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Influence of Elevated Carbon Dioxide and Ozone on the Foliar Nonvolatile Terpenoids in *Ginkgo Biloba*

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Abstract The aim of this study was to determine the effects of elevated O_3 and elevated CO_2, singly and in combination, on the contents of nonvolatile terpenoids in leaves of *Ginkgo Biloba*. The results showed that elevated CO_2, alone and in combination with elevated O_3 increased concentrations of all the determined terpenoids, while elevated O_3 alone only increased concentration of bilobalide. These results demonstrated that the metabolism of terpenoids in ginkgo leaves was more sensitive to elevated CO_2 than elevated O_3.

Keywords *Ginkgo Biloba* · Terpenoids · Elevated CO_2 · Elevated O_3

Atmospheric concentrations of greenhouse gases CO_2 and O_3 are continuously rising due to human activities (IPCC 2001). The elevated atmospheric CO_2 and O_3 concentrations might change the emission of volatile terpenoids (e.g. isoprene, monoterpenes, sesquiterpenes and diterpenes) from plants (Constable et al. 1999; Heiden et al. 1999). These changes may affect the interactions between plant and herbivore or microbes and may affect also atmospheric compositions, then lead to an indirect impact on global changes (Langenheim 1994; Litvak and Monson 1998; Monson 2002). Previous studies, with different gas exposure systems (indoor growth chamber, open-top chamber or free-air enrichment), different plant species (woody or herbaceous) and different growth conditions (in pots or on ground), showed that elevated CO_2, O_3, alone or in combination, had variable effects on the emissions of isoprene, monoterpenes and diterpenes from plants (Loreto et al. 2001; Sallas et al. 2001; Rosenstiel et al. 2003; Possell et al. 2005; Vuorinen et al. 2005). However, most of the attention has been paid to the volatile terpenoids. We have little knowledge about the effect of elevated CO_2 and O_3 on the nonvolatile terpenoids, especially in deciduous trees. Therefore it is important to evaluate the response of nonvolatile terpenoids to elevated ozone and carbon dioxide, so as to have an in-depth understand about the effects of the increasing greenhouse gases on secondary metabolism in plant.

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Materials and Methods

In this study, the four-year-old saplings of *Ginkgo Biloba* L., which is a geologically old tree species with high content of nonvolatile terpenoids in leaves (1–3 mg/g dry weight), were exposed to the elevated ozone and carbon dioxide in open top chambers. This experiment was conducted at Shenyang Arboretum of Chinese Academy of Sciences (41°46 ′N, 123°26 ′E) in an urban area. The factorial design has been reported (He et al. 2006). In April 2006, twelve open-top chambers (OTC) were assigned to four different treatments with three replicates each. Twenty soil-grown trees were randomly planted in each chamber.

The treatments were elevated CO_2 (700 μmol mol^{-1}, EC), elevated O_3 (80 nmol mol^{-1}, EO) and elevated CO_2 plus elevated O_3 in combination (700 μmol mol^{-1} + 80 nmol mol^{-1}, EC + EO); the chambers with ambient air were used as control. Gas cylinders containing pure liquid CO_2 were used as a source of CO_2 gas. Ozone was generated by O_3 generator (GP-5J, China) that used oxygen-enriched dry air as the source gas. Ozone and CO_2

Table 1 Recorded mean values of O_3 and CO_2 concentrations in OTCs

Ambient O_3 (ppb)	Elevated O_3 (ppb)	Ambiet CO_2 (ppm)	Elevated CO_2 (ppm)
40.3 ± 4.6	84.2 ± 1.6	390 ± 17	727 ± 32

Ozone and carbon dioxide exposure are based on hourly mean values. O_3 concentration was elevated only in day time (from 8:00 to 17:00). CO_2 was elevated all day

concentrations inside the chambers and in the ambient air were monitored by O_3 analyzers (S-900 Aeroqual, New Zealand) and CO_2 sensor (SenseAir, Sweden). The concentrations of CO_2 and O_3 were controlled by computers, using a professional program for CO_2 and O_3 dispensing and monitoring.

The trees were exposed to high CO_2 for 24 h day^{-1} in EC chambers, and exposed to O_3 for 9 h (08:00–17:00) day^{-1} in EO chambers. The fumigation period was 25 June–10 October in 2006. The mean CO_2 and O_3 concentration in this period are given in Table 1.

Fully developed leaves were sampled from all individual trees in all the chambers at 9 A.M. on 15 June, 7 July, 27 July, 17 August, 7 September and 27 September. Samples were air-dried at room temperature and then ground to powder and stored at −20°C until analyses (Peltonen et al. 2005). Parallel samples were dried at 80°C to calculate the dry weight per leaf.

For the terpenoids determination, a high performance liquid chromatography electrospray mass spectrometry method was used (Zhou et al. 2005) (In Chinese). The terpenoids were quantified on the basis of the commercial standards: Ginkgolide A from *Ginkgo Biloba* leaves (Sigma-Aldrich, Steinheim, Germany) for ginkgolides A; Ginkgolide B from *Ginkgo Biloba* leaves (Sigma-Aldrich, Steinheim, Germany) for Ginkgolide B and Ginkgolide C; (−)-Bilobalide from *Ginkgo Biloba* leaves (Sigma-Aldrich, Steinheim, Germany) for Bilobalide.

