Elevated ozone negatively affects photosynthesis of current-year leaves but not previous-year leaves in evergreen *Cyclobalanopsis glauca* seedlings

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ABSTRACT

To assess the effects of leaf age/layer on the response of photosynthesis to chronic ozone (O_3), *Cyclobalanopsis glauca* seedlings, a dominant evergreen broadleaf tree species in sub-tropical regions, were exposed to either ambient air (AA) or elevated O_3 (AA + 60 ppb O_3 , E- O_3) for two growing seasons in open-top chambers. Chlorophyll content, gas exchange and chlorophyll *a* fluorescence were investigated three times throughout the 2nd year of O_3 exposure. Results indicated that E- O_3 decreased photosynthetic parameters, particularly light-saturated photosynthesis rate, stomatal conductance and effective quantum yield of PSII photochemistry of current-year leaves but not previous-year leaves. Stomatal conductance of plants grown under ambient conditions partially contributed to the different response to E- O_3 between leaf layers. Light radiation or other physiological and biochemical processes closely related to photosynthesis might play important roles. All suggested that leaf ages or layers should be considered when assessing O_3 risk on evergreen woody species.

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1. Introduction

Ground-level ozone (O_3) has received global concern due to its strong toxicity on agricultural crops, semi-natural vegetation and forest trees, and as a result of rising global background and regional peak O_3 concentrations (e.g. Asia) (Feng and Kobayashi, 2009; The Royal Society, 2008). Numerous experimental studies have demonstrated the detrimental effects of O_3 on tree species growth and physiological function, e.g. photosynthesis (Bagard et al., 2008; Feng et al., 2011; Schaub et al., 2005; Wittig et al., 2009; Zhang et al., 2010). The reduction in photosynthesis rate by O_3 could be attributed to stomatal closure, decreased chlorophyll content and electron transport rate, impaired carboxylation capacity and Rubisco content (Paoletti et al., 2009; Reich, 1983). Although the reduction in Rubisco activity was assumed to be the primary cause in photosynthetic decline under O_3 stress, stoma appeared to an important factor controlling O_3 entering the leaf interior (Novak et al., 2005). Therefore, the response of stomatal conductance (g_s)

to O_3 at different leaf ages could influence the stomatal O_3 uptake and further leaf tissue injury.

Previous studies have demonstrated that the effects of O_3 on gas exchange are influenced by leaf age (Bohler et al., 2010; Coleman et al., 1995; Pääkkönen et al., 1996) and leaf position within canopy (Samuelson and Edwards, 1993; Schaub et al., 2005) when growing under the same conditions. For hybrid poplar and Aspen, elevated O_3 concentrations altered the patterns of change in leaf area, photosynthetic rate and carbon gain with leaf position from root to apex and the older leaves were more sensitive to O_3 than younger leaves (Bagard et al., 2008; Greitner et al., 1994). At the canopy level, ambient O_3 concentrations caused reductions in both light-saturated photosynthesis rate (A_{sat}) and g_s of the lower canopy that were about 30% more than those of the upper canopy in black poplar (*Populus nigra* L.) species (Novak et al., 2005). Young poplar trees also showed that reduced photosynthetic parameters related to chlorophyll content, maximum rate of photosynthetic electron transport and net photosynthetic assimilation appeared only on fully expanded leaves, but not on young and undeveloped leaves, which was partly attributed to differences in leaf anatomy between expanding and fully expanded leaves (Bagard et al., 2008). Similarly, for an evergreen broad-leaved species, *Castanopsis sieboldii*, elevated O_3 significantly reduced the net photosynthetic rate

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of previous-year leaves but not that of expanding current-year leaves (Watanabe et al., 2008).

However, given the differences of leaf growth habit between deciduous and evergreen species, and lower g_s in old (previous-years) leaves compared to young (current-year) leaves for evergreen species, it can be inferred that the response to O_3 among leaf ages of evergreen species might be different from that of deciduous species. Therefore, it is necessary to further investigate the effects of O_3 on the photosynthesis characteristics of evergreen species at different leaf ages/layers, which is critical for ozone risk assessment since most leaves of evergreen species are exposed to O_3 for many years.

