Effects of ozone on soil respiration rate of Siebold's beech seedlings grown under different soil nutrient conditions

Makoto WATANABE^{a,†}, Shigeaki OKABE^a, Yoshiyuki KINOSE^{a,b}, Hiroka HIROSHIMA^a and Takeshi IZUTA^a

(^aInstitute of Agriculture, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8509, Japan
^bFaculty of Life and Environmental Sciences, University of Yamanashi, Kofu, Yamanashi 400-8510, Japan)

Abstract

Ozone (O₃) is an air pollutant that negatively affect carbon budget in woody plants. In the present study, we aimed to clarify the effects of ozone on soil respiration rate of Siebold's beech seedlings (*Fagus crenata*) grown under different soil-nutrient conditions. Seedlings were grown under three levels of O₃ fumigation (charcoal-filtered air or O₃ at 1.0 or 1.5 times ambient concentration) in combination with three levels of nutrient supplies (non-, low- or high-fertilised) for two growing seasons. We determined soil respiration rate in July, August, September, and October of the second growing season. The seedlings were harvested to determine the dry mass in October. Significant effect of O₃ on soil respiration rate was not observed in all measurements. There was a significant interaction between O₃ and nutrient supply for whole-root dry mass. The dry mass in non-fertilised and low-fertilised treatments was reduced by O₃, whereas O₃ did not affect dry mass in the high-fertilised treatment. On the other hand, neither significant effects of O₃, nor a significant interaction between O₃ and nutrient supply for the biomass allocations were observed. Coefficient of positive correlation in the relation of soil respiration rate with dry mass of fine-root across the all treatments was higher than that in the relation of soil respiration rate with coarse-root and whole-root dry mass. These results indicate that no significant effect of O₃ on soil respiration was mainly attributable to no response of fine root dry mass to elevated O₃. Soil nutrient supply decreased soil respiration rate in August. Our results emphasize the importance of fine root in the response of soil respiration to elevated O₃. To clarify the response of soil respiration to elevated O₃, future researches on the effect of O₃ on fine root dynamics including turnover and indirect effect on soil microbial respiration are needed.

Key words: Fine root, Ozone, Siebold's beech, Soil fertilization, Soil respiration

1. Introduction

Ozone (O₃) is a phytotoxic air pollutant and the concentration has been increasing over the last several decades in Japan (Matussek and Sandermann, 2003; Akimoto *et al.*, 2015). Many studies have demonstrated O₃-induced negative impacts on tree photosynthesis and dry matter growth (Wittig *et al.*, 2007, 2009; Watanabe *et al.*, 2017a). Although effect of O₃ on carbon budget of trees canopy was estimated based on the detailed physiological processes (Kitao *et al.*, 2012; Watanabe *et al.*, 2014), carbon budget in belowground is not well understood.

Soil respiration contributes greatly to the carbon budget of forest ecosystems and mainly comprises root respiration and microbial respiration; the fraction of root respiration to soil respiration is considered to be between 40% and 60% (Hanson *et al.*, 2000). Because several papers indicated decrease of root growth and fraction of biomass allocation to roots under elevated O₃ (Wittig *et al.*, 2009; Agathokleous *et al.*, 2016), soil respiration may decrease when O₃ is elevated. Actually, O₃-induced reductions in soil respiration were observed in loblolly pine (*Pinus taeda*) seedlings and aspen forests (Edwards, 1991; Pregitzer *et al.*, 2006). However, the opposite response

also was reported in ponderosa pine (*Pinus ponderosa*) seedlings, silver birch (*Betula pendula*) seedlings, and beech/spruce (*Fagus sylvatica*/*Picea abies*) forests (Scagel and Andersen, 1997; Kasurinen *et al.*, 2004; Nikolova *et al.*, 2010). Tingey *et al.* (2006) reported no significant effect of O₃ on the soil respiration of ponderosa pine seedlings. These studies indicated that the effects of O₃ on soil respiration are quite different among tree species and growing conditions.

