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# Changes of main secondary metabolites in leaves of *Ginkgo biloba* in response to ozone fumigation

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**Abstract**

To investigate the effect of elevated $O_3$ on the accumulation of main secondary metabolites in leaves of *Ginkgo biloba* L., four-year-old trees were exposed in open-top chambers with ambient air and the air with twice ambient $O_3$ concentration in Shenyang in 2006. Elevated $O_3$ increased the concentrations of terpenes, but decreased the concentrations of phenolics in *G. biloba* leaves. The results showed that secondary compounds from *G. biloba* leaves responded to the elevated $O_3$ exposure in a different way when compared to previous studies which showed elevated $O_3$ increased the concentrations of phenolics but had no effect on the terpenes in leaves of other deciduous trees. Furthermore, reduced synthesis of phenolics may decrease the resistance of *G. biloba* to $O_3$ and other environmental factors. On the other hand, the induced synthesis of terpenes may enhance the antioxidant abilities in *G. biloba* leaves at the end of $O_3$ fumigation.

**Key words**: *Ginkgo biloba*; elevated $O_3$ concentration; open-top-chamber; secondary metabolites
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## Introduction

Despite increasing environmental awareness and regulations designed to limit industrial and vehicle emissions, ozone levels are potentially harmful to human health and vegetations. Background concentrations in the troposphere have doubled compared to the pre-industrial years and there is also evidence of an increase in annual mean concentrations estimated ranging from 0.1 to 1 nmol/mol per year (Coyle *et al*., 2003).

Secondary metabolites, including phenolic compounds, terpenes and alkaloid, are the compounds produced in plant for different functions in plant tissues, such as growth regulators, antioxidants, enzyme inhibitors, pigments and UV light screens. Some interact with herbivores, microbes, fungi and nematodes as chemical signals and toxins (Koes *et al*., 1994; Cooper-Driver and Bhattacharya, 1998; Seigler, 1998).

Ozone has been demonstrated to affect not only on the primary, but also on secondary metabolism of vegetation (Jordan *et al*., 1991; Kangasjärvi *et al*., 1994; Eckey-Kaltenbach *et al*., 1994; Booker and Miller, 1998). Ozone fumigation has been shown to increase the activities of phenylalanine-ammonium lyase and chalcone synthase enzymes controlling phenylpropanoid and flavonoid biosynthesis pathways (Kangasjärvi *et al*., 1994). These pathways play significant roles in plant defence responses because they synthesize many potentially protective compounds such as condensed tannins, flavonoids and other phenolics, which act as scavengers of various oxygen species such as superoxide anion, singlet oxygen, hydroxyl radical (Harborne and Williams, 2000). Ozone-caused increases have been reported especially in phenolics (such as condensed tannins, low molecular phenolics and flavonoids concentrations) in deciduous trees species and terpenes (monoterpenes and sesquiterpenes) emissions in coniferous tree species (Rosemann *et al*., 1991; Jordan *et al*., 1991; Lavola *et al*., 1994; Wellburn and Wellburn, 1996; Oksanen and Saleem, 1999; Saleem *et al*., 2001; Yamaji *et al*., 2003; Peltonen *et al*., 2005), providing protection against oxidative damage (Langenheim, 1994; Foy *et al*., 1995; Ormrod *et al*., 1995). It has been suggested that ozone-induced accumulation of phenolics in plants are needed for defense in response to ozone stress (Richter and Wild, 1988; Foy *et al*., 1995; Lavola, 1998).

*Ginkgo biloba* L., a deciduous gymnosperm species, is the only remaining species of the once large order Ginkgoales, with geological records indicating this plant has been growing on the Earth for 150–200 million years. In this study, we hypothesized that: (1) elevated $O_3$ increases the concentrations of antioxidant phenolic compounds (flavonoids and condensed tannins) in *G. biloba* leaves like

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detected in other deciduous trees; (2) elevated $O_3$ increases the concentration of terpenes in the leaves like detected in other coniferous gymnosperm trees.

