

CO-ORDINATION OF PHOTOSYNTHESIS AND STOMATAL RESPONSES OF MONGOLIAN OAK (*QUERCUS MONGOLICA* FISCH. EX LEDEB.) TO ELEVATED O₃ AND/OR CO₂ LEVELS

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Abstract. Four-year-old trees grown in open-top chambers (OTCs) were exposed to elevated O₃ (80 nmol/mol, EO) or/and elevated CO₂ (700 μmol/mol, EC) levels. The object is to study the responses of photosynthesis and stomata in leaves of Mongolian oak (*Quercus mongolica*) to EO and/or EC for two successive growing seasons in an urban area. The experiment was conducted in the arboretum located at the center of Shenyang city. The results show that: (1) EO decreased P_n (Light-saturated net photosynthesis rate), g_s (stomatal conductance) and T_r (transpiration rate). The stomata closure maybe associated with the direct effect of EO on guard cells. The decreases of F₀, F_m and F_v/F_m indicated that EO limits the capability of the plant to use photon energy and thus alters photosynthesis. (2) EC increased P_n and C_i (intercellular CO₂ concentration) of leaves, but decreased g_s and T_r slightly. Stomatal density decreased significantly at the end of the second season. Perhaps, EC was detected by mature leaves in the first season which signaled to the developing leaves in the next season, the stomatal developments of which altered accordingly. (3) Under EC+EO, EC relieved most of the O₃-induced adverse effects to P_n. The decreases of g_s and T_r were mainly caused by EO. There was no significant difference of F_v/F_m detected compared to the control, EC can largely relieve the adverse effects of EO on the PSII reaction center. Changes of stomatal parameters were just like the changes under EC. Perhaps the morphological changes of the stomata were mainly caused by EC.

Keywords: photosynthetic CO₂ exchange, chlorophyll fluorescence, stomatal density, stomatal area

Abbreviations: C_a: concentration of ambient CO₂; C_i: concentration of intercellular CO₂; F_m: fluorescence maximum; F₀: fluorescence origin; F_v/F_m: maximum quantum yield of PSII photochemistry; g_s: stomatal conductance; P_n: light-saturated net photosynthesis rate; T_r: transpiration rate

Introduction

Since the industrial revolution, accumulating greenhouse gas (O₃ and CO₂) levels have increased over two-fold (Guo et al., 2017). The atmospheric concentrations of CO₂ have increased since 1750. In 2011, the concentration of CO₂ was 391 μmol/mol and exceeded the pre-industrial levels by about 40%. The rise primarily resulted from emissions of fossil fuel burning and secondarily from net land use change (IPCC, 2013). This rise has been accompanied by the increase in troposphere O₃ concentrations, which is not only a significant greenhouse gas but is also recognized as a serious phytotoxic air pollutant that damages plants (Serengil et al., 2011). As concentrations of NO_x and VOCs increase through industrialization and vehicle exhausts, the daily surface O₃ levels in industrialized countries have increased remarkably from 10 nmol/mol prior to the industrial revolution to 60 nmol/mol in the current summer seasons, and are predicted to increase by another

20% by 2050 (IPCC, 2007). Ground-level O₃ concentrations have significantly increased over the past decades, especially in northeast Eurasian region (Akimoto et al., 2015; Feng et al., 2015; Verstraeten et al., 2015).

