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Title	Induced Defense in Japanese White Birch Seedlings against Insect Herbivores
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Citation	Eurasian Journal of Forest Research, 13(2), 49-55
Issue Date	2010-12
Doc URL	https://hdl.handle.net/2115/44670
Type	departmental bulletin paper
File Information	EJFR13-2_002.pdf



Induced Defense in Japanese White Birch Seedlings against Insect Herbivores

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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

herbivores (*Fenusa pusilla*) or chewers, such as gypsy moths (*Lymantria dispar*), occasionally feed on Japanese white birch (e.g. Hara 2001).

There are two types of plant defense against herbivores: physical defense and chemical defense (e.g. Nabeshima *et al.* 2001, Matsuki *et al.* 2004). Physical defense involves an increase in leaf hardness, trichomes, spines, etc. Chemical defense mainly involves production of carbon-based chemicals having harsh flavor and bad taste, and also poisonous substances. For broad-leaved trees (i.e. angiosperm), there can be a trade-off between the production of defense chemicals (i.e. phenolics) and growth (mainly lignin synthesis), since both chemical substances derive from the same metabolic product, phenylalanine (Coley 1988). Growth and reproduction may therefore be impossible for plants if they produce entirely defense chemicals from their photosynthate. To attain adequate growth and reproduction for survival in competition with other plants, they must use their resources for defense effectively. As soon as plants are grazed by herbivores, they begin to manufacture physical and/or chemical defense, i.e. induced defense (Karban and Baldwin 1997). Induced defense is a less costly and efficient form of plant defense because plants set up defenses only as needed.

The present study looks at induced defense in white birch seedlings. It is generally considered that induced defense derives from the chemical stimulation of herbivory (e.g. by saliva or vomited liquid). Previous studies have found that similar defense responses are induced by mechanical injury (Faeth 1986). In Oak saplings, chemical stimulation is more pronounced than physical stimulation (Nabeshima *et al.* 2001). In a mechanical excision study, Karban and Baldwin (1997)

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



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 hardness (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

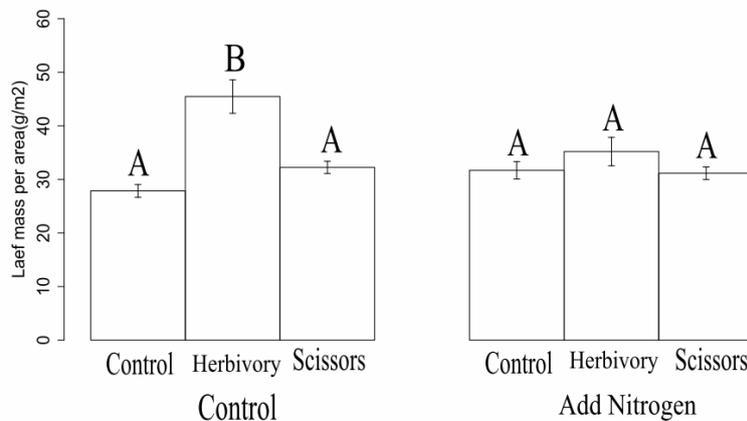


Fig. 1. Response of LMA (leaf mass area) to feeding treatment (control, herbivory, and scissors) and nutrient treatment (control and add nitrogen). Means and SE are presented. Different capital letters indicate significant difference (Tukey-Cramer test).

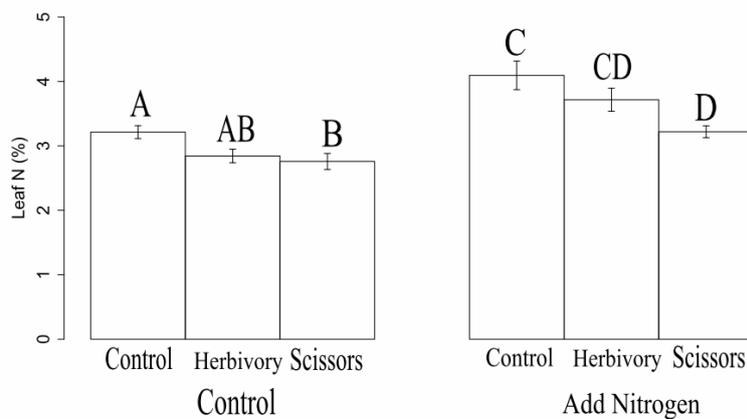


Fig. 2. Response of leaf N contents (g g^{-1}) to feeding treatment (control, herbivory, and scissors) and nutrient treatment (control and add nitrogen). Means and SE are presented. Different capital letters indicate significant difference (Tukey-Cramer test).

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.

