Elevated CO₂ offsets the alteration of foliar chemicals (*n*-icosane, geranyl acetate, and elixene) induced by elevated O₃ in three taxa of O₃-tolerant eucalypts

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Abstract Eucalypts are important forest resources in south-western China, and may be tolerant to elevated ground-level ozone (O₃) concentrations that can negatively affect plant growth. High CO₂ may offset O₃-induced effects by providing excess carbon to produce secondary metabolites or by inducing stomatal closure. Here, the effects of elevated CO₂ and O₃ on leaf secondary metabolites and other defense chemicals were studied by exposing seedlings of *Eucalyptus globulus*, *E. grandis*, and *E. camaldulensis* × *E. deglupta* to a factorial combination of two levels of O₃ (< 10 nmol mol⁻¹ and 60 nmol mol⁻¹) and CO₂ (ambient: 370 μmol mol⁻¹ and 600 μmol mol⁻¹) in open-top field chambers. GC-profiles

of leaf extracts illustrated the effect of elevated O₃ and the countering effect of high CO₂ on compounds in leaf epicuticular wax and essential oils, i.e., *n*-icosane, geranyl acetate and elixene, compounds known as a first-line defense against insect herbivores. *n*-Icosane may be involved in tolerance mechanisms of *E. grandis* and the hybrid, while geranyl acetate and elixene in the tolerance of *E. globulus*. Elevated O₃ and CO₂, singly or in combination, affected only leaf physiology but not biomass of various organs. Elevated CO₂ impacted several leaf traits, including stomatal conductance, leaf mass per area, carbon, lignin, *n*-icosane, geranyl acetate and elixene. Limited effects of elevated O₃ on leaf physiology (nitrogen, *n*-icosane, geranyl acetate, elixene) were commonly offset by elevated CO₂. We conclude that *E. globulus*, *E. grandis* and the hybrid were tolerant to these O₃ and CO₂ treatments, and *n*-icosane, geranyl acetate and elixene may be major players in tolerance mechanisms of the tested species.

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Introduction

High concentrations of tropospheric ozone (O_3) can induce damage in plants and reduce forest productivity (Broadmeadow et al. 1999; Percy et al. 2003; Manning 2005; Proietti et al. 2016; Yuan et al. 2015, 2016). As a result of increasing O_3 concentrations, 50% (17 million km²) of the world's forest may be exposed to O_3 levels > 60 nmol mol⁻¹ and, thus, decrease photosynthetic productivities by the year 2100 (Fowler et al. 1999; Sitch et al. 2007). The considerable genetic variability among plants results in substantially different levels of damage in response to particular O_3 levels (Booker et al. 2009). At the same time, atmospheric carbon dioxide (CO_2) concentrations have also increased and are predicted to reach 600 μ mol mol⁻¹ near the year 2060 (IPCC 2007). Elevated CO_2 may enhance photosynthetic rates and increase plant growth (e.g., Ainsworth and Long 2005). Hence, it is imperative to determine the interactive effects of CO_2 and O_3 on such representative species for future afforestation practices (e.g., Karnosky et al. 2003; Kitao et al. 2015; Shi et al. 2017).

Exposure to O_3 generates a response comparable to that of hypersensitive response of plants to pathogen attack; thus, O_3 is considered an abiotic “elicitor” of plant defense reaction, with the potential to alter the chemistry and metabolism of plant tissue (Sandermann et al. 1998; Matyssek et al. 2012; Agathokleous et al. 2019a). At elevated concentrations, O_3 is a strong oxidant, which reacts with leaf apoplast components and induces high production of reactive chemical species, leading to death of cells, inhibition of carbon assimilation and acceleration of senescence (Singh and Agrawal 2017). Conversely, elevated CO_2 can reduce water loss through transpiration, enhance carbon assimilation and the apparent quantum yield of CO_2 uptake and plant growth and yields, and improve water- and light-use efficiency (Ainsworth and Long 2005; Eguchi et al. 2008). Elevated CO_2 and O_3 can also alter leaf chemistry, including secondary metabolites, in multiple ways (Booker and Maier 2001; Kopper and Lindroth 2003; Oksanen et al. 2005; Lindroth 2010; Novriyanti et al. 2012a; Shi et al. 2016, 2017; Araminiené et al. 2018).

Because CO_2 is a substrate of photosynthesis, elevated CO_2 may increase C availability for secondary metabolite production (Bryant et al. 1983; Herms and Mattson 1992; Mattson et al. 2005); thus, the altered secondary metabolites are more likely due to resource availability rather than induced defense. Hence, it is important to understand whether enhancement of growth and secondary metabolite concentration caused by elevated CO_2 can counteract damage caused by O_3 . Several studies have provided evidences of elevated CO_2 alleviating

the adverse effects of elevated O_3 (Booker and Maier 2001; Karnosky et al. 2003; Kopper and Lindroth 2003; Paoletti and Grulke 2005). Moreover, as stomatal conductance can be suppressed by elevated CO_2 (e.g., Larcher 2003; Schulze et al. 2005), O_3 absorption is expected to be somewhat lessened. CO_2 amelioration of O_3 adverse effects is assumed to be related with the so-called fertilizer effect of elevated CO_2 by which net photosynthesis increases and a C surplus is available for secondary metabolite production.

Eucalypts have been widely used in commercial plantation due to their fast growing traits and broad adaptability (e.g., Orwa et al. 2009) and are one of the most important forest resources in southwestern China (Wang and Koike 2019). Numerous studies have therefore investigated the effects of CO_2 on different eucalypt species, singly or in combination with other factors such as ambient air temperature, light availability, nitrogen deposition, soil nutrients, and water deficit (Lawler et al. 1996; Roden and Ball 1996; McKiernan et al. 2012; Novriyanti et al. 2012a; Murray et al. 2013; Plett et al. 2015; Ghini et al. 2015; Quentin et al. 2015; Xu et al. 2019). Despite the many studies on CO_2 interactive effects with other factors, the interactive effects of CO_2 and O_3 on eucalypts remain unknown, and studies on single effects of O_3 on eucalypts are very limited (Monk and Murray 1995). Furthermore, elevated CO_2 and O_3 alter the concentrations of secondary metabolites in trees in a gas-specific manner and with significant interactive effects of the two gases (Gleadow et al. 1998; Oksanen et al. 2005; McKiernan et al. 2012; Xu et al. 2019). The effects on leaf chemical defense, including secondary metabolites, are of high ecological relevance because they can drive plant–herbivore interactions (Gleadow et al. 1998; Bidart-Bouzat and Imeh-Nathaniel 2008). It is therefore important to investigate the effects of O_3 singly or in combination with CO_2 on leaf chemical defense, and especially secondary metabolites, in eucalypt species. If elevated CO_2 and O_3 affect the leaf chemicals of these promising afforestation species, their defense traits and fitness to grow and succeed in the changing environments should be of concern.

