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HOKKAIDO UNIVERSITY
Induced Defense in Japanese White Birch Seedlings against Insect Herbivores

AOYAMA Chiho, NOVRIYANTI Eka and KOIKE Takayoshi*

Silviculture & Forest Ecological Studies, Hokkaido University, Sapporo 060-8589, Japan

Abstract

Plants defend themselves against herbivores, pests and abiotic stresses. Although some defense traits in plants are constitutive, some are switched on, or increased, only when the plants have suffered from stresses (i.e. induced defense). Growth (i.e. lignin) and the synthesis of most defensive compounds in broad-leaved trees derive from phenylalanine. If, therefore, plants allocate photosynthates mainly to defense, these resources would not be available for growth (lignin synthesis). In this study, we examined the defense induced against grazing by the gypsy moth (Lymantria dispar) in seedlings of the white birch (Betula platyphylla var. japonica) grown under two nitrogen levels. We exposed white birch leaves to be grazed by gypsy moths, and we cut the leaves with scissors in early spring. The defenses of white birch seedlings, especially the amount of total phenolics and condensed tannin, were found to be induced by cutting, to extents that were influenced by the soil nitrogen condition. Induced defense of white birch seedlings may depend on the heterophyllous type of shoot development.

Key words: White birch, induced defense, gypsy moth, mechanical damage, nitrogen condition

Introduction

Plant-insect interactions are a vital component of biological interactions, and contribute to biological conservation (Ohgushi et al. 2007). Bottom-up improvement of plant defense is important in maintaining biological diversity, and in particular the diversification of plant defense mechanisms (e.g. Kagata et al. 2005, Nakamura et al. 2005). In general, plant defense is affected by soil nutrient conditions. Bryant et al. (1983) proposed the Carbon-Nutrient Balance (CNB) hypothesis. The CNB hypothesis explains well the extent of plant defense for various soil nutrient conditions. The defense level of plants grown in fertile soil is low, because plants can develop more leaves to compensate for the loss of leaves eaten by insects. Atmospheric nitrogen (N) deposition in northern Japan has recently been increasing, especially in regions facing toward the Sea of Japan (Yamaguchi et al. 2009). The increase in N deposition will increase nutrient availability in the soil and may induce changes in plant defense strategy. What of the defense of woody plants under increasing N deposition (Galloway et al. 2004)?

Matsuki and Koike (2006) found large differences in defense capacity between woody species even within the same family. Among Betulaceae species, the Japanese white birch (Betula platyphylla var. japonica) was more committed to defense strategies (Matsuki et al. 2004, Matsuki and Koike 2006). The Japanese white birch is a typical deciduous broad-leaved tree; it is light demanding and has early successional traits (Koike 1995). This heterophyllous species has two types of leaf: early and late (Clausen and Kozlowski 1965, Koike 1995). The Japanese white birch usually forms a pure stand after forest fires or landslides. Miner

(Received; Aug. 11, 2010: Accepted; Dec. 14, 2010) * Corresponding author: tkoike@for.agr.hokudai.ac.jp
noted that defense was induced when the damaged area exceeds about 15% of the total leaf blade.

We hypothesize that plant defense is induced by insect herbivores by both mechanical damage and chemical stimulation through their saliva. To test this hypothesis, we studied induced defense in white birch after excision by scissors, acting as mechanical damage, and after grazing by the gypsy moth species (*Lymantria dispar*), which is a generalist herbivore. We determined the amounts of total phenolics and condensed tannin as the particular defense assumed to be effective against grazing by generalist herbivores (Aoyama *et al.* 2009, Hinata *et al.* 2009).

We also examined how N conditions in the soil affect the ability of the plant to produce defense. In this work we used N as a major macro nutrient for plant growth, because it is usually the factor limiting plant growth in forests (Schulze *et al.* 2005). If the N content in leaves increases, production of carbon-based defense chemicals (total phenolics including condensed tannin) should increase because of the increase in photosynthetic production. To study this, we divided our test site into two compartments (N addition vs. no N application). These two sites enabled us to measure the effect of N application on leaf defense in the

**Materials and Methods**

1. **Study site and plant material**

The study was conducted at the Sapporo Experimental Forest of the Field Science Center for Northern Biosphere (43°1'N, 141°3'E) of Hokkaido University, beginning in April 2008. The soil is classified as a brown forest soil. We used 24 individual 3-year-old seedlings of white birch (*Betula platyphylla var. japonica*). The seed source was Naganuma town, near Sapporo.