In this study, we selected *Cyclobalanopsis glauca*, an evergreen broadleaf tree species widely distributed in sub-tropical forests, to investigate the negative effects of elevated O_3 on photosynthetic parameters, including chlorophyll content, gas exchange and photosynthetic electron transport at different leaf ages/positions. The objectives of this study are to determine whether impacts of O_3 on photosynthesis depend on leaf age or leaf layer, and to explore possible mechanisms.

2. Materials and methods

2.1. Experimental site

The experimental site was located at the Tiantong National Field Observation and Research Station for Subtropical Forest Ecosystems (29°48'N, 121°47'E), Ningbo, Zhejiang Province, China. It is typical of the humid subtropical monsoon climate with cold dry winters and warm wet summers. The annual mean temperature is 16.2 °C and the warmest month is July with a mean temperature of 28.1 °C. Average annual precipitation is 1375 mm, concentrated between June and August.

2.2. Plant materials

One-year-old seedlings of *Cyclobalanopsis glauca* L. were individually planted into 6 L circular plastic pots in a temperature controlled and double glazed greenhouse (25 ± 2 °C, air relative humidity 70–90%) from November 2008 until the middle of April 2009. Pots were filled with native yellowish brown lateritic soil (soil organic C 0.98%, total N 1.36 g kg⁻¹, total P 0.27 g kg⁻¹, total K 1.41%) in mixture with litter collected under fir forest in the ratio of 1:1 (v/v). Seedlings with similar height and basal diameter were selected for this study and pre-adapted to open-top chambers (OTCs) conditions for two weeks before O_3 fumigation. During cultivation and O_3 exposure, plants were well watered with tap water to avoid drought stress.

2.3. Ozone exposure

There were two treatments: ambient air (AA) and ambient air with the addition of 60 ppb O_3 (E- O_3), with two replicate chambers for each treatment. Four OTCs (octagonal base, 7 m² of growth space and 2.6 m in height) were randomly used as AA and E- O_3 in the field. For E- O_3 , O_3 was generated from pure oxygen by an electrical discharge O_3 generator (HY003, Chuangcheng Co. Jinan, China) and then mixed with ambient air to achieve the target O_3 concentration. The average air velocity in the chambers corresponded to approximately two complete air change per minute. The concentration of O_3 within the OTCs was monitored at approximately 10 cm above the plant canopy using a UV absorption O_3 analyzer (Model 49i-Thermo, USA).

For each treatment, seedlings were transposed into two fumigation chambers with 8 plants per chamber. The position of the plants was changed every 5–7 days within each OTC to eliminate positional effects. Every 10–15 days, the chambers were emptied and randomly reassigned to any O_3 treatment, and the seedlings were replaced back according to their specified treatment levels. Plants were fumigated for two growing seasons from 25 May to 10 September 2009 and 2 May to 13 October 2010. There was a daily maximum O_3 exposure of 8 h (from 08:00 to 16:00), because O_3 exposure was stopped when there was rain, fog, mist or dew. There were a total of 70 and 79 effective fumigation days during the 2009 and 2010 growing seasons, respectively. During the O_3 fumigation of the 2010 growing season, mean air temperature and relative air humidity within and outside the OTCs were 25.2 and 24.9 °C, 81.8 and 88.2%, respectively, and maximum air temperatures and relative air humidity within and outside the OTCs were 32.7 and 31.8 °C, 99.0 and 93.6%, respectively.

