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Title: Ecophysiological difference in co-existing beech and oak saplings grown in different soil types under a free-air ozone exposure system

Satoshi Kitaoka^{1,2}, Cong Shi³, Toshihiro Watanabe^{*1}, Takayoshi Koike¹

1: Research Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

2: Hokkaido Agricultural Research Center, NARO, Sapporo 062-0045, Japan

3: School of Environmental Science and Engineering, Tiangong University, Tianjin, China

* Correspondence: nabe@agr.hokudai.ac.jp

Abstract

To know effects of elevated ozone (eO₃) response to beech (*Fagus crenata*) and oak (*Quercus mongolica* var. *crispula*) saplings, focusing on shoot elongation and leaf nutrient characteristics, beech and oak were grown in a free-air ozone-enrichment system (concentration: 35 ppb (aO₃) vs. 75 ppb (eO₃)) for two years under different soils (volcanic ash soil vs. brown forest soil). This treatment resulted in lower O₃ tolerance in beech than in oak. The ratio of 2nd shoot flush showed a characteristic response to eO₃ fumigation in beech and oak. For beech, the ratio was less than 0.5, while for oak, it ranged between 0.4 and 0.6. There was no difference in the photosynthetic rate of oak, although the photosynthetic rate of beech was slightly lower under elevated O₃. Principal component analysis provided a good summary of the traits of beech and oak in response to O₃ enrichment and soil combination, with O₃-enriched beech characterized by higher Ca and lower N, P, K and $A_{\max}$ in the 1st component and lower Mg and Mn in the 2nd component. O₃-treated oak in O₃-enriched brown forest soils was characterized by high N, P, K and $A_{\max}$, while the influence of the 2nd component was small. Increased soil acidification will increase soil Mn and Al concentrations and may reduce the photosynthetic activity of beech saplings in eO₃.

Keywords: Ozone, soil fertility, photosynthesis, beech, oak

1. Introduction

Due to Asia' rapid economic growth, nitrogen dioxide (NO₂) as a precursor of ozone (O₃) exhibited levels that were 4 times higher than those of the European Union (EU) and the United States (USA) in 2020 (Feng et al. 2019; Kurokawa and Ohara 2020). Several reports indicate that current O₃ concentrations (approximately 60~80 ppb) could reduce final crop production by 30~60 % (Koike et al. 2013; Feng et al. 2019).

Representative afforestation species of Sugi-cedar (*Cryptomeria japonica*) and Hinoki-cypress (*Chamaecyparis obtusa*) are more O₃-tolerant than beech (*Fagus crenata*) and oak (*Quercus serrata*, and *Q. mongolica* var. *crispula*), which represent the temperate zone, are less tolerant than these conifers (Yamaguchi et al. 2011). The gas-flux data revealed a decline in beech stands in northern Japan have declined but one of the dominant oak species stands remained unchanged (Kitao et al. 2016). In open-system O₃ experiments, Kitao et al. (2016) found that *Q. serrata* seedlings exhibited an increase secondary shoot elongation, while *Q. mongolica* seedlings displayed a slight increase in leaf mass. These results indicate differing interspecific responses to elevated O₃ (eO₃), which may be compensatory growth for growth inhibition. It is also possible that 2nd shoot elongation (flush) is inhibited by eO₃.

Based on a greenhouse-like open-top chamber (OTC) using regular glass (with no UV transmittance) on potted seedlings, it was observed that beech seedlings are more sensitive to eO₃ than oak species when it comes to biomass productivity (Matsumura 2001; Yamaguchi et al. 2011). Additionally, it was found that the sensitivity of beech to O₃ increases with an increase of nitrogen (N) deposition (Yamaguchi et al. 2007). These reports suggest that sensitivity to O₃ may be strongly influenced by soil types. Beech requires moderately fertile soils, whereas oak is relatively tolerant to drought (Matyssek et al. 2012; Leuschner and Ellenberg 2017). These edaphic responses of the two species suggest that they have different access to nutrient elements associated with shoot growth (Schulze et al. 2019). Nutrient conditions affect growth and photosynthesis, but $A_{\max}$ at light and CO₂ saturation directly reflects the effect of nutrients in the plant body, excluding stomatal effects (Kitaoka et al. 2009).

The additional deposition of N, as well as the eO₃, can reduce the allocation of photosynthates to the roots and their amount in many plant species (Agathokleous et al. 2015; Wang et al. 2016; Paoletti et al. 2021) including European beech (Haberer et al. 2008), Siebold's beech, and two oaks (*Quercus serrata* and *Q. mongolica*) native to Japan (e.g., Hoshika et al. 2015). These suggest significant changes in nutrient acquisition by the root system under eO₃. It is essential to understand the photosynthetic response of dominant tree species to eO₃ in relation to the shoot development patterns

as well as soil types, especially nutrients and drought (Haberer et al. 2008; Hoshika et al. 2013; Agathokleous et al. 2015a; 2015b).

A stoichiometry study revealed the important role of re-translocation of foliar nutrients of three deciduous tree species (birch, beech, oak) grown in different soil types at eO₃ (Shi et al. 2017); however, the nutrient composition and the photosynthetic capacity of beech and oak tree saplings have not been examined (Watanabe and Yamaguchi 2011; Watanabe et al. 2013; Kitao et al. 2016; Watanabe et al. 2018). Plant sensitivity to O₃ is affected by soil types as well as specific nutrient balance, including symbiotic microbial activities (Watanabe et al. 2011; Matyssek et al. 2012; Wang et al. 2015). Furthermore, water-soluble elements are strongly influenced by soil moisture conditions, especially for beech species via stomatal regulation (Matyssek et al. 2012; Tobita et al. 2019). However, the growth functions of these deciduous broadleaved trees under eO₃ are influenced by nutrients (Shi et al. 2016).