1.1. **Feeding treatment using an insect herbivore**

When the early leaves of the white birch had finished unfolding, we wrapped the aboveground part of each individual with a net-type bag (the mesh was 0.4 mm; Sanko Chemical Co., Sapporo). Light transmittance into the bag was about 82%, as measured by a PFD sensor (LI-6400, Nebraska, USA). The bag was fixed with wires and soft rubber at the bottom part of the seedlings, so as to prevent attack by external herbivores.

Four individual birch seedlings were planted in soil of each N level, and three types of treatment were used including control plants. As a result, 24 individuals were studied. We used gypsy moths as a generalist herbivore. We collected their eggs in the same forest stand in the experimental nursery and hatched them as larvae (Photo 1). The third to fifth instar larvae of the gypsy moth were first put into the net-bags at the beginning of June. All larvae were then taken out when about 20% of each leaf blade of the shoots had been eaten by the gypsy moths. In mid-June, the leaves of four other individuals in each soil N level were cut off with scissors across the main vein, to simulate mechanical damage. The extent of excision (10-20%) coincides with the amount of grazing in a present-day forest (Hartley and Jones 1997). We made the exposure to herbivore and excision with scissors using early (spring) leaves and late (summer) leaves, both before mid-June.

1.2. **Nutrient treatment**

The study site was divided into two. To one half of the study site, we supplied N at a rate of 30 kg N ha⁻¹ year⁻¹ in the form of ammonium sulfate ((NH₄)₂SO₄), so as to simulate N deposition, at the beginning of May, 2008. This N value was determined according to the maximum amount of N deposition observed recently in Tokyo, in the central part of Japan (Izuta 2006). The other half of the study site did not undergo treatment.

1.3. **Measurements**

We sampled leaves in August 2008, when they were sufficiently mature. The early leaves were too small for

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*Photo 1. Gypsy moth (left: third instars larva on beech and middle: on oak leaves, right: female adults).*
this study, so we sampled the fourth and fifth position of the late leaves. After we determined the leaf mass per area (LMA: mg cm\(^{-2}\)) as an index of leaf hardiness (Koike et al. 2006), the rest of the leaves were used in the chemical analyses. The carbon and N content of the leaf disk after the LMA measurement was analyzed using a NC-Analyzer (NC-900, Sumica-Shimadzu, Kyoto, Japan). In the chemical analyses we measured the amount of total phenolics and condensed tannin, following the standard method of analysis of phenolic compounds as performed by Matsuki et al. (2004). We also measured the amount of lignin (Iiyama and Wallis 1990).

1.4. Statistics

We applied Nested-ANOVA in the analysis of LMA, the amount of total phenolics and condensed tannin, the content of carbon and N, the ratio of carbon to N (C/N ratio) and lignin, after confirming homoscedasticity using the R software for statistical computation (Crawley 2005). We used the parameters specifying the feeding treatment and nutrient treatment. We then examined these with multiple comparisons using the Tukey-Cramer test for each nutrient treatment.

Results

In the study site with no application of N, induced defense was found in the LMA. This increased significantly after exposure to the gypsy moth herbivore (Fig. 1). The same trend in LMA was found in the study site with the N added, although it was not statistically significant. We then looked at the effect of N application on the expression of induced defense in leaves exposed to the herbivore and cut with scissors. The N concentration was higher in the leaves with N application than in leaves without N (Fig. 2). In each N treatment, leaf N concentration rose in the order: control (no treatment), treatment with gypsy moth, and cutting with scissors. The C/N ratio was larger in leaves without N application than in leaves that underwent N

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**Fig. 1.** Response of LMA (leaf mass area) to feeding treatment (control, herbivory, and scissors) and nutrient treatment (control and add nitrogen). Means and SE are presented. Different capital letters indicate significant difference (Tukey-Cramer test).

**Fig. 2.** Response of leaf N contents (g g\(^{-1}\)) to feeding treatment (control, herbivory, and scissors) and nutrient treatment (control and add nitrogen). Means and SE are presented. Different capital letters indicate significant difference (Tukey-Cramer test).
application (Fig. 3). In each N treatment, the C/N ratio in leaves rose in the order: scissors treatment, exposure to herbivore, and control.