The present study aimed to examine the single and combined effects of elevated CO_2 and O_3 on leaf chemical traits, including both primary and secondary chemistry, in representative afforestation eucalypt species. We hypothesized that, under elevated O_3 , a trade-off may occur between growth and secondary metabolites. Both growth and secondary metabolites concentrations would be enhanced by elevated CO_2 due to the fertilizer effect of CO_2 . We predicted that the stimulatory effect of elevated CO_2 will moderate the inhibitory effect of elevated O_3 on leaf chemicals. To test this prediction, three eucalypt species were grown in the field in open-top chambers (OTCs) supplied with ambient air or elevated CO_2 and/or O_3 . The selected eucalypts are fast-growing and widely planted in

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Asian forest plantations (Novriyanti et al. 2012a, b; Wang and Koike 2019) toward informing forestry applications in an O₃-polluted, CO₂-enriched world.

Materials and methods

Study sites and plant materials

The experiment was conducted at the experimental nursery of the Field Science Center of Hokkaido University, Sapporo, Japan (43° 0' N, 141° 2' E, 15 m a.s.l.). The seedlings were grown in 10-L pots filled with a commonly used nursery mixture of pumice soil and clay soil (1:1, v/v), with 200 mL of 500-fold diluted liquid fertilizer (balanced nutrients; 6:10:5, Hyponex Corp. JAPAN, Osaka, Japan) per plant at 2-week intervals to provide 192 mg N per pot. The pots were watered periodically to sustain the soil moisture.

Seedlings of *Eucalyptus globulus* (Glo) and *Eucalyptus grandis* (Gra) and cuttings of hybrid *E. deglupta* × *E. camaldulensis* (Hyb) were used because they are popular for plantations. The seeds were obtained from the Australian Tree Seed Centre of CSIRO, Australia. At the initiation of the experiment, all plants were 5 months old; average height and basal diameter was 25.0 cm and 2.4 mm for Glo, 24.3 cm and 3.0 mm for Gra, and 26.7 cm and 2.4 mm for Hyb. The experiment lasted from June to October 2010.

Gas treatment system

The seedlings were placed in 16 OTCs (1.2 × 1.2 × 1.2 m high; Dalton Co. Ltd. Sapporo, Japan) supplied with one of two levels of O₃ for 7 h during the daytime (ambient [AO]: < 10 nmol mol⁻¹; elevated [EO]: 60 nmol mol⁻¹) in combination with one of two levels of CO₂ (ambient [AC]: about 370 μmol mol⁻¹; elevated [EC]: 600 μmol mol⁻¹) during the daytime. Four chamber replications were deployed for each treatment; therefore, there were 16 OTCs.

Except for the EO chambers, the OTCs were equipped with charcoal filters to first clean the inlet air. The level of EO was selected because similar concentrations have been often observed in many regions in Japan. The CO₂ level of EC is the atmospheric level predicted near the year 2060 (IPCC 2007). The OTCs had an average temperature of 22.3 °C (max 29.8 °C and min 17.8 °C) and relative humidity of 76.4%.

Gas exchange measurements

Gas exchange rates were measured on third and fourth leaves from the shoot top using an open-type gas-exchange system (LI-6400, Li-Cor, Lincoln, NE, USA) with a photosynthetic photon flux of 1500 μmol m⁻² s⁻¹, the light level

corresponding to the light-saturated photosynthetic rate for these plants in the growing environment. Leaf temperature was controlled at 25 °C ± 1 °C and leaf vapor pressure deficit (VPD) maintained at 1.2 ± 0.2 kPa to regulate stomatal conductance during the measurements. The leaves acclimated to the chamber conditions at growth CO₂ concentrations (i.e., 370 μmol mol⁻¹ for AC and 600 μmol mol⁻¹ for EC) for 15–20 min after clipping the leaf to the chamber. After the acclimation, light saturated net photosynthetic rate at growth CO₂ concentration (A_{growth}), stomatal conductance (g_s) and leaf transpiration rate (E) were determined.

To obtain the response curve for net photosynthetic rate (A) to intercellular CO₂ concentration (C_i), i.e., the A_{growth}/C_i curve, 12 levels of external CO₂ concentration were supplied to the chamber (60–1500 μmol mol⁻¹), and the corresponding values for maximum rate of carboxylation (V_{cmax}) and maximum rate of electron transport (J_{max}) were determined (Farquhar et al. 1980; Long and Bernacchi 2003). The Rubisco Michaelis constants for CO₂ (K_c) and O₂ (K_o) and the CO₂ compensation point in the absence of dark respiration (Γ^*) for the analysis of the A_{growth}/C_i curve were obtained from Bernacchi et al. (2001). All gas-exchange variables were expressed on the basis of the projected (one-sided) leaf area covered.

Total phenolic and condensed tannin measurements

Total phenolics were determined using the Folin–Ciocalteu method as modified by Julkunen-Tiitto (1985), and condensed tannins were measured using the proanthocyanidin method of Bate-Smith (1977) (Matsuki et al. 2004). A powdered, freeze-dried leaf sample (20 mg) was placed in 5 mL of 50% methanol in an ultrasonic machine (ST-02M, Sonic Tech, Tokyo, Japan) at 40 °C for 1 h.

For measuring condensed tannins, 1 mL of the methanolic extract solution was placed in a test tube with 4 mL HCl–1-butanol (1: 19). The solution was then boiled for 2 h, then cooled before the optical density at 55 nm was measured with a spectrophotometer (UV-2700, Shimadzu Kyoto, Japan).