This study aims to demonstrate the relationship between nutrient conditions, excluding N, such as representative micro-nutrients, and their effects on plant growth and $A_{\max}$. According to previous research (Wang et al. 2015), even in the presence of eO₃, phosphorus (P) and manganese (Mn) are expected to have a positive correlation with the $A_{\max}$ of beech and oak. This is because both elements are essential, in addition N, for photosynthesis under neutral soil pH (e.g., Kitao et al. 1999).

For this prediction, we measured seasonal changes in leaf mass per unit area (LMA) and leaf chlorophyll content (via SPAD) to determine the leaf maturity time of beech and oak saplings planted in two soil types. Subsequently, we observed 2nd or 3rd shoot growth and examined the $A_{\max}$ and nutrients in the leaves. Based on the secondary shoot growth in summer and the correlation between nutrient concentrations and $A_{\max}$, our data can enhance our understanding of how nutritional differences affect O₃ sensitivity in beech and oak.

2. Materials and Methods

2.1. Plant materials

In mid-July 2014, 2-year-old seedlings of Siebold's beech (*Fagus crenata*; hereafter beech) and oak (*Quercus mongolica* var. *crispula*; hereafter oak), which were obtained from Hokkaido Holti-green Co. Ltd. near Sapporo, were planted at the nursery of the Sapporo Experimental Forest, Hokkaido University, northern Japan (43°04' N, 141°20' E, 15 m a.s.l.). The first tree is a typical late successional species characterized by flush growth of shoot development whereas the second tree is a mid-late successional tree species with a "two to three times flush growth of shoot. Intraspecific competition often

occurs between the two species due to differences in their shoot structure (Kashimura 1978; Yagihashi et al. 2003; Kitao et al. 2019).

The seedlings were exposed to an experimentally enhanced O₃ regime with a free-air O₃ enrichment system (O₃-FACE) (Shi et al. 2017) for two growing seasons. The details of O₃-FACE are elaborated in the following subsection. Monthly sampling of 4 saplings per species for each treatment (8 treatments in total) was performed with 3 replications during the O₃-FACE operated snow-free period. The height of the trees varied between 2.1 m for beech and 2.3 m for oak under ambient O₃ (aO₃) and 1.7 m for beech, 2.5 m for oak under eO₃, respectively. The O₃ exposure took place between early August and mid-November of 2014, from mid-June to early December of 2015, and from mid-June to the end of November of 2016, excluding the snow season. Sapporo's annual average temperatures and total precipitation for 2014, 2015, and 2016 were 8.2°C and 1129 mm, 10.3°C and 1331 mm, and 9.3°C and 1331 mm, respectively.

2.2. Treatments

The experimental site consisted of 6 circular plots, of which 3 were used for (aO₃) and 3 for eO₃. Each circular plot was 6.5 m in diameter with 5.2 m high dispersal pipes surrounding the circle (Kim et al. 2011). The distance between aO₃ plots and eO₃ plots was about 40 m. The method of O₃ exposure applied in this study was based on the system used in the Kranzberg Forest, Germany (Nunn et al. 2005).

We produced O₃ in the field with an O₃ generator (Fuji, Tokyo, Japan). Watanabe et al. (2013) described the free-air O₃ fumigation (O₃-FACE) field station in detail. The O₃ was supplied from the upper part of FACE with Teflon-coated tubes at 80 cm intervals with 0.3 mm holes at 50 cm intervals. In order to simulate the high O₃ concentration in the near future under natural conditions, the O₃ concentration of the eO₃ plots was regulated as $75 \pm 8 \text{ nmol mol}^{-1}$ during daylight hours (about 8 h d^{-1}), while the aO₃ plots were subjected to daytime O₃ concentration of $30 \pm 4 \text{ nmol mol}^{-1}$ as the simulation of the current O₃ condition in urban centers. These were monitored by the O₃ monitor (Type 408, 2B Technologies, Boulder, CO, USA)

Each plot in the experimental system was divided into two sections and set with 2 different soil types: brown forest soil (BF; Dystric Cambisol), which acted as control in this study and was original soil of the nursery, and volcanic ash soil (VA; Vitric Andosol), which was a mixture collected from the Tomakomai Experimental Forest of Hokkaido University, located 60 km south of Sapporo, at about a 25 cm surface depth,. supported the nutrient absorption by the fine roots of two tree species (Shi et al. 2017). BF soil is a fertile soil type containing sufficient nutrients necessary for plant growth (Kim et al. 2011; Kayama et al. 2011). The VA soil was mixed with immature volcanic

ash soil, which is considered highly acidic with a pH of around 4.0; it contains high levels of aluminum (Al) and iron (Fe), but low levels of primary and secondary nutrients (Watanabe et al. 2010; Kayama et al. 2011). The pH of the soil mixture was about 4.38. More details on soil nutrients and pH are given in Table 1. pH was measured with a portable sensor (LAQUA D-220P-S, Horiba, Kyoto, Japan) in each soil type where beech and oak saplings were grown.

2.3. Measurements

Shoots of beech and oak trees can elongate multiple times during the summer season (Kashimura, 1978). To assess the impact of O₃ and soil types on this process, we monitored 100-120 cm shoots located from stem base of individuals in each treatment, recorded the secondary elongation of shoots as a ratio of total individuals, and evaluated the results.

