Title

Growth and photosynthetic response of *Fagus crenata* seedlings to ozone and/or elevated carbon dioxide

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Erratum

The original version of this article contained errors unfortunately, 1 Prof. Takeshi Izuta should be indicated as the corresponding author. 2 The unit for the vertical axis in Fig. 1 should be (cm^2), not (cm^{-2}). We sincerely apologize for the errors.

Abstract

We investigated the effects of ozone (O_3) and/or elevated CO_2 concentration ([CO_2]) on the growth and photosynthetic traits of *Fagus crenata* seedlings. Two-year-old seedlings were grown in four experimental treatments comprising two O_3 treatments (charcoal-filtered air and 100 nmol mol^{-1} O_3; 6 h/day, 3 days/week) in combination with two CO_2 treatments (350 and 700 μmol mol^{-1}) for 18 weeks in environmental control growth chambers. The four treatments were designated as control, elevated O_3, elevated CO_2, and elevated CO_2 + O_3. Dry matter growth of the seedlings was greater in elevated CO_2 + O_3 than in elevated CO_2. In elevated CO_2 + O_3, a marked increase of second-flush leaves, considered a compensative response to O_3, was observed. The net photosynthetic rate of first-flush leaves in elevated CO_2 + O_3 increased earlier and was maintained for a longer period of time than that in elevated CO_2. Because emergence of second-flush leaves of *F. crenata* is greatly affected by the amount of assimilation products of first-flush leaves in current year, we consider that an early increase in the net photosynthetic rate of first-flush leaves contributed to the marked increase in second-flush leaf emergence under elevated CO_2 + O_3. These results imply that we must account for changes in compensative capacity with respect not only to morphological traits but also phenological traits and physiological functions such as photosynthesis for evaluating O_3 effects on *F. crenata* under elevated [CO_2].

Key words

Compensative response, second-flush, Combined effects, Ecophysiological response, Air pollution

1. Introduction

Fagus crenata is one of the most common and widely distributed deciduous broad-leaved tree species in the cool temperate forests of Japan (Nakashizuka and Iida, 1995; Peters 1997). *F. crenata* is an important tree species in Japan because its forests help to conserve forest soil and to maintain biodiversity, and is planted for afforestation as well as for ceremonial plantations (e.g., Murai et al. 1991; Nakashizuka 2004; Terazawa and Koyama 2008). Natural forests of *F. crenata* on the Shirakami Mountains (northeast Japan) were registered by UNESCO as a World Natural Heritage sites in December 1993. However, forest decline and dieback of *F. crenata* have been markedly observed in several mountainous areas in Japan since the late 1980s, including the Tanzawa Mountains in the Kanto region and Tateyama in the Hokuriku region. Several researchers have implicated ozone (O_3) as an important factor in these symptoms of decline (Kume et al., 2009; Takeda and Aihara, 2007).

Tropospheric O_3 is recognized as a widespread phytotoxic gaseous air pollutant, and its concentrations have been increasing in the Northern Hemisphere (ADORC 2006; Akimoto 2003). In Japan, the annual average daytime concentration of photochemical oxidant, of which the main component is O_3, increased at a rate of about 0.33 nmol mol^{-1} $year^{-1}$ from 1985 to 1999 and was about 31 nmol mol^{-1} as an average from 1999 to 2002 (ADORC 2006; Ohara and Sakata 2003). Furthermore, relatively high O_3 concentrations ($[O_3]$) above 100 nmol mol^{-1} have been frequently detected not only in the suburbs of metropolitan areas such as Tokyo and Osaka, but also in several mountainous areas (Network Center for EANET 2007; Takeda and Aihara 2007; Wakamatsu et al. 1998;

Yoshikado 2004).

The impact of O_3 on tree species is greatly affected by soil and/or atmospheric conditions (Matsumura et al. 2005; Matyssek and Sandermann 2003; Pearson and Mansfield 1993; Watanabe et al. 2005; Yamaguchi et al. 2007). Whether recent increasing CO_2 concentration ($[CO_2]$) can ameliorate the adverse effect of O_3 on tree species is currently a subject of debate (Karnosky et al. 2001). Although no evidences of amelioration of O_3 impact by elevated $[CO_2]$ have been reported (Barns et al. 1995; Kull et al. 1996), many studies have demonstrated ameliorating effects by elevated $[CO_2]$ with respect to O_3-induced reduction in photosynthesis and growth (Grams et al. 1999; Lütz et al. 2000; Manes et al. 1998; Volin et al. 1998) Numerous experiments have indicated stomatal closure induced by elevated $[CO_2]$ (Ainsworth and Rogers 2007; Saxe et al. 1998). Therefore, one of the most conceivable ameliorations of adverse O_3 effect by elevated $[CO_2]$ is decrease of stomatal O_3 uptake in leaves because of elevated CO_2-induced stomatal closure (McKee et al. 1995; Volin et al. 1996; Volin et al. 1998). On the other hand, compensatory growth of new leaves may occur to prevent the O_3-induced decline of whole-plant photosynthesis even in beech, which usually emerges leaves once during one growing season (Kikuzawa 1983; Kozovits 2003; Landolt et al. 1997; Matyssek and Sandermann 2003; Pell et al. 1994). Because assimilates are needed for producing new leaves (Matyssek and Sandermann 2003; Pell et al. 1994), there is a possibility that the compensative new leaf emergence in response to O_3 is stimulated under elevated $[CO_2]$, which will increase production of assimilates in leaves.