For measuring total phenolics, 50 μL of the filtered methanolic extract solution was placed in a test tube with 2.25 mL of deionized water, 0.25 mL of 50% phenol reagent and 2.5 mL of 20% w/v Na₂CO₃, and the mixture was mixed thoroughly. After the solution rested for approximately 15 min, the optical density at 760 nm was measured with the spectrophotometer.

The total condensed tannins (mg g⁻¹) content was calculated as $(4.5A/4 + 0.011)/(20.255 \times 5000)/B$, and total phenolics (mg g⁻¹) as $(A/2 - 0.03)/12.281 \times 100,000/B$, where A is the absorbance reading and B is the mass of the sample (± 20 mg).

Gas chromatography

A powdered sample of fresh leaves was soaked 3 times in 15 mL methanol (MeOH), then 1 mL of the MeOH extract was rotoevaporated (< 35 °C and 80 Pa), dissolved in 1 mL CHCl_3 , then added to 3 mL Sep-Pak silica cartridge (Waters Corp., Milford, MA, USA) eluted with CHCl_3 . The eluate was then collected and evaporated. This fraction contained the less-polar phenolics such as waxes, fats, and other hydrocarbons. A MeOH standard solution was made by dissolving 100 mg *m-tert*-butylphenol in 100 mL MeOH, then diluted 10 times with MeOH. The extract was then dissolved in the MeOH-standard solutions to make 1 mL (volumetric) of sample solution.

One microliter of sample solution was injected into a gas chromatograph (GC-2025, Shimadzu, Kyoto, Japan) with a DB-1 column (20 m $\times$ 0.25 mm i.d., 0.25 μm , silica coated; J&W Scientific, Folsom, CA, USA) and helium as the carrier gas. The injector and detector temperature were maintained at 250 °C. The column temperature was initially 70 °C, with a hold-time of 2 min, then increased at 5 °C/min to 275 °C, and finally at 2.5 °C/min to 280 °C, with a final hold-time of 5 min. Peaks detected during the 58 min procedure were compared and analyzed to check the effects of the treatments. There were four replications of each sample solution, and an analysis of variance (ANOVA) was carried out to determine the significant difference among peak patterns in the GC profiles ($\alpha = 0.05$).

Gas chromatography–mass Spectrometry

After the GC-profiles revealed peak patterns affected by the treatments, he representative sample solutions from each species were subjected to gas chromatography–mass spectrometry (GC/MS) using Agilent Technologies 6890 series GC (Hewlett-Packard, Palo Alto, CA, USA) coupled to an electron ionization-mass spectrometer (EI-MS) (JEOL JMS-700TZ) with He as the carrier gas. Data acquisition parameters were flow rate 1.5 mL/min; injection volume 1 μL (splitless); inlet temperature 250 °C; ZB-1MS (phenomenex) 30 m $\times$ 0.25 mm i.d. $\times$ 0.25 μm column; temperature program: 70 °C for 2 min, increasing 5 °C/min to 275 °C, then 2.5 °C/min to 280 °C with a final hold time of 5 min; electron ionization (EI) mode, electron energy 70 eV, 300 μA , source temperature 230 °C, scan mode, mass range at m/z 40–500 Da. The acquired mass spectra were used to search the mass spectral library NIST/EPA/NIH/EINECS/IRDB to identify compounds.

Data analysis

The statistical significance was set a priori to an alpha level of 0.05. The data for each trait and species were averaged

per OTC to provide four replicates per gas treatment. Data were tested against the requirements of parametric statistical tests, and, when needed, subjected to a Box–Cox transformation (Box and Cox 1964) according to the methodology explained by Agathokleous et al. (2016a). Statistical hypothesis testing was done with Spiegel's Method I sum of squares-adjusted (Howell and McConaughy 1982) general linear models (GLM) where species and gas treatment were fixed factors and OTC was a random factor. For significant species, treatment or species $\times$ treatment interactions, Bonferroni post-hoc test was applied for multiple comparisons among the experimental groups. The results are shown as means $\pm$ SD. Data processing and statistics were performed with EXCEL 2010 (Microsoft, Redmond, CA, USA) and STATISTICA v.10 (StatSoft, Tulsa, OK, USA).

Results

Biomass

Biomasses varied significantly among species but were not significantly affected by gas treatment; the species $\times$ treatment interaction was also not significant (Fig. 1).

Gas exchange, leaf mass per area (LMA) and photosynthetic pigments

A_{growth} , g_s , V_{cmax} and J_{max} varied significantly among species; and values were greater for the hybrid than for the other two species, except that V_{cmax} for *E. globulus* did not differ significantly from *E. grandis* and the hybrid (Table 1). Gas treatment was a significant factor only for g_s . In particular, EC did not significantly affect g_s in AO but did increase g_s in EO, suggesting a significant interaction between CO_2 and O_3 (Table 1).

LMA varied among species and among gas treatments (Table 1). *E. grandis* and the hybrid had a higher LMA than *E. globulus* did. EC tended to increase LMA, compared to AC, in AO; however, variation was large, and the difference was not significant. The EC-induced increase in LMA was significant in EO (Table 1).

Chlorophyll *a*, *b* and *a* + *b* contents did not vary significantly among species (Table 1). However, the chlorophyll *a* to *b* ratio (*a/b*) was in the order hybrid > *E. globulus* > *E. grandis*. Gas treatment was a significant factor for chlorophyll *a*, *b* and *a* + *b* contents but not for chlorophyll *a/b*. EC significantly decreased chlorophyll *a* and *a* + *b* levels, but not chlorophyll *b* in AO (Table 1). However, EC significantly decreased chlorophyll *a*, *b* and *a* + *b* content in EO. EO per se did not affect the photosynthetic pigments.

Fig. 1 Biomass of leaves, stems and roots of *Eucalyptus globulus* (Glo), *E. grandis* (Gra), and *E. deglupta* × *E. camaldulensis* (Glo × Gra). Error bar is standard deviation with $n = 4$. AO ambient O₃ (< 10 nmol mol⁻¹), EO elevated O₃ (60 nmol mol⁻¹), AC ambient CO₂, EC elevated CO₂ (600 μmol mol⁻¹). Only species was a significant factor according to general linear model tests (statistical results are provided in Supplementary Material, Table S1)

The interaction between species and gas treatment was not significant for any trait for gas exchange and photosynthetic pigments as for LMA (Table 1).

