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Effects of Elevated CO₂ and Nitrogen Loading on the Defensive Traits of Three Successional Deciduous Broad-Leaved Tree Seedlings

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Abstract: To elucidate changes in the defensive traits of tree seedlings under global environmental changes, we evaluated foliar defensive traits of the seedlings of successional trees, such as beech, oak, and magnolia grown in a natural-light phytotron. Potted seedlings were grown under the combination of two CO₂ concentrations (360 vs. 720 ppm) and two nitrogen (N) treatments (4 vs. 15 kg N ha⁻¹ yr⁻¹) for two growing seasons using quantitative chemical analyses and anatomical method. We hypothesized that the effects of CO₂ and N depend on the successional type, with late successional species providing greater defense of their leaves against herbivores, as this species exhibits determinate growth. Beech, a late successional species, responded the most to both elevated CO₂ concentration (eCO₂) and high N treatment. eCO₂ and low N supply enhanced the defensive traits, such as the high leaf mass per area (LMA), high carbon to N ratio (C/N ratio), and increase in the concentrations of total phenolic and condensed tannin in agreement with the carbon–nutrient balance (CNB) hypothesis. High N supply decreased the C/N ratio due to the high N uptake in beech leaves. Oak, a mid–late successional species, exhibited different responses from beech: eCO₂ enhanced the LMA, C/N ratio, and concentration of total phenolics of oak leaves, but only condensed tannin increased under high N supply. Magnolia did not respond to all treatments. No interactive effects were observed between CO₂ and N supply in all species, except for the concentration of total phenolics in oak. Although the amounts of phenolic compounds in beech and oak varied under eCO₂ and high N treatments, the distribution of these compounds did not change. Our results indicate that the changes in the defensive traits of forest tree species under eCO₂ with N loading are related to the successional type.

Keywords: deciduous broad-leaved tree species; successional species; carbon–nutrient balance hypothesis; localization; plant defense chemicals



Citation: Watanabe, Y.; Hinata, K.; Qu, L.; Kitaoka, S.; Watanabe, M.; Kitao, M.; Koike, T. Effects of Elevated CO₂ and Nitrogen Loading on the Defensive Traits of Three Successional Deciduous Broad-Leaved Tree Seedlings. *Forests* **2021**, *12*, 939. <https://doi.org/10.3390/f12070939>

Academic Editor: Timo Domisch

Received: 28 May 2021

Accepted: 14 July 2021

Published: 16 July 2021

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1. Introduction

In recent decades, global environmental changes, such as an increase in atmospheric CO₂ concentration and increases in nitrogen (N) deposition due to high human activities, have raised concern about changes in plant–insect relationships, as these environmental factors modify the defensive traits of leaves through physiological changes in plants [1,2]. These environmental changes will induce a new relationship between insect and woody plants in the mixed deciduous broad-leaved forest based on the free air elevated CO₂ experiment [3].

The responses of plant defense to environmental changes have been frequently explained by the well-known carbon–nutrient balance (CNB) hypothesis [4]. The CNB hypothesis implies that the synthesis of the carbon (C) as a secondary metabolite depends on the balance between C and nutrient. When a plant grows under low nutrient availability, enhanced C availability promotes the accumulation of carbohydrates [5], and then the accumulated carbohydrates are used for the synthesis of C-based defense compounds. The CNB hypothesis also predicts that increased nutrient availability induces allocation of C to the growth of plants, resulting in the decrease in C-based defense compounds. In the leaf habit of broad-leaved tree species, a trade-off relationship between the synthesis of plant defense chemicals (tannin) and growth (mainly producing lignin) will occur, as both are originated from phenylalanine [6]. Therefore, defense phenolics should be efficiently distributed in the leaf tissues.

According to the CNB hypothesis, elevated CO₂ conditions (eCO₂) or low nutrient (i.e., nitrogen in this study) availability in soil will induce an increase in C-based defense chemicals such as total phenolics and condensed tannin due to the enhancing production of carbohydrates [7–10]. A meta-analysis by Robinson et al. [11] revealed that eCO₂ enhanced the concentrations of total phenolics and condensed tannin, the carbon to nitrogen ratio (C/N ratio), and the leaf toughness in forest trees. These changes affect the performance of herbivorous insects: increase in relative consumption rate and total consumption and decrease in pupal weight and relative growth rate under eCO₂ [12–14], as these insects must feed on more low-quality foliage to get sufficient nutrients for their growth under eCO₂.

However, N deposition or over fertilization increases foliage N contents and reduces C-based defensive compounds, which results in the production of high-quality foliage for herbivorous insects [15–17]. However, the combined effects of eCO₂ and N deposition vary among species [5,13,18,19]. Forest tree species have various successional types and shoot growth patterns. Therefore, this successional type would affect the changes in foliar defensive traits as affected by storage of C and nutrients [20] under global environmental changes.

We investigated the effects of the combination of two CO₂ concentrations and two N loadings on the defensive traits of successional deciduous broad-leaved tree seedlings in a natural-light phytotron [5,21]. We analyzed the defensive traits such as the LMA, C/N ratio, and concentrations of total phenolics and condensed tannin of leaves after two growing seasons. As is well described in the reviews [13,22], the levels of both physical and chemical defense are enhanced by grazing (i.e., induced defense). Therefore, phytotron should be used for pest management to avoid induced defense and to obtain the potential capacity of change in the defensive traits under eCO₂ and N loadings. Furthermore, the distribution of phenolic compounds, such as total phenolics and condensed tannin, in leaf tissues also provides important information on the defensive traits of forest tree leaves [23–25]. However, little is known about the changes in the distribution of phenolic compounds in leaves with various leaf phenologies under eCO₂ and N treatments. Therefore, intensive analyses were conducted to elucidate the change in plant defensive traits without induced defense using not only quantitative chemical methods but also anatomical methods.

