Changes in effects of ozone exposure on growth, photosynthesis, and respiration of *Ginkgo biloba* in Shenyang urban area

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Abstract

An open-top chamber experiment was carried out from April through October 2006 to examine the effects of elevated (80 nmol mol^{-1}) atmospheric O_3 on *Ginkgo biloba* (4-years-old) in urban area. The air with ambient O_3 (AA, ≈ 45 nmol mol^{-1}) was used as control. The leaf mass and size, leaf area index, net photosynthetic rate (P_N), apparent quantum yield, transpiration rate, and stomatal conductance were decreased by elevated O_3 (EO) exposure. Visible foliar injury, which is light-brown flecks, was observed in the EO OTCs after 90 d of exposure. Carboxylation efficiency (Φ_{CO2}) and photorespiration and dark respiration rates were enhanced by EO exposure in the first half of the season, but all of them turned to be lower than those of the AA control at the end of experiment. Stomata limitation of photosynthesis was significantly higher than control in the whole season ($p<0.05$). Chlorophyll (Chl) content was lower in EO variant than in the control and the difference became more and more apparent through the season. Hence the decrease in P_N of *G. biloba* exposed to EO was the result of both stomatal and non-stomatal limitations. In the early season, the inhibition of photosynthesis was mainly caused by the stomatal limitation, and the earliest response was photoprotective down-regulation of photosynthesis but not photodamage. However, at the end of the season, the non-stomatal limiting factors such as decrease in Chl content, decrease in Φ_{CO2}, and anti-oxidative enzyme activity became more important.

Additional key words: carboxylation efficiency; chlorophyll; dark respiration; gas exchange; intercellular CO_2 concentration; leaf area index; non-stomatal limitations; photorespiration; stomatal conductance; transpiration rate.

Introduction

Tropospheric O_3 is the most widespread atmospheric pollutant that has a major impact on plant growth and productivity (Reich 1987, Musselman and Massman 1999, Plażek *et al.* 2000, Degl'Innocenti *et al.* 2002). Ambient concentrations of O_3 have increased 1–2 % per year during the past 20 years (Fuhrer and Booker 2003). Research on the effects of O_3 on plants has been carried out on a wide range of plant groups, including annual crops, conifers, and broad-leaf trees (Mikkelsen *et al.* 1995). O_3 can reduce leaf area, plant dry mass, and side-shoot development (Volin *et al.* 1998, Ollerenshaw *et al.* 1999). O_3 action frequently results in yield and productivity losses, which is of special importance in agricultural and horticultural plants.

Photosynthesis is severely affected by the oxidative stress of O_3 (Heath 1994, Ciompi *et al.* 1997, Farage and Long 1999). Alterations in the photosynthetic performance can occur before visible symptoms of injury appear on leaf surface and the extent of O_3 effects is dependent on plant species, leaf age, O_3 concentration, duration of exposure to O_3, and other environmental conditions. Increased stomatal limitations may be the cause of decreased photosynthesis following exposure to O_3 (Moldau *et al.* 1993). However, the reduction in leaf

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Abbreviations: AA – ambient air; C_i – intercellular CO_2 concentration; Chl – chlorophyll; E – transpiration rate; EO – elevated O_3 concentration; g_s – stomatal conductance; L_s – stomatal limit value; OTC – open-top chamber; P_N – net photosynthetic rate; PPFD – photosynthetic photon flux density; R_D – dark respiratory rate; R_P – photorespiration rate; Φ_a – apparent quantum yield; Φ_{CO2} – carboxylation efficiency.

conductance has been found, at least in mature leaves, not to be the primary cause for the reduction in photosynthesis following O_3 exposure (Grandjean Grimm and Fuhrer 1992). A reduction in carboxylation efficiency has been considered a main factor in the impairment of photosynthesis (Pell *et al.* 1992, 1994, Farage and Long 1999).

