Leaf phenology and insect grazing of Japanese white birch saplings grown under free-air ozone exposure

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Abstract

Ground-level ozone (O_3) increases yearly and negatively affects the growth of birches via leaf dynamics. Leaf area is directly related to plant production and is usually reduced by elevated O_3 (eO_3) and insect herbivores. The objective of this study was to explore factors affecting change in leaf number of white birch saplings and insects grazing under eO_3 . In order to accomplish this objective, we observed seasonal changes in leaf phenology and determined foliar defense chemicals in saplings grown under ambient air and free-air O_3 enrichment (60 nmol mol^{-1} for daytime). The leaf phenology showed the total number of emerged leaves was about 10 per shoot in the control and eO_3 plots. However, the number of attached leaves per shoot at ambient air decreased after mid-July, probably due to grazing by larvae of the leaf beetle. In addition, the concentration of condensed tannin in early leaves was higher in the eO_3 plot than that in the control plot. Such leaves with decreased nutritional value under eO_3 may prevent feeding of leaf beetles. These findings lead to the plausible understanding of an indirect effect of eO_3 on leaf phenology via foliar defense of white birch against insect herbivores.

Key words: Free- O_3 enrichment, Grazing, Leaf beetle, Plant defense, White birch.

1. Introduction

Ground-level ozone (O_3) has been increasing in Japan because of high emissions of its precursors under rapid economic development (Akimoto, 2003; Yamaji *et al.*, 2008; Ohara, 2011). The Acid Deposition and Oxidant Research Center (ADORC, 2006) suggested that elevated O_3 (eO_3) levels in Japan might have negative impacts on forest trees and their photosynthetic production. According to other studies (Watanabe *et al.*, 2013, 2014), eO_3 can cause a decline of photosynthetic capacity and an increase of respiration in Siebold's beech (*Fagus crenata*) and oak (*Quercus mongolica* var. *crispula*) trees. Plant production is highly related to the leaf area index (LAI) and the photosynthetic activity (e.g. Larcher, 2003). As, LAI is strongly influenced by biotic and abiotic stresses (Orcutt and Nilsen, 2000), more knowledge is required regarding factors affecting LAI in the field.

Izuta *et al.* (2001) reported that eO_3 alters the physiological functions of plants and their defense against pests, probably because eO_3 leads to increase concentrations of phenolics via oxidation of fatty acid of leaf cell membrane (Yamaji *et al.*, 2006; Valkama *et al.*, 2007; Oksanen *et al.*, 2009; Lindroth, 2010; Matyssek *et al.*, 2012). Therefore, eO_3 may affect the LAI by impacting the defense against insect herbivores.

White birch is a typical pioneer tree species in cool temperate regions and widely used for re-vegetation in northern Japan (Koike, 1995). It has heterophyllous leaves; it flushes early

leaves in spring and then, after the complete expansion of early leaves, develops late leaves (Koike, 1995). Nowadays, the grazing damage to white birch by larvae of leaf beetle (*Agelastica coerulea*) is increasing in northern Japan (Sayama *et al.*, 2014).

In Hokkaido, northern Japan, O_3 concentration is increased from spring to early summer (Ohara, 2011). The preference in oviposition and larval feeding of the leaf beetle might be altered by eO_3 due to the diminishing of photosynthetic production (Hoshika *et al.*, 2013) and a the consequently reduced plant defense. To test this, we observed the leaf phenology and determined the concentrations of foliar defense chemicals in leaves of white birch saplings grown in a free-air O_3 fumigation system. Based on our findings, we discuss a plausible understanding of leaf phenology under eO_3 in relation to the insect herbivores.

2. Materials and Methods

2.1 Experimental plot and Plant material

This study was conducted in the free-air- O_3 enrichment system established at Sapporo Experimental Forest, Hokkaido University, in northern Japan ($43^{\circ}04' \text{ N}$, $141^{\circ}20' \text{ E}$, 15 m a.s.l.). This forest is located on flatland. The study sites were equipped with brown forest soil, ambient air and another for eO_3 , with a distance of 24 m between them. Each size was $5.5 \times 7.2 \text{ m}$. Three-year-old saplings of Japanese white birch (*Betula platyphylla* var. *japonica*) were planted in each plot in 2011. In the spring of 2013, the mean tree height was $3.0 \pm 0.4 \text{ m}$, and the mean stem diameter at breast height was $15.5 \pm 2.1 \text{ mm}$. Ten saplings were subjected to O_3 treatments from August to November 2011, and from May to November 2012 and 2013.

