forests

MDPI

Article

Nitrogen Use Efficiency for Growth of *Fagus crenata* Seedlings Under Elevated Ozone and Different Soil Nutrient Conditions

Makoto Watanabe [1,*], Hiroka Hiroshima [1], Yoshiyuki Kinose [2], Shigeaki Okabe [1] and Takeshi Izuta [1]

[1] Institute of Agriculture, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8509, Japan; hirokahiroshima@yahoo.co.jp (H.H.); shige.okabe0602@gmail.com (S.O); izuta@cc.tuat.ac.jp (T.I.)
[2] Graduate Faculty of Interdisciplinary Research, University of Yamanashi, Kofu, Yamanashi 400-8510, Japan; ykinose@yamanashi.ac.jp
* Correspondence: nab0602@cc.tuat.ac.jp; Tel.: +81-42-367-5820

Received: 15 January 2020; Accepted: 21 March 2020; Published: 26 March 2020

Abstract: Ozone is a phytotoxic gaseous air pollutant and its negative effects on forest production are a major concern. To understand the effects of ozone on forest production, it is important to clarify the nitrogen use efficiency (NUE) for tree growth under elevated ozone conditions, because nitrogen is a primal limiting factor of forest production in many cool-temperate forests. Soil nutrient conditions are considered factors affecting ozone susceptibility of tree growth. Therefore, in the present study, we investigated the effects of ozone on NUE for the growth of Siebold's beech (*Fagus crenata* Blume) seedlings grown under different soil nutrient conditions. Seedlings of Siebold's beech were grown under three gas treatments (charcoal-filtered air or ozone at 1.0 or 1.5 times the ambient concentration) in combination with three soil nutrient conditions (non-fertilised, low-fertilised or high-fertilised) for two growing seasons. Based on the dry mass and nitrogen concentration in each plant organ, we calculated NUE and its components, including nitrogen productivity (NP) and the mean residence time of nitrogen (MRT) during the second growing season. Ozone did not decrease the NUE of the seedlings during the second growing season, whereas leaf level photosynthetic nitrogen use efficiency (PNUE), a component of NP, was decreased by ozone. On the other hand, the soil nutrient supply decreased the NUE of the seedlings. Reductions in both NP and MRT were attributed to the decrease in NUE because of soil nutrient supply, whereas PNUE did not respond to soil nutrient supply. There was no significant interaction of ozone and soil nutrient supply on the NUE, or its components, of the seedlings. Our results indicated that there is a difference in the response between the NUE for individual growth and that of leaf level PNUE of Siebold's beech seedlings to ozone and soil nutrient supply.

Keywords: *Fagus crenata*; growth; ozone; nitrogen use efficiency; nutrient condition

1. Introduction

Ozone (O_3) in the troposphere is recognised as a phytotoxic gaseous air pollutant. The atmospheric concentration of tropospheric O_3 has been increasing since the Industrial Revolution [1–3]. Moreover, this increasing trend will continue especially in East Asia in the near future [4–6]. Many experimental studies have demonstrated negative effects of O_3 on growth and physiological function, such as photosynthesis, of tree species [7–10]. Therefore, O_3 is considered one of the most critical factors in decreasing forest production [11,12].

Nitrogen use efficiency (NUE) is an important trait for tree growth because nitrogen is a primary limiting factor for forest production in many temperate climate zones [13–16]. NUE comprises nitrogen productivity (NP: dry mass growth per unit plant N [17]) and mean residence time of nitrogen (MRT: $\overline{N}$ divided by nitrogen uptake rate) [18]. Longer MRT generally occurs in the plant grown under infertile conditions, whereas higher NP is favoured under fertile conditions [19]. The clarification of the responses of NUE and its components to elevated O_3 will be useful in the elucidation of O_3-induced growth reduction of forest tree species.

There are several studies on the effects of O_3 on leaf photosynthesis considering nitrogen use traits of the forest tree species. Watanabe et al. [20] reported a reduction in nitrogen allocation to the photosynthetic apparatus in leaves of Siebold's beech (*Fagus crenata*) saplings exposed to O_3. A decrease in photosynthetic nitrogen use efficiency (PNUE: net photosynthetic rate divided by leaf nitrogen content) has also been reported in many tree species [21–25]. Because PNUE is a parameter composed of NP, we expect a reduction in NUE under elevated O_3 due to a PNUE reduction. However, no study on the effects of O_3 on NUE of tree species has been conducted.

Soil nutrient status affects the susceptibility of trees to O_3 stress. Yamaguchi et al. [24] reported an increase in susceptibility to O_3 of Siebold's beech seedlings with the increasing supply of nitrogen to the soil, whereas a decrease in O_3 susceptibility of the same species occurred with balanced nutrients (i.e., not only nitrogen but also other elements required) was also observed [26]. There are also some evidences that soil nutrient status affects O_3 susceptibility of woody species [27–32]. Thus, it is important to consider the soil nutrient conditions in understanding the effects of O_3 on the NUE of tree species.

Siebold's beech (*Fugus crenata* Blume) is widely distributed deciduous broadleaved tree species in the cool-temperate forests of Japan [33] and is a culturally and ecologically important species [34,35]. However, this tree species has high susceptibility to O_3 compared to other Japanese forest tree species [9,36].

In the present study, we investigated the effects of O_3 on NUE and its components for Siebold's beech seedlings grown under different soil nutrient conditions. In this experiment, we observed a decrease in the O_3 susceptibility under higher nutrient conditions [26]. Therefore, we hypothesized that (1) O_3 decreases the NUE of Siebold's beech seedlings because of a reduction in NP and (2) the O_3-induced decrease in NUE is mitigated by higher nutrient conditions.

2. Materials and Methods

2.1. Plant Materials and Experimental Design

We used greenhouse-type O_3-fumigation chambers (length: 3.6 m; width: 2.2 m; height at centre: 2.0 m) located at the Field Museum (FM) Tamakyuryo of Tokyo University of Agriculture and Technology (TUAT, 35°4′N, 139°2′E, and 144 m a.s.l., Hachioji, Tokyo, Japan). Details of the O_3 fumigation system have been previously described [26,37]. We planted 2-year-old seedlings of Siebold's beech in 1/2000 Wagner's pots (bulk: 12 L; width: 228–240 mm; depth: 259 mm) filled with brown forest soil. They were planted on 7 May 2014 and grew until 14 May 2014 under field conditions. The soil was brown forest soil (Cambisol, according to the international classification system [38]), and was collected for the experiment from the A-horizon of a deciduous forest floor in the FM Karasawayama of TUAT (Sano, Tochigi, Japan). Brown forest soil is the most general forest soil in Japan. The collected soil was passed through a 5 mm sieve before use in the experiment. Total nitrogen and available phosphorous concentrations in the soil at the start of the experiment were 2.4 g N kg^{-1} soil and 10.5 mg P kg^{-1} soil, respectively [39]. After being transplanted to the potting soil, we moved all the seedlings to nine O_3-fumigation chambers and continued to grow them for two growing seasons until 26 October 2015 (529 days). The average height and stem base diameter of the seedlings at the start of the experiment were 49 cm and 6.3 mm, respectively. The seedlings were regularly irrigated to maintain the soil moisture.