2.4. Measurements

To investigate the leaf response to E- O_3 at different canopy layers during the growing season, we used a non-destructive approach to conduct measurements on

gas exchange, leaf chlorophyll *a* fluorescence and total chlorophyll contents. Generally, *C. glauca* produces leaves in spring (April) and summer (July) during the growing season. We recorded the leaf number of the whole plant in monthly censuses, and marked current-year leaves on the main stem for six seedlings from each O_3 treatment. The leaves that flushed in spring were fully expanded before the O_3 fumigation started, and the number of these leaves averaged 47 and 49 in AA and E- O_3 , respectively. The leaves that flushed in summer developed to be fully expanded during the experiment. Leaf number gradually increased but the position of the leaf in the main shoot changed from upper to lower within the plant canopy during the course of the O_3 fumigation. We conducted three measurements from July to September. Here, all measurements were performed between the sixth layers of leaves from the base at main shoot to apex since the lowest layer (1–5) at the base showed very low values of photosynthesis rate. The fully expanded leaf layer at the top of canopy was Layer 17 at the first measurement (18th July) and Layer 21 at the second (17th August) and third (14th September) measurements. At the 3rd measurement, the number of leaves flushed in summer (including fully expanded) was 201 and 190 in AA and E- O_3 , respectively. All selected leaves were defined into three leaf ages, with the lowest and oldest leaves (from Layer 6 to Layer 11) as the mature leaves (ML) formed in the previous year, the leaves in the middle of the canopy (from Layer 13 to Layer 17) as young leaves formed in spring (YL), and the three uppermost leaves (from Layer 19 to Layer 21) were classified as newly formed leaves in summer (NFL).

2.4.1. Gas exchange and chlorophyll *a* fluorescence measurement

Three plants per chamber were randomly selected and two summer-flushed new leaves, three spring-flushed leaves and three previous-year leaves of each plant were marked for measurements. Gas exchange was determined with a portable photosynthesis system fitted with a 6400-40 leaf chamber fluorometer (LCF) (LI-6400, LI-COR Inc. Lincoln, NE, USA). The system controlled saturating PPFD (photosynthetic photon flux density) at 1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The block temperature was set to the ambient average of 32 °C, CO_2 at 380 $\mu\text{mol mol}^{-1}$, and relative humidity (RH) at 50–65%. All measurements were conducted during 09:00–11:00. During gas exchange measurements, chlorophyll *a* fluorescence parameters were also recorded. All gas exchange and fluorescence measurements were recorded when the stability or steady state indicated by total coefficient of variation was $\leq 3\%$. For chlorophyll fluorescence, steady state fluorescence yield (F_s) was recorded at saturating PPFD of 1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The intensity of the saturation pulses was 6000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 0.8 s, which was then used to produce the maximum fluorescence yield by temporarily inhibiting photosystem II (PSII) photochemistry. Measurements of minimum fluorescence yield were carried out in the presence of far-red light ($5 \mu\text{mol m}^{-2} \text{s}^{-1}$) in order to fully oxidize the PSII acceptor side. We determined A_{sat} , g_s , intercellular CO_2 concentration (C_i) and transpiration rate (T_r). Foliar water use efficiency (WUE) was expressed as the ratio of A_{sat} and T_r . The fluorescence parameters including actual photochemical efficiency of PSII in the saturated light (F_v'/F_m'), quenching of photochemical efficiency of PSII (qP) and effective quantum yield of PSII photochemistry (Φ_{PSII}) were calculated as follows: $F_v'/F_m' = (F_m' - F_o')/F_m'$, $qP = (F_m' - F_s)/(F_m' - F_o')$ (Bradbury and Baker, 1984; Quick and Horton, 1984) and $\Phi_{\text{PSII}} = (F_m - F_s)/F_m'$ (Genty et al., 1989).

2.4.2. Total chlorophyll content measurement

Total chlorophyll content was non-destructively measured on leaves used for gas exchange and fluorescence analysis with a SPAD chlorophyll meter (SPAD-520, Minolta, Japan). Three readings per leaf were averaged to give value.

2.5. Statistical analysis

As gas exchange parameters were not significantly different between chambers within O_3 treatment, the values for each plant were used as an independent experimental unit throughout the statistics. The original data were checked for normality and homogeneity of variance, then subjected to analysis of variance with general linear model to test the effects of O_3 , leaf age, measurement date and their interaction using SPSS 16.0 software package for Windows. The Tukey's HSD test was used to compare response difference among leaf age, measurement date and the interaction between O_3 , leaf age and measurement date. Furthermore, the means of AA and E- O_3 at any leaf layer was compared using Student's *t*-tests. $P \leq 0.05$ was regarded as significant. Data shown in Figures were means $\pm$ SE ($n = 5$ or 6).