These two co-occurring pollutants are quite interesting as they act in diametrically different directions. Stomatal aperture and density influence the rate of photosynthesis and transpiration of plant directly. Regulation of stomatal development and stomatal conductance is a key factor for plant in its adaptation to the environment changes and plants have evolved sophisticated mechanisms to control stomata density and behavior (Engineer et al., 2014). The stomatal control is achieved by the regulation of stomatal aperture through changes in guard cell turgor, and by alteration of stomatal density through modification of stomatal initiation and leaf expansion during leaf development (Haworth et al., 2013). Research of Frey et al. (1996) showed that stomatal density of birch could be increased under a consistently O₃ exposure. A reduction in stomatal conductance is commonly observed in response to elevated CO₂ (Woodward, 1987). Reported changes in stomatal density with growth at elevated CO₂ include increases, decreases, and no change (Drake and González-Meler, 1997). Those species with little or no control of stomatal aperture were more likely to exhibit a reduction in stomatal density under elevated CO₂ (Haworth et al., 2013). But how can stomatal response to both elevated O₃ and elevated CO₂? The stomatal development and stomatal conductance of leaves would how to be regulated to adapt to the combinational environments? The objective of this study was to measure photosynthetic and stomatal parameters of leaves of *Q. mongolica* (*Quercus mongolica*) trees grown in open-top chambers (OTCs) for two growing seasons to evaluate the photosynthesis and stomata responses of *Q. mongolica* to elevated concentrations of O₃ and/or CO₂. Cities are with concentrated industrial plants and heavy traffic. Trees grown in urban regions are more likely affected by elevated O₃ and CO₂, so the OTCs of this experiment were built in the center of the city to simulate the O₃ and CO₂ effects on leaves of *Q. mongolica*.

Research design and methods

Site and OTCs

The experiment site is located in Shenyang Arboretum of Chinese Academy of Sciences (41°46' N, 123°26' E and 41 m above sea level), which is located in the metropolitan area of Shenyang, Liaoning province, China). In this area, average annual precipitation is 755.4 mm and average annual temperature is 7.4 °C. The mean daytime temperature and the mean relative humidity in the OTCs showed no significant differences among treatments (T_{mean} = 26 °C H_{mean} = 70%). Ambient rainfall was occasionally supplemented to minimize water stress. The soil in the OTCs was loamy, supplemental fertilizer was provided and weeds were removed regularly. The factorial design of the OTCs has already been reported, they are 4 m in diameter and 3 m in height with a 45° sloping frustum, and distance among them is at least 4 m (He et al., 2006). There are twelve OTCs provided for four treatments, each with 3 replicates (in 3 OTCs randomly). The treatments were (1) control (ambient air, CK), (2) elevated O₃ (80 nmol/mol O₃ + ambient CO₂, EO), (3) elevated CO₂ (ambient O₃ + 700 µmol/mol CO₂, EC), (4) elevated O₃ + elevated CO₂ (80 nmol/mol O₃ + 700 µmol/mol CO₂, EO+EC).

O₃ was produced from bottled pure oxygen with an ozone generator (GP-5J, China) and pure CO₂ was injected into the chambers from cylinders. In order to monitor O₃ and CO₂ concentrations, O₃ transducer (S-900, Aeroqual, New Zealand) and CO₂ infrared

transducer (SenseAir, Sweden) were used inside the chambers. Elevated CO₂ was applied for 24 h/day. Elevated O₃ was applied for 9 h/day (08:00-17:00).

Plant material

In April, uniform and healthy *Q. mongolica* trees (four-year-old) from local nursery were randomly planted in the soil of twelve OTCs (three OTCs were used as replicates for each treatment), 20 trees per chamber. These young trees were exposed to CK, EO and/or EC from 18 June to 10 October in the first season, overwintered in the OTCs and then re-exposed from 18 June to 10 October in the second season.

Gas exchange measurements

One leaf per plant and three plants per OTC (three OTCs per treatment, $n = 9$) were measured by a portable photosynthesis system (LI-6400, Li-Cor Inc., Lincoln NE, USA) to attain a mean value every 15 days. The same method of gas exchange measurements has been reported (Wang et al., 2009).

Chlorophyll a fluorescence measurements

Chlorophyll a fluorescence was measured once every 15 days at ambient temperature in the OTC's in the second year, using a portable fluorometer (Handy-PEA, Hansatech, England). One leaf per plant and three plants per OTC (three OTCs per treatment, $n = 9$). The environmental conditions were similar to gas exchange measurement. The same method of Chlorophyll a fluorescence measurement has been reported (Wang et al., 2009).