The long-term dynamic effect of O_3 on the photosynthesis of trees in urban area, however, has received little attention. The data in literature were absent from monitoring the changes synchronously, so it is difficult to reveal the dynamic variation to elevated ozone systematically (Reichenauer *et al.* 1997, Vandermeiren *et al.* 2005, Calatayud *et al.* 2006). In fact, realistic O_3 has a remarkable change in the urban atmosphere, and response of photosynthesis to elevated O_3 has a dynamic feature (Legge *et al.* 1995, McPherson and Simpson 1998, Ding *et al.* 2001, Manninen *et al.* 2003). A dynamic monitor can really reveal the dynamic nature of plant response to increasing ambient O_3, and allow more convincingly evaluate the yield loss of plants.

Ginkgo biloba is one of the oldest living tree species. Geological records indicate this plant has been growing on earth for 150–200 million years, and was referred by Darwin as "a living fossil" (Major 1967). To date, there have been dozens reports on its medical use that are related to the anti-oxidative secondary metabolite effects (Bridi *et al*. 2001, Gail 2001). *G. biloba* is indigenous to China, and it is popular for lining streets and for parks, which is important in urban forestry (Paul *et al*. 2002). We have reported responses of the anti-oxidative system in leaves of *G. biloba* to elevated O_3 concentration (EO) in the urban area (He *et al.* 2006), while the effects in terms of photosynthesis changes have not been tested. In order to better understand how the main species of urban forest respond to EO, *G. biloba* was exposed to 80 nmol mol^{-1} O_3 for a growing season in urban area. Both photosynthesis parameters, *i.e*. net photosynthetic rate (P_N), transpiration rate (E), stomatal conductance (g_s), stomatal limit (L_s), intercellular CO_2 concentration (C_i), apparent quantum yield (Φ_a), and quantum efficiency (Φ_{CO2}), as well as dark respiration rate (R_D) and phorespiration rate (R_D) were measured. The aim of this investigation was to find the dynamic response of gas exchange of *G. biloba* to EO and to clarify the possible mechanism.

Materials and methods

Plants and site description: The experiment site was established in Shenyang Arboretum of Chinese Academy of Sciences (41°46′ N, 123°26′ E) which is located in an urban environment. The factorial design has already been reported (He *et al.* 2006). Four-year-old *G. biloba* trees were planted in the soil (loamy type, no extra fertilizer) of six open top chambers (OTCs), 3 were O_3 enriched (80 nmol mol^{-1}, EO) and 3 with ambient air (AA) in April 2006. The trees were randomly distributed among the chambers, 20 trees per chamber. These young trees were exposed to AA or EO from 17 June to 10 October 2006. Healthy *G. biloba* leaves were collected at 09:00 every 10 d and then were immediately used for analyses. To calculate dry mass, parallel samples were dried at 80 °C for 8 h. Mean temperature was about 25 °C (the maximum temperature was about 35.7 °C, the minimum temperature about 11.4 °C). The mean relative air humidity was 50.2 % in OTCs. Gas exposure data are shown in Fig. 1. The day time (08:00–17:00 when EO was given to the OTCs) mean O_3 concentrations were 84.1±1.6 nmol mol^{-1} in the EO-OTCs and 40.9±4.9 nmol mol^{-1} in AA-OTCs (Fig. 1).

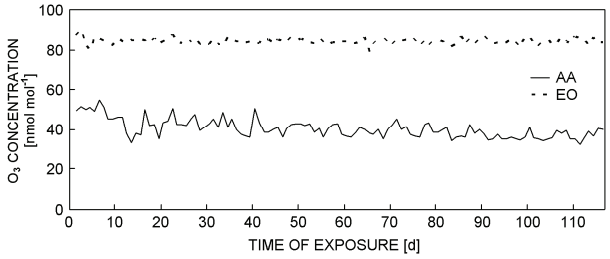

Fig. 1. Seasonal variations in O_3 concentration in OTCs with ambient air (AA) and with elevated O_3 (EO) concentrations. Each point represents a daily mean value of three OTCs (08:00–17:00).