We hypothesized the following. (1) The response of seedlings to these treatments depends on the successional type: late successional species with determinate growth have higher defensive traits than early to mid-successional species with indeterminate growth. Therefore, late successional species produce more phenolic compounds under eCO₂ and/or low N deposition than early to mid-successional species, as predicted by Koike et al. [5]. (2) The changes in phenolic compound concentrations induce changes in the localized deposition of these compounds in a leaf. It is predicted that if the amount of defense chemicals in leaves increases under eCO₂, they can be more widely distributed in leaves.

2. Materials and Methods

2.1. Plant Materials

We studied three deciduous tree species, i.e., Siebold's beech (*Fagus crenata*), oak (*Quercus mongolica* var. *crispula*), and magnolia (*Magnolia hypoleuca*). These species are representative deciduous broad-leaved tree species that can be found in the cool temperate zone of Japan. The successional traits of these species are roughly classified as follows [26]: beech is a late successional species; oak, a mid-late successional species; and magnolia, a mid-successional species. Two-year seedlings of three species were obtained from Hokkaido Horti-green Center, near Sapporo, Japan, and these seedlings were grown under full sunlight in the previous year.

2.2. Treatments

In early May 2007, before leaf emergence, 24 two-year-old seedlings of each species except beech were planted in pots (about 7 L) filled with Kanuma pumice soil and clay loam (1:2 in volume). To avoid insect grazing and disease, the seedlings without induced defense reaction were grown under a natural-light phytotron (KG type, Koito Industries, Yokohama, Japan) in Hokkaido Research Center, FFPRI, Sapporo, Japan. The sunlight transmittance of the glass plate of this phytotron was 380–2000 nm; a total of six cabinets were used for each CO₂ level (360 ppm vs. 720 ppm) at a regulated temperature (day/night; 26 °C/20 °C). The 12 seedlings of each species were grown at three cabinets of 380 ± 20 μmol mol⁻¹ (ambient CO₂ treatment) and at three cabinets of 720 ± 20 μmol mol⁻¹ (elevated CO₂) (four seedlings of each species per cabinet).

Seedlings grown under each CO₂ treatment were supplied with two levels of ammonium sulfate ((NH₄)₂SO₄) of 15 kg N ha⁻¹ yr⁻¹ (high N treatment) or 4 kg N ha⁻¹ yr⁻¹ (low N treatment) (two seedlings with high N treatment and two seedlings with low N treatment per cabinet). We maintained the nutrient conditions of the seedlings by applying 1000-fold diluted Hyponex solution (comprehensive fertilizer, Hyponex, Osaka, Japan) for all seedlings in every two weeks until mid-October. During the treatments, we rotated the potted plants in each cabinet to avoid position effects. No statistical differences were observed among cabinets during each treatment. The seedlings were cultured for two growing seasons (from mid-May to mid-October).

In beech, 48 two-year-old seedlings were planted in May 2008 and were grown at about each three cabinet of 380 ± 20 μmol mol⁻¹ (ambient CO₂ treatment; n = 24) and at 720 ± 20 μmol mol⁻¹ (elevated CO₂; n = 24) (eight seedlings per cabinet). These seedlings grown under each CO₂ treatment were supplied with two levels of ammonium sulfate ((NH₄)₂SO₄) of 15 kg N ha⁻¹ yr⁻¹ (high N treatment) or 4 kg N ha⁻¹ yr⁻¹ (low N treatment) (four seedlings with high N treatment and four seedlings with low N treatment per cabinet). These seedlings were cultured for two growing seasons (from mid-May to mid-October) in the same way as mentioned above.

2.3. Measurements

One or two mature leaves were sampled from the 3rd to the 5th leaves from the top of one shoot of each seedling in mid-August. Measurements of the parameters, such as defensive traits (LMA, C/N ratio, total phenolics and condensed tannin, and microscopic observation of defense chemicals) were performed using each leaf. After taking some leaf disks from the leaf blade for the measurements of the LMA and C/N ratio as well as microscopic observation, the rest of the leaf blade was immediately freeze-dried (FLEXY-DRY, FTS systems, New York, NY, USA) for the measurement of total phenolics and condensed tannin.

2.4. LMA Measurement

The disks were punched out from each leaf using a leaf puncher (FUJIWARA SCIENTIFIC Co., Ltd., Tokyo, Japan). The disks were dried for 2 days at 60 °C, and the LMA was

calculated from the dry mass. The LMA was positively correlated with leaf toughness and used as an index of leaf toughness (physical defense) [5,27].

2.5. N Content, C Content, and C/N Ratio

The N and C concentrations of leaves were determined using an N/C analyzer (NC-900; Shimadzu, Kyoto, Japan). The method was described in detail by Watanabe et al. [28]. The N and C contents and C/N ratio were calculated from the N and C concentration data.

2.6. Quantitative Analysis of Defense Chemicals

Freeze-dried samples were ground to powder using a mill (Wonder Blender, OSAKA CHEMICAL, Osaka, Japan). The amounts of total phenolics and condensed tannin were quantified. The concentration of total phenolics was determined using the Folin–Ciocalteu method, as modified by Julkunen–Tiitto [29]. The concentration of condensed tannin was determined using the butanol hydrochloride method [30].