The leaf greenness was detected using a SPAD chlorophyll meter (Minolta, Tokyo, Japan) (Eguchi et al. 2006), and leaf mass per unit area (LMA) was used for leaf maturity (Watanabe et al. 2018). For oak, the mean SPAD value was obtained from three leaves from the base of the shoot in the 2nd flush. We also observed the shoot development (leaf flush, new shoot development, leaf yellowing) of beech and oak. Fresh leaves were collected from the second or the third leaf counted from the top of a shoot of beech or oak exposed to sunlight. All collected leaf samples were dried at 70 °C for 5-7 days after rinsing the surface with distilled water and ground to a powder in a mill. For both beech and oak, three individuals were selected for each treatment, and one leaf per individual was tested.

The gas exchange rate of mature sun leaves of beech and the first flush of sun leaves of oak was measured during July and August 2016 using with an open gas exchange system (LI-6400, Li-Cor Inc., Lincoln, NE, USA) to adjust leaf age of around 70~80 days at peak (Koike 1990). Four saplings were randomly selected in each gas plot (aO₃ vs. eO₃) under two soil types (BF soil vs. VA soil) of each species, and one leaf of each selected tree was used for gas exchange measurement. The gas exchange rates were measured between 08:00 and 15:00 hours (except 13:00~14:00) to avoid mid-day depression. The leaf temperature and photosynthetic photon flux density during the measurement were maintained at 25.0 ± 0.5 °C and 1500 μmol m⁻² s⁻¹, respectively. The leaf-to-air vapor pressure deficit (VPD) was 1.5 ± 0.2 kPa. To obtain the intercellular CO₂ concentration (C_i) response curve of the net photosynthetic rate (A) (A/C_i curve), A was determined at 10 CO₂ concentration levels in the chamber (C_a, 60-1700 μmol mol⁻¹). Basic measurement procedure of A/C_i curve was following to Long and Bernacchi (2003).

We determined A , stomatal conductance, and the ratio of C_i to C_a at $C_a = 390 \mu\text{mol mol}^{-1} \text{CO}_2$ (A_{sat} , G_s , and C_i/C_a , respectively). The maximum rates of carboxylation (V_{cmax}) and electron transport (J_{max}) were calculated from the A/C_i curve (Farquhar et al. 1980; Long and Bernacchi 2003). Values of the Rubisco and Michaelis constants for CO_2 (K_c) and O_2 (K_o), and the CO_2 compensation point in the absence of dark respiration (Γ^*) for analysis of the A/C_i curve were according to Long and Bernacchi (2003). All A/C_i fitting parameters were calculated by the Plant-Ecophys package of R language (Duursma 2015). Since $V_{\text{cmax}}/J_{\text{max}}$ is almost constant in young trees under eO_3 (Watanabe et al. 2018), A_{max} is regarded as an adequate parameter for estimating the photosynthetic capacity of both tree species. In addition, we can evaluate the potential capacity of photosynthesis without any stomatal regulation under CO_2 and light saturation like *slach1* mutant (Saji et al. 2017).

To measure the concentrations of Magnesium (Mg), potassium (K), Calcium (Ca), Mn, 50 mg leaf samples were digested with nitric acid and hydrogen peroxide. P was determined according to the Bray II method (Wuenschel et al. 2015). Analysis of leaf concentrations was then performed using an inductively coupled plasma mass spectrometer (ICP-MS; IRIS/IRIS Advantage ICAP, Thermo Fisher Scientific Inc., Waltham, MA, USA). The combustion method with an NC analyzer (NC-900, Sumica, Osaka, Japan) was used to measure the N concentration in the leaf samples.

2.4. Statistics

All statistical analyses were performed using by R language (R Core Team 2016). Two-way analysis of variance (Two-way ANOVA) was used to test the effects of O_3 fumigation and the soil types as well as their combinations. The relationship between photosynthetic traits and leaf nutrient elements was tested by principal component analysis (PCA). Before applying ANOVA, homoscedasticity, normality and outliers were checked. The significance levels were set at $p < 0.05$, although we suggested p -values from 0.05 to 0.10 to indicate marginal significance.

3. Results

3.1. Soil types

The pH of soils at the study site, specifically BF and VA, were 5.25 and 4.38, respectively (see Table 1). The concentrations of nitrogen (N) and phosphorus (P) in leaf tissue were significantly greater in BF soil compared to VA soil ($p < 0.05$). Additionally, Magnesium (Mg) concentration was higher in BF soil than in VA soil ($p < 0.05$), particularly under eO_3 treatment. No interaction effects between soil and O_3 were detected concerning N, P, and Mg. There were no significant differences in K, Mn, and

Al concentration in leaf tissue between O₃ levels and soil types. Moreover, measurements of phenological observations, SPAD, LMA, and photosynthetic traits were taken. Beech species flush their leaves uniformly in early May, and the emergence of new leaves doesn't occur until the leaves begin to senesce around mid-to-late September. The SPAD value sharply increased in mid-June, gradually continued to increase through mid-July, and remained unchanged at the beginning of September. Based on Figure 1, the SPAD value of beech in aO₃ was slightly higher than that in eO₃, regardless of soil type, during July and early August.

3.2. Phenological observation, SPAD, LMA and photosynthetic traits

Beech flushed leaf in early May, followed by a senescence period from mid-to-late September, except very few under aO₃ in July. The SPAD of beech showed a sharp increase in mid-June, followed by a gradual increase in mid-July, and remained constant at the start of September. During July and early August, the SPAD in aO₃ was marginally higher than that in eO₃, regardless of the soil type (see Fig. 1).