Growth parameters (leaf numbers, leaf fresh mass, dry mass, leaf area, leaf area index) were measured after 100 d of exposure. Leaf numbers per plant were counted and leaf area was determined with an area-meter (*LI-COR 3000*, Lincoln, NE, USA).

Gas exchange: If not otherwise indicated, photosynthetic parameters were measured on the fully expanded, upper canopy leaves from lateral branches (one leaf per tree) between 09:30 and 11:00 every 10 d, but rainy days were avoided. Photon-saturated P_N, E, and g_s were measured using a portable photosynthesis system (*LI-6400*, *Li-Cor*, Lincoln, NE, USA). The photosynthetic photon flux density (PPFD) was set at 1 000 μmol m^{-2} s^{-1} in the chamber. The temperature and humidity were not controlled but dependent on the ambient weather conditions. L_s values were calculated by the formula of $L_s = 1 - C_i/C_a$, C_i is intercellular CO_2 concentration and C_a external CO_2 concentration. Both of them are from routine measurements of photosynthesis according to the method of Berry and Björkman (1980).

Irradiance response curves of P_N were made by LED source on the top of cuvette of the *LI-6400* photosynthetic system. Artificial irradiation was supplied to the leaf from red-blue LED source, and ambient CO_2 partial pressure was supplied by the CO_2 mixer. The curve was made using the following procedures: healthy leaf was

irradiated at a PPFD of 0 μmol m^{-2} s^{-1} until a steady state P_N was reached. Irradiance was then changed in a stepwise manner, PPFD increased from 0 to 2 000 μmol m^{-2} s^{-1} (0, 25, 50, 100, 200, 300, 500, 800, 1 000, 1 500, 2 000 μmol m^{-2} s^{-1}), and measurements were made once the leaf attained a steady net CO_2 fixation rate. Air temperature in the leaf chamber was maintained at 25 °C. Φ_a was calculated from the initial slope d_A/d_{PPFD} of the curve by linear regression using values got with PPFD below 300 μmol m^{-2} s^{-1}. Mean value of three chambers is given. The CO_2 efflux rate at PPFD = 0 was considered to be R_D.

CO_2 response curve of P_N (P_N–C_i) was made in a closed system. CO_2 concentrations were controlled by a CO_2 steel bottle supplied with a CO_2 mixer system. PPFD in the leaf chamber were maintained at 1 000 μmol m^{-2} s^{-1} and air temperature of 25 °C. Ambient CO_2 partial pressure in the leaf chamber (C_a) was reduced in steps from 360 μmol mol^{-1} down to 50 μmol mol^{-1}, and then increased up to 1 800 μmol mol^{-1} (360, 300, 200, 150, 100, 50, 360, 600, 800, 1 000, 1 200, 1 500, 1 800 μmol mol^{-1}). The correlation curve was made between P_N and C_i when C_i was below 200 μmol mol^{-1}. The slope d_A/d_{Ci} of the regression equation was taken as Φ_{CO2} of the leaf. R_D estimated under light was so low that P_N at $C_i = 0$ can be approximately regarded as photorespiration rate (R_P) according to the equation (Walker 1989, Reichenauer *et al.* 1997, Cai and Xu 2000, Vu 2005).

Chlorophyll (Chl) was extracted from freeze-dried leaf discs with 5 cm^3 dimethylsulphoxide in darkness over night. Chl contents were determined by *Shimadzu UV-1601* spectrophotometer according to Wellburn (1994).

Statistical analysis: The results presented are the means ($n = 3$) of all the measurements. One-way analysis of variance (ANOVA) was performed using the SPSS computer package (SPSS 1999) for all sets of data, and the mean differences were compared by paired-sample *t*-test ($p<0.05$). Sample variability is given as the standard deviation (S.D.) for presentation with line diagram.