2.7. Microscopy

The localization of defense chemicals was observed using a light microscope (Axioskop2 Plus, Carl Zeiss, Jena, Germany) and ultraviolet microphotometer (UV microscopy) (MPM-80, Carl Zeiss, Jena, Germany) via the following methods. Small pieces of leaves were fixed in 4% glutaraldehyde, then the samples for light microscopy were post-fixed in 1% osmium tetroxide (OsO_4). Later, the samples for both light microscopy and UV microscopy were dehydrated in ethanol series and embedded in epoxy resin (Epok812, Oken, Japan). Transverse sections with about 1- μm thickness were cut using an ultramicrotome (Ultracut N, Reichert, Vienna, Austria). The sections for light microscopy were double-stained with 1% safranin and 1% gentian violet, which stain the cell walls. In the UV microscopy, UV photomicrographs were taken at 280 nm [31]. Phenolics fixed in 1% OsO_4 in the vacuoles are observable [32]. The UV microscopy makes it possible to observe the phenolics that absorb UV at a wavelength of 280 nm.

2.8. Statistical Analyses

Two-way ANOVA was conducted to compare the effects of CO_2 and N treatment on the LMA, C/N ratio, and concentrations of total phenolics and condensed tannin for each species. The `*aov()` function of R were used. Furthermore, to exhibit the effect of CO_2 and nitrogen on each defensive traits of each species, principal component analysis (PCA) was tested by “`prcomp ()`” function with scaling in R. Statistical analyses of variance were conducted using R (version 3.6.2, R foundation for statistical computing, Vienna, Austria) [33].

3. Results

3.1. LMA

The LMA of beech leaves significantly increased under eCO_2 ($p < 0.001$) (Figure 1). In oak, the LMA tended to increase under eCO_2 ($p = 0.055$). No significant differences were observed in both beech and oak treated with N loading. In magnolia, there were no significant differences in response to both CO_2 and N treatments. Moreover, no significant interactive effects of CO_2 and N on LMA were demonstrated by all species (Figure 1).

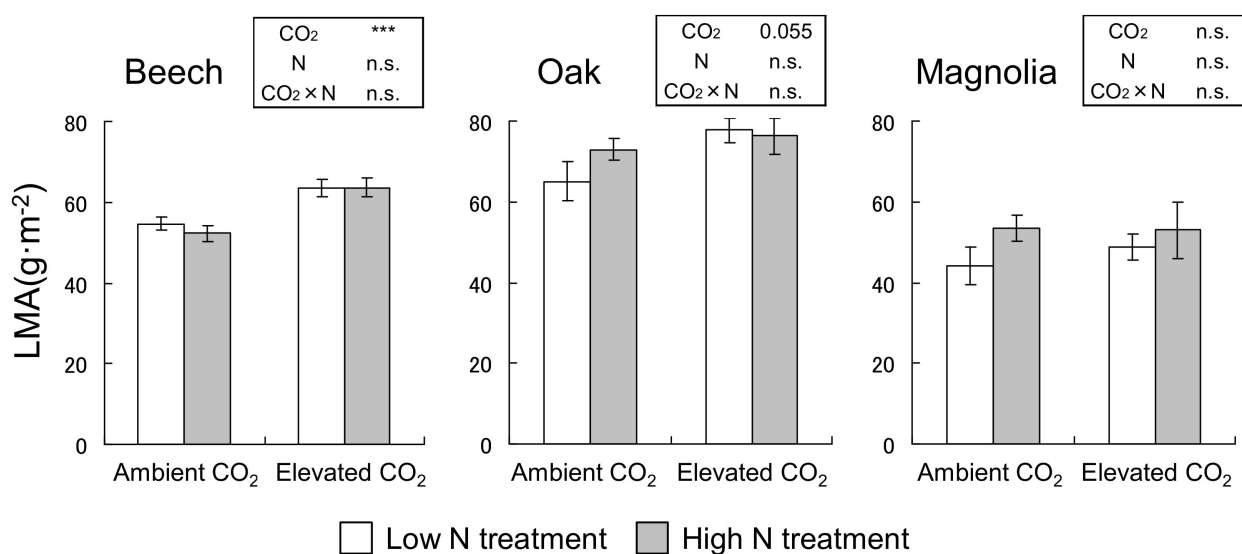


Figure 1. LMA in the leaves of seedlings of three tree species at each treatment. Values are expressed as means \pm SE (beech $n = 12$, oak and magnolia $n = 6$). The results of the two-way ANOVA are also shown (** $p < 0.001$, n.s. $p \geq 0.1$). Actual p values are shown when $0.05 < p < 0.10$. n.s.; not significant.

3.2. N Content, C Content, and C/N Ratio

The N content of beech significantly decreased under eCO₂ and significantly increased with high N treatment ($p < 0.001$) (Figure 2a). No difference in the N content of oak leaves was observed between each CO₂ treatment, but the N content of its leaves significantly increased at high N treatment ($p < 0.05$) (Figure 2a). The C content of beech leaves significantly decreased under eCO₂ ($p < 0.001$) and significantly increased at high N treatment ($p < 0.01$). The C content of oak increased significantly under eCO₂ ($p < 0.05$) and tended to increase at high N treatment ($p = 0.065$) (Figure 2b). The C/N ratio increased under eCO₂ treatment and decreased under high N treatment in both beech and oak (Figure 2c). In magnolia, there were no significant differences in response of the N and C contents, and C/N ratio to both eCO₂ and N treatments. There were no significant interactive effects of CO₂ and N loading on the N content, C content, and C/N ratio in all species (Figure 2).

3.3. Concentrations of Total Phenolics and Condensed Tannin

In beech, total phenolics tended to increase under eCO₂ treatment ($p = 0.067$) and decreased significantly under high N treatment ($p < 0.05$) (Figure 3a). The total phenolics in oak leaves significantly increased under eCO₂ treatment ($p < 0.001$) and tended to increase under high N treatment ($p = 0.068$) (Figure 3a). Moreover, the interactive effects of CO₂ and N on total phenolics were demonstrated. High N increased total phenolics in oak seedlings grown under ambient, but not eCO₂ ($p = 0.071$). In magnolia, no significant differences were observed under both eCO₂ and N treatments.