The availabilities of nutrients vary across forested areas (e.g. Kawada, 1977; Leuschner *et al.*, 2006). Soil-nutrient supply modifies the degree of negative impacts of O₃ in both directions, i.e. enhancement and mitigation (Matussek and Sandermann, 2003; Watanabe *et al.*, 2017b), and therefore, soil-nutrient condition may change the effects of O₃ on soil respiration rate. In fact, according to Andersen and Scagel (1997) and Scagel and Andersen (1997), increases in soil respiration rate of ponderosa pine exposed to O₃ were enhanced under low-nutrient availability. However, there is no other study on the combined effects of O₃ and soil-nutrient conditions on soil respiration rate.

Siebold's beech is a representative tree species native to cool-temperate forests in Japan. Based on several experimental studies carried out previously, Siebold's beech is relatively sensitive to O₃ among the Japanese forest-tree species (Kohno *et al.*, 2005; Watanabe *et al.*, 2008; Yamaguchi *et al.*, 2011). Kinose *et al.* (2017a) reported significant interactive effects of O₃ and nutrient supply to soil for two growing seasons on the root and whole-plant dry mass of Siebold's beech (*Fagus crenata*). The

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[†]Corresponding Author: nab0602@cc.tuat.ac.jp

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O₃-induced reduction in dry mass was observed in the non- and low-fertilised seedlings, but not in the high-fertilised seedlings. Based on this evidence, we hypothesised O₃ decreased soil respiration rate under relatively poor soil-nutrient conditions (i.e. non- and low-fertilised conditions). To test this hypothesis, we investigated effects of O₃ on soil respiration rate of Siebold's beech seedlings grown under different soil nutrient conditions.

2. Materials and methods

2.1 Plant materials and experimental design

Greenhouse-type O₃-fumigation chambers (length: 3.6 m, width: 2.2 m, height in centre part: 2.0 m) with a natural light source located at the Field Museum Tamakyuryo of Tokyo University of Agriculture and Technology (35°4'N, 139°2'E and 144 m a.s.l., Hachioji, Tokyo, Japan) were used in this study (Kinose *et al.*, 2014). On 7 May 2014, two-year-old seedlings of Siebold's beech (*Fagus crenata*) were individually planted in 1/2000 a Wagner's pots (bulk: 12 L, width: 228–240 mm, depth: 259 mm) filled with brown forest soil (Cambisol according to international classification system, IUSS Working Group WRB, 2015) on 7 May 2014. The soil was collected from the A-horizon of the forest floor of a deciduous forest in the Field Museum Mt. Karasawayama of Tokyo University of Agriculture and Technology (Sano, Tochigi, Japan). Before planting the seedlings, the soil was passed through a 5 mm sieve. The concentrations of total nitrogen and available phosphorous in the soil at initiation of the experiment were 2.4 g N kg⁻¹ soil and 10.5 mg P kg⁻¹ soil, respectively (Kinose, 2017). On 15 May 2014, the seedlings were transferred into nine chambers and were grown for two growing seasons until 26 October 2015 (529 days). Mean height and stem base diameter of the seedlings at the start of experiment were 49 cm and 6.3 mm, respectively. All the seedlings were regularly irrigated to keep the potted soil moist.

This experiment had a split-plot factorial design and employed the randomized block method. The whole-plot treatment consisted of three levels of O₃, charcoal-filtered air (CF, mean O₃ removal efficiency: ca. 60%), 1.0 times ambient O₃ concentration (1.0 × O₃), and 1.5 times ambient O₃ concentration (1.5 × O₃), with three chamber replications, giving a total of nine chambers for data analysis. Further details of the fumigation and monitoring systems are described in Kinose *et al.* (2014). The gas treatment was carried out from 15 May

to 30 November 2014 and from 21 April to 26 October 2015. The sub-plot treatment consisted of three levels of soil nutrient conditions. The seedlings were supplied with 500 ml of water (NF: non-fertilised treatment), 2000-fold diluted liquid fertilizer (Hyponex 6-10-5, HYPONeX Japan Co. Ltd., Osaka, Osaka, Japan) (LF: low-fertilised treatment) or 1000-fold diluted liquid fertilizer (HF: high-fertilised treatment) to soil once every 2 weeks during the gas treatment period. Five seedlings were assigned to each O₃-nutrient-chamber combination, for a total of 135 seedlings.