# 1 Materials and methods

## 1.1 Experimental design

The experiment was conducted at Shenyang Arboretum of Chinese Academy of Sciences, China (41°46′N, 123°26′E) located in an urban area. The factorial design was used in the field experiments according to He *et al*. (2006). In April 2006, six open-top chambers (OTCs) were assigned to two different treatments with three replications. Twenty 4-year-old soil-grown *G. biloba* trees were randomly planted in each chamber. The elevated $O_3$ (EO) concentration was 80 nmol/mol. The chambers with ambient air were used as control (CC). The background $O_3$ concentration in ambient air was about 40 nmol/mol. The concentration of $O_3$ were controlled by computers, using a professional program for $O_3$ dispensing and monitoring. The trees were exposed to elevated $O_3$ for 9 h (08:00–17:00) per day in EO chambers. The fumigation period was from 15 June to 10 October in 2006. The mean $O_3$ concentrations in this period were given in Fig. 1.

## 1.2 Sampling

Fully developed leaves from middle canopy were sampled from all individuals as mixed sample in each chamber at 9 a.m. on 15 June, 7 July, 27 July, 17 August, 7 September, and 27 September in 2006. Samples were air-dried at room temperature, ground into powder, and stored at –20°C until analyses (Peltenen *et al*., 2005).

## 1.3 Chemical analyses

Methanol extractable condensed tannins were determined using a butanol-HCl test (Porter *et al*., 1986; Hagerman, 1995). Purified tannin from the leaves of *Betula nana* (L.) was used as a standard.

For flavonoids, an indirect quantitative SPE-HPLC-UV method described by Hasler (1990) was used with some modifications: reflux 100 mg leaf powder with 8 mL MeOH and 2 mL of 25% HCl during 1 h. After cooling, samples were centrifuged (10000 ×*g* for 5 min). The supernatant was transferred into a 15-mL test tube and dried with nitrogen. The dried sample was redissolved in 5 mL of MeOH and filtered through a Bond Elut C18 SPE cartridge equilibrated with MeOH, then eluted with 8 mL MeOH. The eluate was diluted with MeOH in a 10-mL volumetric flask. The samples were analyzed by HPLC-UV (Waters 2695, Palo Alto, USA) with a quarternary solvent delivery system, an autosampler and a photodiode array detector coupled with an HP data system. The analysis was performed at a flow rate 1.5 mL/min. The UV detector was set at λ = 370 nm. Ten microliter of sample were injected and were separated on a Waters XTerra RP-18 ODS column (4.6 × 250 mm, 5 μm particles) maintained at 30°C. Elution with MeOH (solvent A) and 0.5% $H_3PO_4$ in $H_2O$ (solvent B) was carried out in an isocratic manner as follows (A:B, 60:40, *V/V*) (for 15 min).

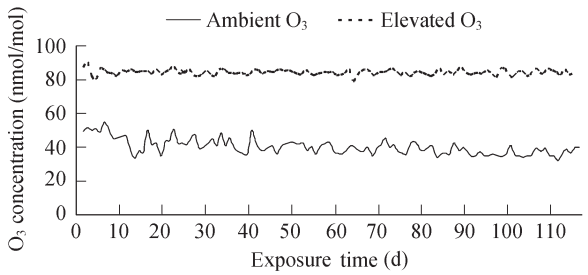


**Fig. 1** Seasonal variations of the $O_3$ concentrations of ambient and elevated $O_3$ in open-top chambers (OTCs) in 2006. Each point represents a daily mean value of three OTCs (08:00–17:00).

For the terpenes determination, a high performance liquid chromatography-electrospray mass spectrometry method was used (Zhou *et al*. 2005). The mass spectrum analysis was performed on a ZQ micromass mass spectrometer (Waters Co., USA). The ionization mode was electrospray ionization (ESI), the polarity mode was negative, and the source temperature was 120°C. The ion-energy was 0.5 V. The capillary voltage was 3.5 kV. The sample cone voltage was 70 V, and the extractor cone voltage was 4.1 V. The cone gas flow-rate was 150 L/h and the desolvation flow rate was 250 L/h. The desolvation temperature was 350°C. The analysis was performed at a flow rate 0.3 mL/min. Ten microliter of sample were injected and were separated on a Waters XTerra RP-18 ODS column (3.9 × 150 mm, 5 μm particles) (USA) maintained at 30°C. Elution with MeOH (solvent A) and $H_2O$ (solvent B) was carried out in a direct gradient manner as follows: 0–15 min, 95%–65% of B in A.

