oil yield.

8 9 10 11 **Discussion**

12 Our results suggest that significant changes in essential oil quality and quantity occur in synchrony with
13 ontogenetic expansion of *E. polybractea* leaves, at least for the two clonal genotypes studied here. The
14 rise in oil per leaf initially appears to be a consequence of increases in both cavity number and size, but
15 once leaves expand beyond approximately 200 mm², oil increases appear to be largely a consequence of
16 secretory cavity size expansion, which appears to reach a maximum in the oldest leaves. The observation
17 that mean secretory cavity size differed between the two clones, particularly in fully expanded leaves,
18 supports recent findings that maximum cavity size is under genetic control and is largely responsible for
19 oil yield differences between *E. polybractea* genotypes with similar-sized leaves (Goodger and Woodrow,
20 2012). The proportion of the pharmaceutical monoterpene 1,8-cineole was also observed to increase with
21 leaf ontogeny, reaching a maximum in the oldest leaves. In the very young leaves, however,
22 sesquiterpenes such as β -elemene were the most abundant constituents of foliar essential oil.

23 The examination of oil quality with leaf ontogeny gives several insights into the mechanism of
24 essential oil production with cavity expansion. It should be noted that total secretory cavity volume per
25 leaf has been shown to be an accurate predictor of total oil per leaf in *E. polybractea* (Goodger and
26 Woodrow, 2012) as only a relatively small proportion of mono- and sesquiterpenes partition into other
27 leaf structures (King et al., 2006). Thus it appears that sesquiterpene synthesis occurs at the earliest
28 stages of leaf and secretory cavity formation, and it is then either greatly diminished or switched off
29 altogether. Monoterpene biosynthesis also begins in early leaf ontogeny and follows as the dominant
30 process driving up essential oil quantity in association with secretory cavity expansion. Monoterpene
31 biosynthesis proceeds with a remarkably tight relationship between 1,8-cineole, sabinene, limonene and
32 myrcene production, albeit with slight, but significant differences in the slopes of these relationships

1 between clones. In fact, the consistency in relationships between monoterpenes irrespective of leaf age
2 within each clone is strong evidence that production of the different monoterpenes is mechanistically
3 linked.

4 There are two possible explanations for such mechanistic links. First, each monoterpene may be
5 produced by a single, specific enzyme, and related compounds may group due to genetic linkage
6 (Wilderman et al., 2004). A simpler and arguably more likely explanation for tight relationships between
7 constituents (see Keszei et al., 2010) is that a single monoterpene synthase, i.e. a 1,8-cineole synthase
8 (CinS), is responsible for biosynthesis of most monoterpenes detected during cavity expansion. In
9 support of this, studies of recombinant CinS from species including *Arabidopsis thaliana* (Chen et al.,
10 2004), *Salvia pomifera* (Kampranis et al., 2007) and *Nicotiana suaveolens* (Roeder et al., 2007) have
11 shown that CinS is capable of producing up to 10 monoterpene products from its single substrate, geranyl
12 diphosphate (GPP). Importantly, production of sabinene, limonene and myrcene is a consistent feature of
13 CinS forms in these studies and the relative amounts of these and other products depend on only relatively
14 small changes in enzyme structure (Kampranis et al., 2007). Such slight changes in enzyme structure can
15 arise from single nucleotide polymorphisms (SNPs), a relatively high proportion of which have recently
16 been found in eight genes from the terpene biosynthetic pathway of four *Eucalyptus* species (Kulheim et
17 al., 2009). From an ecological stand point, the production of a mixture of monoterpenes may advantage
18 *E. polybractea* because *Eucalyptus* terpenes are known to have numerous anti-herbivore properties
19 (Batish et al., 2008), and complex mixtures of defence metabolites may confound the capacity for
20 herbivores to evolve resistance to all of the constituents (Langenheim, 1994).