Stomatal parameters measurements

Q. mongolica leaves were collected after 0, 60 days (in the first season) and 210 days exposure (in the second season). Three leaf per plant, three plants per OTC and three OTCs per treatment ($n = 27$). The top, middle and bottom of leaves were painted with transparent nail polish, and then the films were torn off after it dried. The temporary microscopic slides of dried nail polish were observed and photographed with picture pick-up system of bright-field microscope (Olympus BX-50, 1.3 million pixels), and image processing software (Motic Images Advanced 3.0) was used to measure and calculate the stomatal parameters including stomatal density, stomatal length, stomatal width, stomatal perimeter and stomatal area.

Statistical analysis

ANOVA was carried out using SPSS 13.0 computer package to analyze all sets of data and the means were compared by the Tukey test at 5% probability Levels. Sample variability is given as the standard deviation (S.D.) for presentation.

Results

Effects of elevated O₃ and/or CO₂ on P_n, g_s, C_i and Tr of *Q. mongolica* leaves

EO suppressed P_n of *Q. mongolica* leaves significantly ($p < 0.05$) over two growing seasons compared to the control. The maximum difference (about 72%) was recorded

after 45 days exposure in the first season, while EC increased it generally. P_n of leaves exposed to EO+EC was generally significantly higher than that exposed to EO alone, but lower than control except the last measurement (Fig. 1a and b).

In the first season, there was decrease of C_i detected in the EO environment compared to the control. While in the second season, there was almost no significant difference between EO and control treatments. However, EC and EO+EC treatments increased C_i of leaves compared to the control, and it was higher under EC than EO+EC (Fig. 1c and d).

EO and EO+EC generally decreased g_s of leaves significantly (p < 0.05), compared to the control and EC alone. However, there was no significant difference between EO+EC and EO treatments except after 60 days exposure in the second season. Compared to the control, EC slightly decreased g_s of leaves over the two growing seasons (Fig. 1e and f).

Similar change was shown between T_r and g_s of *Q. mongolica* leaves (Fig. 1g and h).

