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RESEARCH ARTICLE

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Seasonal variation of yield and composition in extracts from immature and mature *Eucalyptus bosistoana* leaves

Chamira Rajapaksha[1] | Luis Alejandro Apiolaza[1] | Marie A. Squire[2] | Clemens Altaner[1]

[1]School of Forestry, Te Kura Ngahere, University of Canterbury, Christchurch, New Zealand

[2]School of Physical and Chemical Sciences, Te Kura Matū, University of Canterbury, Christchurch, New Zealand

Correspondence
Clemens Altaner, School of Forestry, Te Kura Ngahere, University of Canterbury, Christchurch, New Zealand.
Email: clemens.altaner@canterbury.ac.nz

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Abstract

Seasonal variations of yield and composition in microwave extracts from mature and immature *Eucalyptus bosistoana* leaves were monitored for 2 years. The highest yield (average 16.7 μL/g (*fresh*)) and percentage of 1,8-cineole (average 67.1%) were obtained from the leaves collected during summer and the lowest during winter. Mature leaves contained a significantly higher ($p \leq .001$) proportion of 1,8-cineole in their extract (60.9%) than immature leaves (44.6%). *Total yield ranged from 3.0 to 27.0 μL/g (fresh) in mature leaves and from 6.0 to 26.7 μL/g (fresh) in immature leaves. Significant differences between half-sibling families were observed, indicating genetic control of these traits. E. bosistoana* leaf extracts could be comparable in quality and quantity to that of *E. globulus*, the species dominating global essential eucalyptus oil production. Oil quality and quantity could be optimized by harvesting mature leaves during summer from superior families.

KEYWORDS
1,8-cineole, coast grey box, extraction, terpenes, total oil

1 | INTRODUCTION

Eucalyptus bosistoana originates from the coastal regions of New South Wales and Victoria, Australia, and produces naturally ground-durable timber.[1] It is the largest and most vigorous species in the eucalyptus box group,[2] and its timber was well-regarded by settlers[1] but never established as a plantation species in Australia. Now the New Zealand Dryland Forests Initiative (NZDFI) aims to establish *E. bosistoana* plantations in New Zealand to supply a hardwood industry producing post and poles for the agricultural sector as well as high stiffness veneers for laminated veneer lumber (LVL).[3] Commercializing an essential oil by-product from foliage could increase the economic viability of such plantations. The species has not been extensively researched for essential leaf oils, but literature suggests that the oil quantity and quality could be comparable to that of *E. globulus*,[4,5] the major source of global essential oil production.[4,6] It is interesting to note that its name is a felicitation for Joseph Bosisto (1824–1898), a pharmacist of Melbourne, Australia, who first realized the commercial export potential of eucalyptus oil.[7]

The concentration and structural characteristics of defensive phytochemicals in plant tissues such as leaves change over time, representing their physiological condition.[8] Leaf age affects essential oil content in *Eucalyptus* species, with lower molecular weight secondary metabolites often more abundant in immature leaves and larger molecular weight secondary metabolites more dominant in mature leaves.[9] These differences in leaf chemistry have been associated with preferences of pests and diseases towards immature leaves; however, physical traits such as foliage toughness or waxes also need to be considered.[10–12] Essential leaf oils also change throughout the year.[13,14] Monoterpenes, the major components of

eucalyptus essential oils, are not only influenced by light intensity[15] and water availability[16] but the temperature drives their evaporation rate.[17]

Additional to tissue age and season, oil traits are also under genetic control.[18,19] For example, the strong genetic control of oil yield and composition is utilized to optimize essential oil production from *E. polybractea* in Australia.[20,21]

This study was aimed to assess the seasonal variation of yield and composition in extracts from immature and mature *E. bosistoana* leaves of 5 half-sibling families.