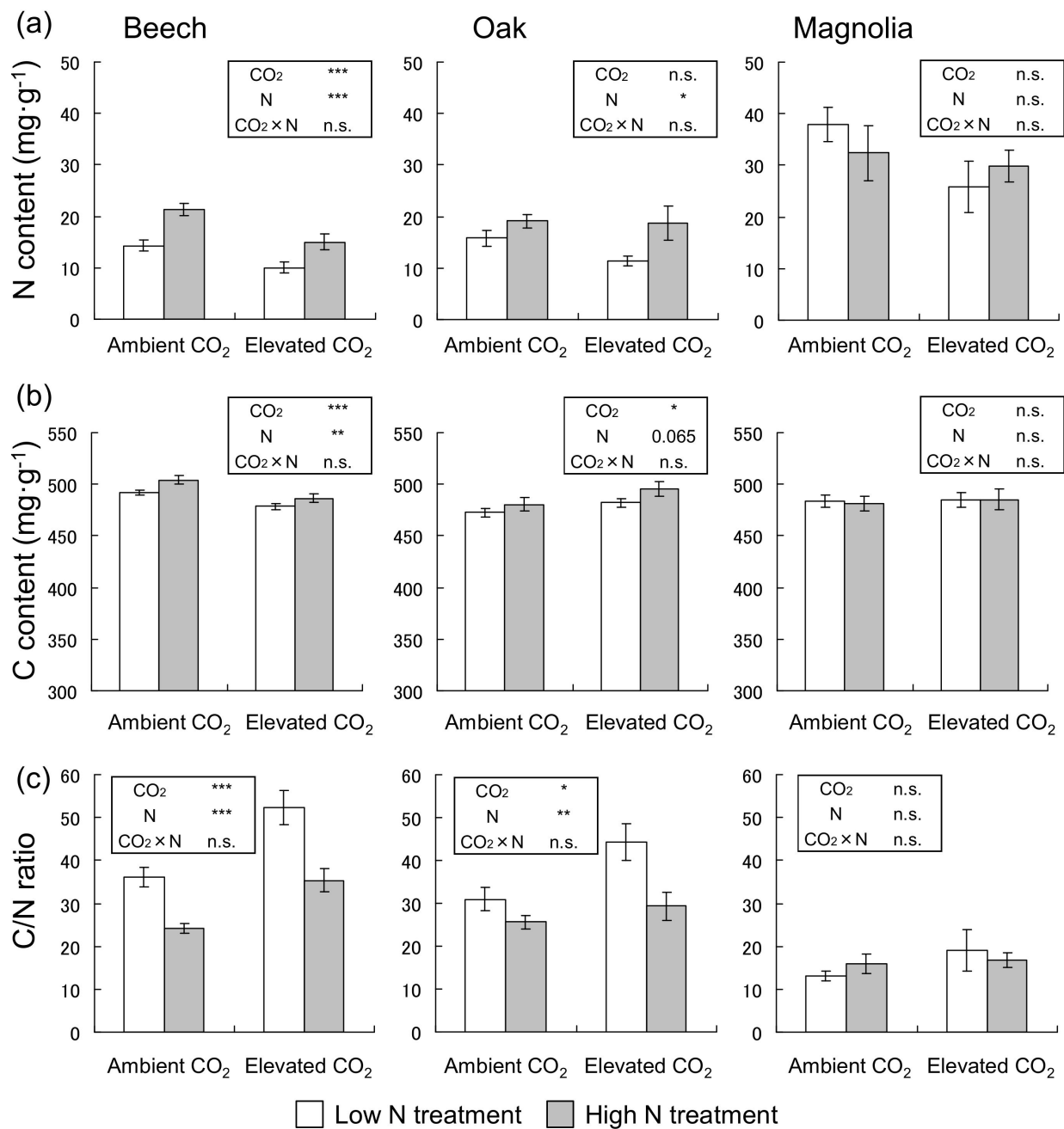


Figure 2. (a) Nitrogen content, (b) carbon content, and (c) C/N ratio in the leaves of seedlings of three tree species at each treatment. Values are expressed as means \pm SE (beech $n = 12$, oak and magnolia $n = 6$). The results of the two-way ANOVA are also shown (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, n.s. $p \geq 0.1$). Actual p values are shown when $0.05 < p < 0.10$. n.s.; not significant.