3. Results

During O_3 fumigation in 2010, the mean of the daily 8 h O_3 concentration (M8) in AA averaged 31 ppb with a maximum of 78 ppb, while M8 in E- O_3 averaged 67 ppb with a maximum of 131 ppb (Table 1). In addition, the accumulated O_3 exposure over a threshold of 40 ppb (AOT40) in AA and E- O_3 by the end of each fumigation season was 5.8 and 34.5 ppm h in 2009 and 3.5 and 43.5 ppm h in 2010, respectively.

Table 1

8-h mean O₃ concentration (M8) and AOT40 for ambient (AA) and elevated O₃ (E-O₃) treatments (8:00–16:00) during the 2009 and 2010 growing seasons.

	2009 (25th May–10th Sep.)		2010 (2nd May–13th Oct.)	
	AA	E-O ₃	AA	E-O ₃
Average of M8 (ppb)	32.0	60.3	32.1	66.9
Maximum M8 (ppb)	97.6	159.7	78.0	131.1
Minimum M8 (ppb)	14.9	74.9	9.2	69.1
AOT40 (ppm h)	5.8	34.5	3.5	43.5

Across the three measurement campaigns, significant differences in total chlorophyll contents were detected in response to O₃, leaf age and measurement date, but not in interactions between O₃ and leaf age or O₃ and date (Fig. 1, Table 2). However, the impacts of E-O₃ on leaves with three ages differed at the different measurement dates, as indicated by the significant interaction between O₃ × age × date ($P < 0.001$, Table 2). As can be seen from Fig. 1, E-O₃ induced a larger reduction in chlorophyll content in the upper leaves (Layer 14 to Layer 20, NFL and YL) than in the lower canopy of plants (ML) at the 3rd measurement, whereas all leaf ages showed a similar response to E-O₃ at both the 1st and 2nd measurements.

Across all measurements, A_{sat} and g_s were significantly affected by E-O₃, leaf age and their interactions, but not O₃ × age × date (Table 2), suggesting that the reduced A_{sat} and g_s with E-O₃ differed between leaf ages throughout the measurements. Fig. 2 shows that E-O₃ significantly decreased A_{sat} only in NFL at the 2nd measurement, NFL and YL at the 3rd measurement, and g_s only in YL and NFL leaves at the 2nd measurement. A_{sat} and g_s in ML showed little response to E-O₃. Moreover, the leaves in lower part of canopy (ML) had a much lower A_{sat} and g_s than the upper leaves (NFL and YL) (Fig. 2, Table 2). In contrast, E-O₃ did not significantly affect C_i or WUE at any leaf age or leaf layer throughout the course of the measurements (Table 2, Fig. 2).

For chlorophyll *a* fluorescence, F_v/F_m' and qP were significantly reduced by E-O₃ across all measurements, but were not affected by the interaction of O₃ × age or O₃ × age × date (Fig. 3, Table 2). However, E-O₃ significantly reduced the Φ_{PSII} in both NFL and YL but not ML, as shown by the significant interaction of O₃ and age (Fig. 3, Table 2). For all three parameters, ML at any leaf layer did not show a significant response to E-O₃ at any measurement.

Table 2

Main and interactive effects of elevated O₃, leaf age and measurement date on leaf photosynthesis parameters of *Cyclobalanopsis glauca* seedlings in the 2010 growing season.