21 Another notable finding was that key (pharmaceutical) undesirables α - and β -phellandrene were
22 not detected at any ontogenetic leaf stage in either clone. A similar lack of the phellandrenes in *E.*
23 *polybractea* foliage was noted by Goodger *et al.* (2007) for mature leaf extracts from 40 saplings and
24 coppice, and by Brophy et al., (1991) for bulk extracts of leaves from over 2000 coppice plants. In
25 addition, the aforementioned CinS forms from *Arabidopsis*, *Salvia* and *Nicotiana* were also incapable of
26 phellandrene production, but a recently characterized form from *Lavandula* catalyzed production of 6.6%
27 α -phellandrene together with 80% 1,8-cineole from GPP (Demissie et al., 2012). Interestingly, α -
28 phellandrene (Wildy et al., 2000) and β -phellandrene (King et al., 2006) have also been reported at low
29 levels in some *E. polybractea* individuals, adding further support for the existence of multiple forms of
30 monoterpene synthases within the species.

31 The process of sesquiterpene formation in *E. polybractea* has received little attention, presumably
32 because studies have tended to focus on mature, fully expanded leaves in which levels of sesquiterpenes
33 are very low. Our results show that there is a period in young leaves when the sesquiterpene β -elemene –

1 and to a lesser degree δ -cadinene and β -caryophyllene – is the major essential oil constituent of leaves,
2 and that reduction or cessation of sesquiterpene biosynthesis, together with increased and protracted
3 monoterpene synthesis, reduces the concentration of these compounds to < 0.2% in older, fully expanded
4 leaves. Similarly low levels of these and other sesquiterpenes were reported in fully expanded, mature
5 leaves from *E. polybractea* saplings (King et al., 2006) and in a bulk sample from over 2000 *E.*
6 *polybractea* coppice trees (Brophy et al., 1991). Interestingly, in the latter study, δ -cadinene and β -
7 caryophyllene each comprised some 0.1% of the total oil but no β -elemene was detected. King et al.
8 (2006) also found that β -elemene and δ -cadinene were lacking in some, but not all trees examined.

9 One interpretation of these findings is that like CinS, different forms of a sesquiterpene synthase
10 can produce qualitatively different combinations of products, possibly due to SNPs in sesquiterpene
11 synthase genes. There is some evidence to support the existence of multi-product sesquiterpene
12 synthases. For example, the recombinant protein expressed by cDNA of the *Arabidopsis* flower
13 At5g44630 gene catalysed the production of 15 different sesquiterpenes from farnesyl diphosphate (FPP;
14 Tholl et al., 2005). Moreover, a recombinant β -caryophyllene synthase from *Oryza sativa* has been
15 shown to be capable of producing several additional sesquiterpenes, including β -elemene and δ -cadinene,
16 from FPP (Cheng et al., 2007). In the rice leaf, the β -caryophyllene synthase was transcriptionally
17 regulated, exhibiting both circadian rhythm fluctuations and induction in response to methyl jasmonate
18 application (Cheng et al., 2007). It is likely that transcriptional regulation also occurs in *E. polybractea*
19 with one or more sesquiterpene synthases being highly active at the very early stages of leaf development
20 and then being substantially reduced or switched off resulting in a relatively constant total amount of
21 sesquiterpenes in fully expanded leaves. In contrast, the expression of CinS appears to be prolonged from
22 early leaf ontogeny onwards with its activity ceasing or being reduced to low levels (possibly to account
23 for monoterpene volatilization) only after full secretory cavity expansion is attained in mature leaves.