2 | EXPERIMENTAL

2.1 | Chemicals

Analytical grade ethanol (≥99.5%), the internal standard n-hexadecane (99%) and reference standards of major components of eucalyptus essential oil, 1,8-cineole (99%), limonene (97%), α-phellandrene (≥95%), p-cymene (99%), α-terpeneol (≥90%), α-pinene (98%), aromadendrene (≥97%), β-myrcene (≥90%), caryophyllene (≥98%), trans-pinocarveol (≥96%), ocimene (≥90%) and linalool (97%) were purchased from Sigma Aldrich, New Zealand.

2.2 | Material

E. bosistoana leaf samples were collected from a nursery trial planted in 2012 located at 43°28′02.2" South latitude and 172°35′20.2" East longitude, New Zealand. The mean annual temperature for 2019 and 2020 was 12.2°C and total annual rainfall was 574 mm and 517 mm,[22,23] respectively. Three trees each from five different *E. bosistoana* half-sibling families (seed of a known mother tree but with unknown father tree) were sampled. Mature (fully lengthened leaves) and immature leaves (from branch tips) were collected in the last week of each month from March 2019 to April 2021. Each time approximately 8 to 10 similar sized leaves were collected from each tree.

2.3 | Extract analysis

While microwave solvent extraction (MSE) extracts are not identical with commercial essential oils obtained by distillation, the volatile compounds are representative of commercial oils[24–28] The quick MSE method uses less solvent, time and energy than steam distillation and was used for this study as it is amenable to a large number of samples.

1 g of fresh leaves cut into ~1 cm^2 pieces was immersed for 1 h in 2 mL of ethanol, which contained 0.025% n-hexadecane as internal standard, before being microwaved for 10 s at 1000 W. The filtered (0.45 μm, PTFE) extracts were further analysed by gas chromatography (GC, Agilent, model 7820A) through an Agilent DB-wax-polyethylene glycol (PEG) column (30 m × 0.250 mm × 0.25 μm). GC settings were splitless; injection volume1 μL; injector temperature 250°C; initial oven temperature 35°C for 3 min, increasing to 70°C at 3°C min^{-1}, increasing to 110°C at 5°C min^{-1}, increasing to 240°C at 50°C min^{-1} and finally held for 3 min; detector temperature 300°C. Total elution time was 29.3 min. Instrument control and data analysis were conducted using Chemstation software (Agilent Technologies).

Thirteen compounds with typical peak areas larger than 200 mV*min were quantified using peak areas normalized by the internal standard (n-hexadecane) and individual response factors for identified standard compounds as well as the average response factor of the identified compounds for unidentified terpenoids. Each compound was then expressed as the percentage of total extract. Total extract was defined as the internal standard normalized sum of all peak areas larger than 20 mV*min in a chromatogram, except the internal standard, multiplied by the concentration of the internal standard in the sample.

The peak assignments were confirmed by mass spectrometry. A GC/MS (Shimadzu GCMS-QP2010) in conjunction with the GCMSsolution software fitted with a Rtx-5MS (30 m × 0.25 mm × 0.25 μm) column and running temperature programme detailed above was used to record mass spectra at 70 eV and a mass range from m/z 50 to 400 amu.

Table 1 lists the retention times as response factors of standard compounds in the two GC systems. The GC–MS fingerprints of the standard compounds were compared with those of the peaks at the relevant retention time in the leaf extracts (additionally the library in the MS software also verified the identity of the compounds).

TABLE 1 Retention times (RT) and response factors (Rf) of compounds in *E. bosistoana* leaf extracts in GC-FID and GC–MS.

	GC-FID		GC–MS	
Compound	RT (min)	Rf (μL/g)	RT (min)	Identified by MS
β-Myrcene	5.8	1.24	6.3	yes
α-Pinene	7.3	0.72	7.8	yes
Limonene	7.9	1.16	10.6	yes
1,8-Cineole	9.3	1.39	10.8	yes
Ocimene	9.9	1.42	11.0	yes
Terpenoid 1	10.5	1.05[a]	11.6	no
Linalool	20.4	0.97	21.6	yes
Caryophyllene	22.1	0.66	22.4	yes
Aromadendrene	22.7	0.93	22.9	yes
Trans-pinocarveol	23.5	0.94	24.9	yes
Terpenoid 2	24.4	1.05[a]	25.3	no
α-Terpineol	24.6	1.06	25.9	yes
Terpenoid 3	26.4	1.05[a]	26.5	no

[a]Average response factor of all identified compounds.