This experiment had a split-plot factorial design and employed the randomized block method. The whole-plot consisted of three O_3 levels: charcoal-filtered air (CF), 1.0- and 1.5-fold ambient O_3

concentration (1.0 × O_3 and 1.5 × O_3, respectively), with three-chamber replications, providing a total of nine chambers for data analysis. Further details of the O_3 fumigation and monitoring systems are described in Kinose et al. [37]. We conducted gas treatment from 15 May to 30 November 2014 in the first growing season and from 21 April to 26 October 2015 in the second growing season. The subplot consisted of three levels of soil nutrient treatments. We supplied 500 mL of water as non-fertilised treatment (NF), 2000-fold diluted liquid fertiliser (Hyponex 6–10–5, HYPONeX Japan Co. Ltd., Osaka, Japan) as the low-fertilised treatment (LF) or 1000-fold diluted liquid fertiliser as the high-fertilised treatment (HF) to the seedlings at 2-week intervals during the gas treatment period. Ten seedlings were assigned to each O_3 nutrient chamber combination, for a total of 270 seedlings.

The indices of O_3 fumigation in each growing season are shown in Table 1. The daily average of air temperature and relative air humidity inside the three chambers during the second growing season were 21.3 °C and 84.5%, respectively. The details of measurements of O_3 concentration, air temperature and relative air humidity were reported previously by Kinose et al. [37]. Pot rotations among the chambers and within the chamber to reduce position effects on the seedlings were conducted at 3-week and 1-week intervals, respectively.

Table 1. Average concentration and accumulated exposure over a threshold of 40 nmol mol^{-1} (AOT40) [40] of ozone in each gas treatment during the period of ozone fumigation.

		Concentration (nmol mol^{-1})		**AOT40 (µmol mol^{-1} h)**
		24 h	**Daylight Hours**	**Daylight Hours**
2014	CF	8.1 (1.1)	13.1 (1.2)	0.1 (0.0)
15 May–30 Nov.	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	CF	8.1 (0.6)	11.2 (1.7)	0.1 (0.1)
21 Apr.–26 Oct.	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)

Each value is the mean of three chamber replicate (standard deviation) (n = 3). Gas treatments: CF: charcoal-filtered air, 1.0 × O_3: 1.0 time ambient ozone concentration, 1.5 × O_3: 1.5 times ambient ozone concentration. Daylight hours: solar radiation > 50 W m$^{-2.}$

2.2. Measurement of Plant Growth and Nitrogen Concentration

During the first and second growing seasons, we measured the monthly height and stem base diameter of the seedlings and calculated the stem volume index as the product of the square of the diameter (D) and height (H) (i.e., D^2H) for all seedlings. Fifteen seedlings in each treatment (five seedlings per treatment in each chamber; three chamber replicates) were harvested on 25 October 2014 and 26 October 2015. The harvested seedlings were separated into the leaves, stems (trunk and branch), buds and roots, and were dried at 80 °C in an oven for 1 week. After drying, we measured the dry mass of each seedling organ. The dried samples were ground into a fine powder using a sample mill (Wonder Blender, Osaka Chemical Co., Osaka, Japan). Nitrogen concentration of powdered samples was determined with a C/N analyser (MT-700, Yanaco, Tokyo, Japan). We established allometries between stem dry mass and D^2H and between root dry mass and D^2H based on the harvest dataset.

2.3. Determination of Nitrogen Use Efficiency and Its Components

The NUE (g g^{-1} N), NP (g g^{-1} N day^{-1}) and MRT (day) were determined according to Hirose [16]. The NUE of the seedlings during a given period was calculated as follows,

$$NUE = \frac{\Delta W}{\Delta N} \tag{1}$$

where ΔW (g) and ΔN (g) are the dry mass increment and the amount of nitrogen uptake from the soil for a given period, respectively. The NUE comprises NP and MRT, and the components are calculated as follows,

$$NUE = NP{\cdot}MRT \tag{2}$$

$$NP = \frac{\Delta W}{\overline{N}{\cdot}\Delta T} \tag{3}$$

$$MRT = \frac{\overline{N}}{\Delta N}{\cdot}\Delta T \tag{4}$$

where ΔT (day) is the experimental duration and $\overline{N}$ (g) is the mean whole-plant nitrogen content during the experimental period, which is determined by

$$\overline{N} = \frac{1}{\Delta T}\int_0^T [N_0 + f(t) - g(t)]dt \tag{5}$$

where N_0 is the initial nitrogen content, and the functions *f(t)* and *g(t)* represent the whole-plant nitrogen uptake and loss at a given time point, respectively. The conceptual model of this calculation is shown in Figure 1. The area with hatching indicates the PND as proposed by Hirose [16], which is the product of $\overline{N}$ and ΔT, and is equal to the length of the time integral in Equation (5). In the present study, we determined N_0 and the time course of whole-plant nitrogen content and nitrogen loss during the second growing season, based on the measurement data.

The ΔT in the NUE analysis of the present study was 188 days from 21 April (just before leaf emergence) to 26 October 2015. We assumed that leaf emergence was completed on the next day of leaf emergence (i.e., 22 April). To calculate N_0, we first determined the dry mass of the seedlings on 21 April from D^2H using allometries for woody tissues. Then, we multiplied the estimated dry mass on 21 April and nitrogen concentration of woody tissues on 25 October 2014. The N_0 was calculated as the sum of this product and the resorbed nitrogen from the leaves before abscission in the end of previous growing season. The resorption rate was determined from the nitrogen concentration of leaf litter in November–December 2014.

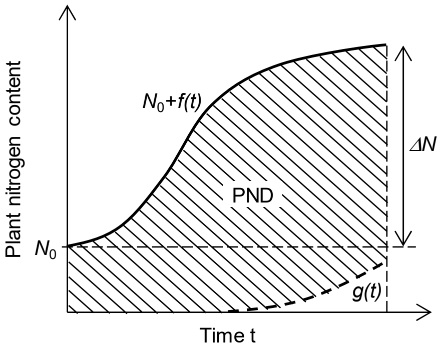

Figure 1. Diagram of time course in plant nitrogen content. N_0, initial plant nitrogen content; *f(t)*, nitrogen uptake; *g(t)*, nitrogen loss; ΔN, total N uptake. The area with hatching indicates the plant nitrogen duration (PND). Redrawn from Hirose [16].

To estimate the dry mass and nitrogen contents in the first-flush leaves at a given time point of the growing season, the dry mass and nitrogen content of abscised leaves due to leaf fall and sampling for biochemical analysis as in Kinose et al. [26] during the growing season were determined and added to that of the leaves in the final sampling. The same method was applied for second and third flush leaves. Then, we estimated mean dry mass and nitrogen content ($\overline{N}_L$, g) of the whole leaves during the second growing season. The dry mass of stems and roots on the date of D^2H measurements were calculated using allometry based on the integrated data of samplings in October 2014 and 2015. We applied a logistic model to describe the growth curves of stems and roots during the second growing season, and calculated mean dry mass of stems and roots. Mean nitrogen content of stems and roots was calculated as products of mean dry mass and nitrogen concentration determined in the final sampling (October 2015). The $\overline{N}$ of the whole seedling and PND were calculated as the sum of mean nitrogen contents of the leaves, stems and roots and the product of $\overline{N}$ and ΔT, respectively. The NP was composed of the leaf nitrogen productivity (LNP, g g^{-1} N day^{-1}) and leaf nitrogen fraction (LNF) as follows.