Table 1 shows indices of O₃ fumigation in each growing season. Average O₃ concentration and accumulated exposure over a threshold of 40 nmol mol⁻¹ of O₃ (AOT40, Agathokleous *et al.*, 2018) for daylight hours (> 50 W m⁻²) during the second growing season were 11.2 nmol mol⁻¹ and 0.1 μmol mol⁻¹ h in CF, 32.9 nmol mol⁻¹ and 8.9 μmol mol⁻¹ h in 1.0 × O₃, and 49.3 nmol mol⁻¹ and 26.8 μmol mol⁻¹ h in 1.5 × O₃, respectively. Air temperature and relative air humidity of one chamber in each gas treatment, totally three chambers, were continuously measured at 10 min intervals using a TR-72U Thermo Recorder (T&D Corporation, Nagano, Japan). The daily average air temperature and relative air humidity measured inside the three chambers during the second growing season were 21.3°C and 84.5%, respectively. Rotation of the pots between chambers to reduce chamber effects was carried out at three-week interval. We also conducted pot rotation within a chamber at two-week interval to reduce position effect in a chamber.

2.2 Measurement of soil respiration rate

We determined soil respiration rate of the potted soil where Siebold's beech seedlings were planted on 7–10 July, 4–11 August, 2–10 September and 7–13 October of the second growing season (2015). The measurements were collected five times (2:00, 10:00, 14:00, 18:00 and 22:00) each day. To select the seedlings for the measurement of soil respiration rate, we first determined stem basal diameter (D) and height (H) of all seedlings on 2 June 2015, then calculated volume index (D²H). Three seedlings with median values of D²H were selected to measure soil respiration rate in each O₃-nutrient-chamber combination.

Dynamic closed chamber method with an acrylic plastic chamber (W 8 cm × D 17 cm × H 11 cm) equipped with an

Table 1. Average concentration and accumulated exposure over a threshold of 40 nmol mol⁻¹ (AOT40) of ozone in each gas treatment during the period of ozone fumigation.

		Concentration (nmol mol ⁻¹)		AOT40 (μmol mol ⁻¹ h)
		24 hours	Daylight hours	Daylight hours
2014 15 May–30 Nov.	CF	8.1 (1.1)	13.1 (1.2)	0.1 (0.0)
	1.0 × O ₃	16.9 (0.7)	22.6 (0.5)	4.1 (0.0)
	1.5 × O ₃	24.4 (0.6)	33.5 (0.7)	14.4 (0.7)
2015 21 Apr.–26 Oct.	CF	8.1 (0.6)	11.2 (1.7)	0.1 (0.1)
	1.0 × O ₃	23.9 (0.3)	32.9 (0.4)	8.9 (0.4)
	1.5 × O ₃	34.9 (0.2)	49.3 (0.1)	26.8 (0.0)

CF: charcoal-filtered air, 1.0 × O₃: 1.0 times ambient ozone concentration, 1.5 × O₃: 1.5 times ambient ozone concentration.

Each value is the mean of three chamber replicates, and the standard deviation is shown in parenthesis.

Daylight hours: solar radiation > 50 W m⁻².

infrared gas analyser (IRGA, GMP222, Vaisala, Helsinki, Finland) was applied to determine soil respiration rate (Rochette *et al.*, 1997). The head space of the chamber was 1.46 L. The chambers were gently placed on the potted soil, between rim of pot and stem of seedlings, and a small amount of soil was mounted to seal the space between the chamber and the soil surface. First, we checked leaks of the chamber in each measurement by breathing outside of the chamber and confirming no change of the CO₂ concentration in head space of the chamber. Then, CO₂ concentration inside the chamber was monitored at one second intervals for ten minutes. After the monitoring of CO₂ concentrations, soil temperature was determined using a soil conductivity and temperature tester (Soil Test HI 98331, Hanna Instruments Japan, Chiba, Japan). The linear portion of the slope of the CO₂ concentration increment with time (usually from 60 s to 360 s) was used for the calculation of soil respiration rate in a unit of g CO₂ m⁻² h⁻¹. To calculate the dependency of soil respiration rate to soil temperature (Q_{10}), the soil respiration rate was approximated as a function of soil temperature with exponential function (Jones, 2013)

2.3 Measurement of plant growth

All the seedlings were harvested on 26 October 2015 to determine their biomass. We first separated the harvested seedlings into above-ground (leaf + stem) and roots. Then, the roots were separated into fine (diameter < 2 mm) and coarse roots (diameter ≥ 2 mm). The plant organs were dried in an oven at 80°C for five days and weighed.