Parameters	Main effects			Interactive effects		
	O ₃	Age	Date	O ₃ × date	O ₃ × age	O ₃ × age × date
A_{sat}	<0.001	<0.001	0.177	0.84	0.04	0.11
g_s	<0.001	<0.001	<0.001	0.23	0.01	0.88
C_i	0.65	0.07	<0.001	0.77	0.64	0.59
WUE	0.94	0.008	<0.001	0.71	0.61	0.55
F_v/F_m'	<0.001	<0.001	<0.001	0.22	0.21	0.18
Φ_{PSII}	<0.001	<0.001	<0.001	0.69	0.003	0.13
qP	0.052	<0.001	0.003	0.61	0.35	0.22
Chl	<0.001	<0.001	<0.001	0.44	0.58	<0.001

4. Discussion

Elevated O₃ induced significant decreases in total chlorophyll content, net photosynthesis rate and g_s across all leaf ages/layers of *C. glauca* (Table 2), in accordance with responses of other tree species, e.g. *Cinnamomum camphora* (Feng et al., 2011), *Viburnum lantana* (Calatayud et al., 2010), poplar (Bohler et al., 2010), aspen (Noormets et al., 2001) and beech (Bortier et al., 2008). Also, F_v/F_m' and Φ_{PSII} but not qP were significantly reduced by E-O₃, suggesting that E-O₃ increased light energy dissipation of the antenna pigment, thus leading to a reduction in the efficiency of excitation energy captured by the open PSII reaction center, but no change on the proportion of open PSII reaction center. All these indicated that both dark and light reactions of photosynthesis of *C. glauca* were negatively affected by elevated O₃ after two years exposure (Figs. 2 and 3). On the basis of Farquhar and Sharkey (1982), the response of gas exchange parameters (reduced A_{sat} and g_s , and no change of C_i) to E-O₃ indicated that the decrease in net photosynthetic rate might not be fully explained by stomatal closure but by the decrease in the assimilation capacity. No difference in WUE between O₃ treatments was found for any measured leaves during the growing season (Fig. 2), suggesting that O₃ exposure may reduce photosynthetic decline in parallel with stomatal closure.

From Table 2, the lack of a significant interaction between O₃ and date indicated that the effects of E-O₃ on all tested parameters were fairly constant over time, i.e. the relative negative impacts of E-O₃ on photosynthesis did not change with leaves and canopy development. However, a significant interaction between O₃ and leaf age was found on A_{sat} , g_s and Φ_{PSII} , suggesting that the negative effect of E-O₃ was strongly dependent on leaf age, as demonstrated by larger effects of E-O₃ on NFL and YL than ML (Figs. 2 and 3). A_{sat}

Fig. 1. The SPAD value of three age-classes leaves of *Cyclobalanopsis glauca* seedlings grown in ambient (AA) and elevated O₃ (E-O₃) treatments. An asterisk indicates a significant difference between O₃ treatments at any leaf layer ($P \leq 0.05$). Different letters at each leaf age and O₃ combination mean a significant difference ($P \leq 0.05$) across all measurement dates through Tukey's HSD test. The values are shown as means $\pm$ SE ($n = 5$ or 6). DAF: days after fumigation; ML: mature leaves; YL: young leaves; NFL: newly formed leaves.

Fig. 2. Light-saturated photosynthesis (A_{sat}), stomatal conductance (g_s), intercellular CO_2 concentration (C_i) and water use efficiency (WUE) of three age-classes leaves of *Cyclobalanopsis glauca* seedlings grown in ambient (AA) and elevated O_3 (E-O_3) treatments (means $\pm$ SE, $n = 5$ or 6). An asterisk indicates a significant difference between O_3 treatments at any leaf layer. Different letters at each leaf age and O_3 combination mean a significant difference ($P \leq 0.05$) at each measurement date through Tukey's HSD test as there was no significant interaction between O_3 and date (Table 2). The values are shown as means $\pm$ SE ($n = 5$ or 6). DAF: days after fumigation; ML: mature leaves; YL: young leaves; NFL: newly formed leaves.

and Φ_{PSII} was significantly reduced by E-O_3 in both NFL and YL, and stomatal closure by E-O_3 was only detected in YL leaves on the middle canopy. However, the results on the deciduous species indicated that E-O_3 caused larger effects on old leaves of the lower canopy than young leaves of the upper canopy e.g. poplar, silver birch (Bortier et al., 2000; Mäenpää et al., 2011; Reich, 1983), and the developing leaves of silver birch (*Betula pendula* Roth.) were more tolerant to O_3 than expanded leaves (Pääkkönen et al., 1996). Therefore, the response of evergreen tree species *C. glauca* to E-O_3 at different leaf ages or leaf layers was a bit different from that of tested deciduous species, possibly attributable to the difference in leaf development traits between evergreen and deciduous species.