The identification of the secondary components in *Ginkgo* leaves was based on their retention time and UV spectra or *m/z* (mass-to-charge ratio). The quantification was based on commercial reference standards as follows: quercetin dehydrate, keampferol, isorhamnetin, Ginkgolide A and B from *G. biloba* leaves and (–)-Bilobalide from *G. biloba* leaves. All the standards were bought from Sigma-Aldrich (Steinheim, Germany).

## 1.4 Statistical and graphical analysis

All data were averaged from 3 replications and processed with univariate analysis of variance (ANOVAR) using SPSS 11.5. One-way ANOVA was used to determine statistically significant differences in concentrations of secondary metabolites by treatments in each sampling date (Chen *et al*., 2008).

# 2 Results

## 2.1 Phenolics

Condensed tannin concentrations varied in response to $O_3$, which was 15% lower in elevated $O_3$ treatments relative to unenriched treatments. But the effects of $O_3$ were strongly time-dependent, with the largest difference between treatment and control occurring in July, 2006 (Fig. 2).

Different flavonoids responded to elevated $O_3$ in different ways. Levels of quercetin in $O_3$-enriched foliage

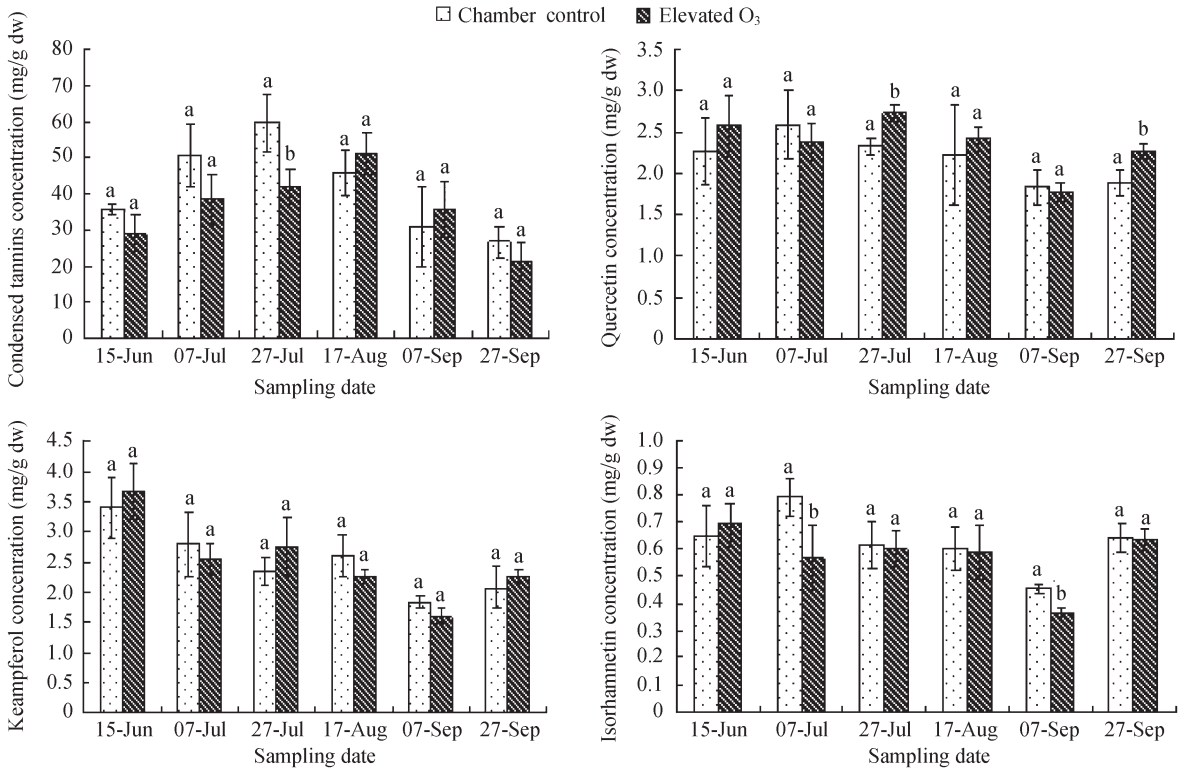


**Fig. 2** Effects of elevated $O_3$ on concentration of condensed tannin, flavonoids quercetin, keampferol, and isorhamnetin in *Ginkgo* leaves at different sampling times in 2006. Values are means ± SE ($n$ = 3 chambers). Different letters show significance at the 0.05 level for each sampling time. dw: dry weight; CC: chamber control; EO: elevated $O_3$.

tended to be higher than in unenriched foliage. The concentrations of quercetin were increased significantly in July and September; the concentration of keampferol in *Ginkgo* leaves showed no response to elevated $O_3$; and the concentration of isorhamnetin was decreased significantly in early July and early September, which was averaged 10% lower in elevated $O_3$ treatment relative to unenriched treatment (Fig. 2).

### 2.2 Terpenes

Terpenes concentrations in *Ginkgo* leaves also varied in response to $O_3$ (Fig. 3). The effects of $O_3$ were strongly time-dependent, with the largest difference occurring in September compared to control. Elevated $O_3$ increased concentration of bilobalide by 220% ($p < 0.001$), of Ginkgolide C by 69.6% ($p < 0.01$), of Ginkgolide A by 34.1% ($p < 0.01$) and of Ginkgolide B by 34.3% ($p < 0.01$) on 27 Septemeber. But elevated $O_3$ reduced concentration of bilobalide by 13.2%, of Ginkgolide C by 28.1% and of Ginkgolide B by 15.2% on 27 July (Fig. 3).