2.4 Statistical analysis

Statistical analyses were undertaken using R software, version 3.4.0 (R Development Core Team, 2017). The analyses were conducted using one average value per soil nutrient treatment per chamber, thus giving a total of 3 values per experimental condition. The effects of O₃, nutrient supply, and soil temperature during the measurement of soil respiration rate on soil respiration rate were tested by generalized linear model (GLM). The GLM was also applied to test the effect of O₃ and nutrient supply on Q_{10} of soil respiration rate. Because we confirmed the normality for all parameters by Shapiro-Wilk Normality test, response variables were assumed to follow Gaussian distribution in the model. AOT 40 and amount of fertilizer until the measurement period of the second growing season were used as explanatory variables for the effects of O₃ and nutrient treatments, respectively. A two-way analysis of variance (ANOVA) was applied to test the effect of O₃ and nutrient supply on growth parameters of the seedlings because dry mass of whole-root and whole-plant of this study was already analysed by two-way ANOVA and published in Kinose *et al.* (2017a). Pearson's correlation test was used to determine the relationship between soil respiration rate in each measurement period (July, August, September and October) and dry mass of fine-root, coarse-root and whole-root of the seedlings at the end of the experiment. The mean soil respiration rate of five determinations (2:00, 10:00, 14:00, 18:00 and 22:00) was used to this analysis. We also used Pearson's correlation test for the relationship between Q_{10} and AOT40.

3. Results

There was no significant effect of O₃ on soil respiration rate in all measurement periods (Fig. 1, Table 2). Soil nutrient supply significantly decreased soil respiration rate in August. Soil respiration rate was significantly increased with increasing soil temperature. We observed significant interactions between O₃ and nutrient supply, and between O₃, nutrient supply and soil temperature for soil respiration in October.

There was an antagonism between O₃ and nutrient supply for Q_{10} of soil respiration rate in August (Fig. 2). The Q_{10} was positively correlated with the amount of fertilizer in CF and 1.0 × O₃ treatments ($r = 0.715$, $P = 0.030$ in CF; $r = 0.679$, $P = 0.044$ in 1.0 × O₃), while there was no significant correlation in 1.5 × O₃ treatment ($r = -0.260$, $P = 0.499$).

We observed significant reductions of fine-root dry mass and dry mass ratio of fine roots to coarse roots due to the soil nutrient supply, whereas the nutrient supply did not induce reductions of whole-root and whole-plant dry mass of the seedlings (Table 3, Kinose *et al.*, 2017a). There were significant interactions between O₃ and nutrient supply for whole-root dry mass and whole-plant dry mass. The dry mass in NF and LF treatments was reduced by the exposure to O₃, whereas O₃ did not affect dry mass in the HF treatment. The nutrient supply significantly reduced the ratios of fine-root or whole-root dry mass to whole-plant dry mass (Table 4). Neither significant effects of O₃, nor a significant interaction between O₃ and nutrient supply for the dry mass ratios, were observed.

As shown in Table 5, soil respiration rates in July and September were significantly and positively correlated with fine root dry mass when we analysed pooled data across gas and soil-nutrient treatments. On the other hand, there was no significant correlation between soil respiration and coarse-root or whole-root dry mass at any measurement periods.

4. Discussion

Soil respiration is mainly the results of root respiration (autotrophic) and processes of the microorganisms (heterotrophic) involved in the decomposition of organic materials (Cao and Woodward, 1998; Munoz *et al.*, 2010). Soil nutrient treatment can directly affect both root and microorganisms processes. On the other hand, O₃ does not physically penetrate soil (Matyssek *et al.*, 2010) and all the leaf litter in the present study was removed. Thus, the primary effect of O₃ on soil respiration is considered as the result of the autotrophic respiration (i.e. root respiration) although there is a possibility that O₃-induced changes in quantity and/or quality of root exudation affect heterotrophic respiration (Andersen, 2003).

Little effect of O₃ on soil respiration rate was observed in the present study even in the NF treatment which shows relatively large reduction of root dry mass. Many papers suggested that fine root dry mass is one of the most important factors determining soil respiration (e.g. Lee and Jose, 2003; Pregitzer *et al.*, 2008). Correlation analysis of this study also supports this suggestion (Table 5) although the biomass data was determined only in the end of growing season. The correlations of soil respiration rate with fine root dry mass of Siebold's beech seedlings were

Fig. 1. Diurnal variation of soil respiration rate of Siebold's beech seedlings in July, August, September, and October 2015.