In mid-May, oak partially displayed 3-5 leaves as the 1st flush, and by early June, it successfully developed 3-4 leaves as the 2nd flush. No difference in the SPAD value was observed between the 1st and 2nd flushes of oak. Furthermore, in August, the LMA value of beech tended to be larger independent of soil type at eO₃ ($p = 0.11$). Regardless of soil type, an increase in LMA was seen in oak under eO₃ conditions during the early growing season. However, there were no significant differences in LMA during leaf senescence or after early frost past mid-September. Leaf senescence of oak in the 1st and 2nd flush began in early October and mid-October, respectively.

The ratio of 2nd flush demonstrated a distinctive response to O₃ exposure in beech and oak. Regardless of soil, one to two individuals of beech exhibited 2nd flush in mid-July in aO₃. No 2nd flush was observed in August. In contrast, 3 to 4 oak individuals per ring showed 2nd flush in July in aO₃, 1 to 2 trees in eO₃ showed fall growth in July regardless of soil, and 1 to 2 trees in eO₃ showed 2nd flush in August regardless of soil and O₃ levels.

During the leaf senescence period, LMA gradually decreased, ranging from 62-63 g m⁻² around August to 56-58 g m⁻² towards mid-October ($P < 0.05$). The decline of LMA in both beech and oak trees was higher in aO₃ surroundings as compared to eO₃, regardless of soil types, towards October. In 2nd flush of oak growth, the mean LMA of three leaves from the bottom of the shoot, grown in VA, was approximately 5 g m⁻² larger and maintained the highest value of 63-66 g m⁻² in early October when the leaves were destroyed by early frost.

In beech trees, $A_{\max}$ tended to decrease with exposure to eO_3 (Table 2). Additionally, both $V_{C_{\max}}$ and $J_{\max}$, which are biochemical characteristics of photosynthesis, were significantly reduced by eO_3 . However, there were no significant effects observed within the BF and VA soil. In contrast, oak did not show a significant decrease in $A_{\max}$ due to eO_3 , and no significant effects of eO_3 were observed in $V_{C_{\max}}$ and $J_{\max}$, as shown in $A_{\max}$. Additionally, there were no significant differences between BF and VA soils.

3.3. Principal component analysis (PCA)

The PCA indicated that the 1st principal component (pc) had a contribution ratio of 0.51, whereas the 2nd pc contributed 0.22 (Figure 2). The 1st pc comprised of $A_{\max}$ and N, P, K with Ca as the 2nd pc. When N, P, K, and $A_{\max}$ were high, the score was low, and the value of Ca was greater when the score was high.

The 2nd pc score was defined by the secondary elements Mg, as well as the trace elements of Mn and Al. Higher scores indicated higher values for Mn and Al, while larger values for Mg indicated lower values for the second primary component score. Beech was more easily distinguished by eO_3 than oak. O_3 exposure with BF resulted in lower levels of N, P, K, and $A_{\max}$, as well as higher Ca content, as characterized by the 1st pc having little influence on the 2nd pc. Similar trends were observed in eO_3 with VA. Additionally, the N, P, K, and $A_{\max}$ were high in aO_3 with BF.

Oak in aO_3 with both BF and VA exhibited comparable responses, with the small influence of the 1st pc and the amount of Mg in the 2nd pc being the defining characteristics. Oak exhibited lower variation within treatments in the 1st pc, while the differences in treatment were distinguished by the variation in the 2nd pc. No discernible pattern was observed for eO_3 . Oak trees in aO_3 with BF and eO_3 with VA soils were less impacted by the 1st pc. Moreover, in eO_3 with forest soils, oaks were identified by their Al content.

4. Discussion

Previous studies have reported that various tree species with distinct shoot development patterns - flush type, succeeding growth type, and intermediate type - display unique resource retranslation characteristics in temperate regions (Larcher 2003; Watanabe et al. 2013; Shi et al. 2017). According to data from SPAD and LMA (Fig. 1), beech leaves reach leaf maturation under eO_3 within 1.5 months after emergence, while oak leaves complete maturation in August. Although research indicates that oak has a higher tolerance for O_3 than beech (Yamaguchi et al. 2011), the delayed maturation of oak leaves suggests that unmaturing oak leaves may have been exposed to O_3 for longer periods than beech. Leaf Mass per Area (LMA) demonstrates a strong correlation with

$A_{\max}$ and is influenced by light conditions. Additionally, it characterizes distinct traits of beech and oak saplings following exposure to eO_3 , even during free-air exposure in July and August. The second flush for oak occurred in mid-July and was unaffected by soil type or O_3 levels, as seen in the results of two oak species at the O_3 -FACE near Tokyo, documented by Kitao et al. (2015). Similar findings were also present in beech and two oak species grown in Andisol soil originating from volcanic ash under eO_3 , as Tobita et al. (2019).