24 Differential expression of synthases for mono- and sesquiterpenes with leaf ontogeny has not
25 been directly assessed in any plants to date. Nonetheless, a number of studies have shown differential
26 patterns in concentrations of these terpene classes with leaf ontogeny. For example, in a study on the
27 shrub *Micromeria fruticosa*, increasing leaf age was shown to have little influence on the proportions of
28 various sesquiterpenes in the oil or their total amount, but it had a large influence on monoterpenes such
29 as isomenthol which increased from 0% in young leaves to more than 60% in older leaves (Dudai et al.,
30 2001). Moreover, in a manner reminiscent of the data presented here, a study on *Melissa officinalis* found
31 total sesquiterpenes constituted 39% of oil in apical leaves, but diminished to only 10% of oil in the older,
32 basal leaves (Hose et al., 1997). Furthermore, in *Salvia officinalis*, the sesquiterpenes viridiflorol, α -
33 humulene, and β -caryophyllene accounted for up to 20% of the essential oil in leaf primordia, but

1 constituted less than 8% in mature leaves (Dudai et al., 1999). It is known that the two terpene classes
2 have different sites of synthesis within cells with monoterpenes produced in plastids and sesquiterpenes in
3 endoplasmic reticulum and cytoplasm (Gershenzon, 1994), but it also seems they are capable of
4 differential regulation with leaf ontogeny.

5 Although not related to leaf ontogeny, a study on wound inducibility of oleoresin in *Abies grandis*
6 also found differential transcriptional regulation of mono- and sesquiterpene synthesis (Steele et al.,
7 1998). In that study, RNA-blot analyses indicated that synthase genes were transcriptionally activated in
8 response to wounding, with monoterpene synthases up-regulated within 2 h after wounding, followed by
9 the appearance of sesquiterpene synthases beginning after 3 days. The authors speculated that the early
10 and prolonged synthesis of monoterpenes may relate to their role in defence from insects, whereas the
11 sesquiterpene production could be delayed to coincide with the construction of egg galleries by infesting
12 beetles or with spore germination of pathogenic fungi vectored by infesting beetles (Steele et al., 1998).
13 The reason why sesquiterpenes and monoterpenes appear to be synthesized at different times during *E.*
14 *polybractea* leaf ontogeny is not known, but may also relate to different roles in leaf defence. It is
15 relevant to mention the results of a study on *Artemisia annua* in which expression of β -caryophyllene
16 synthase was induced in response to fungal elicitation, whereas a synthase for the monoterpene linalool
17 was induced in response to wounding (Cai et al., 2002). Perhaps in a similar manner, the early expression
18 of sesquiterpenes in young *E. polybractea* leaves may relate to their vulnerability to fungal attack at a
19 stage prior to the full formation of protective epicuticular wax layers (Wirthensohn and Sedgley, 1996),
20 whereas the prolonged synthesis of monoterpenes may relate to a role in defence from herbivores such as
21 insects throughout leaf ontogeny.

22 The significant negative relationship observed between cavity size and number (Fig. 6D) suggests
23 that any increase in cavity number as leaves develop may be offset by a decrease in mean cavity volume
24 such that the total amount of space dedicated to secretory cavities remains relatively constant. This
25 notion is supported by the data from the abnormally sickle-shaped leaf found on Clone 2 (see * in Fig. 5).
26 This leaf had a proportionally greater number of cavities initiated for its leaf size (Fig. 1B), but its average
27 cavity volume was greatly reduced resulting in the maintenance of a relatively constant total cavity
28 volume for the leaf (Fig. 5B). In addition, we have recently reported that as *E. polybractea* leaves
29 expand, there may be a lower limit of some 2 μ l of non-secretory cavity leaf volume (e.g. photosynthetic
30 mesophyll) that is required to sustain each μ l of secretory cavity volume (Goodger and Woodrow, 2012).
31 The significant exponential decrease observed here between cavity number initiated and the extent of
32 cavity expansion appears consistent with this finding - reflecting a leaf's requirements to maintain
33 photosynthetic capacity to both service the secretory cavities and maintain carbon export.