Paludan-Muller et al. (1999) reported a considerable variability in O_3 sensitivity among *Fagus sylvatica* seedlings from 12 provenances. This indicates that while the studies on the combined effects of O_3 and elevated [CO_2] on tree species conducted in Europe and North America provide useful information, we cannot directly apply that information to Japanese tree species. Thus, little information is available on the combined effects of O_3 and elevated [CO_2] on tree species native to Japan (Matsumura et al. 2005).

Therefore, in the present study, we investigated the effects of O_3 and/or elevated [CO_2] on the growth and photosynthetic traits of *F. crenata* seedlings in order to provide a basic understanding of these issues for conservation of *F. crenata* forests. Our hypothesis is that the negative impact of O_3 is ameliorated under elevated [CO_2] because of elevated [CO_2]-induced reduction in the O_3 uptake of leaves and stimulation of compensative response to O_3.

2. Materials and methods

2.1 Plant material

Two-year-old seedlings of *F. crenata* were transplanted into 2-L pots (11-cm diameter) filled with brown forest soil collected from a mixed deciduous forest at the University Forest of Tokyo University of Agricultural and Technology (Midori, Gunma Prefecture, Japan). We obtained *F. crenata* seedlings of the Toyama Prefecture provenance, meaning that the seedlings have traits related to survival in a heavy snow region (Koike et al. 1998; Koike and Maruyama 1998). Moreover, symptoms of *F. crenata* forest decline have been observed in this prefecture (Ishida 2004; Kume et al. 2009). All the seedlings were transferred into

four environmental control growth chambers (0.815 × 1.200 × 1.815 m^3, Koito Co. Ltd., Japan) and grown for 22 weeks (from 4 May to 7 October, 1995). The photoperiod in the chamber was 15 h (05:00–20:00) and the photosynthetic photon flux (PPF) at the canopy of the seedlings in the chambers was controlled at 600 μmol m^{-2} s^{-1}. We used 14 fluorescent lamps and incandescent lamps as light sources in each chamber to simulate natural sunlight. The temperature in the growth chamber was gradually increased from 16˚C to 22˚C during the time period 5:00–7:00 and was gradually decreased from 22˚C to 16˚C from 18:00 to 20:00. During the periods 7:00–18:00 and 20:00–5:00, the temperature was maintained at 22˚C and 16˚C, respectively. The relative humidity was regulated at 75%. The rate of air change in the growth chambers was set at 13.6 m^3 h^{-1} (i.e., the air volume was changed approximately 8 times h^{-1}). Liquid fertilizer (N:P:K = 5:10:5, Hyponex Japan, Osaka, Japan) was supplied at the rate of 280 mg of N pot^{-1} $week^{-1}$.

2.2 CO_2 and ozone treatments

The seedlings were grown in four factorial treatments consisting of two levels of [CO_2] and two levels of [O_3]. We started the CO_2 treatment on 4 May. The charcoal-filtered ambient air (350 ± 20 μmol mol^{-1} CO_2) was introduced into the two growth chambers and 700 μmol mol^{-1} CO_2 was introduced into the other two chambers. Chamber [CO_2] was regulated by a control unit (MC-F20/S, Koito Co. Ltd., Japan).

The O_3 treatment was started on 4 June, when the expansion of the first-flush leaves of the seedlings had completed. We conducted the exposure of

O_3 at 100 ± 10 nmol mol^{-1} for 6 h (10:00–16:00) per day, 3 days per week. The O_3 was generated from ambient air with an electrical discharge O_3 generator (IO-1A5, Nippon Ozone Co., Ltd., Tokyo, Japan) and injected into the chambers through a water trap to remove nitrogen by-products produced by the O_3 generator such as N_2O_5 (Brown and Roberts, 1988). The [O_3] in the growth chambers were continuously monitored with a UV absorption O_3 analyzer (TUV-1100, Tokyo Industries inc., Tokyo, Japan). In the chambers not treated with O_3, the [O_3] was below 10 nmol mol^{-1}.

The four treatments were designated as control (350 μmol mol^{-1} CO_2 + < 10 nmol mol^{-1} O_3), elevated O_3 (350 μmol mol^{-1} CO_2 + 100 nmol mol^{-1} O_3), elevated CO_2 (700 μmol mol^{-1} CO_2 + < 10 nmol mol^{-1} O_3), and elevated CO_2 + O_3 (700 μmol mol^{-1} CO_2 + 100 nmol mol^{-1} O_3). There was no CO_2 or O_3 replication because of the limited number of growth chambers. Therefore, all the seedlings were switched between chambers at 2-week intervals during the growth period (Dutilleul, 1993; Potvin and Tardif, 1988). To reduce position effects in the chamber (Miao et al., 1992), the position of the seedlings was randomized at the same interval as that of the chamber switching.

2.3 Growth analysis

On 4 June, the starting date of the O_3 exposure, the two seedlings from each treatment (four seedlings from each CO_2 treatment) were randomly harvested (initial sampling). On 7 October, in the 18th week after the initiation of O_3 exposure (WAE), we harvested five seedlings from each treatment (final sampling). Harvested seedlings were separated into leaves, branches, stems and

roots. Because the second-flush was observed after the sixth WAE, we separated the leaves of the second-flush and that of the first-flush. Leaf area was measured with an area meter (AAM-7, Hayashidenkoh, Tokyo, Japan). The plant organs were dried at 80˚C for 10 days and weighed.