Independent of the N treatment, lignin content tended to be higher in leaves that were exposed to the insect herbivore (P<0.10) (Fig. 4). In the synthesis of total phenolics (Fig. 5) and condensed tannin (Fig. 6), induced defense in leaves was clearly observed in the seedlings that underwent N application (P<0.01). With the N application, the total phenolic and condensed tannin contents were greater in leaves that experienced only cutting (Fig. 5, 6), but the condensed tannin content was significantly greater in leaves that were both cut and exposed to gypsy moths (P<0.01); see Fig. 6. All statistics are listed in Table 1.

Fig. 3. Response of CN ratio to feeding treatment (control, herbivory, and scissors) and nutrient treatment (control and add nitrogen). Means and SE are presented. Different capital letters indicate significant difference (Tukey-Cramer test).

Fig. 4. Response of Lignin ratio to feeding treatment (control, herbivory, and scissors) and nutrient treatment (control and add nitrogen). Means and SE are presented. Different capital letters indicate significant difference (Tukey-Cramer test).
Induced defense in white birch

Fig. 5. Response of Total phenolics (mg g⁻¹) to feeding treatment (control, herbivory, and scissors) and nutrient treatment (control and add nitrogen). Means and SE are presented. Different capital letters indicate significant difference (Tukey-Cramer test).

Fig. 6. Response of Condensed tannin (mg g⁻¹) to feeding treatment (control, herbivory, and scissors) and nutrient treatment (control and add nitrogen). Means and SE are presented. Different capital letters indicate significant difference (Tukey-Cramer test).

Table 1. Two-way ANOVA for the effects of feeding treatment (Ftreat) and nutrient treatment (Ftreat) on LMA, leaf N contents, CN ratio, lignin, total phenolics and condensed tannin. The value is F. Asterisks show significant difference (* P < 0.05, ** P < 0.01, *** P < 0.001, n.s. P > 0.05)

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<th>Total phenolics</th>
<th>Condensed tannin</th>
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<td>Ftreat</td>
<td>6.7645(*)</td>
<td>55.4142(***)</td>
<td>39.0487(***)</td>
<td>0.4185(n.s.)</td>
<td>3.3431(n.s.)</td>
<td>3.9661(*)</td>
</tr>
<tr>
<td>Ntreat</td>
<td>34.3894(***)</td>
<td>14.8683(***)</td>
<td>12.5489(***)</td>
<td>2.1169(n.s.)</td>
<td>8.1953(***</td>
<td>5.6096(**)</td>
</tr>
<tr>
<td>F×N</td>
<td>12.7191(***)</td>
<td>2.2396(n.s.)</td>
<td>0.5569(n.s.)</td>
<td>0.4413(n.s.)</td>
<td>2.4997(n.s.)</td>
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Nitrogen treatment : Ntreat
Feeding treatment : Ftreat
F×N: interaction
Discussion

To test our hypothesis, we studied defense of white birch seedlings induced by the saliva of gypsy moths and with cutting by scissors at two N levels. We selected N as the nutrient variable because we wish to consider the effect of the increasing N deposition taking place in northern Japan (Yamaguchi et al. 2009). Since no LMA responses were observed in leaves cut with scissors, independent of N treatment (Fig. 1), an increase in LMA may demand the presence of gypsy moth saliva (Agrawal 1999). An increase in LMA was, however, found in leaves exposed to gypsy moths without N; this clearly supports the CNB hypothesis (Bryant et al. 1983).

We observed a decrease in the N content and an increase in the C/N ratio in leaves cut with scissors. This trend was found in leaves of the seedlings irrespective of the N treatment, which does not provide evidence for the CNB hypothesis (Bryant et al. 1983). The decrease in leaf N may be due to the retranslocation of N in the cut leaves before leaf shedding. Recycled N appears to translocate to newly expanding leaves. This point should be taken into account later, in relation to the specific shoot development pattern in heterophyllous species of Betula (Koike 1995). These results show that induced defense may occur in leaves after mechanical damage, as well as after chemical stimulation from (for example) the saliva of herbivores. Total phenolics in the added-N treatment (Figs. 4-6) over the exposure to the insects did not increase the total in the leaves and reduced the C/N ratio (Figs. 2, 3), however, found in leaves exposed to gypsy moths without N; this clearly supports the CNB hypothesis (Bryant et al. 1983).

We deliberately damaged early leaves of the white birch in order to simulate herbivore grazing during the spring season. Since birch is characterized by heterophyllous shoot development (Coly, P.D. 1988) effects of plant growth rate and leaf

References


lifetime on the amount and type of anti-herbivore defense. Oecologia 74: 531-536.


