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

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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 (O₃) in the troposphere is recognised as a phytotoxic gaseous air pollutant. The atmospheric concentration of tropospheric O₃ 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 O₃ on growth and physiological function, such as photosynthesis, of tree species [7–10]. Therefore, O₃ 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: \( N \) 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 (\textit{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 (\textit{Fagus 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) Tamakuryo 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 × O₃ and 1.5 × O₃, respectively), with three-chamber replications, providing a total of nine chambers for data analysis. Further details of the O₃ 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 O₃ nutrient chamber combination, for a total of 270 seedlings.

The indices of O₃ 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 O₃ 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 × O₃                 | 16.9 (0.7)            | 22.6 (0.5) | 4.1 (0.0) |
|                | 1.5 × O₃                 | 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 × O₃                 | 23.9 (0.3)            | 32.9 (0.4) | 8.9 (0.4) |
|                | 1.5 × O₃                 | 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 × O₃: 1.0 time ambient ozone concentration, 1.5 × O₃: 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., D²H) 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 D²H and between root dry mass and D²H 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,

\[ NUE = \frac{\Delta W}{\Delta N} \] (1)
where $\Delta W$ (g) and $\Delta N$ (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,

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

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

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

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

$$
\bar{N} = \frac{1}{\Delta T} \int_{0}^{\Delta T} [N_{0} + f(t) - g(t)]dt \tag{5}
$$

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 $\bar{N}$ and $\Delta T$, 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 $\Delta T$ 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$^{2}$H 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.

![Diagram of time course in plant nitrogen content.](image)

**Figure 1.** Diagram of time course in plant nitrogen content. $N_{0}$, initial plant nitrogen content; $f(t)$, nitrogen uptake; $g(t)$, nitrogen loss; $\Delta N$, 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 ($\bar{N}_{L}, g$) of the whole leaves during the second growing season. The dry mass of stems and roots on the date of D$^{2}$H 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 $N$ 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 $N$ and $\Delta T$, respectively. The NP was composed of the leaf nitrogen productivity (LNP, g g$^{-1}$ N day$^{-1}$) and leaf nitrogen fraction (LNF) as follows.

$$NP = LNP \cdot LNF$$  \hspace{1cm} (6)

$$LNP = \frac{\Delta W}{N_L \cdot \Delta T}$$  \hspace{1cm} (7)

$$LNF = \frac{N_L}{N}$$  \hspace{1cm} (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 O₃ or the interaction of the two factors (Figure 2). As shown in Table 2, O₃ did not significantly affect all of the NUE components. We observed a significant increase in ∆W, ∆N 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 O₃ and nutrient supply was detected for ∆W. Ozone significantly decreased ∆W of the seedlings in NF treatment, whereas the ∆W was not decreased by the exposure to O₃ in both LF and HF treatments. Neither a significant effect of O₃ and soil nutrient supply nor a significant interaction of the two factors was observed for N₀ and the time integral of nitrogen loss (∫N.loss). The time integral of nitrogen uptake (∫N.uptake) had the same trend as PND because of the small amount of ∫N.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 × O₃: 1.0 time ambient ozone concentration; 1.5 × O₃: 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 ($\Delta W$), nitrogen uptake ($\Delta N$), 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 ($\int N_{\text{loss}}$) of Siebold’s beech seedlings in the second growing season.

| Nutrient | Gas              | $\Delta W$ (g) | $\Delta N$ (g N$^{-1}$ N) | LNF (g g$^{-1}$ N) | LNP (g g$^{-1}$ N day$^{-1}$) | $N_0$ (g N) | PND (g N day$^{-1}$) | $\int N_{\text{loss}}$ (g N day$^{-1}$) |
|----------|------------------|----------------|---------------------------|-------------------|-------------------------------|-------------|---------------------|-----------------------------|
| 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 $\times$ $O_3$ 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 $\times$ $O_3$ 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 $\times$ $O_3$ 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 $\times$ $O_3$ 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 $\times$ $O_3$ 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 $\times$ $O_3$ 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 $\times$ $O_3$ 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 $\times$ $O_3$ 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) |
|          | 1.0 $\times$ $O_3$ 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) |
|          | 1.5 $\times$ $O_3$ 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. |
|          | $O_3 \times$ N | * | 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 $\times$ $O_3$: 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. The actual $p$ value is shown when 0.05 $< p < 0.10$. Different letter indicates significant difference between the treatments in gas $\times$ nutrient combination or gas pooled with a $p < 0.05$. 