Since there was no significant difference in the leaf area and dry mass of the seedlings between the two CO_2 treatments at the initial sampling, all values of leaf area and dry mass across the CO_2 treatments were averaged as the initial values. Based on the values of leaf area and dry mass of plant organs in the initial and final samplings, we calculated the relative growth rate (RGR), net assimilation rate (NAR), leaf area ratio (LAR), specific leaf area (SLA), and leaf mass ratio (LMR) according to the following formulas (Hunt, 1978):

$$\mathrm{RGR}\ (\%\ \mathrm{day}^{-1}) = [(\ln WDM_2 - \ln WDM_1) / (t_2 - t_1)] \times 100$$

$$\mathrm{NAR}\ (\mathrm{g\ cm}^{-2}\ \mathrm{day}^{-1}) = [(\ln LA_2 - \ln LA_1) \times (WDM_2 - WDM_1)] / [(LA_2 - LA_1) \times (t_2 - t_1)]$$

$$\mathrm{LAR}\ (\mathrm{cm}^2\ \mathrm{g}^{-1}) = \mathrm{RGR} / \mathrm{NAR}$$

$$\mathrm{SLA}\ (\mathrm{cm}^2\ \mathrm{g}^{-1}) = LA_2 / LDM_2$$

$$\mathrm{LMR}\ (\%) = (LDM_2 / WDM_2) \times 100$$

where *WDM*, *LA*, and *LDM* are whole-plant dry mass (g), leaf area per plant (cm^2), and leaf dry mass per plant (g), respectively. The numbers next to the abbreviations indicate the harvest timing (i.e., 1 and 2 mean initial and final samplings, respectively). Time is indicated by t and $t_2 - t_1$ equals 126 days.

2.4 Measurement of gas exchange rate

The leaf gas exchange rate of sun leaves was measured using an assimilation chamber at one week intervals from the first WAE. We randomly selected three seedlings per treatment and then measured the gas exchange rates of the same leaves throughout the experiment.

The air temperature in the assimilation chamber was maintained at 25 ± 0.5˚C. The light was supplied from a cold lighting system (PICL-NEX twin, Nippon P.I., Tokyo, Japan), and the PPF at the leaf surface was maintained at 600 μmol m^{-2} s^{-1}. The [CO_2] and air humidity were measured with an infrared gas analyzer (IR 59-07, Yokogawa, Tokyo, Japan) and a humidity sensor (HUP35A, Vaisala, Helsinki, Finland), respectively. To obtain the intercellular [CO_2] (C_i)-response curve of the net photosynthetic rate (A), i.e. the A/C_i curve, the A was determined for [CO_2] in the chamber at 10, 100, 350, 700, and 1400 μmol mol^{-1}.

We determined the A and stomatal conductance at the growth [CO_2] (A_{growth} and G_s, respectively), i.e., 350 μmol mol^{-1} in the control and elevated O_3 treatments and 700 μmol mol^{-1} in the elevated CO_2 and elevated CO_2+O_3 treatments. The carboxylation efficiency (CE) of photosynthesis was determined as the initial slope of the linear portion of the A/C_i curve. The A at 1400 μmol mol^{-1} CO_2 was regarded as the maximum net photosynthetic rate at saturated [CO_2] (A_{max}).

2.5 Statisitical analysis

Statistical analyses were performed with the SPSS software (SPSS, Inc,

Chicago, IL, USA). Two-way analysis of variance (two-way ANOVA) was used to test the effects the of elevated CO_2 and O_3 treatments. When a significant interaction between elevated CO_2 and O_3 treatments was detected, Duncan's new multiple range test was performed to identify significant differences among the four treatments.

3. Results

3.1 Growth of seedlings

Little difference in leaf area, dry mass of plant organs, or the ratio of above-ground dry mass to below-ground dry mass (T/R ratio) was found between control and elevated O_3 treatments (Fig. 1; Table 1). However, the dry mass of second-flush leaves tended to increase. In the elevated CO_2 treatment, dry mass was increased as compared to that in the control treatment (Table 1). Significant interaction between O_3 and CO_2 was detected for the dry mass of second-flush leaves, branches, stems, coarse roots, and whole-plant and for the leaf area of second-flush leaves. Although the dry mass and leaf area of second-flush leaves of seedlings grown under a [CO_2] of 350 μmol mol^{-1} were not significantly changed by exposure to O_3, those grown under a [CO_2] of 700 μmol mol^{-1} were significantly increased.

The RGR under elevated CO_2 and elevated CO_2 + O_3 treatments was 25% and 54% higher than that under control treatment, respectively, whereas there was no change in RGR under elevated O_3 treatment (Fig. 2). The NAR in the elevated O_3 treatment was 20% lower than that in the control treatment, while the LAR was increased by 23%. Although the increased NAR under elevated CO_2 and

elevated CO_2 + O_3 treatments was similar (approximately 30%), the increase of LAR was only observed under elevated CO_2+O_3 treatment.

There was no significant effect of O_3 and/or elevated [CO_2] on the SLA and LMR of first-flush leaves (Fig. 3). On the other hand, the LMR of second-flush leaves was significantly increased by elevated [CO_2].