The seedlings were grown under three levels of ozone fumigation in combination with three levels of nutrient supplies. CF: charcoal-filtered air, $1.0 \times O_3$: 1.0 times ambient ozone concentration, $1.5 \times O_3$: 1.5 times ambient ozone concentration. The result of the statistical analysis is shown in Table 2.

Table 2. Result of generalized linear model analysis for the effects of ozone, nutrient supply, and soil temperature on soil respiration rate of Siebold's beech seedlings in July, August, September, and October 2015.

	Jul	Aug	Sep	Oct
Ozone (O_3)	n.s.	n.s.	n.s.	n.s.
Nutrient (N)	n.s.	*	n.s.	n.s.
Temperature (T)	***	*	***	**
$O_3 \times N$	n.s.	n.s.	n.s.	*
$O_3 \times T$	n.s.	n.s.	n.s.	n.s.
$N \times T$	n.s.	n.s.	n.s.	n.s.
$O_3 \times N \times T$	n.s.	n.s.	n.s.	*

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n.s. not significant. Actual P value is shown if $0.05 < P < 0.10$.

higher than those with coarse root and whole-root dry mass. On the other hand, neither significant effect of O_3 nor significant interaction between O_3 and nutrient supply for the fine root dry mass was observed (Table 3). These results indicate no effects of O_3 on fine root was one of the reasons of no response of soil respiration to elevated O_3 .

Edwards (1991) and Nakaji and Izuta (2001) reported that O_3 -induced reduction of fine root dry mass was greater than that of coarse root dry mass in seedlings of loblolly pine (*Pinus taeda*) and Japanese red pine (*Pinus densiflora*), respectively, although the different responses were also observed (Agathokleous *et al.*, 2016). The biomass of fine root is determined as a difference between growth and mortality of fine root. Matyssek *et al.* (2010) indicated an increased fine root turnover in mature

Fig. 2. Temperature sensitivity (Q_{10}) of soil respiration rate of Siebold's beech seedlings in July, August, September, and October 2015. CF: charcoal-filtered air, $1.0 \times O_3$: 1.0 times ambient ozone concentration, $1.5 \times O_3$: 1.5 times ambient ozone concentration. NF: non-fertilised, LF: low-fertilised, HF: high-fertilised. * $P < 0.05$, *** $P < 0.001$, n.s. not significant.

Table 3. Dry mass of roots and whole plant, and dry mass ratio of fine root to coarse root (Fine/Coarse) of Siebold's beech seedlings grown under three levels of ozone fumigation in combination with three levels of nutrient supplies.

Nutrient	Gas	Fine-root (g)	Coarse-root (g)	Fine/Coarse	Whole-root (g)	Whole-plant (g)
NF	CF	34.3 (2.5)	43.4 (2.2)	0.79 (0.10)	77.8 (0.6)	186.9 (4.2)
	$1.0 \times O_3$	30.3 (0.9)	36.3 (1.0)	0.83 (0.03)	66.6 (1.6)	163.1 (6.2)
	$1.5 \times O_3$	24.3 (2.8)	37.1 (2.2)	0.65 (0.04)	61.4 (5.1)	151.3 (9.6)
LF	CF	30.2 (6.4)	43.2 (3.0)	0.70 (0.13)	73.4 (8.5)	189.7 (14.2)
	$1.0 \times O_3$	30.8 (6.8)	42.3 (2.6)	0.72 (0.12)	73.0 (9.3)	183.7 (8.6)
	$1.5 \times O_3$	26.4 (8.5)	36.6 (3.2)	0.73 (0.26)	63.0 (8.2)	171.5 (17.2)
HF	CF	23.8 (2.3)	43.4 (5.4)	0.56 (0.12)	67.2 (4.1)	182.7 (4.7)
	$1.0 \times O_3$	24.2 (3.8)	40.6 (6.9)	0.61 (0.15)	64.8 (6.3)	184.4 (18.8)
	$1.5 \times O_3$	26.7 (3.7)	45.1 (5.0)	0.60 (0.11)	71.8 (5.9)	191.0 (15.0)
Two-way ANOVA						
	Ozone (O_3)	n.s.	n.s.	n.s.	n.s.	n.s.
	Nutrient (N)	*	n.s.	*	n.s.	**
	$O_3 \times N$	n.s.	n.s.	n.s.	*	*

NF: non-fertilised, LF: low-fertilised, HF: high-fertilised.