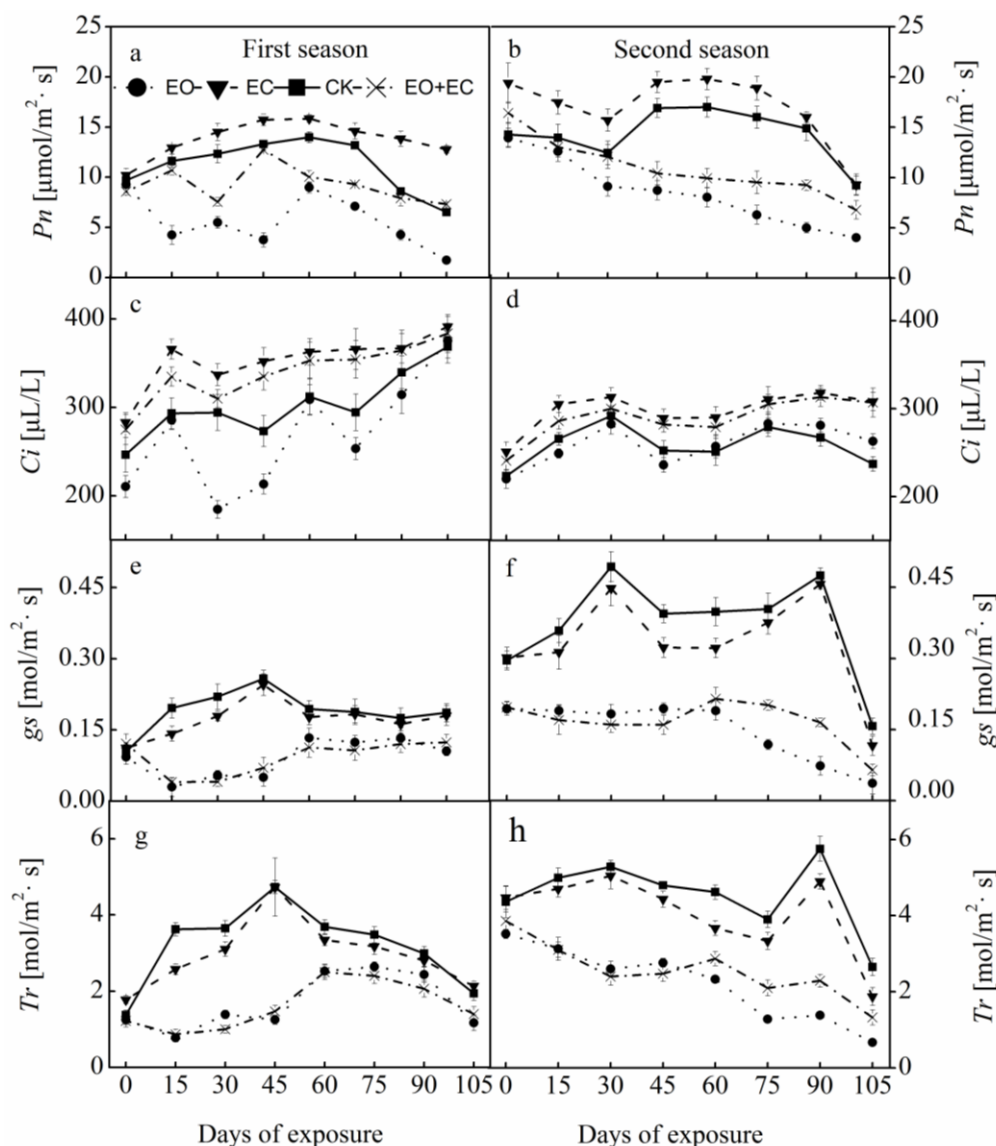


Figure 1. Effect of elevated CO₂ and/or O₃ on the seasonal changes of P_n, g_s, C_i and T_r of *Q. mongolica* leaves. Each value represents the mean ± SD

Effects of elevated O₃ and/or CO₂ on chlorophyll fluorescence parameters of *Q. mongolica* leaves

EO decreased F_0 , F_m and F_v/F_m of *Q. mongolica* leaves after 75 days in the second season compared to the control. The differences were significant in the later dates ($P < 0.05$), but there was no significant differences detected in other treatments (Fig. 2).