3.2 Gas exchange rate

Regardless of the O_3 exposure, the A_{growth} of seedlings grown under a [CO_2] of 700 μmol mol^{-1} was higher than that of seedlings grown at a [CO_2] of 350 μmol mol^{-1} (Fig. 4). The A_{growth} under elevated O_3 treatment was reduced after the ninth WAE as compared to that under the control treatment. The peak of A_{growth} occurred at the seventh WAE under the elevated CO_2 treatment, while it occurred much earlier (at the fifth WAE) under the elevated CO_2 + O_3 treatment. The G_s in elevated O_3 treatments was lower than that in the control treatment in most measurements. In the elevated CO_2 and elevated CO_2+O_3 treatments, the level of G_s was similar but lower as compared to the control treatment. The *CE* under the control and elevated O_3 treatments was decreased beginning at about the fourth WAE, while the decrease started at the 7th and 11th WAE under elevated CO_2 and elevated CO_2 + O_3 treatments, respectively. There was no clear effect of O_3 on the *CE* of seedlings grown under a [CO_2] of 350 μmol mol^{-1}. An O_3-induced reduction in the A_{max} was found from the 11th to the 14th and from the 5th to the 10th WAE in the 350 and 700 μmol mol^{-1} CO_2 treatments, respectively.

Figure 5 shows the relationship between G_s and A_{growth} in the first-flush leaves of *F. crenata* seedlings. No correlation was observed between G_s and A_{growth}

in the seedlings grown under a [CO_2] of 350 μmol mol^{-1} during the experimental period. In the 700 μmol mol^{-1} CO_2 treatments, there was relatively high correlation between G_s and A_{growth} until the ninth WAE. However, the correlation was not found in the 10th to the14th WAE.

4. Discussion

Leaves of *F. crenata* are usually flushed one time during the growing season because this tree is classified as a fixed-growth-type species (Kikuzawa 1983), However, subsequent leaf emergence (the second-flush) has also been found to occur under well-fertilized and good light conditions (Hashizume 1982; Hashizume and Yamamoto 1975). Therefore, the seedlings of *F. crenata* in the present study were grown under sufficient nutrient and light conditions.

The reduction in NAR under elevated O_3 treatment (Fig. 2) indicates that the efficiency of dry matter production in the leaves was reduced by exposure to O_3. The O_3-induced reduction of NAR has been reported in many tree species (e.g., Izuta et al. 1996; Oksanen and Saleem 1999; Shimizu and Feng 2007). The reduction in net photosynthetic rate is considered to be one of the most important factors leading to NAR reduction in O_3-exposed trees (Izuta et al. 1996; Shimizu and Feng 2007). In the present study, the A_{growth} in the first-flush leaves under the elevated O_3 treatment was reduced as compared to that under the control treatment after the ninth WAE (Fig. 4) contributing to NAR reduction.

The diffusion of atmospheric CO_2 into leaves depends mainly on the behavior of stomata, as well as on the difference in [CO_2] between the atmosphere and the leaf interior (e.g., Lambers et al. 2008). In the present study, although no

clear relationship between A_{growth} and G_s was observed under a [CO_2] of 350 μmol mol^{-1} (Fig. 5), the G_s in the elevated O_3 treatment from the 10th to the14th WAE was lower than that in the control treatment. Thus, stomatal closure is considered to be a reason for the reduction of A_{growth} in elevated O_3 treatment. The O_3-induced stomatal limitation of photosynthesis was also reported by Kitao et al. (2009). On the other hand, the reduction in photosynthetic activity was indicated through the analysis of A/C_i curve. The A_{max}, which reflects the RuBP regeneration rate in the Calvin cycle, depends mainly on the electron transport rate in the thylakoid membrane (Farquhar et al 1980; Sharkey 1985). The *CE* corresponds to the activity of CO_2 fixation in the Calvin cycle and depends primarily on the activity and/or quantity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (von Caemmerer and Farquhar 1981). In the present study, exposure to O_3 reduced A_{max} in the leaves of *F. crenata* seedlings, while there was no clear effect of O_3 on *CE* (Fig. 4). These results strongly suggest that O_3 inhibits RuBP regeneration. Although the net photosynthetic under a [CO_2] of 350 μmol mol^{-1} is generally restricted by the activity of carboxlation by Rubisco, there is a possibility that the reduction in the RuBP regeneration also contribute to the O_3-induced reduction in A_{growth}. The reduction in A_{max} in *F. crenata* and *F. sylvatica* were also reported by Yonekura et al. (2001) and Lippert et al. (1996), respectively.

Because the emergence of first-flush leaves of deciduous tree species depends mainly on nutrients that reserved in the previous year (Kikuzawa 1983), the area growth and morphological traits of first-flush leaves of seedlings would not be affected by the current year O_3 exposure (Figs. 1 and 3). On the other hand, the LMR of the second-flush leaves was increased by the exposure to O_3 (Fig. 5),

and this increase lead to the LAR increase. The enhancement of new leaf emergence by O_3 exposure has been reported by several researchers and is considered to be one of the compensation responses to O_3 (Landolt et al. 1997; Pell et al. 1994). Consequently, the O_3-induced reduction in NAR of *F. crenata* seedlings was cancelled by the increase of LAR through the increase in LMR of the second-flush leaves.