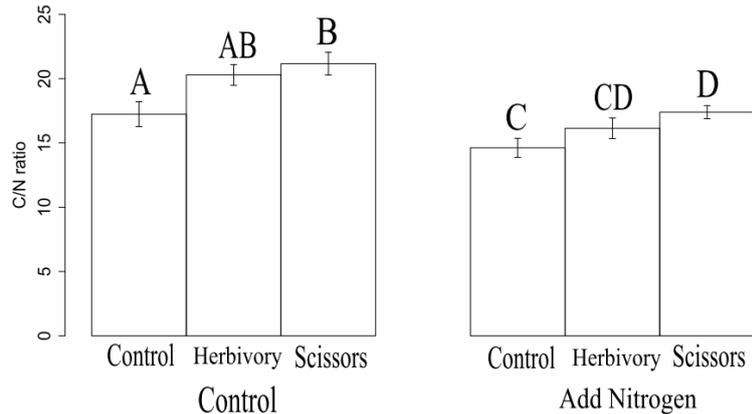


Fig. 3. Response of C/N ratio to feeding treatment (control, herbivory, and scissors) and nutrient treatment (control and add nitrogen). Means and SE are presented. Different capital letters indicate significant difference (Tukey-Cramer test).

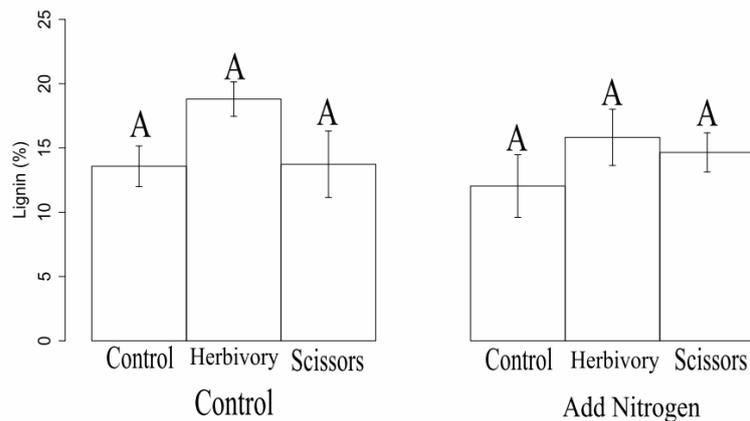


Fig. 4. Response of Lignin ratio to feeding treatment (control, herbivory, and scissors) and nutrient treatment (control and add nitrogen). Means and SE are presented. Different capital letters indicate significant difference (Tukey-Cramer test).

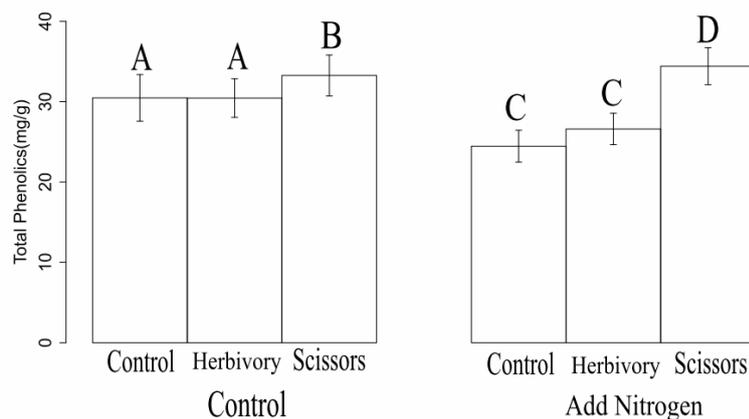


Fig. 5. Response of Total phenolics (mg g^{-1}) to feeding treatment (control, herbivory, and scissors) and nutrient treatment (control and add nitrogen). Means and SE are presented. Different capital letters indicate significant difference (Tukey-Cramer test).

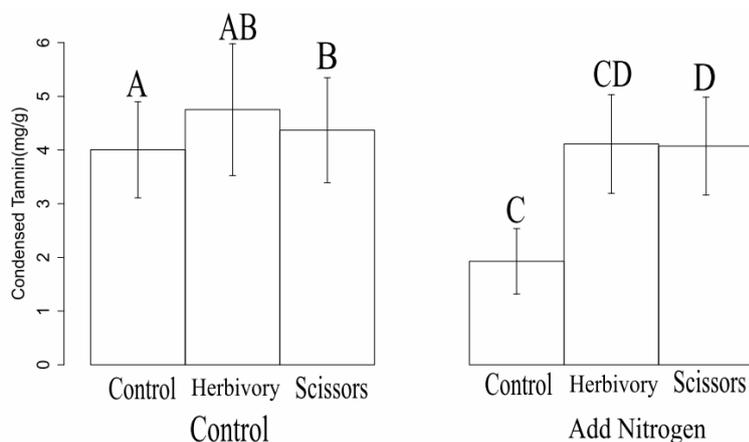


Fig. 6. Response of Condensed tannin (mg g^{-1}) to feeding treatment (control, herbivory, and scissors) and nutrient treatment (control and add nitrogen). Means and SE are presented. Different capital letters indicate significant difference (Tukey-Cramer test).