3.2. Leaf Photosynthetic Traits

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

We observed a significant decrease in PNUE by the exposure to O\textsubscript{3} in July and September (Table 5). The PNUE at 1.5 \times O\textsubscript{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 ($\mu$mol 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.s.      |
| N × LP                               |         | n.s.     | 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 × O\(_3\) | 1.5 (0.2) | 1.6 (0.1) | 1.4 (0.1) |
|          | 1.5 × 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 × O\(_3\) | 1.6 (0.2) | 1.8 (0.3) | 1.5 (0.1) |
|          | 1.5 × O\(_3\) | 1.7 (0.2) | 1.8 (0.2) | 1.5 (0.1) |
| HF       | CF        | 1.7 (0.1) | 1.9 (0.1) | 1.7 (0.1) |
|          | 1.0 × O\(_3\) | 1.8 (0.2) | 1.8 (0.1) | 1.6 (0.1) |
|          | 1.5 × 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 × O\(_3\) | 0.9 (0.0) | 1.1 (0.1) | 1.0 (0.1) |
|          | 1.5 × 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 × O\(_3\) | 1.0 (0.2) | 1.1 (0.1) | 1.0 (0.1) |
|          | 1.5 × 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 × O\(_3\) | 1.1 (0.0) | 1.3 (0.1) | 1.2 (0.0) |
|          | 1.5 × 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 × O\(_3\) | 1.2 (0.5) | 1.4 (0.5) | 1.2 (0.4) |
|          | 1.5 × 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\(_3\) × N | n.s. | n.s. | n.s. |
| O\(_3\) × LP | n.s. | n.s. | n.s. |
| N × LP | n.s. | 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. 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  | bcd          | 85 (23)      | 71 (4)        |
| 1.0 × O\(_3\) | 94  | (5)          | 83 (13)      | 67 (4)        |
| 1.5 × O\(_3\) | 92  | (12)         | 68 (2)       | 57 (3)        |
| LF CF    | 97  | bcd          | 87 (7)       | 72 (13)       |
| 1.0 × O\(_3\) | 98  | (9)          | 82 (12)      | 72 (3)        |
| 1.5 × O\(_3\) | 96  | (9)          | 78 (21)      | 58 (7)        |
| HF CF    | 102 | (7)          | 82 (8)       | 75 (14)       |
| 1.0 × O\(_3\) | 92  | (9)          | 74 (8)       | 65 (6)        |
| 1.5 × O\(_3\) | 88  | (1)          | 72 (2)       | 65 (4)        |
|          |     | Lower canopy leaves |             |               |
| NF CF    | 138 | a            | 100 (3)      | 78 (13)       |
| 1.0 × O\(_3\) | 126 | (3)          | 84 (4)       | 78 (7)        |
| 1.5 × O\(_3\) | 122 | (2)          | 73 (14)      | 68 (9)        |
| LF CF    | 110 | abcd         | 100 (5)      | 89 (10)       |
| 1.0 × O\(_3\) | 121 | (20)         | 103 (12)     | 91 (5)        |
| 1.5 × O\(_3\) | 118 | (11)         | 84 (10)      | 66 (5)        |
| HF CF    | 107 | bcd          | 93 (0)       | 85 (7)        |
| 1.0 × O\(_3\) | 118 | (3)          | 86 (4)       | 78 (6)        |
| 1.5 × O\(_3\) | 108 | (4)          | 88 (5)       | 64 (7)        |
|          |     | Nutrient pooled |             |               |
| CF 103  | (30)| 86 (24)      | a 74 (21)    | a             |
| 1.0 × O\(_3\) | 102 | (30)         | 81 (23) abcd | 71 (20)       |
| 1.5 × O\(_3\) | 98  | (28)         | 73 (21) b    | 60 (16)       |
|          |     | 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\(_3\) | Nutrient (N) | Leaf position (LP) | O\(_3\) × N | O\(_3\) × LP | N × LP | O\(_3\) × N × LP |
|--------------------------|---------|--------------|--------------------|-------------|-------------|--------|-----------------|
|                          | n.s.    | n.s.         | ***                | n.s.        | 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 ∆W in NF treatment, whereas there was no significant O\(_3\)-induced reduction in ∆W 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 ∆W, A\(_{Sat}\)
(Table 3) and other photosynthetic parameters such as maximum carboxylation rate and maximum electron transport rate [45] showed greater reduction by the O₃ exposure in higher nutrient condition. Kinose et al. [26] concluded the reduction in the leaf level photosynthetic activity by the O₃ exposure in higher nutrient condition was offset by the O₃-induced increase in the area of first-flush leaves in the second growing season.

There was no significant effect of O₃ or the interaction of O₃ 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 O₃ (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 O₃ 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 O₃. 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 O₃ during a growing season, being considered compensation response to O₃ [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 O₃, 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 ∆N (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 ∆N 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\textsubscript{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\textsubscript{3} and soil nutrient supply on NUE and its components, although there was a significant interaction on the growth of seedlings (i.e., $\Delta W$). These results suggested that NUE might not be primary factor to explain the growth response of Siebold’s beech to O\textsubscript{3}. However, this experiment is the first to evaluate the individual level NUE under elevated O\textsubscript{3}. It is important to accumulate the results to obtain a robust understanding about the growth responses of trees to O\textsubscript{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\textsubscript{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\textsubscript{3} by various tree species over a wide range of soil nutrient conditions are highly important.

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Abbreviations

The following abbreviations are used in this manuscript:

- NUE: Nitrogen use efficiency
- NP: Nitrogen productivity
- MRT: Mean residence time of nitrogen
- $\Delta W$: Dry mass increment for a given period
- $\Delta N$: Amount of nitrogen uptake from the soil for a given period
- $N_0$: Initial nitrogen content
- PND: Plant nitrogen duration
- $\bar{N}$: Mean whole-plant nitrogen content during a given time period
- $\bar{N}_{RL}$: Mean whole-leaf nitrogen content during a given time period
- LNF: Leaf nitrogen fraction
- LNP: Leaf nitrogen productivity
- $\int N_{loss}$: Time integral of nitrogen loss
- $\int N_{uptake}$: Time integral of nitrogen uptake
- CF: Charcoal-filtered air
- 1.0 $\times$ O\textsubscript{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

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