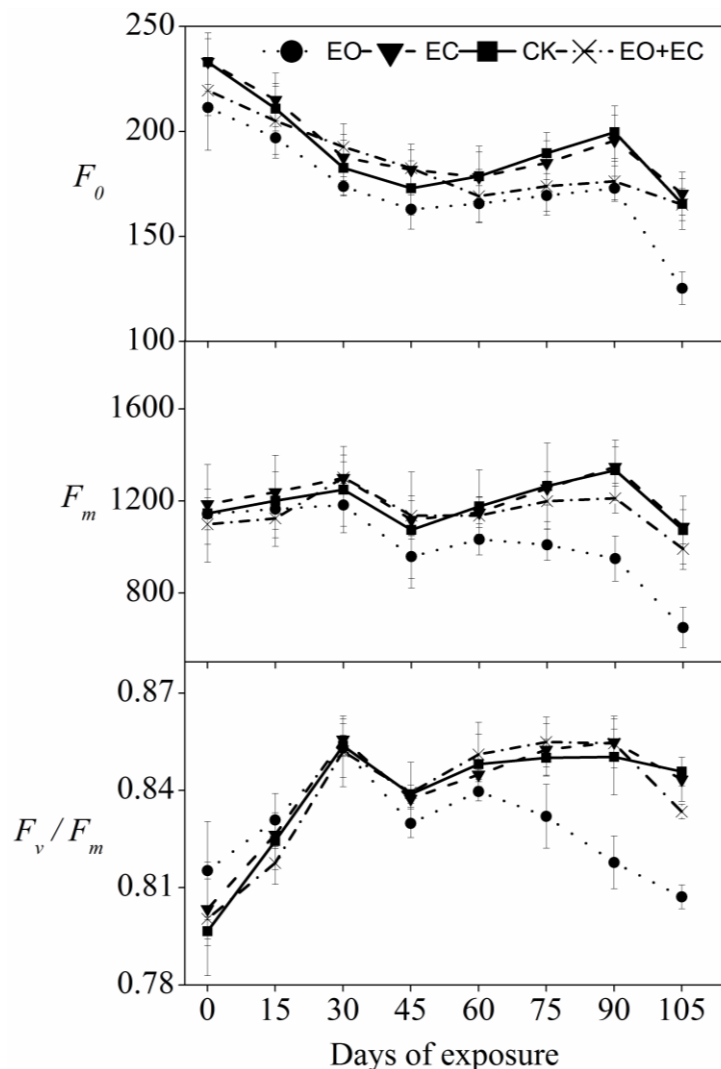


Figure 2. Effect of elevated CO₂ and/or O₃ on the seasonal changes of F_0 , F_m and F_v/F_m (the maximum quantum yield of PSII photochemistry) of *Q. mongolica* leaves. Each value represents the mean $\pm$ SD

Effects of elevated O₃ and/or CO₂ on stomatal parameters of *Q. mongolica* leaves

There was no difference of stomatal density of leaves detected in all the treatments in first season (0th, 60th day in the first season), but in the end of the second season (210th day), EC and EO+EC significantly decreased it compared to the control ($P < 0.05$). There was only a slight increase of stomatal width of leaves by EC compared to the control on 60th day ($P < 0.05$). There were no significant differences of stomatal length, stomatal perimeter and stomatal area of leaves in the all treatments (Table 1).

Table 1. Effect of elevated CO₂ and/or O₃ on stomatal density, length, width, perimeter and area of *Q. mongolica* leaves.

Days of exposure	Treatments	Stomatal density (No/mm ²)	Stomatal length (μm)	Stomatal width (μm)	Stomatal perimeter (μm)	Stomatal area (μm ²)
0 days	CK	293.00±22.03 ^a	72.72±5.21 ^a	23.20±1.42 ^a	204.91±18.80 ^a	1553.1±197.1 ^a
	EO	293.05±19.01 ^a	73.91±4.01 ^a	23.51±1.73 ^a	205.70±16.51 ^a	1555.0±173.6 ^a
	EC	293.01±20.16 ^a	74.13±5.72 ^a	24.05±1.91 ^a	206.27±11.59 ^a	1548.3±178.4 ^a
	EO+EC	293.00±18.05 ^a	73.50±6.51 ^a	23.47±1.58 ^a	206.05±25.62 ^a	1545.5±113.0 ^a
60 days	CK	293.05±25.02 ^a	76.06±7.22 ^a	21.05±1.53 ^b	203.04±16.72 ^a	1541.0±179.7 ^a
	EO	293.00±19.01 ^a	76.11±4.03 ^a	22.21±1.66 ^{ab}	204.72±12.12 ^a	1597.3±166.2 ^a
	EC	291.47±10.10 ^a	77.10±6.20 ^a	23.58±1.31 ^a	205.41±20.01 ^a	1646.6±105.8 ^a
	EO+EC	284.86±24.32 ^a	76.04±7.14 ^a	22.02±2.15 ^{ab}	203.60±18.88 ^a	1597.0±102.7 ^a
210 days	CK	366.00±16.90 ^a	65.92±6.82 ^a	18.09±1.11 ^a	174.21±19.12 ^a	913.8±167.5 ^a
	EO	350.43±22.60 ^a	66.07±4.38 ^a	18.22±1.44 ^a	175.83±13.64 ^a	923.3±108.4 ^a
	EC	325.67±12.00 ^b	67.03±4.31 ^a	18.53±1.62 ^a	181.11±16.63 ^a	967.2±117.2 ^a
	EO+EC	319.09±15.70 ^b	67.31±5.90 ^a	18.61±1.40 ^a	181.21±11.21 ^a	968.6±134.9 ^a