1 The variation in oil yield and quality with leaf ontogeny that we have recorded for *E. polybractea*
2 branches has implications for commercial harvesting practices in which mechanical harvesters remove all
3 above ground biomass. Our results show that the desirable attributes of oil yield and 1,8-cineole
4 proportion both reach their maximum in leaves of the same ontogenetic stage - full expansion. Although
5 only the basal leaves appeared fully expanded on the branches examined here, the quantity of oil and 1,8-
6 cineole proportion is consistent with values reported for fully expanded leaves found at all positions along
7 branches (Goodger and Woodrow, 2009, 2012; King et al., 2006), suggesting the unexpanded apical
8 leaves will develop in a similar manner to the basal leaves, albeit possibly to different final leaf sizes.
9 The short cycle time of annual to biannual harvest rotation (depending on growth rates) means that, on the
10 positive side, potential loss of oil via volatilization in old and senescent leaves (List et al., 1995) is likely
11 to be reduced, but on the negative side, it may be challenging to avoid the harvesting of immature and
12 expanding leaves. It can be argued that young and expanding leaves comprise only a small proportion of
13 the total leaf mass of harvested leaves and that their lower yields of poorer quality oil are unlikely to
14 affect the overall harvested oil quality greatly. Nonetheless, *Eucalyptus* leaves are generally produced in
15 major seasonal flushes (Doran and Bell, 1994), thus there are times throughout the year when the numbers
16 of such young leaves are likely to be proportionally increased. Manipulation of harvesting to avoid such
17 times could ensure the greatest amount of high quality oil is harvested.

18

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22

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25 The authors declare that they have no conflict of interest.

26

27

1 **Figure and Table Captions**

2 **Fig. 1.** Area of leaves along developing branches of saplings of two *Eucalyptus polybractea* clones. Two
3 branches were harvested from Clone 1 (panel A) and Clone 2 (panel B) and leaves of one branch per tree
4 cleared for microscopic examination (open symbols) and those of the other extracted for oil analyses
5 (cross hair symbols).

6 **Fig. 2.** Oil quantity extracted from leaves along developing branches of saplings of two *Eucalyptus*
7 *polybractea* clones. Total oil is presented on both mass per leaf (A) and concentration per unit dry mass
8 (B) bases. Cross hair circle symbols represent Clone 1 and grey cross hair triangles Clone 2.

9 **Fig. 3.** Quality of oil extracted from leaves along developing branches of saplings of two *Eucalyptus*
10 *polybractea* clones. Proportion (A) and concentration (B) of the pharmaceutical monoterpene 1,8-cineole
11 in total oil - the most abundant constituent of the oldest leaves on both clones. Proportion (C) and
12 concentration (D) of the sesquiterpene β -elemene - the most abundant constituent of the youngest leaves
13 on both clones. Cross hair circle symbols represent Clone 1 and grey cross hair triangles Clone 2.

14 **Fig. 4.** Differential patterns of mono- and sesquiterpenes in leaves along developing branches of saplings
15 of two *Eucalyptus polybractea* clones. Concentration of 1,8-cineole in each leaf extract plotted against
16 the monoterpenes sabinene (A), limonene (B) and myrcene (C), and the sesquiterpenes β -elemene (D), δ -
17 cadinene (E) and β -caryophyllene (F). Cross hair circle symbols represent Clone 1 and grey cross hair
18 triangles Clone 2. Linear regressions performed on the monoterpene data were significant with dashed
19 and solid lines representing Clones 1 and 2, respectively.

20 **Fig. 5.** Microscopic examination of secretory cavities in leaves along developing branches of saplings of
21 two *Eucalyptus polybractea* clones. Cavity parameters quantified were: (A) average cavity volume (\pm 1
22 SE); (B) cavity density per unit leaf area; (C) total cavity number per leaf; and (D) total cavity volume per
23 leaf. Open circles represent Clone 1 and grey triangles Clone 2. * denotes an unusually sickle-shaped
24 leaf on Clone 2 that had numerous small cavities compared with similar sized and aged leaves on the
25 same branch.