Many plant species absorb P, water, and N through the functional activities of symbiotic ectomycorrhizae (Schulze et al. 2019), which are affected by soil pH and fertility (Marschner 2011). The $A_{\max}$ for beech and oak species in VA under eO_3 levels was slightly higher than that of the BF species. This increase can be attributed to the production of antioxidants in both species under nutrient-limited conditions. According to further research, the nutrient utilization traits of beech and oak differ. Beech commonly requires fertile soil, as noted by Kitao et al. (2016). Additionally, beech utilizes K in conjunction with P (Supple-Tab.1). Synergy and antagonism effects among elements must be taken into account (Marschner 2011). Larcher (2003) found that despite Mg serving as the skeleton material of chlorophyll, the positive correlation between Mg and $A_{\max}$ was weaker in oak than in beech. The nutrient balances found between Mg and Ca (Shi et al. 2016; Shi et al. 2017) may create a synergy effect while playing no role in the contrast of Mn between beech and oak. Additionally, oak had a higher $A_{\max}$ than beech, due to the influence of soil nutrient conditions (Table 1). In truth, tree growth is affected not only by the chemical or nutritional properties of the soil but also by physical factors (Schulze et al. 2019).

Recently, Watanabe et al. (2020) suggested that the photosynthetic nitrogen use efficiency ($PNUE$) in beech does not change with nutrient application, but $PNUE$ may increase photosynthesis per N and residence time in leaves. This research may add another explanation for the increase in N, which will decrease the net photosynthetic activity at aCO_2 levels under eO_3 (Yamaguchi et al. 2007).

The correlation between $A_{\max}$ and Mn, Al varied between beech and oak (Supple-Tab. 1). Mn and Al exhibited negative correlation with $A_{\max}$ in beech, indicating that soil acidification poses a greater threat to beech than O_3 stress in suburban regions (Gregg et al. 2003; Kohno 2017; Moser-Reischl et al. 2019). On the other hand, Supple.-Table 1 revealed that while oak did not exhibit a clear correlation between Mn and $A_{\max}$, a weak positive correlation was uncovered between Al and $A_{\max}$ in oak leaves. This trend appears uncommon. Notably, Watanabe and Azuma's (2021) screening experiments on 819 plant species indigenous to Japan recently uncovered Al's unique

characteristics. According to them (2021), the coefficient of variation (CV) for Al was approximately 5.5 among species, while the CV for macro-elements was less than 1.0, and that for micro-elements ranged from 1 to 6, excluding Ni. It is worth noting that some species do require Ni as a micro-element. The Al tendency in oak trees, as suggested by the study, is backed by the concept that even though there are substantial dissimilarities in the absorption of nutrients containing Al between various plant species (Watanabe and Azuma 2021), oak can be categorized as a species that has a need for Al, which is depicted in the advancement of root growth (Tomioka et al. 2007; Tomioka and Takenaka 2007).

The PCA results demonstrated distinct features between beech and oak in BF soil with exposure to eO₃ (Fig. 2). Oak exposed to eO₃ in brown forest soil retained larger K, N, and P concentrations than in aO₃, with no suppression in $A_{\max}$. Although, there was an indication of a decreasing trend in Ca accumulation in leaves. This trend suggests that the deposition on leaf cell walls is delayed due to Ca absorption by the transpiration stream. This may occur because of transpiration suppression, which avoids O₃ absorption (Hoshika et al. 2011). In contrast, beech did not show notable suppression in K, N, P, and $A_{\max}$ compared to the control. Despite that, high Ca accumulation indicates that the leaf cell walls may have matured early and turned tough, which may inhibit future photosynthesis. In populated areas, individuals with excessive Ca supply exhibit lower NPK concentrations than those with optimal Ca supply (Weng et al., 2022). Additionally, Ca has been found to function as a signaling response to pollutants, particularly O₃, in stomata (McAinsh et al., 2002). These discoveries suggest that beech is affected by eO₃ levels since it absorbs O₃ through its stomata. In contrast, oak is highly tolerant to O₃ (Yamaguchi et al., 2011) and achieves higher $A_{\max}$ than beech.

Ca is a reliable indicator of soil fertility (Marschner, 2011; Foth and Ellis, 2020). According to Kitao et al. (2016), beech prefer to grow in fertile and moist soil. Ca serves to strengthen the cell walls of leaves, and once deposited in the cell wall, it remains largely immobile (Marschner, 2011; Watanabe and Azuma, 2021). Whilst oak underwent a 2nd flush even by September, beech did not, even in aO₃ conditions. During the process of leaf aging or shedding, crucial elements are re-translocated from aging leaves to new or non-photosynthetic organs (Marschner 2011; Shi et al. 2016). A weak correlation between Ca and $A_{\max}$ of oak observed in this study can be attributed to the pattern of shoot development, specifically the presence of a 2nd flush. It is important to consider the carryover effects of O₃ on the growth of perennial plants, particularly fixed-growth tree types such as beech (Yonekura et al., 2001; Yonekura et al., 2004).

However, future changes in beech and oak under rapidly changing environmental conditions such as N deposition and soil acidification remain unclear.

The decline in vegetation function in East Asia is partly due to changes in NO₂ emissions (Kurokawa and Ohara 2020; Qu et al. 2022). NO₂ is the precursor of O₃ under ultraviolet radiation, which suppresses photosynthetic function through oxidation (e.g., Gregg et al. 2003). In particular, the agricultural system in Northeast China has been changing, especially soybean cultivation; farmers use commercial chemical fertilizers instead of *Rhizobium* sp. (N₂-fixing microbe) (Qiu 2009; Ju et al. 2009; Lu et al. 2015; Li et al. 2017; Hou et al. 2018). As a result, additional N leads to N deposition, causing an imbalance of N cycling in this region (Izuta 2017; Takahashi et al. 2020; Qu et al. 2022).