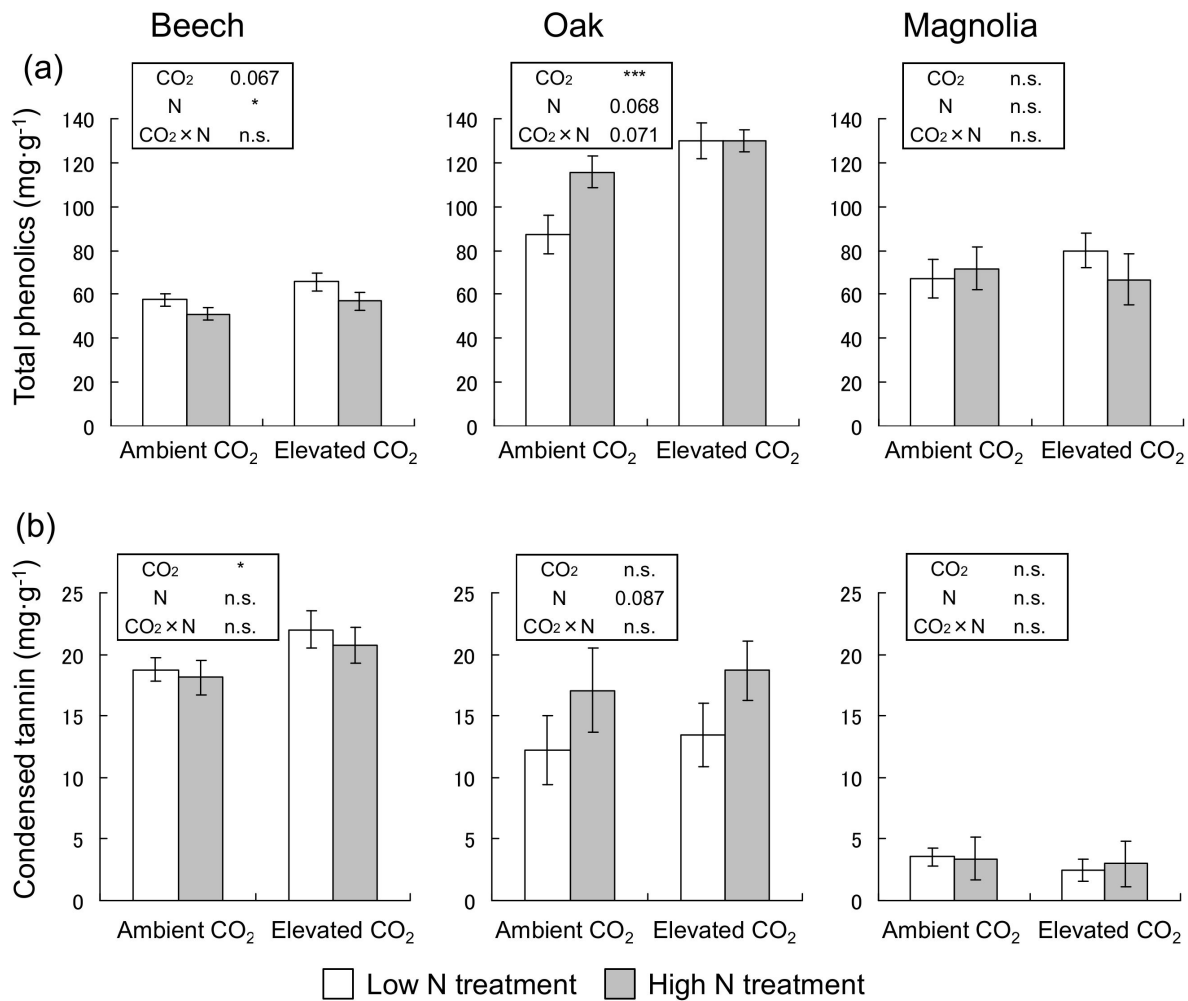


Figure 3. Concentrations of (a) total phenolics and (b) condensed tannin in the leaves of seedlings of three tree species at each treatment. Values are expressed as means \pm SE (beech $n = 12$, oak and magnolia $n = 6$). The results of the two-way ANOVA are also shown (** $p < 0.001$, * $p < 0.05$, n.s. $p \geq 0.1$). Actual p values are shown when $0.05 < p < 0.10$. n.s.; not significant.

The condensed tannin of beech leaves significantly increased under eCO₂ treatment ($p < 0.05$), but no differences were observed between each N treatment (Figure 3b). Although there were no significant differences in the response of oak leaves to both CO₂ and N treatments, condensed tannin tended to increase at high N treatment ($p = 0.087$). In magnolia, there were no significant differences in the condensed tannin under both eCO₂ and N treatments (Figure 3b). The concentration of condensed tannin in magnolia was remarkably lower than in the other two species. No significant interactive effects of CO₂ and total phenolics and condensed tannin in all species were observed, except in oak (Figure 3).

Furthermore, we calculated the amount of total phenolics and condensed tannin per area (Table 1). In beech, eCO₂ enhanced the amounts of both phenolic compounds per area. In oak, eCO₂ enhanced the amount of total phenolics per area, and condensed tannin tended to increase at high N treatment (Table 1). In magnolia, there were no significant differences in the amounts of both phenolic compounds per area under both eCO₂ and N treatments (Table 1).

Table 1. The amounts of total phenolics and condensed tannin per area in the leaves of three tree species grown in each treatment. Values in parentheses are standard errors of the means. Results of two-way ANOVA are also shown (** $p < 0.001$, n.s. $p \geq 0.01$), n.s.; not significant. Actual p values are shown when $0.05 < p < 0.10$.

Species	Defense Chemicals	Treatments				Two-Way ANOVA		
		360 ppm		720 ppm		CO ₂	N	CO ₂ × N
		Low N	High N	Low N	High N			
Beech	Total phenolics (g m ⁻²)	3.15 (±0.17)	2.72 (±0.20)	4.21 (±0.33)	3.63 (±0.27)	***	0.055	n.s.
	Condensed tannin (g m ⁻²)	1.01 (±0.05)	0.97 (±0.08)	1.41 (±0.12)	1.32 (±0.10)	***	n.s.	n.s.
Oak	Total phenolics (g m ⁻²)	5.72 (±0.70)	8.46 (±0.70)	10.12 (±0.87)	9.90 (±0.66)	***	n.s.	0.061
	Condensed tannin (g m ⁻²)	0.86 (±0.26)	1.23 (±0.23)	1.05 (±0.21)	1.38 (±0.16)	n.s.	0.087	n.s.
Magnolia	Total phenolics (g m ⁻²)	2.98 (±0.52)	3.80 (±0.46)	3.82 (±0.29)	3.32 (±0.35)	n.s.	n.s.	n.s.
	Condensed tannin (g m ⁻²)	0.16 (±0.03)	0.18 (±0.09)	0.11 (±0.03)	0.15 (±0.03)	n.s.	n.s.	n.s.