Phenolics, tannins, C and N

Total phenolics varied only among species; the hybrid had lower phenolic content than the other two species (Table 2). Condensed tannins varied among species and among gas treatments; however, the species × treatment interaction was not significant. The hybrid and *E. grandis* had similar content, significantly more than that of *E. globulus*. While EC did not significantly affect tannin content in AO, it increased tannin content in EO. EO did not significantly affect the total phenolics or tannins in AC or EC.

Regarding C and N traits, there were significant differences among species and among gas treatments, but the interaction between species and treatments was not significant (Table 2). In both AO and EO, EC significantly increased the area-based C, but there was a trend toward lower mass-based C content, which was significant only in EO. EO did not affect C_{area} and C_{mass} in either AC or EC. EC decreased N_{area} and N_{mass} in both AO and EO, although for N_{area} the difference was not significant in EO. EO decreased significantly both N_{area} and N_{mass} in AC, but this effect was offset by EC (no significant differences between AO × EC and EO × EC). Interestingly, EC significantly increased the C/N ratio in both AO and EO, but this effect was mainly due to a decrease in N content than an increase in C content by EC. EO increased the C/N ratio in AC but did not affect C/N ratio in EC where C/N ratio was driven by EC.

Lignin content was significantly decreased by EC in EO but was not significantly affected by EC in AO (Table 2).

However, this difference seems to be due to higher lignin content in EO × AC (compared to AO × AC), although the difference between the two groups was not significant. EO did not significantly affect lignin. While species and treatments had significant effects on lignin, the interaction of the two factors was not significant. Interestingly, the hybrid that tended to have high biomass was also the species with the highest lignin content and lowest phenolic content; it also had high tannin content (across gas treatments).

GC profiles of leaf extracts

Peaks in the GC profile for *E. globulus* were the most abundant, compared with those of hybrid and *E. grandis* (Fig. 2). Although the GC profiles differed among the species and hybrid, all showed the same peak pattern at retention time (RT) 34.8 min (hereafter compound 1).

Regarding *E. grandis*, EO increased the peak height of compound 1 in AC but not in EC (Fig. 3a), indicating that EC offsets the EO-induced effect. Regarding the hybrid, EO increased the peak height of compound 1 in both AC and EC, while EC decreased it in both AO and EO; nonetheless, EC did not fully offset the EO-induced effect because the value was still higher in EO × EC than in AO × EC (Fig. 3b). As for *E. globulus*, there were no significant differences in the relative peak height of compound 1 among treatments (Fig. 4a). However, *E. globulus* displayed an EO-induced increase in the relative concentration of compounds at RT 17.2 min (hereafter compound 2; Fig. 4b) and 24.1 min (hereafter compound 3; Fig. 4c) in AC. This EO-induced effect did not appear in EC, where there were no significant differences between AO × EC and EO × EC, due to CO₂ mediation. Similarly with EO, EC significantly increased

Table 1 Photosynthetic rate (A_{growth}), stomatal conductance (g_s), maximum rate of carboxylation (V_{cmax}), maximum rate of electron transport (J_{max}), leaf mass per unit area (LMA) and photosynthetic pigments of *Eucalyptus globulus*, *E. grandis*, and hybrid *E. deglupta* × *E. camaldulensis*

Plant	Treatment	A_{growth} ($\mu\text{mol m}^{-2} \text{s}^{-1}$) ^B	g_s ($\text{mol m}^{-2} \text{s}^{-1}$) ^B	V_{cmax} ($\mu\text{mol m}^{-2} \text{s}^{-1}$) ^{AB}	J_{max} ($\mu\text{mol m}^{-2} \text{s}^{-1}$) ^B	LMA (g m^{-2}) ^B	Chl <i>a</i> ($\mu\text{g mg}^{-1}$) ^A	Chl <i>b</i> ($\mu\text{g mg}^{-1}$) ^A	Chl <i>a/b</i> ($\mu\text{g mg}^{-1}$) ^B	Chl <i>a+b</i> ($\mu\text{g mg}^{-1}$) ^A
<i>E. globulus</i>	AO × AC	9.41 ± 5.34 ^A	0.20 ± 0.11 ^{AB}	45.37 ± 23.80 ^A	74.81 ± 23.4 ^A	72.93 ± 14.43 ^{AB}	3.98 ± 1.19 ^A	1.68 ± 0.48 ^{AB}	2.40 ± 0.21 ^A	5.66 ± 1.66 ^A
	AO × EC	10.85 ± 2.94 ^A	0.25 ± 0.09 ^A	38.57 ± 15.19 ^A	72.39 ± 17.18 ^A	93.61 ± 19.98 ^{AB}	2.81 ± 0.63 ^B	1.39 ± 0.44 ^{BC}	2.14 ± 0.14 ^A	4.20 ± 1.07 ^B
	EO × AC	6.81 ± 3.05 ^A	0.14 ± 0.08 ^B	34.92 ± 14.95 ^A	67.89 ± 23.66 ^A	74.22 ± 6.61 ^B	3.41 ± 0.22 ^A	1.55 ± 0.25 ^A	2.25 ± 0.31 ^A	4.97 ± 0.42 ^A
	EO × EC	10.25 ± 2.26 ^A	0.37 ± 0.19 ^A	45.52 ± 11.74 ^A	89.62 ± 25.29 ^A	89.55 ± 15.37 ^A	2.78 ± 0.48 ^B	1.23 ± 0.17 ^C	2.28 ± 0.17 ^A	4.01 ± 0.64 ^B
<i>E. grandis</i>	AO × AC	8.80 ± 1.40 ^A	0.19 ± 0.05 ^{AB}	38.07 ± 4.17 ^A	63.71 ± 5.44 ^A	77.37 ± 15.44 ^{AB}	3.71 ± 0.33 ^A	1.93 ± 0.46 ^{AB}	1.98 ± 0.30 ^A	5.63 ± 0.79 ^A
	AO × EC	8.38 ± 0.77 ^A	0.23 ± 0.02 ^A	31.63 ± 6.68 ^A	58.16 ± 3.43 ^A	97.49 ± 6.86 ^{AB}	2.39 ± 0.60 ^B	1.21 ± 0.25 ^{BC}	1.98 ± 0.21 ^A	3.60 ± 0.83 ^B
	EO × AC	8.07 ± 1.14 ^A	0.19 ± 0.03 ^B	38.05 ± 7.67 ^A	73.88 ± 9.64 ^A	80.12 ± 6.72 ^B	3.54 ± 0.31 ^A	1.84 ± 0.19 ^A	1.93 ± 0.23 ^A	5.38 ± 0.41 ^A
	EO × EC	8.64 ± 2.95 ^A	0.16 ± 0.04 ^A	36.01 ± 7.50 ^A	72.37 ± 6.54 ^A	106.95 ± 10.26 ^A	2.51 ± 0.27 ^B	1.16 ± 0.27 ^C	2.23 ± 0.09 ^A	3.68 ± 0.46 ^B
Hybrid	AO × AC	13.53 ± 1.53 ^A	0.39 ± 0.26 ^{AB}	56.71 ± 8.73 ^A	83.84 ± 14.98 ^A	95.73 ± 5.87 ^{AB}	3.19 ± 0.27 ^A	1.22 ± 0.13 ^{AB}	2.62 ± 0.19 ^A	4.41 ± 0.37 ^A
	AO × EC	16.50 ± 1.77 ^A	0.47 ± 0.36 ^A	42.38 ± 11.16 ^A	84.75 ± 15.55 ^A	96.95 ± 6.30 ^{AB}	3.27 ± 0.25 ^B	1.18 ± 0.13 ^{BC}	2.76 ± 0.08 ^A	4.45 ± 0.38 ^B
	EO × AC	13.16 ± 5.81 ^A	0.52 ± 0.48 ^B	53.3 ± 11.11 ^A	94.81 ± 13.21 ^A	79.22 ± 8.82 ^B	4.02 ± 1.00 ^A	1.67 ± 0.64 ^A	2.54 ± 0.33 ^A	5.69 ± 1.61 ^A
	EO × EC	13.45 ± 3.94 ^A	0.64 ± 0.49 ^A	44.90 ± 12.00 ^A	87.39 ± 16.34 ^A	101.05 ± 11.06 ^A	2.79 ± 0.18 ^B	1.11 ± 0.10 ^C	2.53 ± 0.09 ^A	3.90 ± 0.28 ^B