All the data were averaged from three replicates. Terpenoid concentrations were pooled from six sampling dates to calculate the yearly mean concentrations. Then data were processed with univariate analysis of variance (ANOVAR) using SPSS 11.5 for Windows statistical software (SPSS, Chicago, IL, USA). The terpenoids concentrations were used as within-subjects factors, and CO_2 and O_3 were used as between-subjects factors. Between-subjects factors effects were also estimated using graphical vector analysis (Peltonen et al. 2005).

Results and Discussion

The treatments had significant effects on the leaf dry weight: the EC increased the leaf dry weight by 26%, whereas EO decreased leaf dry weight by 22%. Almost like the EC treatment, EC + EO increased 20% in leaf dry weight (Table 2).

Elevated CO_2 significantly increased yearly means of concentrations of terpenoids in ginkgo leaves: increased the concentration of BB by 35%, GA by 65%, GB by 31% and GC by 31% ($p = 0.003$, $p = 0.003$, $p = 0.003$ and $p = 0.007$, respectively) (Tables 3 and 4). According to the graphic vector analysis, the increase in concentration of BB, GA, GB and GC by EC was a result of induced accumulation of these compounds (Fig. 1). Whether EC affects the emissions of volatile terpenoids in woody plant species is still controversial, but according to Li et al. (unpublished data), EC significantly enhanced isoprene and

Table 2 Yearly means (±SD) and variance analysis of leaf dry weight (mg) of ginkgo leaves

	CC	EC	EO	EC + EO
Leaf dry weight	146.8 ± 28.9	184.1 ± 45.2	114.2 ± 19.8	176.5 ± 38.2
		CO_2	O_3	$CO_2 + O_3$
Leaf dry weight		↑**	↓*	↑*

↑, increase; ↓, decrease; NS, non significant

* $p < 0.1$; ** $p < 0.05$; *** $p < 0.001$ (Peltonen et al. 2005)

CC, chamber control; EC, elevated CO_2; EO, elevated O_3

Table 3 Yearly mean concentrations of terpenoids in ginkgo leaves

	CC	EC	EO	EC + EO
BB	1.54 ± 0.16	2.08 ± 0.15	2.14 ± 0.04	2.15 ± 0.07
GA	0.26 ± 0.02	0.43 ± 0.02	0.32 ± 0.05	0.35 ± 0.03
GB	0.16 ± 0.01	0.21 ± 0.02	0.18 ± 0.01	0.19 ± 0.02
GC	0.32 ± 0.05	0.42 ± 0.05	0.34 ± 0.01	0.41 ± 0.01
Total terpenoids	2.28 ± 0.23	3.15 ± 0.24	2.97 ± 0.03	3.11 ± 0.09

Mean value was averaged from six sampling dates (mg g^{-1} dry mass ± SD)

Table 4 Univariate variance analysis (ANOVAR) result of between-subjects main effects and their interactions for yearly mean concentrations of individual terpenoids in ginkgo leaves

Compounds/Group	CO_2	O_3	$CO_2 \times O_3$
BB	↑**	↑***	↑**
GA	↑***	NS	↑**
GB	↑**	NS	↑*
GC	↑**	NS	NS
Total terpenoids	↑***	↑**	↑**

↑, increase; ↓, decrease; NS, non significant

* $p < 0.1$; ** $p < 0.05$; *** $p < 0.001$ (Peltonen et al. 2005)