$$NP = LNP \cdot LNF \tag{6}$$

$$LNP = \frac{\Delta W}{\overline{N}_L \cdot \Delta T} \tag{7}$$

$$LNF = \frac{\overline{N}_L}{\overline{N}} \tag{8}$$

2.4. Gas Exchange Measurement of Leaves

In the second growing season, we vertically divided the crown of seedlings into five layers with 30 cm intervals from the potted soil surface (i.e., Layer 1: 0–30 cm; Layer 2: 30–60 cm; Layer 3: 60–90 cm; Layer 4: 90–120 cm; Layer 5: >120 cm). Approximately 85% of the first-flush leaves belonged to layers 1–3. Nine seedlings per treatment (three seedlings per treatment in each chamber; three chamber replicates) were randomly selected, and the leaf gas exchange rates were measured using an open-pass gas exchange system (LI-6400, Li-Cor, Inc., NE, USA). The light-saturated net photosynthetic rate (A_{sat}) of the first-flush leaves in Layer 3 and Layer 1 was determined as upper and lower canopy leaves, respectively, in May, July and September 2015. The conditions for gas exchange measurements were 24, 29 and 23 °C for leaf temperatures in May, July and September, respectively, with 400 μmol mol^{-1} (ppm) of CO_2 concentration, 1.3 ± 0.1 kPa of vapour pressure deficit (VPD) from leaf to air and 1500 μmol m^{-2} s^{-1} of photosynthetic photon flux density. After the measurement of leaf gas exchange, we determined the nitrogen concentration in the leaves with the C/N analyser as described above. The PNUE (μmol mol^{-1} s^{-1}) was calculated as A_{sat} divided by leaf area-based nitrogen content (N_{area}, g m^{-2}).

2.5. Statistical Analyses

Statistical analyses were performed using R 3.4.0 software [41]. The analyses were performed with one mean value per soil nutrient treatment per chamber, giving three 3 values (n = 3) per experimental condition were used for the analyses. First, we confirmed the normality for all variables by Shapiro–Wilk test. Then, we applied a two-way analysis of variance (ANOVA) to test the effects of O_3 and soil nutrient supply on NUE and its components. A three-way ANOVA was used to test the effects of O_3, soil nutrient supply and leaf position (upper and lower canopies) on leaf gas exchange traits. In both procedures, the homogeneity of residual variance was confirmed with Levene's test. When a significant single effect of O_3 or soil nutrient supply was detected, post hoc multiple range test with Tukey–Kramer method was performed to identify significant differences between three O_3 treatments or three soil nutrient treatments, respectively. When we found a significant interaction of two or three explanatory factors, the same multiple range test was performed to identify significant differences among the all treatments and leaf positions.

3. Results

3.1. Nitrogen Use Efficiency and Its Components

Nutrient supply significantly decreased NUE, NP and MRT of Siebold's beech seedlings, whereas there was no significant effect of O_3 or the interaction of the two factors (Figure 2). As shown in Table 2, O_3 did not significantly affect all of the NUE components. We observed a significant increase in ΔW, ΔN and PND, and a significant decrease in LNP by soil nutrient supply. Although LNF showed an increasing trend with soil nutrient treatment, the effect was marginal. A significant interaction of O_3 and nutrient supply was detected for ΔW. Ozone significantly decreased ΔW of the seedlings in NF treatment, whereas the ΔW was not decreased by the exposure to O_3 in both LF and HF treatments. Neither a significant effect of O_3 and soil nutrient supply nor a significant interaction of the two factors was observed for N_0 and the time integral of nitrogen loss ($\int N_{\mathrm{loss}}$). The time integral of nitrogen uptake ($\int N_{\mathrm{uptake}}$) had the same trend as PND because of the small amount of $\int N_{\mathrm{loss}}$ (data not shown).

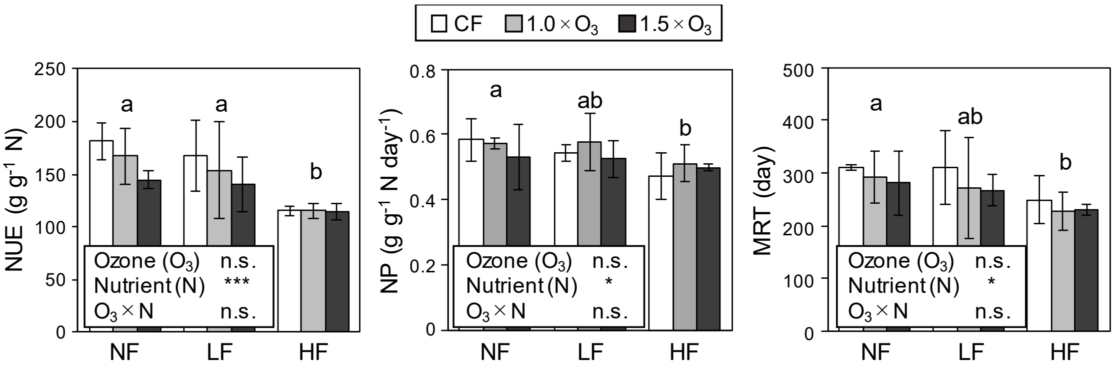

Figure 2. Nitrogen use efficiency (NUE), nitrogen productivity (NP) and mean residence time of nitrogen (MRT) of Siebold's beech seedlings in the second growing season. The seedlings were grown in nine treatments comprised of three levels of ozone treatments (CF: charcoal-filtered air; 1.0 × O_3: 1.0 time ambient ozone concentration; 1.5 × O_3: 1.5 times ambient ozone concentration) in combination with three levels of soil nutrient treatments (NF: non-fertilised; LF: low-fertilised; HF: high-fertilised). Each value is the mean ± standard deviation (n = 3). Two-way ANOVA: * $p < 0.05$, *** $p < 0.001$, n.s. not significant. Different letter indicates significant difference between the nutrient treatments (ozone treatments are pooled) with a $p < 0.05$.

Table 2. Components of nitrogen use efficiency: whole-plant biomass growth (ΔW), nitrogen uptake (ΔN), leaf nitrogen fraction (LNF), leaf nitrogen productivity (LNP), initial whole-plant nitrogen content (N_0), plant nitrogen duration (PND) and time-integral of nitrogen loss ($\int N_{\mathrm{loss}}$) of Siebold's beech seedlings in the second growing season.