Each value represents the mean ± SD. (Different letters indicate significant differences between treatments at the same time at P < 0.05)

Discussion

Effects of elevated O₃

Many authors have found that photosynthetic processes are very sensitive to high O₃, and a decrease in the rate of light-saturated net photosynthesis as a result of exposure to elevated O₃ has been reported (Farage and Long, 1999). In our experiment, from the apparent decreases in P_n of *Q. mongolica* leaves under EO fumigation appear in early of the growing season, It can be concluded that EO has a severe impact on P_n of *Q. mongolica* leaves and *Q. mongolica* is an O₃-sensitive tree species. Since Bortier et al. (2000) suggested that faster growing species tend to be more sensitive than slower growing species. Also compared with mature trees, young trees are more susceptible to O₃ stress because of their high metabolic activity (Laurence et al., 1994). The sensitivity of *Q. mongolica* to EO in our experiment may be related to their young age. It has been correlated observed that photosynthesis decreased with a reduction in stomatal conductance under O₃ exposure (Mansfeld, 1998). In other cases it was assumed that the decline in photosynthetic rate was the result of the interaction of O₃ with photosynthetically active mesophyll cells (Farage and Long, 1995).

Stomatal conductance plays an important role in the photosynthetic efficiency of O₃ stressed plants by controlling the amount of pollutant entering the plants through the stomata (Matyssek et al., 2004). It is well known that species with high stomatal conductance are more prone to injury because of enhanced pollutant uptake (Matyssek et al., 2004). Our results showed that g_s of *Q. mongolica* leaves were significantly decreased by EO at the beginning of the experiment, while the similar decrease was appeared in T_r of leaves. These results indicate that the partial closure of stomata can be induced by EO to avoid the further ozone uptake. Stomatal limitation is mainly attributed to stomata closure and, subsequently, to the decrement of C_i under normal conditions (Salazar-Parra et al., 2015), however, many authors suggested

that stomata closure is not a direct response to O₃ insult, but a reaction to an increased intercellular CO₂ concentration resulted from the inhibition of carbon assimilation (Weber et al., 1993; Heath and Taylor, 1997). In our study, there was nearly no increase in C_i of leaves under EO, compared to the control at 15 days when g_s of leaves had decreased to minimum, so we propose that the stomata closure maybe associated with the direct effect of EO on guard cells of *Q. mongolica* leaves.

In low O₃ environments, photosynthesis and stomatal conductance are typically tightly coupled because the stomatal aperture equivalently controls the exchange of CO₂ and water at the leaf surface (Lombardozzi et al., 2012). However, from our study we can see that the typical coupling of stomatal conductance and photosynthesis does not always hold under EO and the decreases in P_n can be independent (in some extent) of g_s, suggesting that decreases in carboxylation, rather than g_s, are responsible for decreases in P_n.