3.4. Principal Component Analysis

The cumulative proportion of PC1 and PC2 variance was 0.80, and the proportion of variance was 0.56 in PC1 and 0.24 in PC2 (Figure 4). Factor loadings of PC1 and PC2 were shown in Table 2. PC1 had strong positive correlation among LMA, C/N ratio, and mass base content of condensed tannin, while strong negative correlation was observed in mass base N content (Table 2). PC2 had positive correlation in mass base C content, but also negative correction in condensed tannin (Table 2).

Table 2. Factor loadings of principal component analysis.

Factor	PC1	PC2
LMA (g m ⁻²)	0.87	−0.38
N content (mg g ⁻¹)	−0.94	−0.19
C content (mg g ⁻¹)	−0.08	0.68
C/N ratio	0.89	0.19
Total phenolics (mg g ⁻¹)	0.49	−0.73
Condensed tannin (mg g ⁻¹)	0.83	0.48

From the PCA analyses, beech located widely, on PC1 from minus to plus region and PC2 > 0. Oak located in the region of PC1 > 0 and PC2 < 0. Magnolia located in the region of both PC1 and PC2 < 0 (Figure 4). The studied three deciduous tree species were characterized as below. Beech was characterized with higher LMA leaves, less nitrogen content, much condensed tannin, and less total phenolic content. Oak was characterized with higher LMA leaves, less nitrogen content, much condensed tannin, and total phenolic content. Magnolia was characterized with lower LMA leaves, much nitrogen content, less condensed tannin, and much total phenolic content.

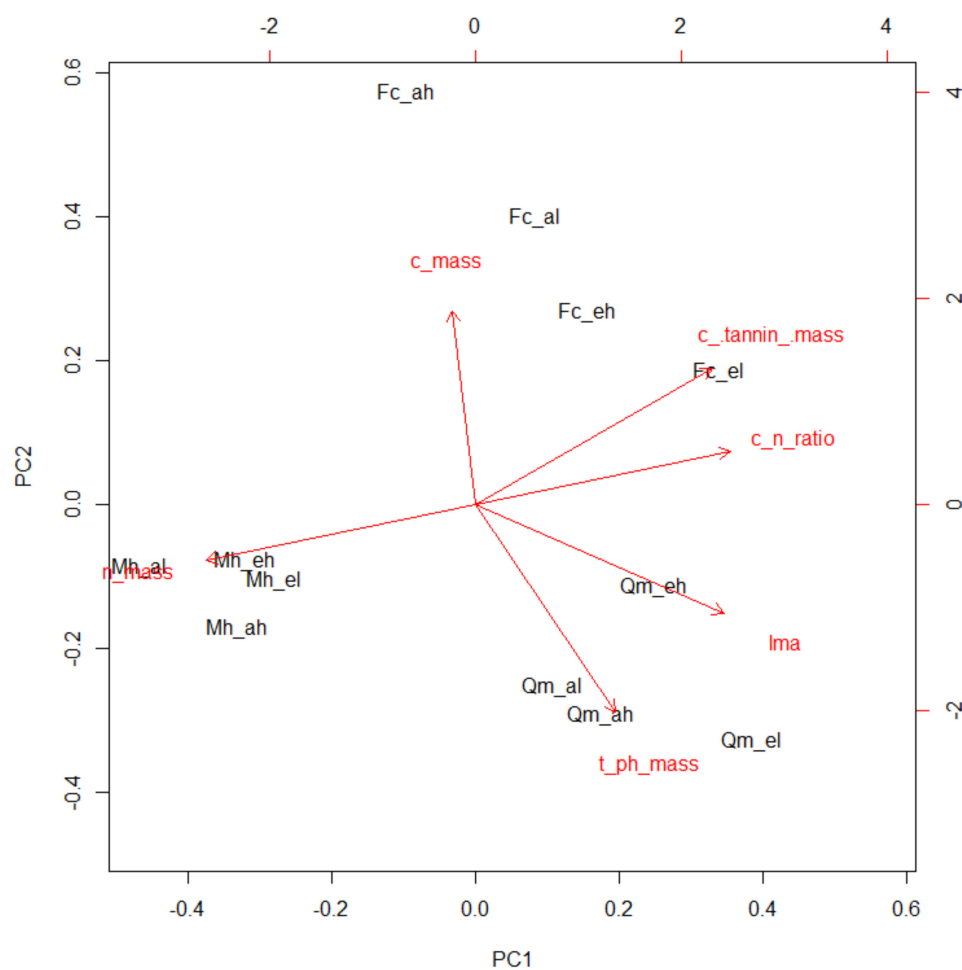


Figure 4. PCA of three deciduous tree species. PC1 indicate principal components 1, and PC2 indicate principal components 2. Symbols indicate “species name _ growth condition”, species name Fc: *Fagus crenata* (beech); Qm: *Quercus mongolica* var. *crispula* (oak); Mh: *Magnolia hyporeuca* (magnolia), in growth condition, al: ambient CO₂ with low nitrogen fertilization; ah: ambient CO₂ with high nitrogen fertilization; el: elevated CO₂ with low nitrogen fertilization; eh: elevated CO₂ with high nitrogen fertilization.