Nutrient	**Gas**	**ΔW (g)**		**ΔN (g N)**		**LNF (g N g^{-1} N)**	**LNP (g g^{-1} N day^{-1})**		**N_0 (g N)**	**PND (g N day)**		**$\int N_{\mathrm{loss}}$ (g N day)**
NF	CF	131 (4)	ab	0.73 (0.05)		0.25 (0.02)	2.36 (0.33)		0.74 (0.04)	226 (19)		3.46 (1.72)
	1.0 × O_3	117 (2)	bc	0.71 (0.17)		0.24 (0.03)	2.42 (0.13)		0.62 (0.16)	204 (8)		3.37 (0.60)
	1.5 × O_3	105 (13)	c	0.73 (0.16)		0.25 (0.03)	2.17 (0.13)		0.62 (0.06)	202 (17)		3.46 (1.36)
LF	CF	130 (4)	abc	0.80 (0.11)		0.28 (0.02)	1.93 (0.13)		0.75 (0.06)	240 (3)		3.12 (1.09)
	1.0 × O_3	136 (8)	ab	0.93 (0.27)		0.27 (0.01)	2.13 (0.35)		0.67 (0.20)	237 (22)		4.94 (1.90)
	1.5 × O_3	122 (10)	abc	0.88 (0.04)		0.25 (0.02)	2.08 (0.10)		0.69 (0.15)	232 (36)		6.31 (0.52)
HF	CF	133 (11)	ab	1.16 (0.10)		0.25 (0.04)	1.90 (0.38)		0.75 (0.09)	284 (21)		4.28 (1.32)
	1.0 × O_3	138 (12)	ab	1.20 (0.13)		0.26 (0.02)	1.93 (0.13)		0.66 (0.11)	272 (7)		4.80 (4.15)
	1.5 × O_3	143 (10)	a	1.25 (0.11)		0.26 (0.02)	1.96 (0.17)		0.69 (0.08)	288 (20)		4.79 (2.73)
Nutrient pooled												
	CF	131 (7)		0.89 (0.23)		0.26 (0.03)	2.06 (0.29)		0.75 (0.09)	250 (29)		3.62 (1.25)
	1.0 × O3	130 (12)		0.95 (0.26)		0.26 (0.02)	2.16 (0.29)		0.65 (0.13)	238 (36)		4.37 (1.35)
	1.5 × O3	124 (19)		0.95 (0.25)		0.25 (0.02)	2.07 (0.24)		0.66 (0.09)	241 (41)		4.85 (2.85)
Gas pooled												
	NF	118 (13)	a	0.72 (0.08)	b	0.24 (0.02)	2.32 (0.28)	a	0.66 (0.08)	211 (18)	c	3.43 (1.21)
	LF	129 (10)	ab	0.87 (0.18)	b	0.27 (0.02)	2.04 (0.22)	b	0.70 (0.14)	236 (13)	b	4.79 (2.69)
	HF	138 (10)	b	1.20 (0.11)	a	0.26 (0.02)	1.93 (0.12)	b	0.70 (0.10)	281 (24)	a	4.63 (1.57)
ANOVA	Ozone (O_3)	n.s.		n.s.		n.s.	n.s.		n.s.	n.s.		n.s.
	Nutrient (N)	**		***		0.058	*		n.s.	***		n.s.
	O_3 × N	*		n.s.		n.s.	n.s.		n.s.	n.s.		n.s.

Each value is the mean of three chamber replicate (standard deviation) ($n = 3$). Soil nutrient treatments: NF: non-fertilised; LF: low-fertilised; HF: high-fertilised. Gas treatments; CF: charcoal-filtered air; 1.0 × O_3: 1.0 time ambient ozone concentration; 1.5 × O_3: 1.5 times ambient ozone concentration. Three-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. not significant. The actual p value is shown when $0.05 < p < 0.10$. Different letter indicates significant difference between the treatments in gas × nutrient combination or gas pooled with a $p < 0.05$.

3.2. Leaf Photosynthetic Traits

The effects of O_3, soil nutrient supply and leaf position on A_{sat} were significant, except for the O_3 effect in May (Table 3). A_{sat} in the upper canopy leaves was higher than that in the lower canopy leaves in all measurement periods. In May, we observed significant two-factor interactions for A_{sat} for all combinations of O_3, soil nutrient supply and leaf position. The A_{sat} at CF and $1.0 \times O_3$ in the upper canopy leaves was increased at HF as compared with NF and LF, whereas no significant difference among the soil nutrient treatments was found at $1.5 \times O_3$. In the lower canopy leaves, there was no significant difference among the all treatments. The A_{sat} in July was significantly decreased by the exposure to O_3 and increased by soil nutrient treatment, although there was no significant difference between treatments according to multiple range test. The effects of O_3 and soil nutrient treatments became clear in September. When we compared gas treatments with nutrient pooled data set, A_{sat} at $1.5 \times O_3$ was significantly lower than that at CF and $1.0 \times O_3$. By contrast, A_{sat} at HF was significantly higher than that at NF according to the gas pooled data set. We also observed a significant interaction of O_3 and soil nutrient supply for A_{sat} in September. In both the upper and lower canopy leaves, the tendency was similar to the interaction observed in the upper canopy leaves in May. A significant increase in A_{sat} by soil nutrient supply was found at CF, whereas no significant nutrient supply-induced enhancement of A_{sat} was observed at $1.0 \times O_3$ and $1.5 \times O_3$.

As shown in Table 4, the soil nutrient supply significantly increased the N_{area} of the seedlings during all measurement periods, although there was no significant difference among the treatment according to multiple range test. The N_{area} of the upper canopy leaves was significantly higher than that of the lower canopy leaves. There was no significant interaction for all combinations of O_3, soil nutrient supply and leaf position for N_{area}.

We observed a significant decrease in PNUE by the exposure to O_3 in July and September (Table 5). The PNUE at $1.5 \times O_3$ was significantly lower than that at CF. The lower canopy leaves have higher PNUE as compared to those in the upper canopy in all measurement periods. In May, there was a significant interaction effect of soil nutrient supply and leaf position on PNUE. The PNUE in the upper canopy leaves was not affected by soil nutrient supply. In the lower canopy leaves, a decreasing trend with increasing soil nutrient supply was observed, although it was not clearly supported by multiple range test.

Table 3. Light-saturated net photosynthetic rate (μmol m^{-2} s^{-1}) in upper and lower canopy leaves of Siebold's beech seedlings.

Nutrient	Gas	May			July		September		
Upper canopy leaves									
NF	CF	10.4	(0.7)	bcd	9.7	(0.6)	7.1	(0.5)	bc
	1.0 × O_3	10.1	(0.8)	cde	9.8	(0.9)	6.7	(0.7)	bcde
	1.5 × O_3	11.0	(0.3)	abc	9.0	(0.4)	6.1	(0.2)	cdef
LF	CF	12.1	(0.3)	b	11.3	(0.6)	7.7	(0.8)	ab
	1.0 × O_3	11.3	(0.5)	abc	10.2	(0.2)	7.4	(0.3)	bc
	1.5 × O_3	11.3	(0.8)	abc	9.6	(1.6)	6.0	(0.3)	cdef
HF	CF	12.3	(0.7)	a	10.8	(1.1)	9.0	(1.0)	a
	1.0 × O_3	11.5	(0.2)	ab	9.5	(0.8)	7.3	(0.4)	bc
	1.5 × O_3	11.1	(0.2)	abc	9.6	(0.2)	7.3	(0.3)	bc
Lower canopy leaves									
NF	CF	8.6	(0.6)	f	7.1	(0.4)	5.3	(0.7)	ef
	1.0 × O_3	8.5	(0.1)	f	6.8	(0.6)	5.4	(0.0)	def
	1.5 × O_3	9.2	(0.4)	def	6.6	(0.9)	5.0	(0.4)	f
LF	CF	8.5	(0.2)	f	8.1	(0.4)	6.8	(0.1)	bcd
	1.0 × O_3	8.3	(0.2)	f	8.1	(0.9)	6.1	(0.4)	cdef
	1.5 × O_3	8.9	(0.2)	ef	7.8	(0.5)	5.1	(0.2)	f
HF	CF	8.6	(0.3)	f	8.5	(1.1)	7.2	(0.3)	bc
	1.0 × O_3	8.7	(0.6)	ef	8.0	(0.4)	6.4	(0.4)	bcdef
	1.5 × O_3	8.6	(0.1)	f	8.0	(0.8)	5.1	(0.6)	f
Nutrient pooled									
	CF	9.5	(2.9)		8.8	(2.7)	6.8	(2.0)	a
	1.0 × O_3	9.2	(2.6)		8.3	(2.4)	6.2	(1.7)	a
	1.5 × O_3	9.5	(2.6)		8.0	(2.3)	5.4	(1.6)	b
Gas pooled									
	NF	9.6	(1.1)		8.2	(1.5)	5.9	(0.9)	b
	LF	10.1	(1.6)		9.2	(1.5)	6.5	(1.0)	ab
	HF	10.1	(1.6)		9.1	(1.2)	7.0	(1.3)	a
Leaf position									
	Upper	11.2	(0.8)		9.9	(1.0)	7.2	(1.0)	
	Lower	8.6	(0.4)		7.7	(0.9)	5.8	(0.9)	
ANOVA									
O_3		n.s.			*		***		
Nutrient (N)		**			***		***		
Leaf position (LP)		***			***		***		
O_3 × N		*			n.s.		*		
O_3 × LP		*			n.s.		n.s.		
N × LP		***			n.s.		n.s.		
O_3 × N × LP		n.s.			n.s.		n.s.		