CF: charcoal-filtered air, $1.0 \times O_3$: 1.0 times ambient ozone concentration, $1.5 \times O_3$: 1.5 times ambient ozone concentration.

Each value is the mean of three chamber replicates, and the standard deviation is shown in parenthesis.

* $P < 0.05$, ** $P < 0.01$, n.s. not significant.

Data of whole-root dry mass and whole-plant dry mass were referred Kinose *et al.* (2017a).

European beech exposed to elevated O_3 . Pregitzer *et al.* (2008) found that elevated ozone increased both fine root production and mortality in aspen community. The increment of root turnover would stimulate decomposition of carbohydrate from root, resulting increase of soil respiration (Matyssek *et al.*, 2010). In the present study, however, there was no significant effect of O_3 on soil respiration rate (Fig. 1, Table 2). Enhanced fine root turnover of Siebold's beech seedlings in the present study may not be a reason of no significant effect of O_3 on fine root dry mass.

The Q_{10} in CF and $1.0 \times O_3$ were increased with increasing the amount of nutrient to the soil, while no significant correlation was observed between Q_{10} and soil nutrient treatment in $1.5 \times O_3$ treatment in August (Fig. 2). It is known that Q_{10} of soil respiration responds to environmental conditions, especially temperature and soil water content (e.g. Hashimoto *et al.*, 2009; Wang *et al.*, 2014). However, the response of Q_{10} to soil nutrient condition is not very understood. Soil respiration rates under different amount of simulated nitrogen depositions were

determined in a *Populus euphratica* community in north-western China (He *et al.*, 2015). However, the correlation between Q_{10} of soil respiration and soil nitrogen concentration was not significant. Burton *et al.* (2002) measured fine root respiration and nitrogen concentration in fine root across biome in North American forests. In this study, there was also no significant correlation between Q_{10} of root respiration and nitrogen concentration in root. Further research is needed to clarify the mechanism behind in the effect of soil nutrient treatment and their interaction with O_3 observed in the present study.

Soil respiration rate in August decreased due to the soil nutrient supply (Fig. 1, Table 2). Nutrient supply significantly reduced fine-root dry mass, dry mass ratio of fine root to coarse root, and dry mass ratio of fine root to whole plant mass, although there was no significant effect on whole-root dry mass (Tables 3 and 4). There are several observations of decreases in fine-root biomass with increasing nitrogen availabilities in mixed-forest (Aber *et al.*, 1985), *Populus* trees (Pregitzer *et*

Table 4. Ratios of fine root, coarse root, and whole root dry mass to whole plant dry mass (FRMR, CRMR and RMR, respectively) of Siebold's beech seedling grown under three levels of ozone fumigation in combination with three levels of nutrient supplies.

Nutrient	Gas	FRMR	CRMR	RMR
NF	CF	0.18 (0.01)	0.23 (0.01)	0.42 (0.01)
	1.0 × O ₃	0.19 (0.01)	0.22 (0.00)	0.41 (0.01)
	1.5 × O ₃	0.16 (0.01)	0.25 (0.01)	0.40 (0.02)
LF	CF	0.16 (0.02)	0.23 (0.01)	0.38 (0.01)
	1.0 × O ₃	0.17 (0.03)	0.23 (0.00)	0.39 (0.03)
	1.5 × O ₃	0.15 (0.03)	0.21 (0.03)	0.36 (0.02)
HF	CF	0.13 (0.01)	0.24 (0.02)	0.37 (0.01)
	1.0 × O ₃	0.13 (0.02)	0.22 (0.02)	0.35 (0.00)
	1.5 × O ₃	0.14 (0.02)	0.24 (0.01)	0.37 (0.00)
Two-way ANOVA				
	Ozone (O ₃)	n.s.	n.s.	n.s.
	Nutrient (N)	**	n.s.	***
	O ₃ × N	n.s.	n.s.	n.s.

NF: non-fertilised, LF: low-fertilised, HF: high-fertilised.