The reduction in photosynthesis may also occur due to structural damage of thylakoids, which affects the photosynthetic electron transport and is indicated by the reduction in F_v/F_m ratio (Rai and Agrawal, 2012). F_v/F_m ratio is an indicator of the photoinhibition to PS II complexes. Under O₃ exposure, there are reports on wheat and rice showing increase in F₀ and a parallel decrease in F_m, suggesting impairment of PS II activity (Feng et al., 2011; Ishii et al., 2004). In our experiment, in the end of season, under EO exposure (after 75 days), there were decreases of F₀, F_m and F_v/F_m. Decreases of F₀ and F_v/F_m are the characteristics of thermal dissipation which depend on xanthophyll cycle and that dissipation reduces the photon energy to the photochemical reaction center (Xu, 2002). This can be seen as an important protection mechanism to avoid the destruction of PSII reaction centre. The decreases of F₀, F_m and F_v/F_m indicate that O₃ can limit the capability of the plant to use photon energy and thus alter photosynthetic processes. Decrease in the F_v/F_m is generally attributed to damage to the PSII reaction centre, apart from the down-regulation of the capacity of PSII electron transport (Chaumont et al., 1995).

Increased stomatal density seems to be a consistent effect related to ozone exposure and has been reported by Matyssek et al. (1991), but the same result was not found in this study, there were nearly no significant differences of stomatal parameters under EO compared to the control.

Effects of elevated CO₂

Rising CO₂ will impact plants through two processes, reducing g_s and increasing P_n (Ainsworth and Rogers, 2007). It has been reported that elevated CO₂ stimulated light-saturated photosynthesis in C₃ plants (Ainsworth and Rogers, 2007; Lahive et al., 2018; Panigrahi et al., 2016). In our experiment, EC also increased P_n of *Q. mongolica* leaves generally in two growing seasons until in the late of the second season (after 90 days).

Decreases in g_s of some plants induced by high CO₂ have been measured (Gao et al., 2012). However, the CO₂-sensing mechanism in guard cells that is responsible for the short-term sensitivity of g_s to elevated CO₂ is still unknown (Ainsworth and Rogers, 2007). In our experiment, EC decreased g_s slightly. Interestingly, unstressed plants tend to express a rather conservative range of C_i during steady-state photosynthesis, C_i is generally maintained at 0.7 C_a (concentration of ambient CO₂) even when C_a is varied (Drake and González-Meler, 1997). In our experiment, C_i of leaves under EC was generally higher than under ambient CO₂ treatments, that means perhaps change in C_i is closely related to C_a, and it also may be the reason of why g_s was decreased by EC.

Shifts in stomatal function and morphological response to CO₂ are likely to affect transpiration rates under rising atmospheric CO₂. In our study, reduction of stomatal aperture and conductance (g_s) explains the reduction in T_r observed in plants grown in EC.

Stomata display a wide range in short-term behavioral and long-term morphological responses to atmospheric CO₂ concentration (Haworth et al., 2013). In the short term, stomatal aperture generally decreases in response to high CO₂, as described earlier. In the long term, decreases in g_s can be caused by changes in stomatal density, as well as stomatal aperture (Ainsworth and Rogers, 2007), in the other hand, changes in leaf stomatal densities which in turn can control maximum values of stomatal conductance.

Ainsworth and Rogers' study (2007) showed that elevation of CO₂ in FACE experiments reduced stomatal conductance by 22%, yet this reduction was not associated with a similar change in stomatal density. However, some researchers believe that plants adapt to the elevated CO₂ by reducing their stomatal density (that is, the number of stomata per unit of epidermal surface area) (Engineer et al., 2014). One expectation at increased C_a is that fewer stomata are required because the rate of CO₂ diffusion into the leaf will be a decreasing limitation to photosynthesis as C_a rises (Drake and González-Meler, 1997). While in our experiment, there were no difference in stomatal density of mature *Q. mongolica* leaves detected among all treatments on 60th day (in the first season), but at the end of the second season (210th day), a significant decrease in stomatal density in response to EC was shown. Perhaps high CO₂ was detected by mature leaves in the first year and signaled to developing leaves in the next year, whose stomatal developments altered accordingly (Lake et al., 2001). Researchers believed that those species with little or no control of stomatal aperture (termed passive) to C_a were more likely to exhibit a reduction in stomatal density than species with active stomatal control when grown in atmospheres of elevated CO₂ (Haworth et al., 2013), that is perhaps why there was only a slight decrease in g_s of leaves detected in our experiment.