Each value is the mean ± standard error ($n = 3$). Soil nutrient treatments: NF: non-fertilised; LF: low-fertilised; HF: high-fertilised. Gas treatments; CF: charcoal-filtered air; 1.0 × O_3: 1.0 time ambient ozone concentration; 1.5 × O_3: 1.5 times ambient ozone concentration. Three-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. not significant. Different letter indicates significant difference between the treatments in gas × nutrient combination, nutrient pooled or gas pooled with a $p < 0.05$. Data were referred by Kinose et al. [26].

Table 4. Area-based nitrogen content (g m^{-2}) in upper and lower canopy leaves of Siebold's beech seedlings.

Nutrient	Gas	May	July	September
Upper canopy leaves				
NF	CF	1.5 (0.2)	1.7 (0.3)	1.5 (0.1)
	$1.0 \times O_3$	1.5 (0.2)	1.6 (0.1)	1.4 (0.1)
	$1.5 \times O_3$	1.7 (0.2)	1.9 (0.1)	1.5 (0.1)
LF	CF	1.8 (0.2)	1.8 (0.1)	1.5 (0.2)
	$1.0 \times O_3$	1.6 (0.2)	1.8 (0.3)	1.5 (0.1)
	$1.5 \times O_3$	1.7 (0.1)	1.8 (0.2)	1.5 (0.1)
HF	CF	1.7 (0.1)	1.9 (0.1)	1.7 (0.1)
	$1.0 \times O_3$	1.8 (0.2)	1.8 (0.1)	1.6 (0.1)
	$1.5 \times O_3$	1.8 (0.0)	1.9 (0.0)	1.6 (0.0)
Lower canopy leaves				
NF	CF	0.9 (0.1)	1.0 (0.1)	1.0 (0.1)
	$1.0 \times O_3$	0.9 (0.0)	1.1 (0.1)	1.0 (0.1)
	$1.5 \times O_3$	1.1 (0.0)	1.3 (0.1)	1.0 (0.1)
LF	CF	1.1 (0.0)	1.1 (0.1)	1.1 (0.1)
	$1.0 \times O_3$	1.0 (0.2)	1.1 (0.1)	1.0 (0.1)
	$1.5 \times O_3$	1.1 (0.1)	1.3 (0.1)	1.1 (0.1)
HF	CF	1.1 (0.1)	1.3 (0.2)	1.2 (0.0)
	$1.0 \times O_3$	1.1 (0.0)	1.3 (0.1)	1.2 (0.0)
	$1.5 \times O_3$	1.1 (0.0)	1.3 (0.0)	1.1 (0.1)
Nutrient pooled				
	CF	1.3 (0.5)	1.4 (0.5)	1.3 (0.4)
	$1.0 \times O_3$	1.2 (0.5)	1.4 (0.5)	1.2 (0.4)
	$1.5 \times O_3$	1.3 (0.5)	1.5 (0.5)	1.2 (0.4)
Gas pooled				
	NF	1.3 (0.3)	1.4 (0.3)	1.2 (0.3)
	LF	1.4 (0.4)	1.5 (0.3)	1.3 (0.3)
	HF	1.4 (0.3)	1.6 (0.3)	1.4 (0.3)
Leaf position				
	Upper	1.7 (0.2)	1.8 (0.2)	1.5 (0.1)
	Lower	1.0 (0.1)	1.2 (0.1)	1.1 (0.1)
ANOVA				
O_3		n.s.	n.s.	n.s.
Nutrient (N)		**	*	***
Leaf position (LP)		***	***	***
$O_3 \times N$		n.s.	n.s.	n.s.
$O_3 \times LP$		n.s.	n.s.	n.s.
$N \times LP$		n.s.	n.s.	n.s.
$O_3 \times N \times LP$		n.s.	n.s.	n.s.

Each value is the mean ± standard error ($n = 3$). Soil nutrient treatments: NF: non-fertilised; LF: low-fertilised; HF: high-fertilised. Gas treatments; CF: charcoal-filtered air; $1.0 \times O_3$: 1.0 time ambient ozone concentration; $1.5 \times O_3$: 1.5 times ambient ozone concentration. Three-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. not significant. Data in July were referred by Kinose et al. [26].

Table 5. Photosynthetic nitrogen use efficiency (µmol mol^{-1} s^{-1}) in upper and lower canopy leaves of Siebold's beech seedlings.

Nutrient	**Gas**	**May**			**July**			**September**		
Upper canopy leaves										
NF	CF	97	(8)	bcd	85	(23)		71	(4)	
	1.0 × O_3	94	(5)	cd	83	(13)		67	(4)	
	1.5 × O_3	92	(12)	cd	68	(2)		57	(3)	
LF	CF	97	(9)	bcd	87	(7)		72	(13)	
	1.0 × O_3	98	(9)	bcd	82	(12)		72	(3)	
	1.5 × O_3	96	(9)	cd	78	(21)		58	(7)	
HF	CF	102	(7)	bcd	82	(8)		75	(14)	
	1.0 × O_3	92	(9)	cd	74	(8)		65	(6)	
	1.5 × O_3	88	(1)	d	72	(2)		65	(4)	
Lower canopy leaves										
NF	CF	138	(16)	a	100	(3)		78	(13)	
	1.0 × O_3	126	(3)	ab	84	(4)		78	(7)	
	1.5 × O_3	122	(2)	abc	73	(14)		68	(9)	
LF	CF	110	(6)	abcd	100	(5)		89	(10)	
	1.0 × O_3	121	(20)	abc	103	(12)		91	(5)	
	1.5 × O_3	118	(11)	abc	84	(10)		66	(5)	
HF	CF	107	(14)	bcd	93	(0)		85	(7)	
	1.0 × O_3	118	(3)	abcd	86	(4)		78	(6)	
	1.5 × O_3	108	(4)	bcd	88	(5)		64	(7)	
Nutrient pooled										
	CF	103	(30)		86	(24)	a	74	(21)	a
	1.0 × O_3	102	(30)		81	(23)	ab	71	(20)	a
	1.5 × O_3	98	(28)		73	(21)	b	60	(16)	b
Gas pooled										
	NF	112	(20)		82	(15)		70	(10)	
	LF	107	(14)		89	(14)		74	(14)	
	HF	102	(12)		82	(9)		72	(10)	
Leaf position										
	Upper	95	(8)		79	(12)		67	(9)	
	Lower	119	(13)		90	(11)		78	(11)	
ANOVA										
O_3		n.s.			**			***		
Nutrient (N)		*			n.s.			n.s.		
Leaf position (LP)		***			***			***		
O_3 × N		n.s.			n.s.			n.s.		
O_3 × LP		n.s.			n.s.			n.s.		
N × LP		*			n.s.			n.s.		
O_3 × N × LP		n.s.			n.s.			n.s.		