CF: charcoal-filtered air, 1.0 × O₃: 1.0 times ambient ozone concentration, 1.5 × O₃: 1.5 times ambient ozone concentration.

Each value is the mean of three chamber replicates, and the standard deviation is shown in parenthesis.

** $P < 0.01$, *** $P < 0.001$, n.s. not significant.

al., 1995) and Japanese red pine (*Pinus densiflora*, Nakaji *et al.*, 2004). We think that the reduction in soil respiration rate of Siebold's beech seedlings in our study was partly due to the reduction in fine-root biomass with high respiration activity (Makita *et al.*, 2009; Chen *et al.*, 2010; Burton *et al.*, 2012).

Soil-nutrient supply may directly affect soil microbial communities, although the impact of O₃ first occurs in leaves of plants as mentioned above. According to a meta-analysis on the responses of soil respiration and its components (i.e., autotrophic and heterotrophic respirations) to nitrogen addition (Zhou *et al.*, 2014), nitrogen addition induces decreases of heterotrophic respiration in temperate and boreal forests. Although the increase in soil nitrogen concentration in this study was relatively small, as compared to that of phosphorous and potassium concentrations (Kinose *et al.*, 2017b), there is a possibility that not only a decrease of root respiration, but also that of microbial respiration contributed to the decrease in soil respiration rate by nutrient supply.

In the present study, the growth enhancement of Siebold's beech seedlings by soil nutrient treatment was little (Table 3). Total soil nitrogen concentration at the initiation of the experiment was 2.4 g N kg⁻¹, and the seedlings in LF and HF treatments were supplied nitrogen at 198 and 396 mg N during one growing season. On the other hand, the nitrogen concentration in the initial soil and amount of nitrogen supply during one growing season in Yamaguchi *et al.* (2007), which demonstrated growth enhancement of Siebold's beech seedlings by the application of ammonium nitrate, were 3.5 g N kg⁻¹ and 100 and 250 mg N per seedling. The nitrogen content and amount of nitrogen application to soil were comparable between our study and Yamaguchi *et al.* (2007). On the other hand, leaf

Table 5. Correlation coefficient between soil respiration rate in each month and dry mass of fine-root, coarse-root and whole-root in Siebold's beech seedling across the three levels of ozone fumigation in combination with three levels of nutrient supplies.

	July	August	September	October
Fine-root	0.475*	0.295	0.445*	0.155
Coarse-root	-0.358	0.139	0.004	-0.083
Whole-root	0.120	0.300	0.324	0.060

See Materials and method for details of the analysis.

* $P < 0.05$.

nitrogen content in the present study (1.6–1.9 g N m⁻²) was higher than that of Yamaguchi *et al.* (2007) (0.8–1.3 g N m⁻²) even in the NF treatment (Kinose *et al.*, 2017a). This result indicates a possibility that the nutrient in the original soil of the present study was sufficient and therefore the fertilization did not affect the growth of Siebold's beech seedlings.

Root growth limitation due to the pot size is an important experimental concern when using pot-grown seedlings (e.g. Arp, 1991). We checked the ratio of root dry mass to shoot dry mass (S/R ratios) of the seedlings in the end of first and second growing seasons (Kinose *et al.*, 2017a). If the size of pot was not enough for root growth, the R/S ratios would become low in the second growing season as compared to the first growing season. However, the R/S ratios in the end of first growing season and second growing season were similar (0.59 and 0.63 on average of all treatments, respectively). Therefore, we consider the restriction of root growth due to the limited soil volume was not serious.

5. Conclusion

Based on the results obtained from this study, our hypothesis was rejected. Fine root biomass of Siebold's beech was an important factor in determining soil respiration rate under elevated O₃ with various soil nutrient conditions in our experiment. On the other hand, neither significant effects of O₃, nor a significant interaction between O₃ and nutrient supply for fine root although significant interaction between O₃ and soil nutrient treatment was observed for whole-root biomass. Our results emphasize the importance of fine root in the response of soil respiration to elevated O₃. There are many reports on O₃-induced reduction in fine root biomass (Edwards, 1991; Nakaji and Izuta, 2001). To clarify the response of soil respiration to elevated O₃ condition, future researches on the effect of O₃ on fine root dynamics including turnover and indirect effect on soil microbial respiration are needed.

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