The increase of RGR under elevated CO_2 treatment was mainly due to the increase of NAR, indicating stimulation of dry matter-production efficiency in the leaves. There have been many reports on the increase of NAR by elevated [CO_2] (e.g., Centritto et al. 1999; Pettersson and McDonald 1992). The increase of A_{growth} under elevated CO_2 treatment contributes to the increase of NAR. However, the stimulation of A_{growth} in elevated CO_2 treatment in the present study was temporary (Fig. 4). It decreased from the seventh WAE to a level of A_{growth} similar to that under the control treatment. There have been many reports of acclimation of photosynthesis to elevated [CO_2] (e.g., Eguchi et al. 2008; Kitao et al. 2005). Because there was no correlation between G_s and A_{growth} in the 10th to the 14th WAE (Fig. 5), the decrease of A_{growth} under elevated CO_2 treatment was not due to stomatal closure but to a decline of photosynthetic activity in the chloroplast. The *CE* and A_{max} in the elevated CO_2 treatment decreased from the 7th and 11th WAE, respectively (Fig. 4). Therefore, we consider that the reduction in CO_2 fixation activity in the Calvin cycle was the first factor that induced reduction in the A_{growth} of *F. crenata* seedlings and that the reduction in the RuBP regeneration rate was an ensuing factor (Farquhar et al 1980; Sharkey 1985; von Caemmerer and Farquhar 1981).

Based on the single effect of O_3 or elevated [CO_2] on the growth and photosynthesis of *F. crenata* seedlings, we thought that we had a plausible understanding of the combined effects of O_3 and elevated [CO_2]. However, a marked increase of dry matter growth was unexpectedly found in the elevated CO_2 + O_3 treatment (Table 1). Average G_s during the O_3 exposure period in elevated CO_2 + O_3 treatment was 15% lower than that in elevated O_3 treatment. Because stomatal conductance to O_3 was considered as proportional to G_s (Emberson et al. 2000), the decrease in leaf O_3 uptake under elevated [CO_2] can be estimated as similar extent. However, the reduction in O_3 uptake cannot explain the growth stimulation in elevated CO_2 + O_3 treatment. Increases of both NAR and LAR contributed to the increase of RGR (Fig. 2). The increase of LAR in the elevated CO_2 + O_3 treatment was mainly due to the increased emergence of second-flush leaves (Fig. 3). The emergence of second-flush leaves of *F. crenata* seedlings depends mainly on assimilation products from first-flush leaves in the current year (Hashizume 1982). In the present study, the A_{growth} of first-flush leaves under elevated CO_2 + O_3 treatment increased earlier and was approximately twice as high in third WAE as compared to the A_{growth} in the elevated CO_2 treatment, although this difference of A_{growth} was not significant because of the large variation (Fig. 3). This early increase of A_{growth} of first-flush leaves would thus contribute to the marked increase in the emergence of second-flush leaves.

Because a relatively high correlation between G_s and A_{growth} in the first through fourth WAE was found under elevated CO_2 + O_3 treatment, the increased stomatal opening would lead to an increase of A_{growh} to some extent (Figs. 4 and

5). However, the A_{growth} reached the maximum value one week earlier than did G_s. On the other hand, *CE* under elevated CO_2 + O_3 treatment was higher than that under elevated CO_2 treatment in the second and third WAE (Fig. 4). Therefore, we consider that the increase in CO_2 fixation activity in the Calvin cycle was the main reason for the early increase of A_{growth} under elevated CO_2 + O_3 treatment. It has been observed that a relatively low concentration of O_3 temporarily increased Rubisco quantity in the leaves of *Betula pendula* and *F. sylvatica* (Lütz et al. 2000; Pääkkönen et al. 1996). This is considered to be one of the compensation responses to O_3 exposure. More carbon would be needed for an extended compensation response (Fuhrer and Booker 2003). In the present study, the possibility exists that the compensative response of *F. crenata* seedlings to O_3 was stimulated under elevated [CO_2] due to the abundant carbon resource.

The timing of the decrease in *CE* under the elevated CO_2 + O_3 treatment was later than in the elevated CO_2 treatment (Fig. 3). This result suggests that a relatively high level of CO_2 fixation activity in the Calvin cycle was maintained under CO_2 + O_3 treatment and contributed to the stimulation of dry matter growth in the seedlings through the increase of NAR.

Matsumura et al. (2005) reported that the amelioration of adverse O_3 effects under elevated [CO_2] was not observed in *F. crenata* seedlings from the open-top chamber experiment; this differs from the result of our study. We maintained O_3 exposure for 6 h per day, 3 days per week, whereas the *F. crenata* seedlings of Matsumura et al. (2005) were exposed to O_3 at 1.5 times ambient concentration for 24 h per day everyday. This difference in O_3 exposure regime could be a reason for the differing results between the studies. Specifically, the