Results

Visible leaf injuries: No visible foliar injury was observed in trees grown in AA (Fig. 2*A*), but was observed in EO-OTCs. Visible foliar injury, which is light-yellow flecks, was first observed after 70-d exposure on trees in the EO-OTCs (Fig. 2*B*), and 13.6 % of the leaves were affected. The rate increased progressively with time of O_3 exposure, reaching 22.5 % at the end of the experiment (after 110-d exposure). The first light brown flecks were recorded after 90-d exposure (Fig. 2*C*).

Growth analysis: Fresh mass, dry mass per leaf, area per leaf, and leaf area index of *G. biloba* were reduced by 50.9, 28.1, 23.1, and 28.9 %, respectively by the EO exposure, whereas leaf number per plant did not change significantly (Table 1).

Table 1. Effects of elevated O_3 exposure on growth parameters of *Ginkgo biloba*. Means±S.D. ($n = 3$) were calculated at 100 d of exposure. Numbers of each row followed by the same letters are not significantly different for p=0.05.

	Leaf number per plant	Fresh mass per leaf [g]	Dry mass per leaf [g]	Area per leaf [cm^2]	Leaf area index
Ambient O_3	26.3±1.86a	1.57±0.34a	0.32±0.03a	28.6±2.07a	1.14±0.37a
Elevated O_3	25.7±1.24a	0.77±0.30b	0.23±0.03b	22.3±1.84b	0.81±0.37b

Gas exchange parameters: P_N was decreased significantly by EO exposure in the whole growing season ($p<0.01$) (Fig. 3*A*). Φ_a showed a pattern similar to P_N, and it was significantly lower than control after 70-d exposure ($p<0.01$) (Fig. 3*B*). The g_s began to decrease significantly after 20 d of EO exposure, whereas there was no significant effect between 60 d and 80 d. In the last 30-d exposure, g_s was lowered significantly once again ($p<0.05$) (Fig. 4*A*). There was significant correlation between g_s and E ($r = 0.808$, $p<0.01$), so the variation in E was similar to that in g_s (Fig. 4*B*). The L_s (Fig. 4*C*) was higher and C_i (Fig. 4*D*) lower during the whole season in the leaves exposed to EO than in AA-leaves.

R_P and R_D: Compared to the plants grown under AA, leaf R_P increased significantly in the first 60 d ($p<0.05$) and then decreased significantly in the following days till the end of the season ($p<0.01$) (Fig. 5*A*). R_D was also significantly increased by the EO-exposure except at the beginning and end of the season ($p<0.01$) (Fig. 5*B*).

Φ_{CO2} and Chl content: Φ_{CO2} in EO-OTCs seemed to be not affected by O_3 in the first 20 d. Then higher Φ_{CO2} was found in the EO-leaves for a short period. After prolonged exposure, Φ_{CO2} in EO-OTCs became lower than that at AA until the end of the experiment. For most part of the season, Φ_{CO2} of *G. biloba* leaves was significantly lowered by EO-exposure ($p<0.01$) (Fig. 5*C*).

After 60 d of EO-exposure, the reduction in Chl content became more and more significant with advancement of the season ($p<0.01$) (Fig. 5*D*).

Discussion

Visible injury has been the criterion used in many interspecies comparisons (Donnelly *et al.* 2001, Woo and Hinckley 2005). The O_3-specific visible injuries were mainly light-brown flecks, which have previously been identified by Bungener *et al.* (1999) and Bassin *et al.* (2004). Hybrid poplars are among the most O_3 sensitive tree species. Typical O_3-induced visible foliar symptoms were consistently observed in poplars (Pell *et al.* 1999). Similar injuries were observed on *G. biloba* leaves in our study. The light-brown flecks were observed after 90-d EO-exposure (Fig. 2), indicating *G. biloba* leaves are also sensitive to high O_3.