Different capital letters beside the trait names indicate statistically significant differences among species (across gas treatments). Different capital letters after values indicate significant differences among gas treatments (across species). Data are mean values ± SD ($n=4$). Data were tested with general linear model at $\alpha=0.05$, followed by a Bonferroni post-hoc test when the main effect was significant. The significance of the main effects is provided in Supplementary Material (Table S2). Species × treatment interaction was not significant for any trait

AO ambient O_3 (< 10 nmol mol⁻¹), EO elevated O_3 (60 nmol mol⁻¹), AC ambient CO_2 , EC elevated CO_2 (600 $\mu\text{mol mol}^{-1}$), A_{growth} light saturated net photosynthetic rate at growth CO_2 concentration, Chl chlorophyll, g_s stomatal conductance, J_{max} maximum rate of electron transport, LMA leaf mass per area, V_{cmax} maximum rate of carboxylation

Table 2 Leaf chemistry of *Eucalyptus globulus*, *E. grandis*, and hybrid *E. deglupta* × *E. camaldulensis*

Plant	Treatment	Total phenolics (mg g ⁻¹) ^B	Condensed tannins (mg g ⁻¹) ^B	C/N ratio (unitless) ^A	C _{area} (g m ⁻²) ^B	C _{mass} (mg g ⁻¹) ^A	N _{area} (g m ⁻²) ^B	N _{mass} (mg g ⁻¹) ^B	Lignin (%) ^B
<i>E. globulus</i>	AO × AC	125.60 ± 14.03 ^A	3.31 ± 1.01 ^{BC}	49.41 ± 10.74 ^C	36.92 ± 7.59 ^A	50.54 ± 1.17 ^A	0.75 ± 0.10 ^A	1.06 ± 0.22 ^A	6.69 ± 0.85 ^{AB}
	AO × EC	148.42 ± 19.00 ^A	3.93 ± 1.39 ^A	96.71 ± 17.53 ^A	44.17 ± 11.17 ^B	49.97 ± 1.88 ^{AB}	0.46 ± 0.06 ^C	0.54 ± 0.12 ^C	6.09 ± 1.46 ^B
	EO × AC	129.08 ± 6.26 ^A	3.39 ± 0.78 ^C	69.71 ± 6.08 ^B	37.31 ± 3.22 ^A	50.29 ± 0.97 ^A	0.54 ± 0.07 ^B	0.72 ± 0.07 ^B	8.53 ± 2.61 ^A
	EO × EC	132.94 ± 14.25 ^A	4.35 ± 1.23 ^{AB}	94.98 ± 10.5 ^A	44.05 ± 7.56 ^B	49.19 ± 0.75 ^B	0.47 ± 0.09 ^{BC}	0.52 ± 0.06 ^C	5.74 ± 0.76 ^B
<i>E. grandis</i>	AO × AC	140.83 ± 12.81 ^A	5.78 ± 1.13 ^{BC}	48.03 ± 8.34 ^C	37.29 ± 7.50 ^A	48.18 ± 0.44 ^A	0.78 ± 0.05 ^A	1.03 ± 0.21 ^A	6.58 ± 2.03 ^{AB}
	AO × EC	133.86 ± 3.37 ^A	8.23 ± 1.56 ^A	100.11 ± 8.01 ^A	47.03 ± 4.10 ^B	48.20 ± 1.04 ^{AB}	0.47 ± 0.07 ^C	0.48 ± 0.04 ^C	5.24 ± 0.69 ^B
	EO × AC	128.33 ± 17.24 ^A	5.21 ± 0.88 ^C	62.32 ± 3.74 ^B	38.76 ± 3.28 ^A	48.38 ± 1.14 ^A	0.62 ± 0.02 ^B	0.78 ± 0.05 ^B	6.10 ± 0.13 ^A
	EO × EC	131.41 ± 17.56 ^A	6.84 ± 2.03 ^{AB}	90.53 ± 9.29 ^A	50.21 ± 4.68 ^B	46.96 ± 0.63 ^B	0.56 ± 0.08 ^{BC}	0.52 ± 0.04 ^C	5.29 ± 0.89 ^B
Hybrid	AO × AC	107.83 ± 5.58 ^A	6.76 ± 0.94 ^{BC}	43.71 ± 8.61 ^C	47.36 ± 3.62 ^A	49.44 ± 1.01 ^A	1.13 ± 0.29 ^A	1.18 ± 0.31 ^A	9.34 ± 0.50 ^{AB}
	AO × EC	109.70 ± 7.61 ^A	7.95 ± 1.19 ^A	78.57 ± 7.89 ^B	47.28 ± 3.39 ^B	48.75 ± 0.54 ^{AB}	0.60 ± 0.06 ^C	0.63 ± 0.07 ^C	8.59 ± 1.37 ^B
	EO × AC	104.57 ± 4.92 ^A	6.40 ± 0.93 ^C	50.49 ± 8.45 ^B	39.05 ± 4.51 ^A	49.27 ± 0.89 ^A	0.79 ± 0.13 ^B	0.99 ± 0.15 ^B	10.79 ± 2.34 ^A
	EO × EC	115.28 ± 6.31 ^A	7.77 ± 1.55 ^{AB}	66.11 ± 14.89 ^A	49.30 ± 4.44 ^B	48.80 ± 0.49 ^B	0.77 ± 0.14 ^{BC}	0.77 ± 0.18 ^C	8.52 ± 1.48 ^B