26 **Fig. 6.** Relationship between secretory cavity size and density in leaves along developing branches of
27 saplings of two *Eucalyptus polybractea* clones. (A) Representative young leaf (Leaf 2, Clone 2), (B)
28 representative intermediate leaf (Leaf 8, Clone 2) and (C) representative mature leaf (Leaf 16, Clone 2)
29 cleared for microscopic secretory cavity measurements showing the marked increase in cavity size and
30 concomitant decrease in cavity density as leaves expand. Scale bars = 200 μ m. (D) Plot of mean cavity
31 size against cavity density for all leaves on branch 1 from Clone 1 (open circle symbols) and Clone 2

1 (grey triangles). An exponential decay regression performed on the combined data from both clones was
 2 significant ($P < 0.0001$)

3 **Table 1.** Regression statistics for data presented in Figure 4.

4

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31 **Table 1.** Regression statistics for data presented in Figure 4.

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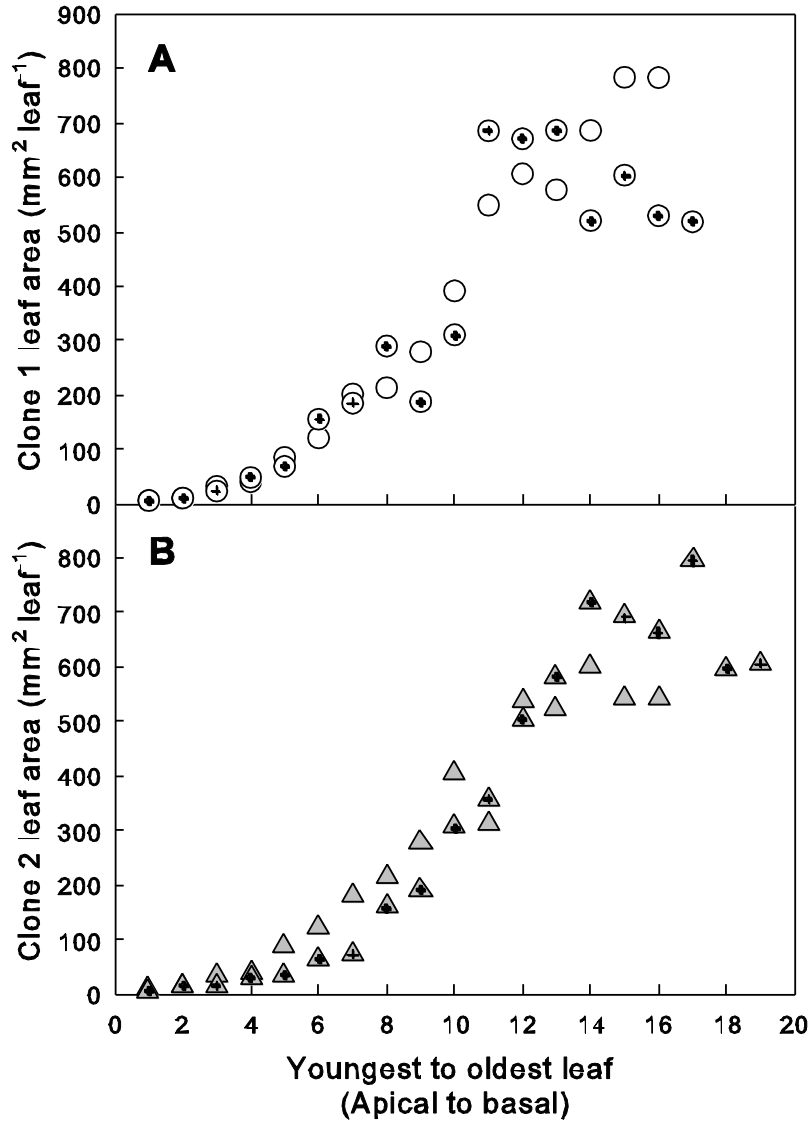
Linear regressions $y = y_0 + ax$			Regression coefficients and significance						ANOVA		
Independent variable	Dependent variable	Tree	y_0	t	P	a	t	P	F	P	r^2
1,8-cineole	Sabinene	1	-0.8	-0.6	ns	0.059	56	<0.0001	3095	<0.0001	0.99
		2	-1.1	-1.0	ns	0.066	114	<0.0001	13041	<0.0001	0.99
	Limonene	1	0.2	0.3	ns	0.041	90	<0.0001	8140	<0.0001	0.99
		2	0.2	0.2	ns	0.042	111	<0.0001	12264	<0.0001	0.99
	Myrcene	1	-0.3	-0.5	ns	0.019	49	<0.0001	2353	<0.0001	0.99
		2	0.1	0.2	ns	0.021	61	<0.0001	3773	<0.0001	0.99
Non-linear regressions $y = a \times (1 - e^{-bx})$			Regression coefficients and significance						ANOVA		
Independent variable	Dependent variable	Tree	a	t	P	b	t	P	F	P	r^2
1,8-cineole	β -elemene	1	47	11	<0.0001	0.007	2.5	<0.05	35	<0.0001	0.72
		2	102	18	<0.0001	0.004	3.4	<0.01	121	<0.0001	0.88
	δ -cadinene	1	56	7	<0.0001	0.001	2.1	ns	61	<0.0001	0.82
		2	44	32	<0.0001	0.001	7.7	<0.0001	678	<0.0001	0.98
	β -caryophyllene	1	23	16	<0.0001	0.003	3.6	<0.01	97	<0.0001	0.87
		2	9	18	<0.0001	0.002	4.4	<0.001	179	<0.0001	0.92