Each value is the mean ± standard error ($n = 3$). Soil nutrient treatments: NF: non-fertilised; LF: low-fertilised; HF: high-fertilised. Gas treatments; CF: charcoal-filtered air; 1.0 × O_3: 1.0 time ambient ozone concentration; 1.5 × O_3: 1.5 times ambient ozone concentration. Three-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. not significant. Different letter indicates significant difference between the treatments in gas × nutrient combination or nutrient pooled with a $p < 0.05$.

4. Discussion

The exposure to O_3 significantly decreased ΔW in NF treatment, whereas there was no significant O_3-induced reduction in ΔW in the LF and HF treatments (Table 2). This result was in agree with the tendency in dry mass of the seedlings at the end of this experiment [26,42]. Similar results of lower O_3 susceptibility under higher nutrient condition were reported in the other studies on larch [30,43] and aspen [22] seedlings, although several studies demonstrated nitrogen supply-induced enhance of the susceptibility to O_3 in Siebold's beech [24] and Scots pine [44]. In contrast with ΔW, A_{sat}

(Table 3) and other photosynthetic parameters such as maximum carboxylation rate and maximum electron transport rate [45] showed greater reduction by the O_3 exposure in higher nutrient condition. Kinose et al. [26] concluded the reduction in the leaf level photosynthetic activity by the O_3 exposure in higher nutrient condition was offset by the O_3-induced increase in the area of first-flush leaves in the second growing season.

There was no significant effect of O_3 or the interaction of O_3 and soil nutrient supply on the NUE and all the components of NUE of Siebold's beech seedlings in the present study (Figure 2; Table 2). These results are different from the response of the whole-plant growth of the seedlings (Table 2) [26]. On the other hand, PNUE, a component of LNP, was significantly reduced by O_3 (Table 5). Ozone-induced reduction in PNUE has been reported in several tree species, including Siebold's beech [20–25]. We consider the contribution of O_3 effects on leaf-level photosynthesis to the response of whole plant nitrogen use was limited in the present study.

The growth type of the tree species may affect the response of NUE to elevated O_3. The plant material in the present study, Siebold's beech, is classified as fixed growth type [46]. This growth type basically produces new leaves in one time in spring. In contrast, indeterminate growth type and multi-flush growth type can produce new leaves during a growing season. These new leaf productions may change nitrogen use traits. Accelerating senescence of old leaves exposed to O_3 during a growing season, being considered compensation response to O_3 [47,48]. This phenomenon requires nitrogen reallocation from old leaves to young leaves and loss of some nitrogen with abscission of old leaves, which also affect NUE. It is important to study on the response of nitrogen use trait of various growth types.

The NUE of the seedlings decreased in relation to soil nutrient supply (Figure 2). Similar decreasing results for NUE with increasing nutrient (or nitrogen) availability have been reported [14]. Both reductions in NP and MRT can be attributed to NUE reduction for Siebold's beech seedlings. NP is composed of LNF and LNP as shown in Equation (6), and the latter is important as an explanation factor in the results of the present study (Table 2), indicating a lower efficiency of leaf N usage for increasing biomass under a higher nutrient supply. On the other hand, the reduction of PNUE, which is considered an important parameter explaining LNP, based on soil nutrient supply was not clear (Table 5). Similar to its response to O_3, discordance between the response at the individual level and that at the leaf level to soil nutrient supply was observed.

The reduction in MRT by soil nutrient supply indicated a smaller increase in PND as compared to that of ΔN (Table 2). In this experiment, the dry mass ratio of fine roots to coarse roots was decreased by increased soil nutrient supply [42], indicating the possibility of lower N uptake efficiency. However, the contribution of lower nitrogen uptake efficiency was smaller compared to that of the greater soil N availability under the high fertilisation condition because we observed a clear increase in the ΔN of the seedlings with an increase in soil nutrient supply.

Studies on the NUE response of tree seedlings to nutrient supply are limited. Zhu et al. [49] reported a decrease in NUE because of increasing ammonium nitrate supply, with 50 and 100 kg N ha^{-1} applied to potted konata oak (*Quercus serrata*) seedlings, with consideration of phosphorous supply. The reduction in the rates of NUE as compared to that with no nitrogen supply in Zhu et al. [49] were 43% and 52% for nitrogen treatments of 50 and 100 kg N ha^{-1}, respectively, as averages for two phosphorous supplies. The extents of the reduction in NUE of our study (6% and 29% reduction in LF and HF treatments, respectively) were smaller than those in Zhu et al. [49], whereas the amount of the nitrogen supply for one growing season in our study (39.6 and 79.2 kg N ha^{-1} in LF and HF, respectively) were comparable. The smaller extent of NUE decrease was explained by the inclusion of nutrients other than nitrogen. The liquid fertiliser used in our study contained both macronutrients and micronutrients, totally 15 nutrients. Furthermore, the soil in the present study (brown forest soil) was fertile [42] as compared to the soil in Zhu et al. [49], which was very poor in nutrients, being a mixture of Kanuma soil (pumice soil) and Akadama soil (clay soil). Although this is a comparison between different tree species, the difference in the reduction in NUE between the two experiments strongly suggests the importance of the availability of other nutrients for determining NUE of trees grown under various soil nitrogen conditions.

5. Conclusions

Based on the results of the present study, our hypothesis was rejected. Ozone did not decrease the NUE and its components of Siebold's beech seedlings, although there were several negative impacts of O_3 on biomass and photosynthetic activity in the same experiment [26,42,46]. On the other hand, the effects of soil nutrient supply on the NUE of the seedlings were clear. Decreases in NP due to lower LNP and MRT contributed to the reduction of NUE. There was no significant interaction of O_3 and soil nutrient supply on NUE and its components, although there was a significant interaction on the growth of seedlings (i.e., ΔW). These results suggested that NUE might not be primary factor to explain the growth response of Siebold's beech to O_3. However, this experiment is the first to evaluate the individual level NUE under elevated O_3. It is important to accumulate the results to obtain a robust understanding about the growth responses of trees to O_3 with special attention of nitrogen use traits. We noted a difference between the response of NUE (individual level) and that of PNUE (leaf level) to O_3, as well as soil nutrient supply. To fill the gap in the responses between the two scales, further research is needed. In addition, studies on the response of NUE to elevated O_3 by various tree species over a wide range of soil nutrient conditions are highly important.

Author Contributions: Conceptualization, H.H. and M.W.; methodology, H.H., Y.K. and M.W.; formal analysis, H.H.; investigation, H.H., S.O. and Y.K.; resources, T.I. and M.W.; visualization, M.W.; writing—original draft preparation, H.H. and M.W.; writing—review and editing, Y.K., T.I.; project administration, T.I. and M.W.; supervision, M.W.; funding acquisition, T.I. and M.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by JSPS KAKENHI, Type B program (17H01868 to M.W.; 18H03410 to M.W. and T.I.) and Type C program (18K11664 to M.W.), and JST SICORP (JPMJSC16HB to M.W. and T.I.).

Acknowledgments: The authors are greatly indebted to Kazuhide Matsuda, Hiroyuki Ozawa and Yoshinobu Fukamachi (Tokyo University of Agriculture and Technology) for the technical support. We are grateful for Rainer Matyseek for the discussion about this study in 2nd Asian Air Pollution Workshop at Beijing in May 2015. The authors thank Editage (www.editage.com) for English language editing.