absence of O_3 exposure at night time in the present study may be an important factor since night is a time for recovery from O_3 injury (De Temmerman et al. 2002). Moreover, Matyssek et al. (1995) reported that the level of reduction in whole-plant growth of *B. pendula* cuttings produced by night time exposure to O_3 was similar to the level produced by day time O_3 exposure. Therefore, the seedlings in the present study would recover more and stimulate the compensative response to adverse O_3 effects during the night under elevated [CO_2] as compared to the results reported by Matsumura et al. (2005). On the other hand, there is a possibility that the provenance of *F. crenata* seedlings is also an important factor that may have induced different responses to O_3 under elevated [CO_2] in the two studies. The provenance of the seedlings used in our study and those of Matsumura et al. (2005) were Toyama (Hokuriku region) and Hokkaido, respectively. Although it is thought that the *F. crenata* in the two regions are genetically similar (Koike et al. 1998; Tomaru et al. 1997), the exposure level of O_3 in Toyama is relatively high as compared to the other regions in Japan, while relatively low levels of O_3 exposure have been observed in Hokkaido (Kohno et al 2005). Paludan-Müller et al. (1999) reported that the O_3 tolerance of *F. sylvatica* seedlings from southeast European provenances was higher than that of *F. sylvatica* seedlings from northwest European provenances. In that report, the authors speculated that the conditions of high O_3 exposure of the southeast European provenances conferred an O_3-tolerance on *F. sylvatica* seedlings. Molinier et al. (2006) indicated that genomic changes in response to environmental stresses could be inherited by successive generations. Therefore, there is a possibility that *F. crenata* seedlings used in the present study have high

congenital compensative capacity to O_3 as compared to those used in the study by Matsumura et al. (2005).

In conclusion, our hypothesis is partly supported. Although the reduction in O_3 uptake in leaves of *F. crenata* seedlings was indicated under elevated [CO_2], it cannot explain the growth stimulation in elevated CO_2 + O_3 treatment. On the other hand, the compensative response to O_3 (i.e., increase of second-flush leaves) was surprisingly stimulated under elevated [CO_2], which may be due to the early increase in the net photosynthetic rate of the first-flush leaves before the emergence of second-flush leaves. As a result, highest growth was observed under CO_2 + O_3 treatment. Based on the results obtained from the present study, we conclude that when evaluating the effects of O_3 on *F. crenata* under elevated [CO_2], we must take into account changes in compensative capacity brought about not only by morphological traits but also by phenological traits and physiological functions such as photosynthesis.

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Figure captions

Fig. 1 Leaf area of *F. crenata* seedlings at the end of the experimental period. Each value is the mean of five determinations. The vertical bar indicates standard deviation. Two-way ANOVA: ** $p < 0.01$; *** $p < 0.001$; ns not significant. Values with different letters are significantly different, with a $p < 0.05$

Fig. 2 Relative growth rate (RGR), net assimilation rate (NAR) and leaf area ratio (LAR) of *F. crenata* seedlings during 18 weeks of O_3 exposure. Values are shown relative to the control treatment. The absolute values of RGR, NAR, and LAR in the control treatment were 1.36×10^{-2} g g^{-1} day^{-1}, 5.22×10^{-4} g cm^{-2} day^{-1}, and 25.98 cm^2 g^{-1}, respectively. Each value is the mean of five determinations

Fig. 3 Specific leaf area (SLA) and leaf mass ratio (LMR) of *F. crenata* seedlings at the end of the experimental period. Each value is the mean of five determinations, The vertical bar indicates standard deviation. Two-way ANOVA: * $p < 0.05$; ns not significant. Values with different letters are significantly different, with a $p < 0.05$

Fig. 4 Net photosynthetic rate at growing CO_2 concentrations (A_{growth}, a, b), stomatal conductance (c, d), carboxylation efficiency (e, f), and maximum net photosynthetic rate (A_{max}, g, h) in the first-flush leaves of *F. crenata* seedlings. Each value is the mean of three determinations. The vertical bar indicates standard deviation. Vertical dashed lines in (a) and (b) indicate the emergence time of second-flush leaves

Fig. 5 Relationship between stomatal conductance and net photosynthetic rate at growing CO_2 concentration (A_{growth}) in the first-flush leaves of *F. crenata* seedlings. Solid and dashed lines indicate the regression line for the elevated CO_2 and elevated CO_2 + O_3 treatments, respectively. WAE = Week after the initiation of O_3 exposure

Table 1

Table 1 Dry mass of plant organs and the ratio of above-ground dry mass to below-ground dry mass (T/R ratio) of *F. crenata* seedlings at the end of the experimental period

Treatment		Dry mass								
		1st flush leaves	2nd flush leaves	Bud	Branch	Stem	Fine root	Coarse root	Whole-plant	T/R ratio
Control		0.361	0.770 a	0.388	0.720 ab	2.064 a	1.708	2.136 a	8.146 a	1.078
Elevated O_3		0.396	1.079 a	0.339	0.603 a	1.683 a	1.859	1.910 a	7.820 a	1.138
Elevated CO_2		0.521	1.295 a	0.618	1.404 b	2.266 a	2.880	3.531 b	12.505 b	0.978
Elevated O_3+CO_2		0.582	3.757 b	0.508	3.130 c	3.338 b	4.562	4.677 c	20.553 c	1.258
ANOVA	O_3	ns	***	ns	***	ns	*	*	***	ns
	CO_2	**	***	**	***	***	***	***	***	ns
	O_3×CO_2	ns	**	ns	***	**	ns	**	***	ns

Each value is the mean of 5 determinations.

Two-way ANOVA: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns not significant.

Within the same parameter, values with different letters are significantly different, with a $p < 0.05$.