2.4 | Data analysis

Data were analysed using R statistical software using linear mixed models.[29] Models were fitted using the nlme package,[30] and graphs were plotted using ggplot2.[31] Variations for each leaf compound were analysed using the following linear mixed effect model (in matrix notation):

$$y = Xb + Z_1 l + Z_2 t + Z_3 f + e$$

where $\boldsymbol{y}$ is the vector of compound assessments and $\boldsymbol{b}$ is the vector of fixed effects containing the overall intercept (b_0), leaf type (b_1 with two levels: immature and mature) and a general sine wave to model seasonality. The wave was expressed as $b_2 \sin(2\pi m) + b_3 \cos(2\pi m)$ where m is number of months since the beginning of the study divided by 12.

The random effects of the model involved leaf type ($\boldsymbol{l}$) nested in tree ($\boldsymbol{t}$) nested in family ($\boldsymbol{f}$) and were assumed to have 0 mean, with variances σ_l^2 (leaf type), σ_t^2 (tree), and σ_f^2 (family). These effects were assumed to be independent of each other.

The incidence matrices $\boldsymbol{X}$, $\boldsymbol{Z}_1$, $\boldsymbol{Z}_2$ and $\boldsymbol{Z}_3$ link the observations to the appropriate effect levels.

The vector $\boldsymbol{e}$ of residuals was assumed to follow a normal distribution with variance σ_e^2 and a first-order autoregressive process between successive assessments. Therefore, the residual correlation between two assessments of the same tree would be r^{lag}, where lag is the number of months between assessments.

3 | RESULTS AND DISCUSSION

Extract yield ranged from 3.0 to 27.0 μL/g (fresh) in mature leaves (Table 2) and from 6.0 to 26.7 μL/g (fresh) in immature leaves (Table 3). Thirteen major compounds were quantified and of those ten were identified: limonene, α-terpineol, α-pinene, aromadendrene, β-myrcene, caryophyllene, trans-pinocarveol, ocimene and linalool. 1,8-Cineole was the dominant compound and its mean percentage of the extract ranged from 40.1% to 92.7% in mature leaves and from 23.7% to 82.0% in immature leaves. α-Terpineol, limonene, aromadendrene, trans-pinocarveol and α-pinene were also present in higher quantities (0.5–20.9%, 1.4–20.5%, 0.04–16.1%, 0.05–17.5% and 0.1% to 8.6%, respectively) (Tables 1 and 2).

The leaf extracts were qualitatively similar to published data for *E. bosistoana*.[32–35] Leaf oil from *E. bosistoana* grown in Morocco was reported to be dominant in 1,8-cineole (68.2–79.2%) and also containing α-terpineol (3.3–7.3%), aromadendrene (0.5–2.2%), trans-pinocarveol (0.4–7.2%) and α-pinene (1.1–6.1%).[32] A similar composition of 55.3–63.9% 1,8-cineole, 2.4–2.6% limonene, 3.2–3.6% trans-pinocarveol and 11.6–12.1% α-pinene was reported for *E. bosistoana* oil trees harvested in Algeria.[33] 1,8-Cineole (59.3%), α-pinene (14.2%), limonene (4.5%) and α-terpineol (6.9%) were the main compounds of *E. bosistoana* oil form Portugal.[34] Three compounds previously reported for *E. bosistoana* oil, namely p-cymene (3.1–6.8%), α- and β-phellandrene (0.1–0.8%, 7%),[5,33,35] were not identified in this study. The absence of phellandrene in *E. bosistoana* essential oil is beneficial as it reduces essential oil quality[36] as phellandrene was associated with adverse health effects.[37]

It should be noted that the in this study obtained MSE extract are not essential oils as defined by ISO 770,[38] however, their volatile compounds are repetitive of essential oils.[26–28] Overall, *E. bosistoana* leaf extracts could be comparable in quality and quantity to that of *E. globulus*, the main source of the global oil production.[4,6]

TABLE 2 Ranges of total oil yield and composition of mature leaves for five *E. bosistoana* families.