In addition, water stress is often caused by changes in precipitation pattern (Yonekura et al. 2001; Yonekura et al. 2004). As a result, photosynthetic activity is certainly affected by plant nutrient load; in addition, plant physiological functions are strongly influenced by soil pH and the status of water-soluble elements. Although Mn is an essential microelement and is released below pH 5.0, additional Mn reduces photosynthetic activity, while Al released below pH 4.5 has a negative effect on root meristem development (Marschner 2011; Schulze et al. 2019; Watanabe and Azuma 2021). Regardless of the O₃ level, the pH of the studied soils was lower than 5.2 (Table 1); consequently, in VA soils, the pH values independent of O₃ levels (about 4.6 and 4.3, respectively) imply that Mn was already released (pH <5.0) and Al was gradually released (pH <4.5).

The amount of SO₂ deposition has decreased with the rapid installation of desulfurization equipment in China, and consequently, SO₄ deposition toward Japan has also decreased (Takahashi et al. 2020). As mentioned above, N deposition is increasing in China (Qu et al. 2022). The increase in NH₄⁺ and SO₄²⁻ is due to the conversion of some of the (NH₄)HSO₄ to (NH₄)₂SO₄, which is already found on the west coast of the USA, where pollution has been worsening (Butler et al. 2016). Compared to oak, beech growth activity is expected to decrease with increasing soil Al content accompanied by decreasing soil pH under elevated ground-level O₃ (Tab. 2).

These nutrient responses of beech and oak species are strongly influenced by the direct effects of eO₃ combined with drought (Haberer et al. 2008) and other environmental stresses (Agathokleous et al. 2015; Paoletti et al. 2021). Although several studies have been conducted on mature European beech exposed to eO₃ or N deposition, an essential role of phytohormones in fine root formation was found (Matyssek et al.

2012). Further studies are needed to analyze the roots of beech and oak to better understand the role of fine roots.

Finally, based on the flux tower experiments, beech stands have already shown O₃ induced symptoms of decline, but there were no clear symptoms of decline in oak stands. In general, beech prefers nutrient-rich and moist sites where water-soluble nutrients play a crucial role for its growth under eO₃ compared to oak stands. From our findings on different soil conditions in the O₃-FACE, the reduced activity of beech under eO₃ may be due to the imbalance of nutrients compared to oak.

Supplementary Materials: The following are available online at Supplementally table 1,

Results of Spearman's rank correlations between $A_{\max}$ and leaf nutrients of beech and oak grown under ozone fumigation treatments with two different soil types.

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Figures and Tables legends,

Table 1. Soil nutrient conditions at an outdoor ozone fumigation site with two soil types. BF, brown forest soil; VA, volcanic ash soil.

Figure 1. Seasonal changes in SPAD (chlorophyll) of beech and oak leaves exposed to ambient vs. elevated O₃ grown in brown forest soil mixed with volcanic ash soil. Different line colors indicate different treatments, ambient with brown forest soil (BF Amb, blue), ambient with volcanic ash soil (VA Amb, gray), ozone fumigation with brown forest soil (BF O₃, orange), and ozone fumigation with volcanic ash soil (VA O₃, sunlight yellow).

Table 2. Leaf nutrients and $A_{\max}$ of beech and oak trees grown under factorial combinations of two O₃ levels and two soil types. Means and results of two-way ANOVA of $A_{\max}$ between nutrient elements of beech and oak leaves grown under combinations of O₃ levels and different soils as shown in Table 1. SE, standard error; $A_{\max}$, maximum photosynthetic rate (nmol m⁻²s⁻¹; under light and CO₂ saturation). N measured in mg g⁻¹, other nutrients (P, K, Mg, Al, Mn, and Ca) in mg 100g⁻¹. Symbols on values indicate significant differences, † p < 0.1; * p < 0.05; ** p < 0.01.

Figure 2. Results of principal component analysis of beech and oak grown under both ambient and O₃ fumigation treatments. Abbreviations: Al: leaf aluminum concentration, $A_{\max}$: photosynthetic rates at light and CO₂ saturation, Ca: leaf calcium c., Fc_abf: beech grown under aO₃ with brown forest (BF), Fc_avl : beech grown under aO₃ with volcanic ash (VA) soil, Fc_obf: beech grown under eO₃ with BF, Fc_ovl: beech grown under eO₃ with VA, K: leaf potassium c., Mg: leaf magnesium c., Mn: leaf manganese c., N: leaf nitrogen c., P: leaf phosphorous c., Qm_abf: oak grown under aO₃ with BF soil, Qm_avl : oak grown under aO₃ with VA, Qm_obf: oak grown under eO₃ with BF, Qm_ovl: oak grown under eO₃ with VA.

Supplementally Table 1

Results of Spearman's rank correlations between photosynthetic rates ($A_{\max}$) and leaf nutrients of beech and oak grown under ozone fumigation treatments with two different soil types. Abbreviations: Al: leaf aluminum concentration, $A_{\max}$: photosynthetic rates at light and CO₂ saturation, Ca: leaf calcium c, K: leaf potassium c., Mg: leaf magnesium