Fig. 1

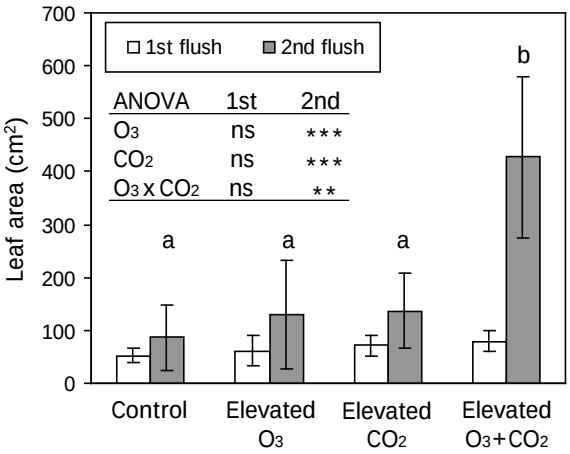

700
600
500
400
300
200
100
0
Leaf area (cm²)
1st flush
2nd flush
ANOVA 1st 2nd
O3 ns ***
CO2 ns ***
O3 x CO2 ns **
a
a
a
b
Control
Elevated O3
Elevated CO2
Elevated O3+CO2

Fig. 2

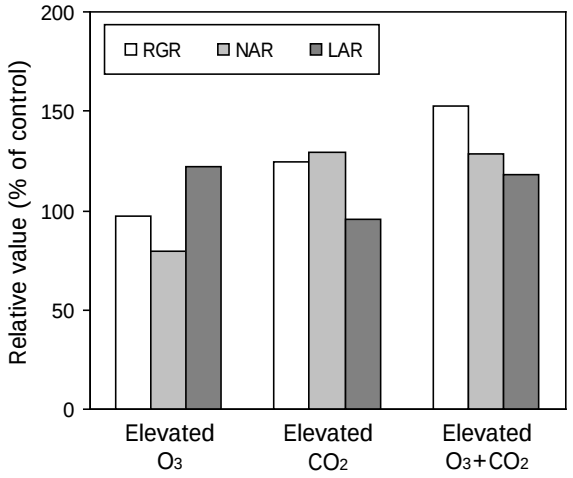

RGR
NAR
LAR
Relative value (% of control)
200
150
100
50
0
Elevated O3
Elevated CO2
Elevated O3+CO2

Fig. 3

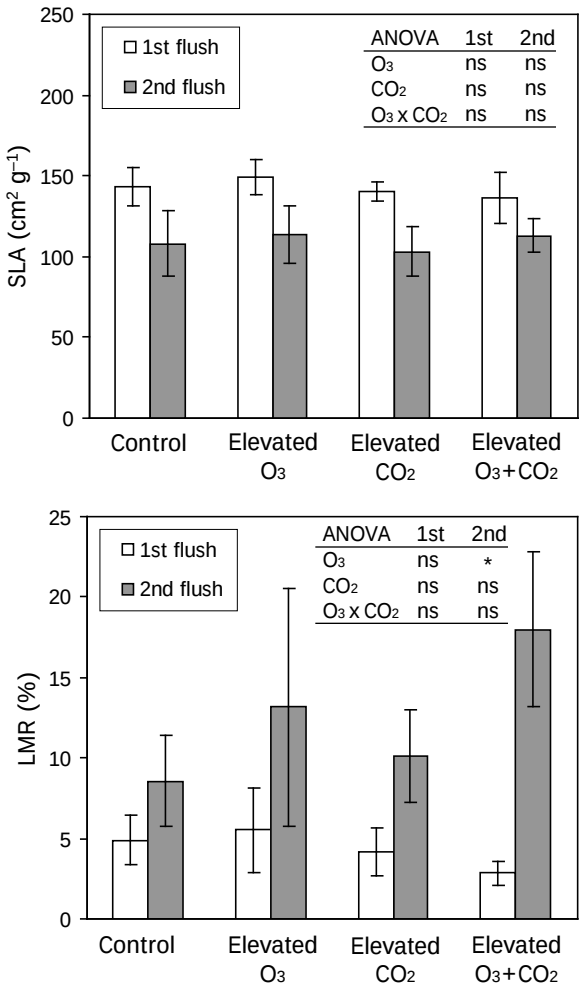

250
200
150
100
50
0
SLA (cm² g⁻¹)
1st flush
2nd flush
ANOVA 1st 2nd
O3 ns ns
CO2 ns ns
O3 x CO2 ns ns
Control
Elevated O3
Elevated CO2
Elevated O3+CO2
25
20
15
10
5
0
LMR (%)
1st flush
2nd flush
ANOVA 1st 2nd
O3 ns *
CO2 ns ns
O3 x CO2 ns ns
Control
Elevated O3
Elevated CO2
Elevated O3+CO2