## 3 Discussion

In this article we investigated in detail the dynamic responses of secondary metabolites in the leaves of *Ginkgo* to elevated $O_3$. The results showed that secondary compounds from *Ginkgo* leaves responded to the elevated $O_3$ exposure in different ways.

Elevated $O_3$ did not elicite increases phenolic compounds in *Ginkgo* in this study, this result was different when compared to other deciduous tree species. Pehnolic compounds, as the antioxidants in plant, which can scavenge various oxygen species caused by ozone (Grace *et al*., 1998; Harborne and Williams, 2000). Numerous publications reported that elevated $O_3$ increased the concentrations of foliar tannins and flavonoids in birch and aspen (Lavola *et al*., 1994; Lindroth *et al*., 2001; Saleem *et al*., 2001; Copper and Lindroth 2003; Valkama *et al*., 2003; Yamaji *et al*., 2003; Peltonen *et al*., 2005). In current study, however, elevated $O_3$ decreased the annual mean concentration of total phenolic compounds by 11%. Elevated $O_3$ only increased the concentration of quercetin, but decreased the condensed tannins and isorhamnetin concentrations in *Ginkgo* leaves. The decrease of the phenolic compounds content in *Ginkgo* leaves may increase the leaf injuries and damages under elevated $O_3$ (Yamaji *et al*., 2003). Furthermore, as the phenolic compounds serving as UV light screens and they interact with herbivores, microbes, fungi and nematodes as chemical signals and toxins (Koes *et al*., 1994; Cooper-Driver and Bhattacharya, 1998; Seigler, 1998), the decrease of concentrations of condensed tannins and flavonoids in *Ginkgo* leaves might reduce the abilities of adaptation to other environmental stress.

Consistent with our hypothesis, elevated $O_3$ increased the concentrations of terpenes in *Ginkgo* leaves. This result is similar to studies on gymnosperm species, such as *Pinus sylvestris*, *Pinus ponderosa*, *Pinus taeda* (Sallas *et al*., 2001; Valkama *et al*., 2007), but not consistence with the results from birch, which showed that elevated $O_3$ have