The targeted O_3 concentration was 60 nmol mol^{-1} during daytime, which is the standard of the Japanese environment (ADORC,

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2006). The regulation of the operation was attained using a light sensor with a cut-off of light compensation point (LCP) over $60 \mu\text{mol m}^{-2}\text{s}^{-1}$, as determined previously (Koike, 1988). The O_3 concentration at the canopy height was monitored continuously by an O_3 monitor (Mod. 202, 2B Technologies, Boulder, CO, U.S.A.), and the observed value was used as a feedback to the three-way valve to regulate the O_3 concentration the PID algorithm. The average O_3 concentrations during daytime in the control and eO_3 treatment plots were 26.5 and $53.8 \text{ nmol mol}^{-1}$, respectively. The targeted O_3 concentration was satisfactorily regulated, especially at relatively low wind speeds. More details can be found in Watanabe *et al.* (2013).

2.2 Monitoring of leaf phenology

The leaf developmental pattern of middle crown exposed to sunshine was monitored in 8 to 9 individuals in each plot, at a 1- to 3-week interval during the growing season of 2013. For each plot, we used steal jungle gym towers with $6.0 \times 7.5 \times 6.0 \text{ m}$ in size. A survey of the number of leaves was conducted at the terminal shoot of the saplings, according to the observation method of Ito *et al.* (2011). Namely, the average number of leaves per shoot was calculated according to Kikuzawa (1978) and Kikuzawa and Lechowicz (2011). The defoliation was measured as the number of shed leaves (Hensel *et al.*, 1993). Finally, this information of the dynamics of leaf number per selected shoot (=leaf phenology) specified the number of total emerged leaves per shoot.

2.3 Insect behavior and defensive chemicals

According to Ikenoya (2006), the life cycle of the leaf beetle (*Agelastica coerulea* Baly) is as follows: adults overwinter under a shallow part of soil and litter; appear and start grazing leaves of alder and birch from late April to early June (in central Hokkaido island); and make ovipositing eggs on the abaxial side of leaves from June. Then, hatched larvae start to graze leaves from early July to early September; they move very slowly in a limited area on a leaf or hatched leaf. Insect grazing by larvae of the leaf beetle was visually observed in July. However, we did not measure the population dynamics of adults and larvae of the beetle at each treatment. Additionally, information on the oviposition behavior, such as the sex identification of adults and oviposition or hatching rate, was not observed.

For the determination of the concentration of total phenolics and condensed tannin, 4 to 6 early and late leaves were collected in August of 2013. On May, 2 or 3 leaves of each bud on the saplings started flush: these are classified as early leaves. Leaves flushed in the first week of June and July were used as late leaves. The Folin-Ciocalteu method and proanthocyanidin method were applied for the determination (Bate-Smith, 1977; Julkunen-Tiitto, 1985).

2.4 Statistical analysis

The effect of eO_3 on the number of leaves per shoot and the concentrations of total phenolics and condensed tannin were analyzed using a t-test. Statistical analyses were performed using SPSS Statistics ver.12 (IBM, USA). Results were considered significant at $P < 0.05$ and marginal at $P < 0.10$.

3. Results

3.1 The number of total leaves per shoot

Fig. 1. Cumulative number of leaves per shoot of white birch saplings. Closed square: ambient, open square: O_3 treatment, Data are represented as mean $\pm$ SE ($n=8$)

In the late growing season, the control and eO_3 treatment plots had a cumulative average of 10 total leaves per shoot, as shown in Fig. 1. There was no significant difference in the cumulative leaf number. By late May, shoots were composed of early leaves. From early June, several late leaves gradually emerged.

3.2 The number of attached leaves per shoot

A marginal difference in the number of attached leaves per shoot was observed between the control and eO_3 plots from mid-August to early October (Fig. 2: $P < 0.10$). In the control plot, the number of attached leaves per shoot decreased rapidly from mid-July. As a result, the average number of attached leaves in the control and eO_3 treatment plots was $5.4 (\pm 1.1)$ and $9.9 (\pm 1.9)$, respectively, from September to October. Leaf falling in both plots lasted until early November.

3.3 Grazing by leaf beetles

Adult leaf beetles were observed in both plots from early May, but very few individuals of larvae were found on each leaf in the eO_3 treatment plot. Accordingly, fewer grazing symptoms of leaves by larvae of the leaf beetle were found in the eO_3 treatment plot than in the control plot.

3.4 Defensive chemical compounds

A marginal difference in the concentration of condensed tannin between the control and eO_3 treatment plots was observed only in early leaves (Fig. 3: $P = 0.08$). As shown in Table 1, the concentration of condensed tannin in early leaves in the eO_3 plot was

Fig. 2. Number of attached leaves per shoot of white birch saplings. Closed square: ambient, open square: O_3 treatment, Data are represented as mean $\pm$ SE ($n=8$). t-test: † : $P < 0.10$

Fig. 3. Concentration of condensed tannin of early and late leaves of white birch saplings.

□: ambient ■: O₃ treatment, Data are represented as mean ± SE (n=4-6). t-test: n.s. no significant, †: $P < 0.10$

Fig. 4. Concentration of total phenolics of early and late leaves of white birch saplings.