Fig. 4

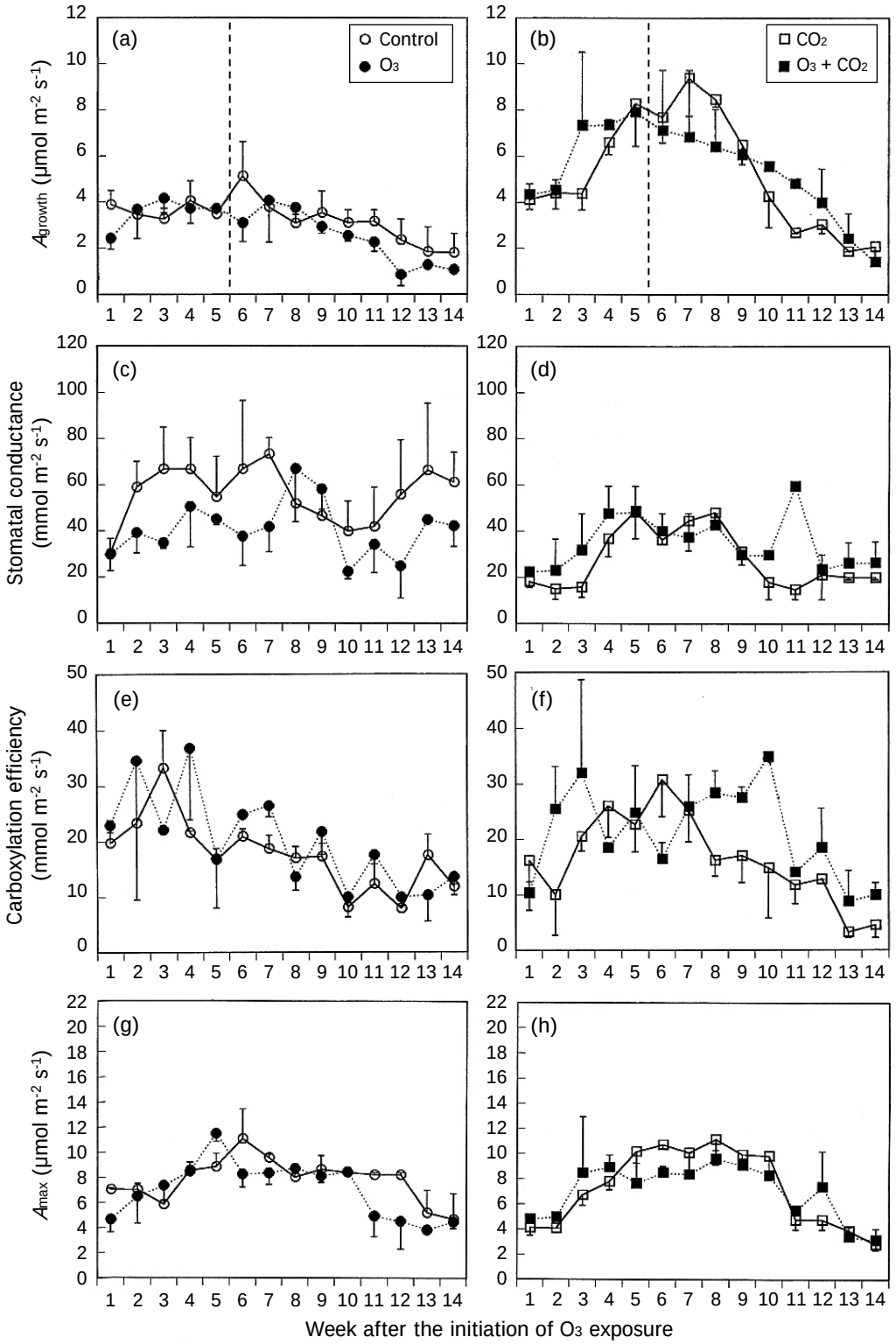
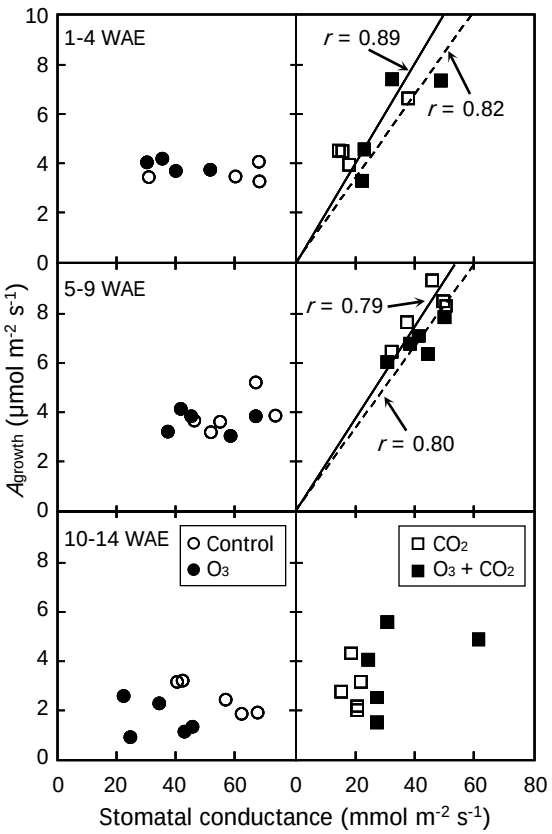

(a)
Control
O3
(b)
CO2
O3 + CO2
(c)
(d)
(e)
(f)
(g)
(h)
Agrowth (μmol m-2 s-1)
Stomatal conductance (mmol m-2 s-1)
Carboxylation efficiency (mmol m-2 s-1)
Amax (μmol m-2 s-1)
Week after the initiation of O3 exposure

Fig. 5


1-4 WAE
r = 0.89
r = 0.82
5-9 WAE
r = 0.79
r = 0.80
10-14 WAE
Control
O3
CO2
O3 + CO2
Agrowth (μmol m-2 s-1)
Stomatal conductance (mmol m-2 s-1)
0
2
4
6
8
10
0
20
40
60
80