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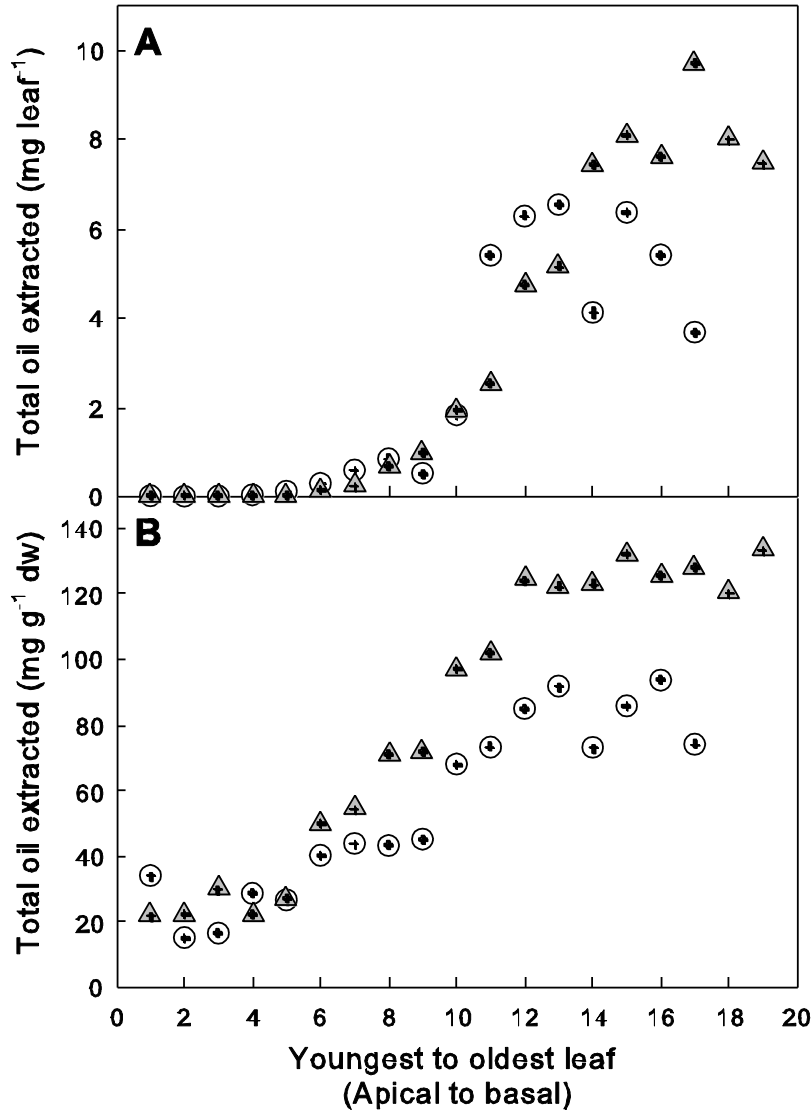