Abbreviations

The following abbreviations are used in this manuscript:

NUE	Nitrogen use efficiency
NP	Nitrogen productivity
MRT	Mean residence time of nitrogen
ΔW	Dry mass increment for a given period
ΔN	Amount of nitrogen uptake from the soil for a given period
N_0	Initial nitrogen content
PND	Plant nitrogen duration
$\overline{N}$	Mean whole-plant nitrogen content during a given time period
$\overline{N}\mathrm{R_L}$	Mean whole-leaf nitrogen content during a given time period
LNF	Leaf nitrogen fraction
LNP	Leaf nitrogen productivity
$\int N_{\mathrm{loss}}$	Time integral of nitrogen loss
$\int N_{\mathrm{uptake}}$	Time integral of nitrogen uptake
CF	Charcoal-filtered air
$1.0 \times O_3$	1.0-fold ambient ozone concentration

$1.5 \times O_3$	1.5-fold ambient ozone concentration
AOT40	Accumulated exposure over a threshold of 40 nmol mol^{-1}
NF	Non-fertilised treatment
LF	Low-fertilised treatment
HF	High-fertilised treatment
A_{sat}	Light-saturated net photosynthetic rate
N_{area}	Area-based nitrogen content in leaves
PNUE	Photosynthetic nitrogen use efficiency
D^2H	Product of the square of the diameter and height
VPD	Vapour pressure deficit

References

1. Akimoto, H. Global air quality and pollution. *Science* **2003**, *302*, 1716–1719. [CrossRef] [PubMed]
2. Stevenson, D.S.; Dentener, F.J.; Schultz, M.G.; Ellingsen, K.; Van Noije, T.P.C.; Wild, O.; Zeng, G.; Amann, M.; Atherton, C.S.; Bell, N.; et al. Multi-model ensemble simulations of present-day and near-future tropospheric ozone. *J. Geophys. Res.* **2006**, *111*, D08301. [CrossRef]
3. Paoletti, E. Ozone Impacts on Forests. In *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources No. 68*; CABI: Wallingford, UK, 2007; pp. 1–13.
4. Ohara, T.; Akimoto, H.; Kurokawa, J.; Horii, N.; Yamaji, K.; Yan, X.; Hatasaka, T. An Asian emission inventory of anthropogenic emission sources for the period 1980–2020. *Atmos. Chem. Phys.* **2007**, *7*, 4419–4444. [CrossRef]
5. Naja, M.; Akimoto, H. Contribution of regional pollution and long-range transport to the Asia-Pacific region: Analysis of long-term ozonesonde data over Japan. *J. Geophys. Res.* **2004**, *109*, D21306. [CrossRef]
6. Yamaji, K.; Ohara, T.; Uno, I.; Kurokawa, J.; Pochanart, P.; Akimoto, H. Future prediction of surface ozone over east Asia using models-3 community multiscale air quality modeling system and regional emission inventory in Asia. *J. Geophys. Res.* **2008**, *113*, D08306. [CrossRef]
7. Wittig, V.E.; Ainsworth, E.A.; Long, S.P. To what extent do current and projected increases in surface ozone affect photosynthesis and stomatal conductance of trees? A meta-analytic review of the last 3 decades of experiments. *Plant Cell Environ.* **2007**, *30*, 1150–1162. [CrossRef]
8. Wittig, V.E.; Ainsworth, E.A.; Naidu, S.L.; Karnosky, D.F.; Long, S.P. Quantifying the impact of current and future tropospheric ozone on tree biomass, growth, physiology and biochemistry: A quantitative meta-analysis. *Glob. Chang. Biol.* **2009**, *15*, 396–424. [CrossRef]
9. Yamaguchi, M.; Watanabe, M.; Matsumura, H.; Kohno, Y.; Izuta, T. Experimental studies on the effects of ozone on growth and photosynthetic activity of Japanese forest tree species. *Asian J. Atmos. Environ.* **2011**, *5*, 65–87. [CrossRef]
10. Watanabe, M.; Hoshika, Y.; Koike, T.; Izuta, T. Effects of ozone on Japanese trees. In *Air Pollution Impacts on Plant in East Asia*; Izuta, T., Ed.; Springer: Tokyo, Japan, 2017; pp. 73–100.
11. Matyssek, R.; Sandermann, H. Impact of ozone on trees: An ecophysiological perspective. *Prog. Bot.* **2003**, *64*, 349–404.
12. Sitch, S.; Cox, P.M.; Collins, W.J.; Huntingford, C. Indirect radiative forcing of climate change through ozone effects on the land-carbon sink. *Nature* **2007**, *448*, 791–794. [CrossRef]
13. Magnani, F.; Mencuccini, M.; Borghetti, M.; Berbigier, P.; Berninger, F.; Delzon, S.; Grelle, A.; Hari, P.; Jarvis, P.G.; Kolari, P.; et al. The human footprint in the carbon cycle of temperate and boreal forests. *Nature* **2007**, *447*, 848–852. [CrossRef] [PubMed]
14. Chapin, F.S., III; Matson, P.A.; Vitousek, P.M. *Principles of Terrestrial Ecosystem Ecology*, 2nd ed.; Springer: Berlin, Germany, 2012; pp. 1–529.
15. Hirose, T. Relations between turnover rate, resource utility and structure of some plant populations: A study of the matter budgets. *J. Fac. Sci. Univ. Tokyo* **1975**, *11*, 355–407.
16. Hirose, T. Nitrogen use efficiency revisited. *Oecologia* **2011**, *166*, 863–867. [CrossRef] [PubMed]
17. Ingestad, T. Nitrogen stress in birch seedlings. II. N, K, P, Ca and Mg nutrition. *Physiol. Plant.* **1979**, *45*, 149–157. [CrossRef]