Total phenolics, condensed tannin, area-based carbon to nitrogen ratio (C/N), C content per unit leaf area (C_{area}), C content per unit leaf mass (C_{mass}), N content per unit leaf area (N_{area}), N content per unit leaf mass (N_{mass}), lignin, and chlorophyll content of the tested eucalypts. Different capital letters above the trait names indicate statistically significant differences among species (across gas treatments). Different capital letters above the SD values indicate statistically significant differences among gas treatments (across species). Data are mean values ± SD (*n* = 4). The data were tested with General Linear Model at a level of significance $\alpha = 0.05$, followed by a Bonferroni post-hoc test when the main effect was significant. The significances of the main effects are provided in Supplementary Material (Table S2). Species × treatment interaction was not significant for any trait

AO ambient O₃ (< 10 nmol mol⁻¹), EO elevated O₃ (60 nmol mol⁻¹), AC ambient CO₂, EC elevated CO₂ (600 μmol mol⁻¹)

Fig. 2 Gas chromatography profile of *Eucalyptus globulus*, *E. grandis*, and hybrid *E. deglupta* × *E. camaldulensis*. *n* = 4. AOAC ambient O₃ + CO₂, EOAC elevated O₃ + ambient CO₂, AOEC ambient O₃ + elevated CO₂, EOEC elevated O₃ + elevated CO₂

the relative concentration of compound 2 in AO; the value decreased when EO and EC were combined in such an extent that it was not significantly different from AO × AC (Fig. 4b). On the other hand, EC significantly decreased the relative concentration of compound 3 in AO, and this

CO₂-induced effect also prevailed in EO, ruling out the EO-induced effect (Fig. 4c).

GC/MS analysis revealed that compound 1 is an alkane, *n*-icosane (Fig. 5). Based on the mass fragmentation resulted from GC/MS analysis, the library provided a match for

Fig. 3 Mean ($\pm$ SD) relative concentration of compound 1 calculated from GC profiles for peak at RT 34.8 min. **a** *Eucalyptus grandis*. **b** *E. deglupta* $\times$ *E. camaldulensis*. Different letters above bars indicate means differed significantly ($n=4$). AOAC ambient O_3 and CO_2 , EOAC elevated O_3 + ambient CO_2 , AOEC ambient O_3 + elevated CO_2 , EOEC elevated O_3 + elevated CO_2 . The data for *E. globulus* are presented in Fig. 4 because of its distinct peaks

compounds 2 and 3: 2,6-octadien-1-ol, 3,7-dimethyl-, acetate, (E)-geranyl acetate or geranyl acetate (Fig. 6) and cyclohexane, 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene) or elixene (Fig. 7). These terpenes are commonly found in plant essential oils.

Discussion

Despite the species-specific biomass A_{growth} , V_{cmax} , and J_{max} , the fact that the eucalypts biomasses A_{growth} , V_{cmax} , and J_{max} were not significantly affected by gas treatments suggests that the three species are not susceptible to EO and EC (Agathokleous and Saitanis 2020). This hypothesis is further supported by lack of a significant effect on the R/S ratio, indicating no adverse single or combined effect of EO and EC (Agathokleous et al. 2016b, 2019b). *E. globulus* has been found to be tolerant to acute O_3 exposures, as indicated by unaffected physiological measures (including photosynthetic traits and lipoxygenase pathway emission

rates) when exposed to $0.3\text{--}2.0\ \mu\text{mol mol}^{-1}$ O_3 for a few hours (O'Connor et al. 1975; Kanagendran et al. 2018). In a different study, *E. globulus* had neither visible injury nor biomass reduction after exposure to diurnally varied concentrations of 26 or $172\ \text{nl l}^{-1}$ for $7\ \text{h day}^{-1}$, 5 days every 14 days, for 18 weeks (Monk and Murray 1995). Although that study with a chronic exposure (Monk and Murray 1995) and other studies with acute exposures (O'Connor et al. 1975; Kanagendran et al. 2018) indicate that *E. globulus* is tolerant to EO, a different study suggested an “extreme sensitivity”, with reduced biomass, A_{growth} and g_s , after seedlings were exposed to $50\ \text{nmol mol}^{-1}$ ($7\ \text{h day}^{-1}$) for 37 days under low light and controlled temperature ($20\ ^\circ\text{C}$) (Pearson 1995); however, the susceptibility of the plants might have been affected by the low light condition and/or the controlled temperature. Chronic and acute exposure to O_3 can differ in their effects on plant physiology due to secondary acclimation responses (Liu et al. 2019). However, our study and the study of Monk and Murray (1995) indicate that *E. globulus* is tolerant to O_3 , and the tolerance of *E. grandis* and the hybrid seem to be similar that of *E. globulus* to EO. It is therefore important to pinpoint biological mechanisms underpinning the tolerance of these eucalypts to the gas treatments.