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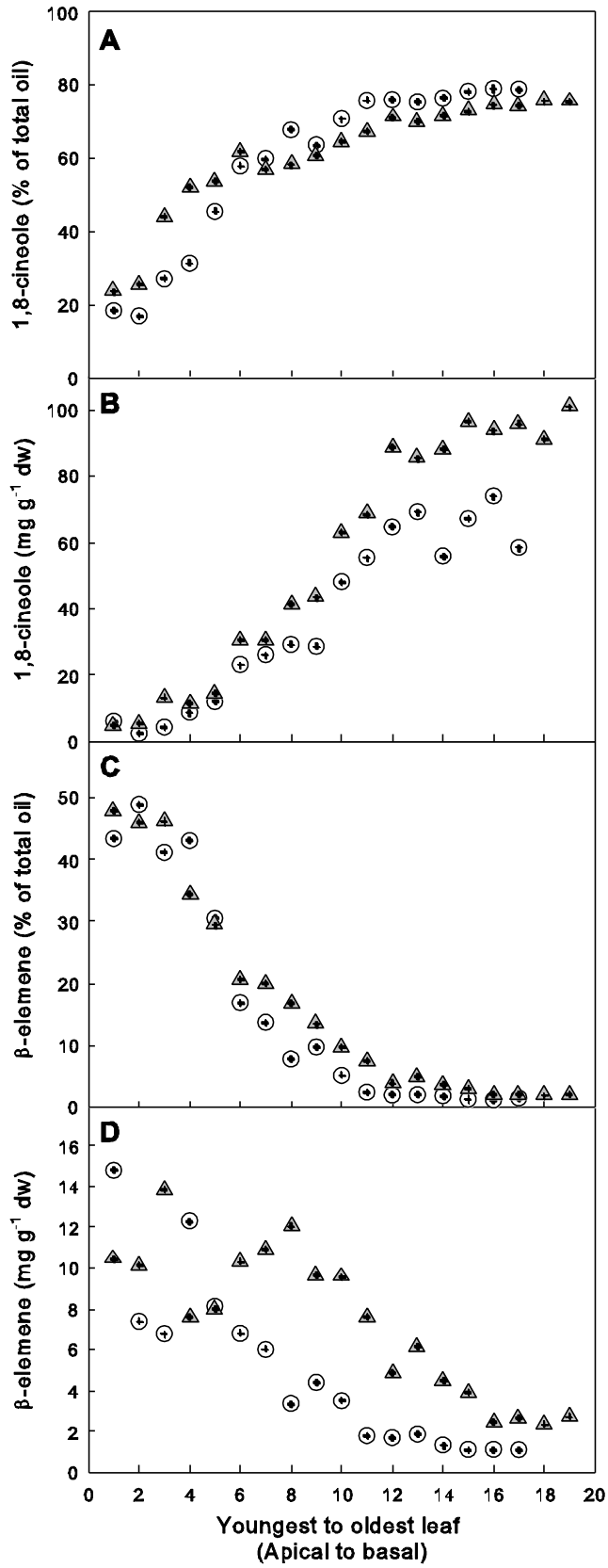
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