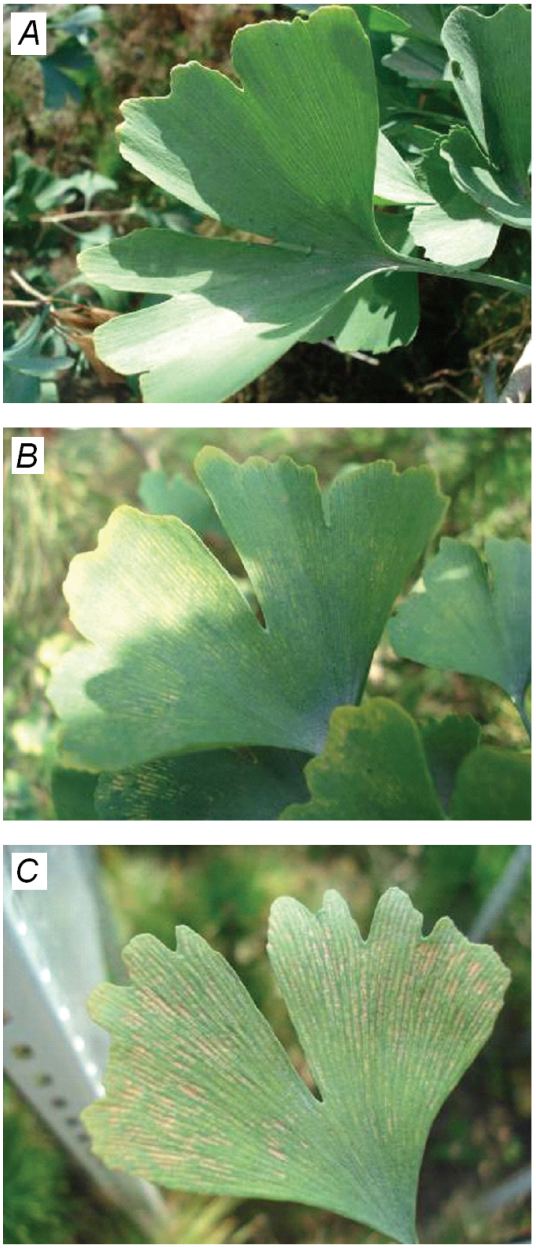

Fig. 2. *Ginkgo biloba* leaves show no visible ozone damage at ambient air (*A*), but light brown flecks after 70 d (*B*) and 90 d (*C*) of high O_3 exposure.

Effects of ozone on growth of *G. biloba* leaves: The reduction in fresh mass/leaf, dry mass/leaf, leaf area/leaf, and leaf area index of *G. biloba* by EO may be caused by decrease in P_N and increase in R_D. This resulted in reduction of plant biomass (Figs. 3 and 5*B*). Similar results were also reported by Keutgen *et al.* (2005) in strawberry. The greater decrease in mass per leaf than the decrease in leaf area indicates that the thickness of leaves or specific leaf mass was decreased by EO-exposure.

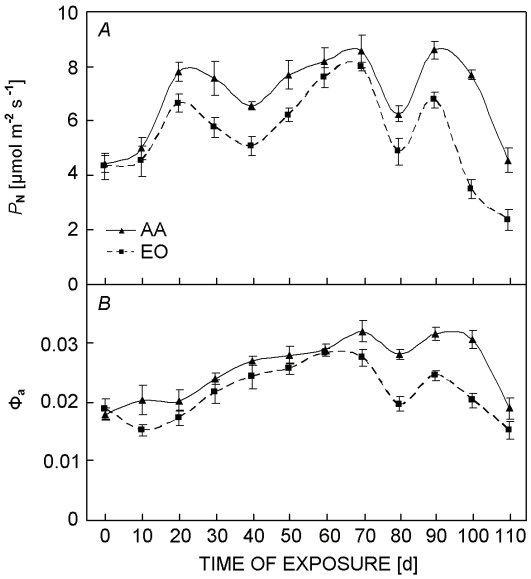

Fig. 3. Time course of (*A*) net photosynthetic rate (P_N) and (*B*) apparent quantum yield (Φ_a) of *Ginkgo biloba* leaves under ambient air (AA) and elevated O_3 (EO) in open-top chambers. Each point represents the daily (09:30–11:00) mean values ±S.E. ($n = 3$). The measurements were done under PPFD of 1 000 μmol m^{-2} s^{-1}.