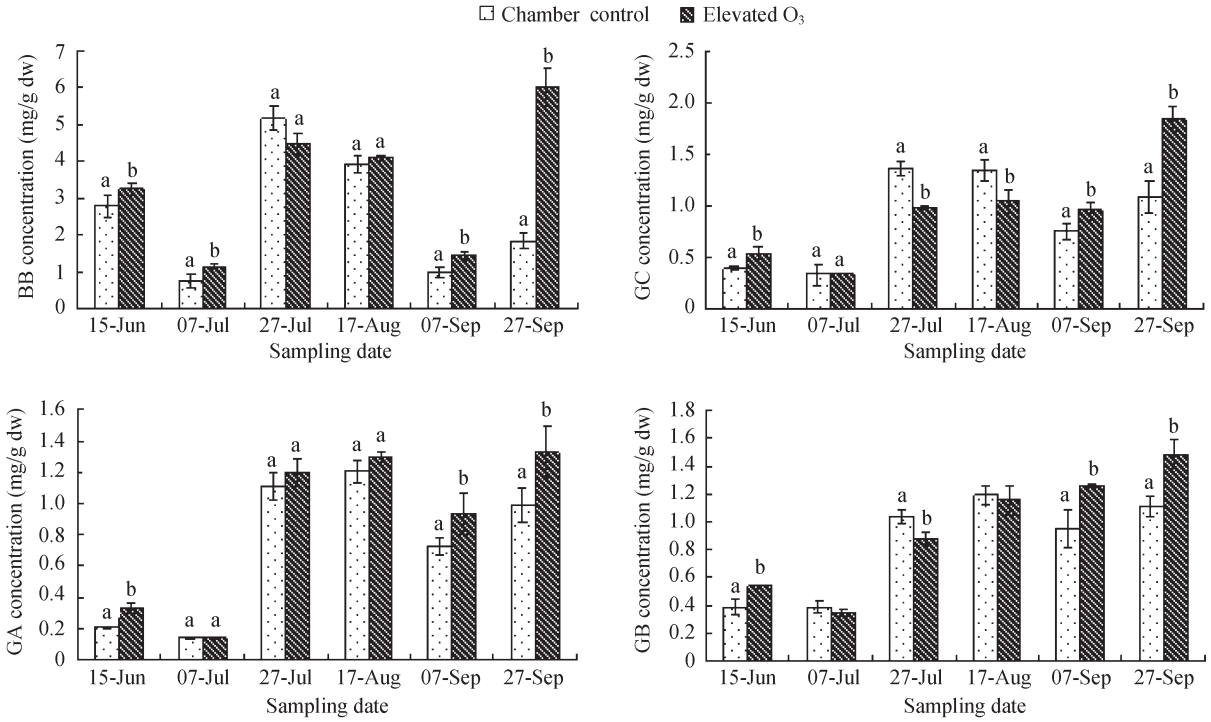


**Fig. 3** Effects of elevated $O_3$ on concentration of terpenes in *Ginkgo* leaves at different sampling times in 2006. Values are means ± SE ($n$ = 3 chambers). Different letters show significance at 0.05 level for each sampling time. BB: bilobalide; GC: Ginkgolides C; GA: Ginkgolides A; GB: Ginkgolides B.

no effects on birch leaf monoterpenes and sesquiterpenes emission (Vuorinen *et al*., 2005). The total mean terpenes concentration was 23% higher than control for elevated $O_3$, especially at the end of fumigation. This probably related to higher level of reactive oxygen species and declined activities of anti-oxidantive system in *Ginkgo* leaves for elevated $O_3$ at the end of growing season (He *et al*., 2006). Langenhein (1994) and Loreto *et al*. (2001) showed that some terpenes in plant can actually protect plants from $O_3$ damage by direct quenching of $O_3$. When the anti-oxidantive system in *Ginkgo* leaves could not resist the long term $O_3$ exposure, the increased concentrations of terpenes might play a role in scavenging the high level of oxygen species caused by ozone at the end of $O_3$ fumigation.

## 4 Conclusions

In this study, *G. biloba* showed a species-special response to elevated $O_3$ concentration paralleling to previous studies on other deciduous trees. Elevated $O_3$ decreased the phenolics synthesis in *Ginkgo* leaves, but induced the terpene synthesis at the end of fumigation. These changes of main secondary compounds in *Ginkgo* leaves caused by elevated $O_3$ may affect the relationships between *Ginkgo*s and other environmental factors.

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