Family	F9	F16	F33	F44	F49
Total oil (μL/g fresh)[a]	4.0–20.0	3.0-16.0	3.0–23.0	3.0–17.0	4.0–27.0
Compounds	**Percentage of the total oil (%)**				
β-Myrcene	0.08–0.93	0.07–3.2	0.09–1.7	0.1–0.9	0.03–1.1
α-Pinene	0.4–5.4	0.1–8.5	0.2–5.2	0.2–4.5	0.2–8.6
Limonene	2.99–19.0	3.05–16.4	3.6–14.8	3.5–19.0	1.4–13.8
1,8-Cineole	43.5–71.5	40.1–72.5	44.0–76.2	40.4–73.6	49.4–92.7
Ocimene	0.1–2.3	0.3–1.13	0.05–1.7	0.08–2.1	0.03–4.2
Linalool	0.1–5.9	0.15–5.1	0.1–6.8	0.2–6.6	0.1–6.3
Caryophyllene	0.2–6.1	0.3–5.8	0.2–3.8	0.2–8.1	0.04–2.9
Aromadendrene	0.3–11.3	0.1–8.7	0.3–11.7	0.15–16.1	0.04–8.8
Trans-pinocarveol	0.15–17.5	0.3–10.4	0.05–10.9	0.2–11.6	0.2–10.8
α-Terpineol	1.3–14.7	1.2–17.5	0.7–16.2	0.6–14.3	0.5–20.9
Terpenoid 1[a]	0.01–2.6	0.07-3.4	0.08–3.3	0.09–2.9	0.03–4.0
Terpenoid 2[a]	0.06–6.0	0.15-6.6	0.05–4.7	0.1–6.5	0.1–2.4
Terpenoid 3[a]	0.16–10.9	0.4-10.4	0.1–10	0.2–14.8	0.1–8.7

[a]Semi-quantitative data.

TABLE 3 Ranges of total oil yield and composition of immature leaves for five *E. bosistoana* families.

Family	F9	F16	F33	F44	F49
Total oil (μL/g fresh)[a]	8.0–25.0	6.0-18.0	7.0–24.0	9.0–19.0	10.0–26.7
Compounds	**Percentage of the total oil (%)**				
β-Myrcene	0.06–2.3	0.07–3.3	0.04–0.6	0.04–1.6	0.07–1.5
α-Pinene	0.6–5.3	0.2–6.0	0.6–5.7	0.8–7.8	0.1–3.0
Limonene	2.7–20.5	4–17.6	4–16.7	5.0–16.1	3.9–16.6
1,8-Cineole	31.6–57.2	27.9–61.9	33.4–55.4	23.7–59.3	31.2–82.0
Ocimene	0.04–0.3	1.1–4.2	0.06–8.9	0.1–5.4	0.08–2.1
Linalool	0.2–11.8	0.2–7.2	0.2–5.5	0.8–5.3	0.07–6.0
Caryophyllene	0.3–12.0	0.13–14.1	0.3–6.4	0.2–5.3	0.2–11.5
Aromadendrene	0.4–11.3	0.1–12.8	0.4–13.6	0.5–12.3	0.15–10.9
Trans-pinocarveol	0.9–11.9	0.13–6.9	0.7–10.2	0.1–10.8	0.3–9.3
α-Terpineol	3.6–18.6	3.1–15.2	1.9–15.1	3.3–15.0	1.4–17.2
Terpenoid 1[a]	0.13–2.9	0.08–3.7	0.1–4.8	0.2–5.0	0.03–3.2
Terpenoid 2[a]	0.07–5.9	0.1–6.9	0.05–4.5	0.1–2.9	0.1–3.3
Terpenoid 3[a]	0.4–11.4	1.1–10.3	1.3–12.2	0.7–12.6	0.9–9.9

[a]Semi-quantitative data.