696 c., Mn: leaf manganese c., N: leaf nitrogen c., P: leaf phosphorous c.. Symbols on the
697 values indicate significant differences, †: $p < 0.1$; *: $p < 0.05$; **: $p < 0.01$.
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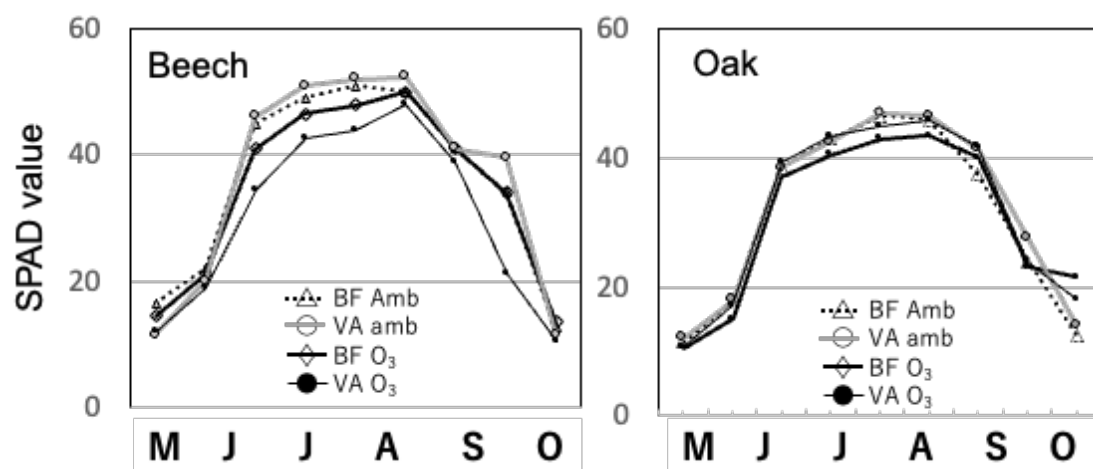
Figure and Tables

Table 1. Soil element (nutrient) conditions at free-air ozone exposure site with two types of soil. Brown forest soil (BF); Volcanic ash soil (VA). Ambient (Amb); Ozone (O₃)

element	mean (SE)				<i>p</i> -value		
condition	Amb-BF	Amb-VA	O ₃ -BF	O ₃ -VA	O ₃	soil	O ₃ × soil
pH	5.24 (0.03)	4.26 (0.04)	5.32 (0.02)	4.57 (0.04)	-	-	-
N	2.98 (0.02)	2.47 (0.29)	2.64 (0.13)	2.34 (0.05)	0.162	0.044*	0.527
P	2.79 (0.29)	2.01 (0.03)	2.65 (0.25)	2.04 (0.14)	0.632	0.022*	0.682
K	21.65 (0.92)	22.2 (1.05)	23.33(1.45)	23.18 (1.51)	0.363	0.763	0.784
Ca	247.97(42.98)	209.87 (34.40)	295.5 (12.90)	273.18 (22.08)	0.290	0.392	0.799
Mg	20.23(1.63)	15.46(1.02)	24.51(1.08)	20.33(0.70)	0.023*	0.013*	0.805

Unit of element: N (mg g⁻¹) and the rest elements (mg 100g⁻¹)

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709 **Figure 1.** Seasonal changes in SPAD (chlorophyll) value of leaves of beech and oak
 710 exposed to ambient vs. elevated O₃ grown in brown forest soil mixed with volcanic ash
 711 soil. Different line colors indicate different treatments, ambient with brown forest soil
 712 (BF Amb, blue), ambient with volcanic ash soil (VA Amb, gray), ozone fumigation
 713 with brown forest soil (BF O₃, orange) and ozone fumigation with volcanic ash soil (VA
 714 O₃, sunlight yellow).

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Table 2. Leaf nutrients and $A_{\max}$ of beech and oak trees are grown under factorial combinations of two O_3 levels and two soil types. The mean values and results of the two-way ANOVA of $A_{\max}$ between nutrient elements of beech and oak leaves grown by combination of O_3 fumigation and different soils as shown in Table 1. SE, standard error; $A_{\max}$, maximum photosynthetic rate ($\text{nmol g}^{-1}\text{s}^{-1}$; under light and CO_2 saturation). N measured in mg g^{-1} , other nutrients (P, K, Mg, Al, Mn, and Ca) in $\text{mg } 100\text{g}^{-1}$. Symbols on the values indicates significant differences, † $p < 0.1$; * $p < 0.05$; ** $p < 0.01$.

Sp.	Mean (SE)				F-value of two-way ANOVA		
	Amb-BF	Amb-VA	O_3 -BF	O_3 -VA	O_3	Soil	$O_3 \times \text{soil}$
Beech	$A_{\max}$	332.83 (26.92)	353.33 (43.62)	214.55 (17.37)	291.61 (42.56)	4.23†	1.56 0.52
	$J_{\max}$	125.89 (7.32)	130.97 (2.52)	96.37 (6.04)	101.52 (7.55)	22.66**	0.68 < 0.01
	$V_{c\max}$	48.95 (4.17)	54.66 (2.12)	37.63 (3.58)	41.99 (5.25)	9.25*	1.63 0.03
	N	43.65 (2.48)	44.77 (1.62)	33.4 (1.45)	38.00 (2.53)	10.66**	1.12 0.45
	P	3.76 (0.19)	3.35 (0.14)	2.56 (0.16)	3.19 (0.28)	8.54*	0.11 4.55
	K	5.38 (0.73)	5.83 (0.86)	4.87 (0.43)	4.93 (0.27)	0.96	0.14 0.08
	Mg	3.8 (0.18)	4.13 (0.32)	3.38 (0.38)	2.90 (0.27)	6.92*	0.04 1.53
	Al	0.19 (0.01)	0.22 (0.02)	0.24 (0.01)	0.26 (0.01)	16.53**	4.03† 0.34
	Mn	0.43 (0.02)	0.49 (0.04)	0.63 (0.03)	0.60 (0.04)	15.66**	0.19 1.41
	Ca	19.15 (1.20)	19.4 (1.18)	25.53 (0.15)	20.95 (1.85)	5.57*	1.91 2.38
Oak	$A_{\max}$	392.09 (55.49)	342.51 (24.72)	332.40 (34.64)	339.06 (42.05)	0.38	0.36 0.40
	$J_{\max}$	135.10 (8.10)	134.47 (3.22)	127.71 (7.92)	136.85 (10.74)	0.10	0.29 0.38
	$V_{c\max}$	46.88 (2.90)	47.97 (1.49)	48.77 (2.26)	50.23 (3.47)	0.62	0.23 < 0.01
	N	45.63 (4.51)	42.8 (0.78)	46.03 (1.28)	47.2 (2.43)	0.41	0.05 0.30
	P	3.46 (0.42)	4.25 (0.20)	3.46 (0.48)	3.97 (0.34)	0.01	1.95 0.1
	K	10.68 (1.24)	12.1 (0.25)	14.57 (1.39)	9.90 (0.99)	0.22	1.50 5.31*
	Mg	3.79 (0.18)	4.11 (0.25)	3.40 (0.38)	2.96 (0.23)	5.82*	0.04 1.36
	Al	0.45 (0.14)	0.21 (0.01)	0.34 (0.14)	0.36 (0.07)	0.001	0.71 0.96
	Mn	0.64 (0.03)	0.34 (0.02)	0.43 (0.05)	0.66 (0.03)	2.13	0.69 49.3**
	Ca	15.78 (0.29)	14.58 (1.22)	14.00 (0.75)	15.98 (1.06)	0.002	0.05 1.69