18. Berendse, F.; Aerts, R. Nitrogen-use-efficiency: A biologically meaningful definition? *Funct. Ecol.* **1987**, *1*, 293–296.
19. Berendse, F. The effects of grazing on the outcome of competition between plant species with different nutrient requirements. *Oikos* **1985**, *44*, 35–39. [CrossRef]
20. Watanabe, M.; Hoshika, Y.; Inada, N.; Wang, X.; Mao, Q.; Koike, T. Photosynthetic traits of Siebold's beech and oak saplings grown under free air ozone exposure in northern Japan. *Environ. Pollut.* **2013**, *174*, 50–56. [CrossRef]
21. Kytöviita, M.; Thiec, D.; Dizengremel, P. Elevated CO_2 and ozone reduce nitrogen acquisition by *Pinus halepensis* from its mycorrhizal symbiont. *Physhiolosia Plant.* **2001**, *111*, 305–312. [CrossRef]
22. Häkiö, E.; Freiwald, V.; Silfver, T.; Beuker, E.; Holopainen, T.; Oksanen, E. Impacts of elevated ozone and nitrogen on growth and photosynthesis of European aspen (*Populus tremula*) and hybrid aspen (*P. tremula* × *Populus tremuloides*) clones. *Can. J. For. Res.* **2007**, *37*, 2326–2336. [CrossRef]
23. Watanabe, M.; Yamaguchi, M.; Tabe, C.; Iwasaki, M.; Yamashita, R.; Funada, R.; Fukami, M.; Matsumura, H.; Kohno, Y.; Izuta, T. Influences of nitrogen load on the growth and photosynthetic responses of *Quercus serrata* seedlings to O_3. *Trees* **2007**, *21*, 421–432. [CrossRef]
24. Yamaguchi, M.; Watanabe, M.; Iwasaki, M.; Tabe, C.; Matsumura, H.; Kohno, Y.; Izuta, T. Growth and photosynthetic responses of *Fagus crenata* seedlings to O_3 under different nitrogen loads. *Trees* **2007**, *21*, 707–718. [CrossRef]
25. Hoshika, Y.; Watanabe, M.; Inada, N.; Mao, Q.; Koike, T. Photosynthetic response of early and late leaves of white birch (*Betula platyphylla* var. *japonica*) grown under free-air ozone exposure. *Environ. Pollut.* **2013**, *182*, 242–247. [CrossRef] [PubMed]
26. Kinose, Y.; Fukamachi, Y.; Okabe, S.; Hiroshima, H.; Watanabe, M.; Izuta, T. Nutrient supply to soil offsets the ozone-induced growth reduction in *Fagus crenata* seedlings. *Trees* **2017**, *31*, 259–272. [CrossRef]
27. Landolt, W.; Günthardt-Goerg, M.S.; Pfenninger, I.; Einig, W.; Hampp, R.; Maurer, S.; Matyssek, R. Effect of fertilization on ozone-induced changes in the metabolism of birch (*Betula pendula*) leaves. *New Phytolosist* **1997**, *137*, 389–397. [CrossRef]
28. Kolb, T.E.; Matyssek, R. Limitations and perspectives about scaling ozone impacts in trees. *Environ. Pollut.* **2001**, *115*, 373–393. [CrossRef]
29. Thomas, V.F.D.; Braun, S.; Flückiger, W. Effects of simultaneous ozone exposure and nitrogen loads on carbohydrate concentrations, biomass, growth, and nutrient concentrations of young beech trees (*Fagus sylvatica*). *Environ. Pollut.* **2005**, *143*, 341–354. [CrossRef]
30. Watanabe, M.; Yamaguchi, M.; Iwasaki, M.; Matsuo, N.; Naba, J.; Tabe, C.; Matsumura, H.; Kohno, Y.; Izuta, T. Effects of ozone and/or nitrogen load on the growth of *Larix kaempferi*, *Pinus densiflora* and *Cryptomeria japonica* seedlings. *J. Jpn. Soc. Atmos. Environ.* **2006**, *41*, 320–334.
31. Marzuoli, R.; Monga, R.; Finco, A.; Gerosa, G. Biomass and physiological responses of *Quercus robur* (L.) young trees during 2 years of treatments with different levels of ozone and nitrogen wet deposition. *Trees* **2016**, *30*, 1995–2000. [CrossRef]
32. Marzuoli, R.; Monga, R.; Finco, A.; Chiesa, M.; Gerosa, G. Increased nitrogen wet deposition triggers negative effects of ozone on the biomass production of *Carpinus betulus* L. young trees. *Environ. Exp. Bot.* **2018**, *152*, 128–136. [CrossRef]
33. Nakashizuka, T.; Iida, S. Composition, dynamics and disturbance regime of temperate deciduous forests in Monsoon Asia. *Vegetatio* **1995**, *121*, 23–30. [CrossRef]
34. Nakashizuka, T. *Story of Forest Trees and Japan*; Tokai University Press: Hatano, Japan, 2004; pp. 1–236. (In Japanese)
35. Terazawa, K.; Koyama, H. *Applied Ecology for Restoration of Beech Forests*; Bun-ichi Sogo Shuppan: Tokyo, Japan, 2008; pp. 1–310. (In Japanese)
36. Kohno, Y.; Matsumura, H.; Ishii, T.; Izuta, T. Establishing critical levels of air pollutants for protecting East Asian vegetation—A challenge. In *Plant Responses to Air Pollution and Global Change*; Omasa, K., Nouchi, I., De Kok, L.J., Eds.; Springer: Tokyo, Japan, 2005; pp. 243–250.
37. Kinose, Y.; Azuchi, F.; Uehara, Y.; Kanomata, T.; Kobayashi, A.; Yamaguchi, M.; Izuta, T. Modeling of stomatal conductance to estimate stomatal ozone uptake by *Fagus crenata*, *Quercus serrata*, *Quercus mongolica* var. *crispula* and *Betula platyphylla*. *Environ. Pollut.* **2014**, *194*, 235–245. [CrossRef] [PubMed]

38. IUSS Working Group WRB. World Reference Base for Soil Resources 2014, Update 2015. International Soil Classification System for Naming Soils and Creating Legends for Soil Maps. In *World Soil Resources Reports No. 106*; FAO: Rome, Italy, 2015; pp. 1–192. Available online: http://www.fao.org/3/i3794en/I3794en.pdf (accessed on 29 November 2019).
39. Kinose, Y. Photosynthetic and Growth Responses to Ozone of *Fagus Crenata* Seedlings Grown under Different Soil Nutrient Levels. Ph.D. Thesis, Tokyo University of Agriculture and Technology, Tokyo, Japan, 16 March 2017.
40. Agathokleous, E.; Kitao, M.; Kinose, Y. A review study on O_3 phytotoxicity metrics for setting critical levels in Asia. *Asian J. Atmos. Environ.* **2018**, *12*, 1–16. [CrossRef]
41. R Development Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2017; pp. 1–3520. Available online: http://www.R-project.org (accessed on 29 November 2019).
42. Watanabe, M.; Okabe, S.; Kinose, Y.; Hiroshima, H.; Izuta, T. Effects of ozone on soil respiration rate of Siebold's beech seedlings grown under different soil nutrient conditions. *J. Agric. Meteorol.* **2019**, *75*, 39–46. [CrossRef]
43. Sugai, T.; Watanabe, T.; Kita, K.; Koike, T. Nitrogen loading increases the ozone sensitivity of larch seedlings with higher sensitivity to nitrogen loading. *Sci. Total. Environ.* **2019**, *663*, 587–595. [CrossRef] [PubMed]
44. Utriainen, J.; Holopainen, T. Nitrogen availability modifies the ozone responses of Scots pine seedlings exposed in an open-field system. *Tree Physiol.* **2001**, *21*, 1205–1213. [CrossRef]
45. Kinose, Y.; Fukamachi, Y.; Okabe, S.; Hiroshima, H.; Watanabe, M.; Izuta, T. Photosynthetic responses to ozone of upper and lower canopy leaves of *Fagus crenata* Blume seedlings grown under different soil nutrient conditions. *Environ. Pollut.* **2017**, *223*, 213–222. [CrossRef]
46. Kikuzawa, K. Leaf survival of woody plant in deciduous broad-leaved forests. 1. Tall trees. *Can. J. Bot.* **1983**, *61*, 2133–2139. [CrossRef]
47. Tjoelker, M.G.; Luxmoore, R.J. Soil nitrogen and chronic ozone stress influence physiology, growth and nutrient status of *Pinus taeda* L. and *Liriodendron tulipifera* L. seedlings. *New Phytol.* **1991**, *119*, 69–81. [CrossRef]
48. Reich, P.B. Effects of low concentrations of O_3 on net photosynthesis, dark respiration, and chlorophyll contents in aging hybrid poplar leaves. *Plant Physiol.* **1983**, *73*, 291–296. [CrossRef]
49. Zhu, B.; Izuta, T.; Watanabe, M. Nitrogen use efficiency of *Quercus serrata* seedlings under different soil nitrogen and phosphorus supplies. *J. Agric. Meteorol.* **2020**, *76*, 11–19.