Table 1. Two-way ANOVA for the effects of feeding treatment (N_{treat}) and nutrient treatment (F_{treat}) 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. $P > 0.05$)

	LMA	N contents	CN ratio	Lignin	Total phenolics	Condensed tannin
F _{treat}	6.7645(*)	55.4142(***)	39.0487(***)	0.4185(n.s.)	3.3431(n.s.)	3.9661(*)
N _{treat}	34.3894(***)	14.8683(***)	12.5489(***)	2.1169(n.s.)	8.1953(***)	5.6096(**)
F×N	12.7191(***)	2.2396(n.s.)	0.5569(n.s.)	0.4413(n.s.)	2.4997(n.s.)	1.9172(n.s.)

Nitrogen treatment : N_{treat}

Feeding treatment : F_{treat}

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 increased with scissor cuttings in the added-N treatment (Fig. 5). We are uncertain of the reason for this observation.

Although the N application increased the N content in the leaves and reduced the C/N ratio (Figs. 2, 3), exposure to the insects did not increase the total phenolics in the added-N treatment (Figs. 4-6) over the specimens with no N application. The photosynthetic rate increases with N application (Schulze *et al.* 2005). Consequently, depending on the availability of N resources, plants increase their growth and also the synthesis of defense chemicals. Mizumachi *et al.* (2004) found in oak seedlings that plants grown in low-nutrient soil (especially N deficient soil) are liable to increase their levels of defense chemicals, relative to plants grown in nutrient-rich soil. Based on these results, some species synthesize defense chemicals upon stimulation by suffering herbivore grazing and/or mechanical damages; but other species do not.

Regardless of the soil N condition, LMA and lignin content tend to be higher in that were exposed to herbivory (Figs. 1, 4). This is probably compensation growth after herbivore grazing. Based on the LMA and lignin content data, an increase in LMA may be related to an increase in lignin content. Condensed tannin is considered to be one of the most effective defense chemicals against herbivores (Nabeshima *et al.* 2001, Mizumachi *et al.* 2004, Eyles *et al.* 2010). After chemical damage (insect grazing) and mechanical damage (by scissors), the C/N ratio and condensed tannin content in leaves clearly increased, especially in the added-N treatment. These carbon-based secondary compounds are synthesized from the photosynthates (Karban and Baldwin 1997). Consequently, the

synthesis of condensed tannin in white birch leaves may require an increase in the photosynthetic rate. This was made possible by the N application.

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 (Clausen and Kozlowski 1965, Koike 1995), induced defense (increase of condensed tannin) may first be synthesized by the partial loss of early leaves. Matsuki *et al.* (2004) found that chemical defense in early leaves of the white birch is very strong, because successive shoot development is closely associated with the production activities of the early leaves. The photosynthates of early leaves are allocated to the succeeding development of shoots bearing late leaves. The growth of shoots of the white birch with late leaves appears to depend strongly on the growth (perhaps the photosynthesis) of early leaves, in contrast to other birch species such as the Monarch birch and the mountain birch (Koike and Sakagami 1985, Koike 1995, Matsuki and Koike 2006). It follows that effective defense chemicals were first induced. Both early and late leaves were also grazed by gypsy moths, which may act to increase the hardness of the late leaves. An increase in the LMA is considered to be a compensation response after the reduction of leaf area due to grazing, because the photosynthetic organs per unit area increase with increasing LMA.

We conclude that the physical and chemical defense of white birch seedlings are induced by herbivore attack or mechanical damage of the leaves, and this induced defense response is influenced by the soil N conditions. Our findings do not clearly support the CNB hypothesis, however. The presence of saliva of herbivores may be important in enhancing the LMA and lignin synthesis. Further studies, involving more detailed analysis of successive leaf development, will be needed to specify in detail the defense characteristics of the white birch.

Acknowledgements

Financial support in part by a Grant-in-Aid from the Japanese Society of Promotion of Science (Type B) to T.K. is gratefully acknowledged. Thanks are also due to Mr. K. Ichikawa for management of the experimental fields, and Dr. M. Watanabe for valuable comments on the early draft of the manuscript.

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