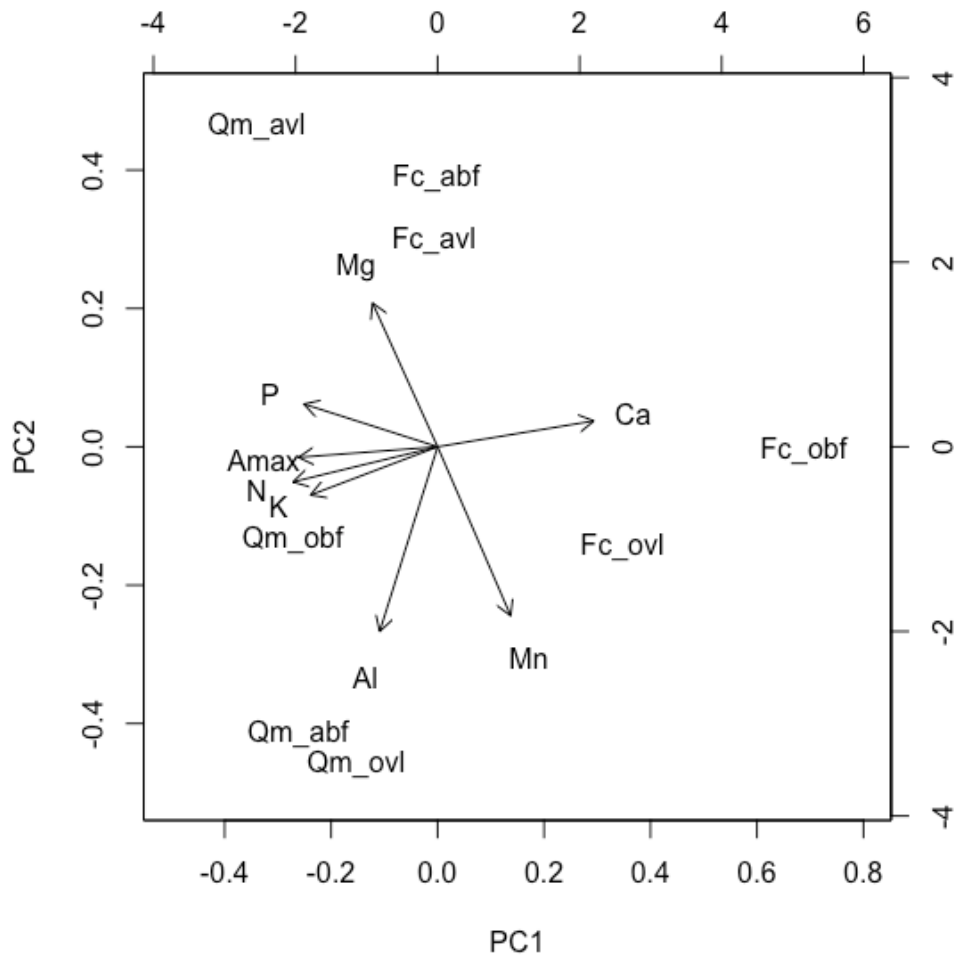


Figure 2. Principal component analysis of beech and oak grown under aO₃ and eO₃. Abbreviations indicate that Al: leaf aluminum concentration, $A_{\max}$: photosynthetic rates at light and CO₂ saturation, Ca: leaf calcium concentration, Fc_abf: beech grown under ambient with brown forest soil, Fc_avl : beech grown under ambient with volcanic ash soil, Fc_obf: beech grown under O₃ fumigation with brown forest soil, Fc_ovl: beech grown under O₃ fumigation with volcanic ash soil, K: leaf potassium concentration, Mg: leaf magnesium concentration, Mn: leaf manganese concentration, N: leaf nitrogen concentration, P: leaf phosphorous concentration, Qm_abf: oak grown under ambient with brown forest soil, Qm_avl : oak grown under ambient with volcanic ash soil, Qm_obf: oak grown under O₃ fumigation with brown forest soil, Qm_ovl: oak grown under O₃ fumigation with volcanic ash soil.