The absence of a significant effect of the gas treatments on A_{growth} , V_{cmax} and J_{max} may indicate acclimation, as reported for many species (Koike et al. 1996; Tissue et al. 1999; Watanabe et al. 2011). This may explain why no significant biomass enhancement or inhibition was induced by EC and EO, respectively. Not only elevated O_3 (Kitao et al. 2009; Koike et al. 2012), but also elevated CO_2 is known to induce stomatal closure (Chater et al. 2015; Dusenge et al. 2019). However, this was not the case in our experiment with the three eucalypts, where EC did not affect g_s in AO but increased g_s in EO. This EC-induced increase in g_s in EO, however, may indicate a permanent stomatal impairment when EO is combined with EC (Hoshika et al. 2019).

Interestingly, EC tended to increase LMA, especially in EO where the effect was significant. This increase has been observed often in plants grown in elevated CO_2 concentrations and may result from accumulation of carbohydrates that are not used for plant growth (Hikosaka et al. 2005). Increased LMA may favor plant growth in atmospheres with elevated CO_2 concentrations by compensating for otherwise reduced leaf N concentration per unit mass (Hikosaka et al. 2005), which may account for the observed EC-induced decrease in N_{mass} in both AO and EO. Increased LMA may also indicate enhanced stored carbohydrates (Booker 2001).

Elevated O_3 exposures have been extensively shown to decrease the level of chlorophylls, an effect associated with inhibition of quantum yield (Saitanis et al. 2001; Li et al. 2017). However, we found no significant effect of the tested EO exposure on the three eucalypt species. We postulate

Fig. 4 Mean ($\pm$ SD) relative concentration of compounds calculated from GC profiles for distinctive peaks on *Eucalyptus globulus*. **a** Peak at RT 34.8 min. The RT is the same as the peaks in Fig. 3. **b** Peak at RT 17.2 min. **c** Peak at RT 24.1 min. Different letters above bars indi-

cate means differed significantly ($n=4$). AOAC ambient O₃ and CO₂, EOAC elevated O₃ + ambient CO₂, AOEC ambient O₃ + elevated CO₂, EOEC elevated O₃ + elevated CO₂

that the three eucalypts might have maintained the chlorophyll content as a tolerance mechanism to maintain the photosynthetic activity at homeostatic levels. Conversely, EC decreased chlorophyll levels in AO and EO. The CO₂ effect on chlorophyll pigments depends on CO₂ concentration (concentration-specific), and our findings agree with those of other studies that show an EC-induced negative effect on chlorophyll levels that can result from reallocation of limiting resources away from the photosynthetic apparatus (Ong et al. 1998; Grams et al. 1999; Ormrod et al. 1999; Pritchard et al. 2000). Since we analyzed chlorophyll levels only at the end of the experiment, the EC-induced decrease in chlorophyll contents in AO and EO may also be due to accelerated development induced by EC (Centritto and Jarvis 1999), although there are no empirical data to support this hypothesis.

The finding that phenolics and tannins were not significantly affected by EO, but tannins were increased by EC when O₃ was elevated, suggests that total phenolics were insensitive but tannins were sensitive to the gas treatment. This finding also suggests that when EO and EC are combined, plant–herbivore interactions might be affected because increased tannins would decrease leaf palatability.

Elevated O₃ and CO₂ can alter C and N metabolism, stock, and allocation to different plant organs (Uddling et al.

2006; Ainsworth et al. 2007; Dusenge et al. 2019; Shang et al. 2019a, b). However, C_{area} and C_{mass} in the three eucalypts were not significantly affected by EO in our study. The results suggest that EC drove the C response in EO as indicated by increased C_{area} and decreased C_{mass}. The fact that the EO-induced decrease in N_{area} and N_{mass} and the increased in C/N ratio were offset by EC, indicates that N response is regulated by EC but not by EO in elevated O₃. The increased C/N ratio (commonly in both AO and EO) by EC was due to decreased N_{area} and N_{mass}. Bloom et al. (2010) reported that elevated CO₂ inhibited the assimilation of nitrate into organic N compound in leaves of wheat and *Arabidopsis*. This inhibition of nitrate assimilation may lead to the decreased organic N content and may play an important role in the photosynthetic acclimation under EO (Bloom et al. 2010). The EC-induced decrease in leaf N might also be due to inhibited N resorption. For example, elevated O₃ substantially impairs N resorption in birch leaves before leaf abscission (Uddling et al. 2006; Shi et al. 2017), causing a significant loss in foliar N. The impairment in N resorption may be due to impaired phloem-loading in leaves due to accumulated starch along leaf veins (Uddling et al. 2006). That the species $\times$ treatment interaction was not significant while the single effects of species and treatment were significant suggests that C and N metabolism was an important

Fig. 5 Mass fragmentation of compound 1 at RT 34.8 min in GC/MS of extracts from *Eucalyptus globulus*. Possible chemical name is *n*-icosane based on comparison with GC/MS library. Insert is from the NIST/EPA/NIH/EINECS/IRDB library

common mechanism among the three eucalypts under the tested gas treatments.