Stomatal limitation to photosynthesis in *G. biloba* leaves and self-regulating mechanism: The fact that EO induced stomata closure and restricted CO_2 amounts entering plant leaves has been reported by Guo *et al.* (2001) and Guidi *et al.* (2001). Similar effect was observed in our study.

Farquhar and Sharkey (1982) considered whether stomatal or non-stomatal factors were the main cause of the reduced P_N that can be judged by the changing pattern of both C_i and L_s. If both C_i and P_N decreased, accompanied by an increase in L_s, the decrease of P_N was mainly caused by stomatal limits. On the contrary, when P_N decreased, C_i may increase or be constant despite lower g_s, and accompanied by a decrease in L_s. The photosynthetic activity of the mesophyll cells rather than g_s was regarded as the critical factor in reducing P_N.

According to the above theory, at the beginning of the experiment the EO-exposure resulted in decreased P_N and C_i and increased L_s, indicating that photosynthesis in *G. biloba* leaves is limited by stomatal factors in early days. This indicated the earliest response was photo-protective down-regulation of photosynthesis but not photodamage.

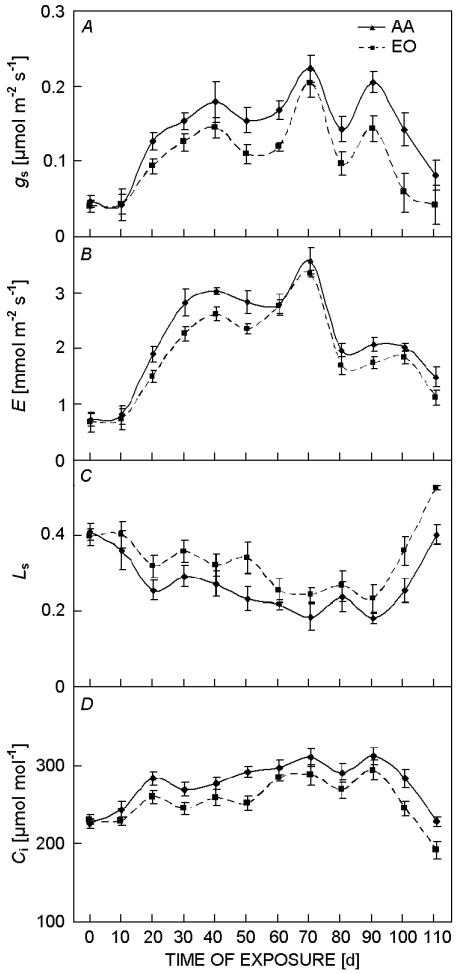

Fig. 4. Effects of elevated O_3 on stomata behaviour of *Ginkgo biloba* leaves under ambient air (AA) and elevated O_3 (EO) in open-top chambers. Each point represents the daily (09:30–11:00) mean values ±S.E. (n = 3).

The biological functions of photorespiration alleviate stress damage, slow the degradation of Chl, and increase ribulose-1,5-bisphosphate carboxylase/oxygenase and glutathione reductase contents (Graham *et al*. 1999, Muraoka *et al*. 2000). We have reported increase of activities of glutathione reductase by EO-exposure in the first half of the season (He *et al.* 2006), which could possibly be related to photorespiration rate of *G. biloba* leaves increased in the early season (Fig. 5*A*).

Reichenaur *et al.* (1997) found that Φ_a of *Populus nigra* decreased, while in our case Φ_a of *G. biloba* was enhanced by high ozone concentration in the first half of the season before falling to levels lower than those of control. This means *G. biloba* had been acclimated to EO with its self-regulating mechanism in the early days of the exposure, and the photosynthetic system in *G. biloba* did respond by acclimating in the early season.