3.5. Anatomical Characteristics and Distribution of Phenolic Compounds

In beech, leaves at ambient CO₂ had one palisade layer, but those at eCO₂ had two (Figure 5). Phenolic compounds were mainly distributed in palisade parenchyma tissues in all treatments for beech. The effects of both eCO₂ and N treatments on the distribution of phenolic compounds were not observed (Figure 5). In oak, phenolic compounds were mainly distributed in both the upper and lower epidermis in all treatments (Figure 6). The changes in the distribution of phenolic compounds were not observed under eCO₂ and N treatments (Figure 6). In magnolia, phenolic compounds were slightly distributed in both the epidermis and mesophyll tissue in all treatments, and no changes in the distribution of defense compounds were observed under eCO₂ and N treatments.

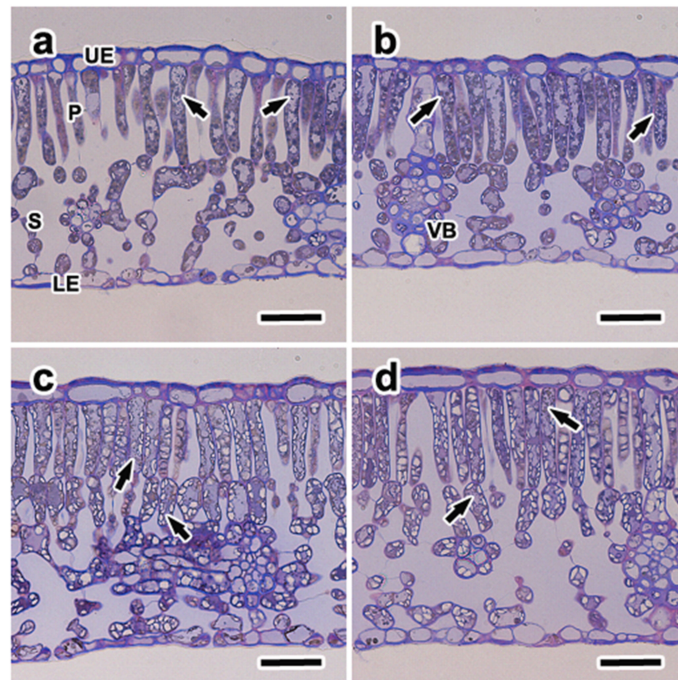


Figure 5. Micrographs of the cross sections of beech (*Fagus crenata*) leaves in all treatments. These sections were double-stained with safranin and gentian violet. (a) Ambient CO₂ and low nitrogen, (b) ambient CO₂ and high nitrogen, (c) elevated CO₂ and low nitrogen, and (d) elevated CO₂ and high nitrogen. The arrows indicate phenolic compounds. LE; lower epidermis, P; palisade layer, S; spongy parenchyma, UE; upper epidermis, VB; vascular bundle, scale bars = 20 μm.

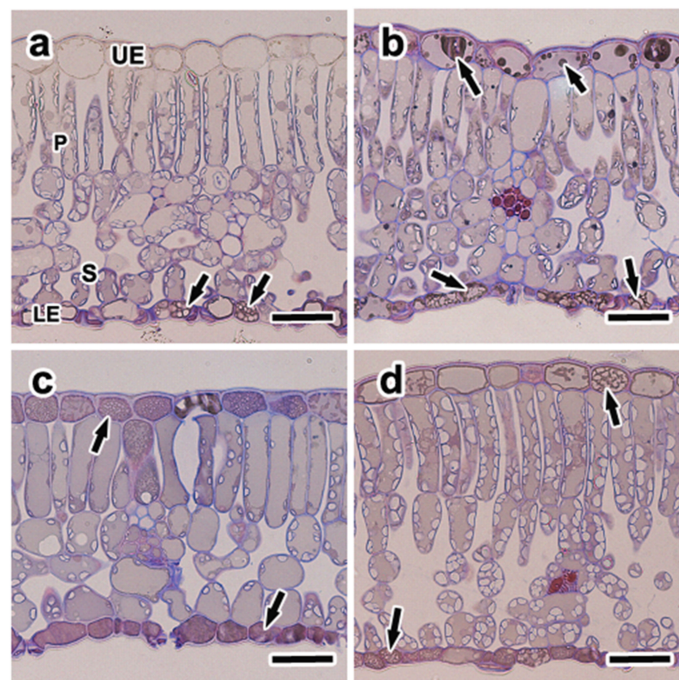


Figure 6. Micrographs of the cross sections of oak (*Quercus mongolica*) leaves in all treatments. These sections were double-stained with safranin and gentian violet. (a) Ambient CO₂ and low nitrogen, (b) ambient CO₂ and high nitrogen, (c) elevated CO₂ and low nitrogen, and (d) elevated CO₂ and high nitrogen. The arrows indicate phenolic compounds. LE; lower epidermis, P; palisade layer, S; spongy parenchyma, UE; upper epidermis, scale bars = 20 μm.

4. Discussion

4.1. Effects of Elevated CO₂ and Nitrogen Loading on Defensive Traits

We hypothesized that late successional species with determinate growth would exhibit high defensive traits under eCO₂ and/or low N treatment to keep leaves with low turnover rate during the growing season, which is suggested by the classic idea of the CNB hypothesis [4,34]. Our results indicated that beech seedlings grown under eCO₂ produced low-quality foliage for herbivorous insects due to the increase in the toughness, C/N ratio, and concentrations of total phenolic and condensed tannin irrespective of the decrease in the C content (Figures 1–3). These results are thought to support the CNB hypothesis [1,4,13,34]. In particular, the reduction in the N content of beech leaves grown under eCO₂ with low N loading indicates the decrease in N uptake as reported by the previous study [35]. Conversely, high N loading induced a significant increase in both the N and C contents of beech leaves. This might be caused by enhanced photosynthesis due to an increase in the N content in the tissues of leaves.