Although we did not measure O_3 concentration along the vertical profile in this experiment, the OTC we used was the same design as that previously used in a rice experiment (Zheng et al., 2007). The O_3 concentration at three heights of 50, 85 and 130 cm from the ground

was 100, 103 and 106 ppb for the target 100 ppb O_3 , and the coefficient of variation was 1.01, 1.07 and 1.07, respectively, indicating that ozone concentration of both vertical and horizontal distribution was equable and stable in our OTCs (Zheng et al., 2007). This indicated that the upper and lower canopies were exposed to similar O_3 concentration during the fumigation.

A significant linear relationship between the response of A_{sat} to E-O_3 and g_s of ambient plants ($r^2 = 0.18$, $N = 22$, $P = 0.048$) suggested that g_s partially contributed to the different response to O_3 among leaf layers or leaves of different ages. Therefore, the observed larger photosynthetic response to E-O_3 in YL can be explained by greater g_s in the upper canopy relative to ML. Apparently, stomatal O_3 uptake was much greater in the upper canopy (i.e. NFL and YL) than lower canopy (i.e. ML). However, it does not imply that cumulative O_3 uptake at upper canopy was more than that at lower canopy because ML had been exposed to O_3

Fig. 3. Actual photochemical efficiency of PSII in the saturated light (F_v/F_m'), quenching of photochemical efficiency of PSII (qP) and light-adapted apparent quantum yield of PSII (Φ_{PSII}) of three age-classes leaves of *Cyclobalanopsis glauca* seedlings grown in ambient (AA) and elevated O_3 ($E-O_3$) treatments. An asterisk indicates a significant difference at any leaf layer. Different letters at each leaf age and O_3 combination mean a significant difference ($P \leq 0.05$) at each measurement date through Tukey's HSD test as there was no significant interaction between O_3 and date (Table 2). The values are shown as means $\pm$ SE ($n = 5$ or 6). DAF: days after fumigation; ML: mature leaves; YL: young leaves; NFL: newly formed leaves.

exposure for one year longer than NFL and YL. Moreover, termination of O_3 exposure may allow previous-year leaves to recover from O_3 damage and confer tolerance to subsequent O_3 exposure. Therefore, lower A_{sat} and g_s at ML may also be a result of adaptation or acclimation of plants to high $[O_3]$. The variation of g_s from the apex along the vertical plant axis has been suggested to correspond with the degree of shading and age of the leaves (Herbinger et al., 2005; Kitao et al., 2006; Paoletti and Grulke, 2005).

Possibly, other factors such as foliar detoxification capacity also play an important role in different responses to $E-O_3$ between leaves of different position or age. Fares et al. (2010) found that a changing defense against O_3 along the plant axis in *Populus nigra* was attributed to a vertical profile of foliar phenolic and volatile compounds. In accordance with their study, concentrations of antioxidants and secondary products were observed higher in sun leaves or upper canopy foliage of *Fagus sylvatica* L., including significantly increased glutathione concentrations under double O_3 concentration across both age classes and canopy levels (Herbinger et al., 2005).

5. Conclusions

From the present study, elevated O_3 decreased the photosynthetic parameters of current-year leaves but not previous-year leaves in evergreen *C. glauca*, suggesting that leaf age should be considered when assessing carbon assimilation loss of evergreen species exposed to long-term O_3 . Stomatal conductance of plants at

different leaf layers partially contributed to the different response to $E-O_3$ between leaf ages. However, information on other physiological and biochemical processes closely related to photosynthesis is needed to determine which factors are crucial to the differential responses to $E-O_3$ among leaves of different ages or layers.

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