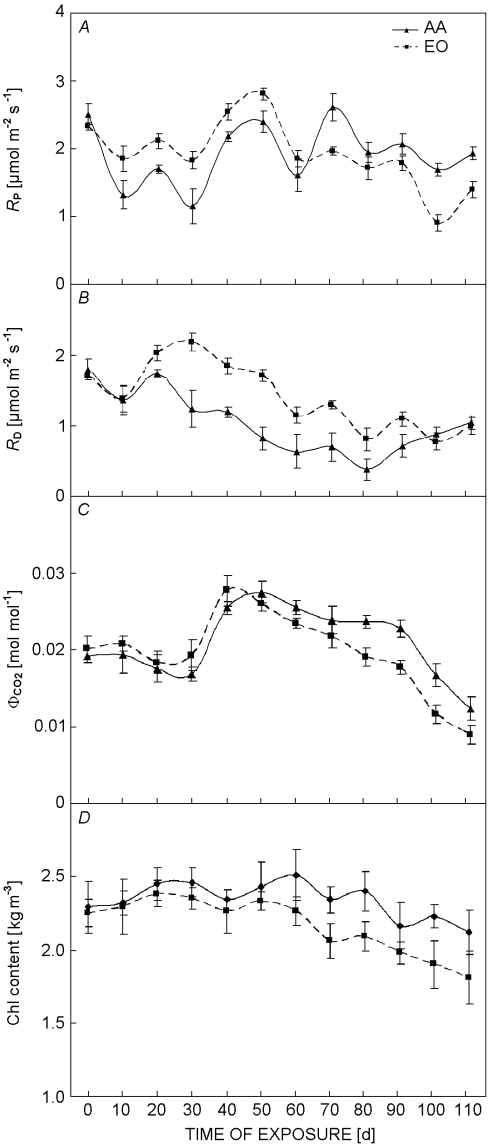

Fig. 5. Photorespiration, R_P (*A*) and dark respiration, R_D (*B*) rates, carboxylation efficiency, Φ_{CO2} (*C*) and chlorophyll (Chl) content (*D*) of *Ginkgo biloba* leaves under ambient air (AA) and elevated O_3 (EO) in open-top chambers. Each point represents a daily (09:30-11:00) mean values ±S.E. (n = 3).

Non-stomatal limitation to photosynthesis in *G. biloba* leaves: O_3 is easily taken up through stomata. Once O_3 enters the leaf, it severely damages the structure and function of photosynthetic apparatus (Lütz *et al.* 2000), accompanied by a series of alterations of physiological and biochemical characters in the plant, such as increased membrane permeability, protein decomposition, and lipid

peroxidation (Jin *et al.* 2000, Calatayud *et al.* 2003). In our study, after 70-d O_3 exposure, R_P became lower than the control. Φ_{CO2}, R_D, and Φ_a were significantly lower than in the control. Chl content was reduced significantly at the 100-d exposure. All this suggests that the non-stomatal factor became more and more important in limiting photosynthesis of *G. biloba* leaves, and light-brown flecks were observed after 90-d exposure (Fig. 2). We reported that EO-exposure induced greater generation of superoxide anion (O_2^{-}), higher H_2O_2 and malondialdehyde contents, and declined activities of anti-oxidative enzymes in the late season (He *et al.* 2006). The visible injury can be easily explained by the enhanced oxidative stress and decreased protection.

In conclusion, EO had a severe impact on photosynthesis and growth, and induced visible injury in leaves of *G. biloba*. The decrease in photosynthesis in the early season was essentially caused by the stomatal limitation. In the early season, photosynthesis showed a down-regulation and protection against the oxidative stress. However, at the end of the season, R_P became lower than in the control and provided much less protection, non-stomatal limiting factors such as damage in cell membrane systems, and decrease in Chl content and in Φ_{CO2} became more important in limiting photosynthesis. The photosynthetic system in *G. biloba* did respond by acclimating in the early season. However, the photosynthetic system could not withstand the long-term exposure and the visible injury was observed late in the season.

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