Oak, a mid-late successional species, demonstrated a different response from beech, in particular, to the concentrations of phenolic compounds (Figure 3). The concentration of condensed tannin increased by N treatment irrespective of CO₂ concentrations unlike the beech. Kitaoka et al. [36] demonstrated that the light-saturated photosynthetic rate (P_{sat}) and mesophyll surface area (S^{mes}) of oak seedlings enhanced under high N supply, even in the field condition. Oak seedlings may prefer to allocate excess photosynthates produced under high N treatment to defense compounds rather than shoot growth owing to its determinate shoot growth trait as reviewed by Lindroth [13].

Magnolia is a mid-successional species with an indeterminate shoot growth pattern but often develops some leaves in an adequate environment [26]. Therefore, it is possible that photosynthates are allocated to growth prior to the defense of leaves. The condensed tannin concentration of magnolia seedlings was much lower than that of the other two species. Aside from beech and oak, magnolia trees are known to contain magnolol and honokiol in their branch, bark, and leaves [37]. These chemicals might be preferentially produced rather than condensed tannins. Further study should elucidate changes in the production of these chemicals under environmental changes.

Our study revealed the differences in the response of species to eCO₂ and high N treatments, as reported by previous studies [5,38] (Figure 4). Beech seedlings responded the most to both CO₂ and N treatments as we expected (Figures 1–4). In particular, N loading is critical for defensive traits of beech leaves (Figure 4). Defensive traits of oak seedlings were greatly affected by N fertilization under eCO₂ (Figure 4). Magnolia seedlings did not significantly respond to all treatments. The differences in the response of species depends on the differences in carbon allocation to defense or growth under environmental changes as suggested by Lindroth et al. [38] and Lindroth [13]. Additionally, our results suggest that foliar defensive traits under environmental changes depend on successional type. Our results with regard to oak leaves differed from those of Koike et al. [5]. This difference might be due, in part, to the duration of the treatments.

4.2. Distribution of Phenolic Compounds under Elevated CO₂ and High N Treatment

We predicted that phenolic compounds would be much more widely distributed in leaves when the amounts of defense chemicals in leaves increase under eCO₂ treatment. Our results indicated no changes in the distribution of phenolic compounds of beech and oak, although amounts of these phenolic compounds per area changed under eCO₂ and high N treatments (Table 1). It is considered that the changes in the amounts of phenolic compounds under eCO₂ treatment were not sufficient to reflect the changes in the distribution of phenolic compounds in the leaf tissues. We did not detect a chemical compound induced by UV-A (320–360 nm), which may be attributed to the transmittance traits of the glass plate of the phytotron.

To protect leaves from herbivores using limited amounts of defense compounds, localization of these compounds in the epidermis is more effective, similar to oak species [39]

(Figure 6). However, the phenolic compounds of beech leaves were mainly distributed in palisade tissues (Figure 5). Beech leaves in August have a well-developed cuticular layer on the upper surface of the leaves. Therefore, it is likely that the distribution of defensive compounds in both sides of the epidermis did not occur. Phenolic compounds also have an antioxidant property against reactive oxygen species (ROS) generated under various environmental stresses [32]. These compounds in palisade tissues would play a role in scavenging ROS.

Furthermore, eCO₂ directly affects the anatomical traits of leaves [40,41]. Miyazawa et al. [41] revealed that eCO₂ affects the development process of new leaves directly or indirectly using poplar. Therefore, we must consider the direct effects of eCO₂ on leaf anatomical traits, as its traits affect the photosynthetic ability that affects the production of defensive chemicals. There is a negative correlation between phenolic compounds and net photosynthetic rate [42]. A recent review [43] indicated an essential role of calcium compounds (i.e., CaOx) for leaf structure and functioning. These evidences are new approaches for understanding more of the leaf functional structure. Moreover, the essential role of trichome [44] should be analyzed in further studies.

5. Conclusions

Our study revealed that the effects of eCO₂ and high N on the defensive traits of successional, deciduous, broad-leaved tree species vary among species and are related to the successional type. Beech, a late successional species, responded clearly to eCO₂ and high N treatment after two growing seasons. In accordance with the CNB hypothesis, eCO₂ significantly enhanced the defensive traits of beech leaves, and high N induced the reduction in the defense compounds. Conversely, the response of oak to eCO₂ and high N differed from that of beech. Although the C/N ratio increased under eCO₂ treatment, the concentrations of total phenolics and condensed tannin increased under high N loading. Magnolia, a mid-successional species, did not respond to all treatments. It has an indeterminate growth of shoots and produces magnolol and honokiol. In this species, photosynthates produced under eCO₂ and high N content might be used for the growth or the production of these compounds.

Our microscopic observation could not clearly show the changes in the distribution of phenolic compounds along with the changes in the amounts of phenolic compounds per area. However, it is important to demonstrate the changes in the distribution of phenolic compounds under global environmental changes, i.e., CO₂ and N loading, as this trait affects the feeding pattern of herbivorous insects. Accumulation of the information on the distribution of phenolic compounds with changes in leaf anatomical traits induced by global environmental changes is required in the future.

Author Contributions: Y.W., K.H. and T.K. conceptualized the study; K.H., L.Q., M.W., M.K. and S.K. did the experiments and data analyses; Y.W. and K.H. did the anatomical studies; K.H. and T.K. prepared the original draft. All are responsible for writing, reviewing, and editing. T.K. supervised this study, and L.Q. and T.K. are responsible for funding. All authors have read and agreed to the published version of the manuscript.

Funding: We are grateful for the financial support of the National Key Research and Development Program of China (No. 2017YFE0127700).

Acknowledgments: We thank H. Utsugi, A. Uemura, and H. Tobita for supporting experiments in the Hokkaido Research Center, FFPRI, Japan.

Conflicts of Interest: The authors declare no conflict of interest.

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