□: ambient ■: O₃ treatment, Data are represented as mean ± SE (n=4-6). t-test: n.s. no significant

about 4.0 mg g⁻¹ higher than that in early leaves in the control plot, although there was no significant or marginal difference between the treatments in late leaves. However, as shown in Fig. 4, no significant difference was found in the total phenolics (≈50 mg g⁻¹) in early and late leaves between the control and eO₃ plots.

4. Discussion

We expected that eO₃ would decrease the number of late leaves per shoot of white birch because late leaves are developed using current photo-assimilates of the early leaves (Koike, 1995). As found by Hoshika *et al.* (2013) in the same species, the photosynthetic activity of early leaves was suppressed by elevated eO₃. Thus, the photo-assimilates of the early leaves may limit the development of late leaves. Likely, the production of defensive chemicals may be diminished.

However, there was no significant difference in the cumulative number of leaves per shoot between the control and eO₃ plots, which implies that eO₃ enrichment of previous years did not have an impact on the formation of leaf primordia. A similar trend was found in another experiment in which poplar clones were fumigated with different O₃ concentrations in open-top chambers in central Europe (Matyssek *et al.*, 1993).

Moreover, the number of attached leaves under ambient air started to decrease sharply from mid-July to August, as shown in Fig. 2. This difference in leaf shedding may be attributed to grazing by the larvae of the leaf beetle. Generally, when leaves are grazed by insect herbivores, plants usually shed the grazed leaves

in order to avoid further damage and the cost of keeping these damaged leaves (Schoonhoven *et al.*, 1998; Kikuzawa, 2005). We observed that leaves were grazed by the leaf beetles, especially by its skeletonizer larvae (grazing leaf surface avoid veins). However, we did not find any leaves grazed by larvae of the leaf beetle under eO₃, which raises a question about what happened to the susceptibility of white birch to leaf beetles with and without eO₃. We cannot clarify that the decrease of attached leaves was mainly a result of the shed of the early or late leaves. Visual observations, however, showed that larvae mainly grazed late leaves.

In general, adult leaf beetles start to graze early leaves from May to June and lay ovipositing eggs on leaves from June. Larvae hatch from the eggs and begin grazing leaves from July, and then adults resume chewing leaves from late August to September (Ikenoya, 2006; Oikawa *et al.*, 2012).

As shown in Fig. 3, saplings under eO₃ produced a high concentration of condensed tannin in early leaves. According to previous meta-analyses, eO₃ treatment has been shown to enhance the activities of phenylalanine-ammonium lyase and chalcone synthase enzymes that regulate phenylpropanoid and flavonoid biosynthesis pathways (grazing leaf surface avoid veins). These pathways play an essential role in plant defense responses because they synthesize several potentially protective compounds, such as flavonoids and other phenolics, which act as scavengers of various oxygen species such as superoxide anion, singlet oxygen, and hydroxyl radicals (grazing leaf surface avoid veins). Tannin strongly inhibits digestion in the leaf beetle (Schoonhoven *et al.*,

Table 1. Data of concentration of total phenolics and condensed tannin. Each value denotes the mean (±SD) of 4 - 6 replicated. The level of significance (P value) of t-test.

		Early leaves	Late leaves (June)	Late leaves (July)
Total phenolics (mg/g)	Ambient	46.0 (11.6)	45.6 (7.2)	53.5 (9.6)
	O ₃	52.8 (6.2)	43.9 (2.4)	45.4 (10.5)
	P value	0.32	0.57	0.27
Condensed tannin (mg/g)	Ambient	10.7 (3.4)	10.2 (3.5)	16.4 (5.7)
	O ₃	14.7 (2.6)	9.9 (1.8)	12.3 (7.0)
	P value	0.08	0.86	0.37

1998), therefore the early leaves of the saplings had decreased nutritional values for the leaf beetles under eO₃ (Hinata, 2010).

It is possible that the adult female of leaf beetles avoid ovipositing on leaves under eO₃, which could explain why no leaves were grazed by the larvae of the leaf beetle under eO₃. The larvae could not move between each plot. A similar study revealed that the leaf beetle avoids ovipositing on the leaves of cottonwood (*Populus deltoids*) exposed to elevated O₃ (Jones and Coleman, 1988). Another study explained that this avoidance by adult females could be due to the production of various substances by plants, such as biogenic volatile organic compounds (BVOCs) (e.g. Yamaji *et al.*, 2006), that act as an “infochemical” (Fares *et al.*, 2010; Lindroth, 2010) used to attract or repel leaf beetles. However, we did not observe the seasonal changes of the rates of mortality and birth of adult beetles as well as their ovipositing behavior. It is also possible that eO₃ or BVOCs emitted from the leaf surface (Niinemets and Monson, 2013) may directly impact leaf beetles and change their behaviors. These possibilities should be tested in future studies.

The results of the present study show that eO₃ altered the leaf phenology via changes in ovipositing preference or larval feeding of the leaf beetle. This probably happened because eO₃ enhanced the foliar defense against insect herbivores.

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