3.1 | Effect of leaf maturity

Average extract yields were significantly different ($p \leq .001$) between immature and mature *E. bosistoana* leaves with averages of 14.6 and 10.2 μL/g (fresh), respectively (Table 4). That immature *E. bosistoana* leaves produced more secondary metabolites than mature leaves was consistent with literature reports for other eucalyptus as was the significantly higher ($p \leq .001$) proportion of 1,8-cineole in mature (60.9%) compared to immature (44.6%) leaves.[9,39,40] Therefore, a purer 1,8-cineole rich essential eucalyptus oil could be obtained from mature leaves. However, separating mature from immature leaves is challenging in a commercial setting.

The concentrations of another six quantified leaf extract compounds (α-terpineol, aromadendrene, linalool and terpenoid 1, 2, 3) also significantly differed between immature and mature leaves, with some being more prominent in immature and others being more prominent in mature leaves (Table 4). No significant difference was found for limonene, β-myrcene, ocimene, trans-pinocarveol, α-pinene and caryophyllene percentages between mature and immature leaves.

TABLE 4 Average composition and amount of *E. bosistoana* essential oil from immature and mature leaves throughout a year (five families represented by three trees each).

	Immature leaves $n = 213$	Mature leaves $n = 360$	
Total oil (μL/g fresh) [a]	14.6	10.2	***
	Percentage of total oil (%)		
β-Myrcene	0.1	0.1	ns
α-Pinene	2.2	2.1	ns
Limonene	9.3	8.8	ns
1,8-Cineole	44.6	60.9	***
Ocimene	0.9	0.6	ns
Linalool	2.1	1.7	**
Caryophyllene	1.1	1.0	ns
Aromadendrene	4.4	2.4	***
Trans-pinocarveol	3.7	4.2	ns
α-Terpineol	8.0	6.9	***
Terpenoid 1[a]	1.1	0.5	***
Terpenoid 2[a]	1.3	0.9	**
Terpenoid 3[a]	4.8	3.7	***

[a]Semi-quantitative data.

***$p \leq .001$, **$p \leq .01$, *$p \leq .05$, ns = not significant.

3.2 | Seasonal variation

Figure 1 shows the seasonal variation and fitted mixed effect model for extract content and 1,8-cineole percentage for five *E. bosistoana* families and two leaf maturity stages over 2 years. The intercepts for extract content in immature leaves and 1,8-cineole were 13.2 μL/g and 43.1%, respectively. Extract amounts and 1,8-cineole percentages showed significant seasonal variation, which was consistent over 2 years. Yield peaked in summer (December) with an average of 16.7 μL/g and was lowest with 7.3 μL/g in winter (July) (Table 5). 1,8-Cineole concentration peaked slightly earlier in January. Similar seasonal patterns were observed for other eucalypts.[14,32,39,41,42] The observed annual variations were consistent