Area-based traits are important for studying photosynthesis-related processes that are measured as a flux per unit of leaf area, whereas mass-based traits are important for studying leaf economy in terms of biomass investment for carbon fixation (Hikosaka 2004; Shang et al. 2019b). Thylakoid N is proportional to the chlorophyll content (e.g., 50 mol thylakoid N per mol chlorophyll); hence, there is a positive correlation between chlorophyll content and total leaf N under typical growth conditions (Evans 1989). A recent study on O_3 effects on two poplar clones revealed that chlorophyll per unit mass was negatively correlated with N_{mass} , whereas chlorophyll per unit area was positively correlated with N_{area} (Shang et al. 2019b). Conversely to the negative correlation that Shang et al. (2019b) found in poplars, a regression analysis with the data of all eucalypt species and gas treatments from our study revealed that chlorophyll per unit mass was positively correlated with N_{mass} ($y = 0.1349x + 0.1456$, $r = 0.571$, $F = 22.2$, $P < 0.001$); interestingly, the R^2 value was nearly identical with that found by Shang et al. (2019b). The finding that EC increased the C_{area} but tended to decrease C_{mass} content (although significant only in EO) and the findings of Shang et al. (2019b) suggest

that care should be exercised when selecting the appropriate unit for assessing C and N responses to gaseous treatments.

Lignin content was significantly decreased by EC in EO but was not significantly affected by EC in AO. However, this difference seems to be due to higher lignin content in $EO \times AC$ (compared to $AO \times AC$),

Elevated CO_2 can modify lignin concentration in leaves, but the effect is species specific, and no general conclusion can be drawn (Coûteaux et al. 1999; Norby et al. 2001; Zheng et al. 2019). In the present study, EC decreased lignin in EO but not in AO; however, this difference seems to be due to more lignin in $EO \times AC$ (compared to $AO \times AC$). Blaschke et al. (2002) reported that lignin in seedlings grown with sufficient nutrients was unaffected or even decreased by elevated CO_2 . Because nutrients were normally supplied in the present study and lignification is a physiologically important process during growth, development and tissue maturation in woody plants (e.g., Blaschke et al. 2002), impairment of lignification may be related to the unaffected biomass in high CO_2 . Interestingly, a regression analysis of the data of all eucalypt species and gas treatments from our study revealed that lignin was negatively correlated with leaf biomass ($y = -0.535x + 11.602$, $r = 0.537$, $F = 18.6$, $P < 0.001$) and with stem biomass ($y = -0.626x + 10.093$,

Fig. 6 Mass fragmentation of compound 2 at RT 17.2 min in GC/MS of extracts from *Eucalyptus globulus*. Possible identity of compound is based on comparison with GC/MS library. Insert is from the NIST/EPA/NIH/EINECS/IRDB library

$r = 0.606$, $F = 26.7$, $P < 0.001$), but was not correlated with root biomass ($y = 0.296x + 6.742$, $r = 0.222$, $F = 2.4$, $P = 0.130$).

GC profiles of the leaf extracts suggest that the compounds *n*-icosane, geranyl acetate and elixene seem to be involved in the eucalypts tolerance to O₃ and CO₂. In particular, *n*-icosane appears to be involved in the response mechanisms of *E. grandis* and the hybrid, and its O₃-induced increase was partly or fully offset by EC. *n*-Icosane is a saturated aliphatic hydrocarbon (a component of wax that is commonly found in leaf epicuticle; Dubis et al. 2001). Epicuticular waxes act as physical barrier against pathogen penetration. Air pollutants, such as O₃, could alter the structure of leaf waxes from crystallite to amorphous with longer carbon chains (e.g., Karnosky et al. 2002). The increased relative concentration of *n*-icosane induced by EO may indicate a defensive mode for preventing O₃ penetration into the leaves. Epicuticular waxes can also be found surrounding the stomata (photo data not shown). EO may enhance the synthesis of *n*-icosane for accumulation in higher concentrations around the stomata (and therefore O₃ uptake through stomata to be reduced). Geranyl acetate and elixene appear

to be involved in the O₃ response mechanisms of *E. globulus*. The relative concentration of geranyl acetate and elixene were increased by EO in AC. However, EC increased the relative concentration of geranyl acetate and decreased the relative concentration of elixene in AO and ruled out an O₃ effect and acted as the primary control of geranyl acetate and elixene in EO. A recent study also revealed that six formylated phloroglucinol compounds (metabolites of Myrtaceae), including five macrocarpals and one sideroxylonal, showed distinct patterns to single and combined effects of acute O₃ exposure ($5 \mu\text{mol mol}^{-1} \times 3 \text{ h}$) and wounding in *E. globulus*; total macrocarpals and total formylated phloroglucinol compounds increased by single elevated O₃ (Liu et al. 2019). Geranyl acetate is a component of plant essential oils that are released from fresh plants as a possible defense mechanism against damage from insects (e.g., Carpino et al. 2004; Peñaflor et al. 2011). Elixene is also a terpene essential oil and insecticidal (e.g., Wang et al. 2011). O₃ can act as an abiotic elicitor of plant defense reaction and generate adaptive responses, which precondition plants for more severe environmental challenges, by activating defense signaling networks and enhancing “stress coping skills”, such as

Fig. 7 Mass fragmentation of compound 3 at RT 24.1 min in GC/MS of extracts from *Eucalyptus globulus*. Possible chemical name is based on comparison with the GC/MS library. Insert is from the NIST/EPA/NIH/EINECS/IRDB library

antioxidative systems (Sandermann et al. 1998; Agathokleous et al. 2019a). Such organismic responses can involve fitness trade-offs between defense and growth/reproduction (Karabourniotis et al. 2014; Agathokleous and Calabrese 2020); however, based on the studied traits, none of the tested eucalypts displayed a negative fitness response to the gas treatments.

Conclusion

Overall, EO and EC, singly and in combination, had no effect on the biomass of the three eucalypts. EO had limited effects on leaf physiology (N_{area} , N_{mass} , n -icosane, geranyl acetate, elixene) in AC. EC affected more traits of leaf physiology (g_s , LMA, tannins, C_{area} , C_{mass} , lignin, n -icosane, geranyl acetate and elixene) and offset most of the limited EO effects on leaf physiology. *E. globulus*, *E. grandis* and the hybrid eucalypt appear to be tolerant to a chronic realistic exposure of O₃ and CO₂. n -Icosane seems to be involved in the tolerance mechanisms of *E. grandis* and the hybrid,

and geranyl acetate and elixene seem to be involved in the tolerance of *E. globulus*. Although the mass fragmentation of the compounds mentioned herein is similar to those in the GC/MS library, further studies are needed to verify whether these specific compounds in plants are involved in the mechanisms of tolerance to O₃ and CO₂.

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