Modification to stomata abundance and size as a stress response is also stated an important pollutant absorption-controlling mechanism, and these parameters mutually affected each other. An inverse relationship between stomatal pore length or size and stomatal density has been observed in the gas exchange responses of plants in response to increased CO₂. However, a similar pattern was not observed in the species analyzed in Haworth's study (2013). While in our study, we can only see a slight increase in stomatal width of leaves in EC on 60th day ($P < 0.05$).

Effects of elevated CO₂ and O₃

It is concluded that season-long exposure to elevated CO₂ had a greater effect on photosynthetic parameters than elevated O₃ (Alison et al., 2001), while this result is opposite to ours. When high O₃ and high CO₂ were given in our experiment, P_n of leaves was still lower than control in the whole growing season except at the end of the season. It is also found that the combination of elevated O₃ and CO₂ can led to a significant decrease in the light-saturated rate of photosynthesis, but compared to elevated O₃ alone, the level of decrease was lower (Kellomäki and Wang, 1997). The study of the combined effects of elevated CO₂ and O₃ on photosynthesis show that elevated CO₂ compensated most of the adverse effects of O₃ (Reid and Fiscus, 1998). In our study, P_n of *Q. mongolica* leaves under EO+EC were nearly all significantly higher than EO in the whole seasons ($p < 0.05$). This suggests that O₃-induced adverse effects

to photosynthesis can be ameliorated, at least partly, by high CO₂, but the complete mechanism behind this interaction is, however, still unclear.

There was no significant difference of g_s between the combination and EO treatments except after 60 days in the second season, which was mainly caused by EO but not EC.

It is found there was a significant interaction between elevated CO₂ and increased O₃, elevated O₃ + CO₂ led to a 14% decrease in F_v/F_m compared to the control (Kellomäki and Wang, 1997). While in this experiment, there was no significant difference in F_v/F_m detected under the combined treatment, compared to the control. High CO₂ can largely relieve the adverse effects of high O₃ to PSII reaction center.

Changes in stomatal parameters of *Q. mongolica* leaves under EO+EC were just like the changes of them under only EC exposure. Perhaps the effect of stomatal morphology was mainly caused by EC but not EO.

Conclusion

Q. mongolica is an O₃-sensitive tree species and EO had a severe impact on P_n of *Q. mongolica* leaves because of their young age. g_s and T_r of leaves was significantly decreased by EO at the beginning of the experiment, which indicates that the partial closure of stomata can be induced by EO to avoid the further ozone uptake. The stomata closure maybe associated with the direct effect of EO on guard cells. Under EO, there were decreases in F_0 , F_m and F_v/F_m at the end of the season (after 75 days). This indicates EO can limit the capability of the plant to use photon energy and thus alter photosynthetic processes; EC generally increased P_n of *Q. mongolica* leaves, but decreased g_s slightly. The decrease of g_s is thought to maintain the ratio of C_i and C_a , and regulate the WUE of trees. A significant decrease in stomatal density in response to EC was shown only at the end of the second season (210th day) but not in the first season. Maybe, EC was detected by mature leaves in the first growing season and signaled to developing leaves in the next growing season, whose stomatal developments altered accordingly; Under EO+EC, P_n of leaves were still lower than that control, but nearly all significantly higher than EO in the whole seasons ($p < 0.05$). This suggests that O₃-induced adverse effects to photosynthesis can be ameliorated, at least partly, by CO₂. The decrease in g_s was mainly caused by EO but not EC. There was no significant difference in F_v/F_m detected under EO+EC, compared to control. EC can largely relieve the adverse effects of EO to PSII reaction center. Changes in stomatal parameters of leaves under EO+EC were just like the changes of them under only EC. Perhaps the effect of stomatal morphology was mainly caused by EC, but not EO.

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