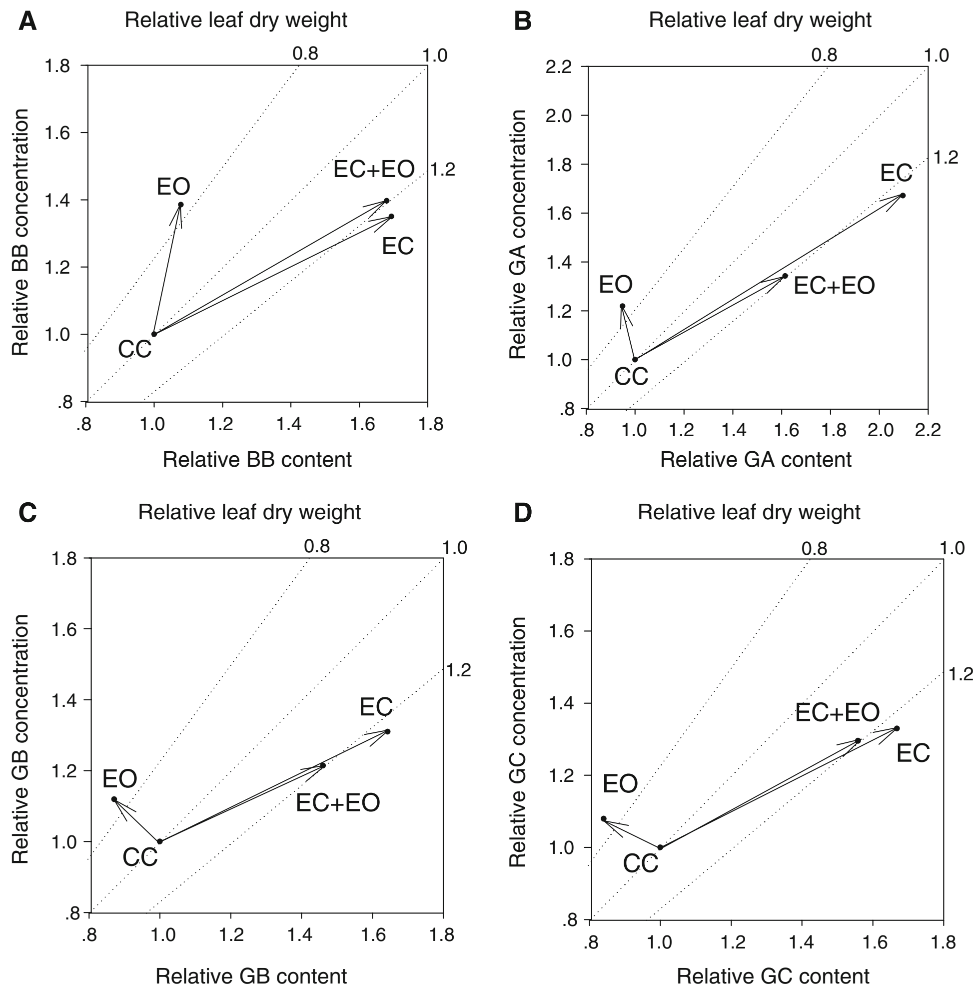

Fig. 1 Effects of treatments on the relative concentration, relative content of nonvolatile terpenoids and the relative leaf dry weight in the ginkgo leaves in 2006. The values for the CC trees were used for the calculation of the relative values and the CC treatment was used as a reference point (1, 1, 1). For the interpretation of the vectors, see the reference in Table 5

Table 5 Interpretations of shifts in the yearly mean of terpenoids content (*x*-axis), concentration (*y*-axis) and leaf dry weight (diagonal lines) in graphical vector analysis

Content	Concentration	Dry weight	Interpretation
+	0	+	Steady increase
+/0	−	+	Dilution effect
−	−	+/−/0	Reduced accumulation
−	0	−	Steady decrease
−/0	+	−	Concentration effect
+	+	+/−/0	Induced accumulation

+, increase; −, decrease; 0, no change. (Peltonen et al. 2005)

monoterpenes emission from *Ginkgo Biloba*. So, increased carbon assimilates due to EC may result in carbon allocation to both volatile and nonvolatile terpenoids synthesis in ginkgo leaves.

Elevated O_3 also increased the concentration of BB by 39%, GA by 22%, GB by 12% and GC by 6% in ginkgo leaves, but the significant increase was only found in BB ($p = 0.001$) (Tables 3 and 4). According to the graphic vector analysis, EO only increased the content of BB, but reduced the content of other three compounds (Fig. 1). The in-significance of increase in concentrations of GA, GB and GC were a result of concentration effect, as the EO significantly reduced leaf dry weight (Table 2). So, content-based expression is a better indicator for carbon allocation to terpnenoids synthesis. In addition, according to the earlier reports on volatile terpenoids which showed that elevated ozone activates defense responses in plants, and may thereby trigger the emission of terpenoids from several defense pathways (Heiden et al. 1999; Vuorinen et al. 2004), the inducible BB might be a protective compound in ginkgo leaves under EO stress in this study.

Elevated CO_2 and O_3 in combination significantly increased the concentrations of BB by 40%, GA by 34% and GB by 19% ($p = 0.005$, $p = 0.004$ and $p = 0.088$, respectively) (Tables 3 and 4). The vector analysis showed that EC + EO resulted in an induced accumulation effect in BB, GA and GB (Fig. 1a, b and c). This result was consistent with earlier report on *Pinus sylvestris* which indicated that EC + EO increased resin acids (diterpenes) concentrations (Valkama et al. 2007). In addition, EC + EO had no effect on sesquiterpenes in *Pinus* *sylvestris,* but our data showed that EC + EO increased the concentrations of sesquiterpenes (GA and GB) in ginkgo leaves.

In summary, elevated CO_2, alone and in combination with elevated O_3 increased concentrations of all the determined terpenoids, while elevated O_3 alone only increased concentration of bilobalide in leaves of *Ginkgo Biloba*. Nonvolatile terpenoids are relatively sensitive to elevated greenhouse gases, especially to elevated CO_2. BB was the most responsive compounds of the nonvolatile terpenoids. The increase in BB may be induced by increased substrate of its synthesis in the environment of elevated CO_2 but resulted by some other different inducing effect by elevated O_3. Elevated CO_2 and elevated O_3 may act on some common metabolic rout in BB synthesis since their effect is not additive.

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