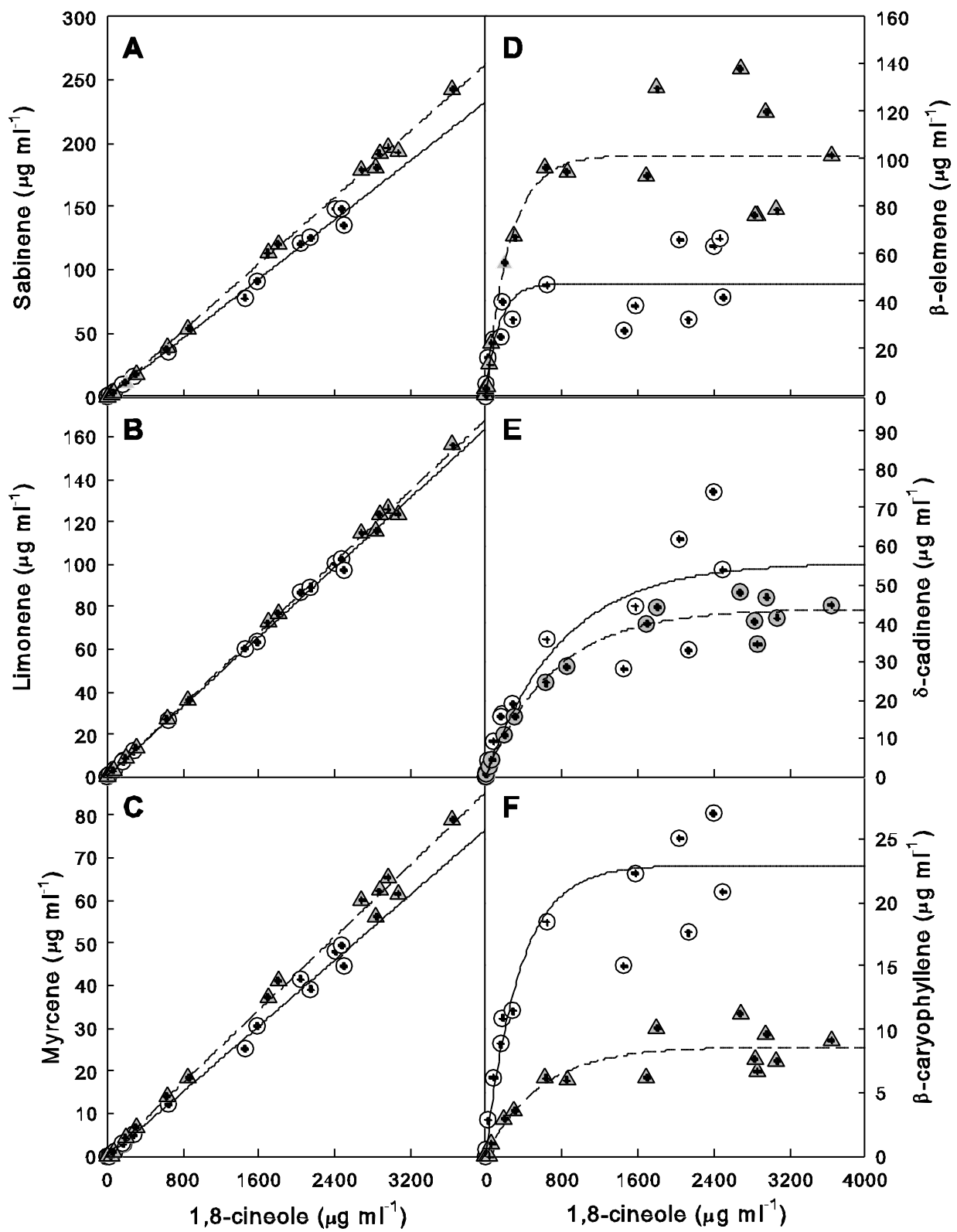


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