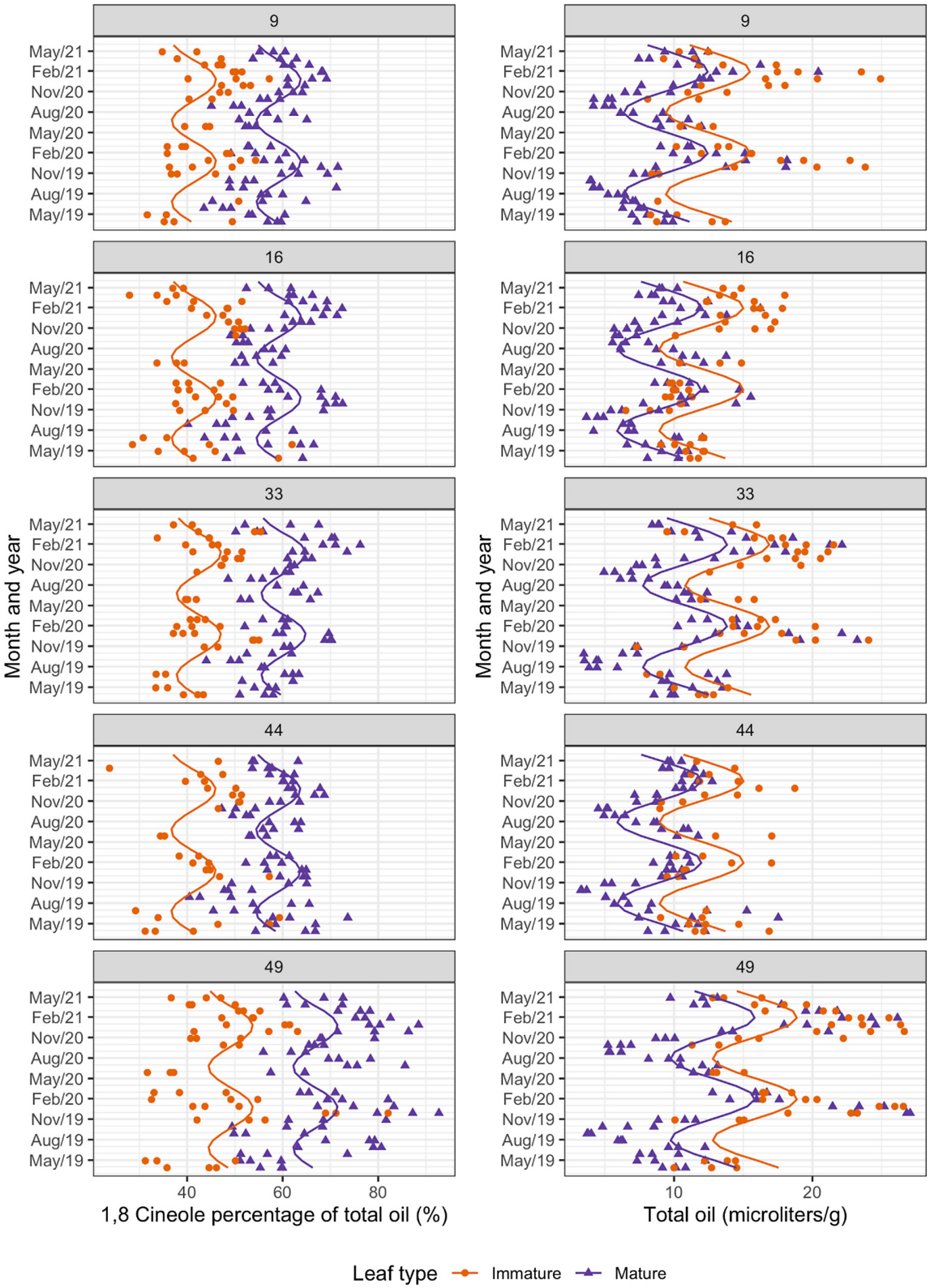

FIGURE 1 Seasonal, leaf type and family variation of extract yield and 1,8-cineole concentration of *E. bosistoana*.

with higher temperatures (≥30°C) facilitating the evaporation of monoterpenes from eucalyptus leaves.[43] The results imply that harvesting foliage during summer would result not only in more abundant but also higher quality 1,8-cineole rich essential oil.

Significant seasonal differences ($p \leq .001$) were also found for β-myrcene, linalool, caryophyllene, aromadendrene, trans-pinocarveol, as well as the terpenoid 2 and 3. While β-myrcene, caryophyllene, aromadendrene and terpenoid 1 peaked like 1,8 cineol and the total oil

TABLE 5 Summary of leaf type, seasonality and family effects.

Compound	Mean immature leaf % of total oil	Difference of mature leaf % of total oil	Seasonal effect and significance (%)	Family random effect[a] (% of total oil)					Peak Month
				F9	F16	F33	F44	F49	
β-Myrcene	0.14	5.91×10^{-5}	0.23 (***)	0.001	-3.48×10^{-5}	0.02	−0.01	−0.009	November
α-Pinene	2.09	0.01	0.35 (ns)	0.04	0.33	−0.006	−0.13	−0.24	February
Limonene	9.22	−0.41	0.59 (ns)	0.60	0.21	−0.15	0.22	−0.88	February
1,8-Cineole	43.10	17.76	9.30 (***)	−1.72	−1.73	−0.70	−1.81	5.96	January
Ocimene	0.76	−0.15	0.49 (ns)	-2.15×10^{-9}	0.49	2.13×10^{-5}	8.18×10^{-5}	-8.65×10^{-9}	February
Linalool	2.23	−0.51	0.98 (***)	0.18	0.06	0.20	0.07	−0.52	May
Caryophyllene	0.88	0.09	1.19 (***)	0.15	0.17	−0.11	0.18	−0.39	December
Aromadendrene	3.80	−1.38	3.37 (***)	0.04	−0.04	0.09	0.03	−0.11	January
Trans-pinocarveol	4.45	−0.29	4.24 (***)	0.34	−0.03	0.05	0.59	−0.95	July
α-Terpineol	8.05	−1.14	0.17 (ns)	−0.01	0.56	0.64	0.36	−1.55	February
Terpenoid 1[b]	0.93	−0.38	0.63 (ns)	−0.0002	0.06	0.04	0.02	−0.11	January
Terpenoid 2[b]	1.36	−0.43	0.73 (***)	0.06	0.27	−0.03	−0.07	−0.23	June
Terpenoid 3[b]	5.06	−1.32	1.83 (***)	0.32	−0.15	0.28	0.31	−0.75	June
Extract (μL/g)[b]	13.19	−3.04	6.09 (***)	−0.76	−1.24	0.62	−1.23	2.61	December

[a]Family random effects are the deviations from the predictions including overall mean, leaf type and seasonality.

[b]Semi-quantitative data.

***$p \le .001$, **$p \le .01$, *$p \le .05$, ns = not significant.

content in summer, linalool, trans-pinocarveol, as well as the terpenoid 2 and 3 peaked in colder months (Table 5). Linalool and trans-pinocarveol percentages in leaf essential oils from *E. globulus* grown in Nigeria were reported higher during the rainy season, while other compounds (including α-pinene, limonene, β-myrcene and caryophyllene) were found in higher quantities during the dry season.[44]

3.3 | Variations between families

The timing of seasonal patterns for *E. bosistoana* extracts was similar across all families (Figure 1). While within family (between trees) variation for leaf extract yields and 1,8-cineole concentration in both leaf types were not significant ($p > .05$), differences between families were significant. In particularly, family 49 expressed both, higher extract yield (2.61 μL/g above average) as well as 1,8-cineole content (6% above average) than the other families (Figure 1 and Table 5). It should be noticed that this family concurrently expressed the lowest amounts of all other compounds except for β-myrcene. Genetic control of essential oil yield and 1,8-cineole percentage has been reported for other eucalypts.[42,45,46] The results indicate that oil quantity and oil quality of *E. bosistoana* could be optimized through breeding.

4 | CONCLUSION

This study showed that mature leaves of *E. bosistoana* possessed higher 1,8-cineole proportions but contained less extract than immature leaves. The highest extract yield and 1,8-cineole percentage were obtained from the leaves collected during summer, suggesting summer is the best period for leaf harvesting for essential oil production. The significant difference in extract quantity and quality between the five tested families in this experiment indicated that these traits are under genetic control. As yield and quality could be comparable to that of *E. globulus* there is potential for essential oil production as a by-product of *E. bosistoana* timber plantations. Exact quantification of *E. bosistoana* essential oil yields and composition need to be determined.

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CONFLICT OF INTEREST STATEMENT

The authors have no relevant financial interests to disclose. Clemens Altaner is Science Team Leader of the New Zealand Dryland Forests Initiative (NZDFI).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

ORCID

Chamira Rajapaksha https://orcid.org/0009-0004-0433-3387
Luis Alejandro Apiolaza https://orcid.org/0000-0003-0958-3540
Marie A. Squire https://orcid.org/0000-0002-8658-921X
Clemens Altaner https://orcid.org/0000-0003-4542-4432

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