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Ingestion and excretion of nitrogen by larvae of a cabbage armyworm: the effects of fertilizer application

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Abstract

1 Insect frass has significant impacts on decomposition and soil nitrogen dynamics. Although the frass contains various forms of nitrogen that may differently influence nitrogen dynamics in the decomposition process, how the nitrogen form in the insect frass is influenced by host plant quality remains poorly understood.

2 The present study examined the effects of application of fertilizer on leaf quality of *Brassica rapa* L. var. *perviridis* Bailey (Brassicaceae), and on the consumption, frass excretion and frass quality of its insect pest *Mamestra brassicae* (L.) (Lepidoptera: Noctuidae), with a particular focus on the dynamics of inorganic nitrogen.

3 *Brassica rapa* increased total nitrogen concentration, and accumulated inorganic nitrogen [i.e. leaf nitrate-nitrogen (NO_3^--N) and ammonium-nitrogen (NH_4^+-N)] in the leaves in response to the application of fertilizer.

4 Although leaf consumption and frass excreted by *M. brassicae* was not affected by fertilizer treatment, frass quality was influenced by host plant quality as altered by fertilizer applications. Frass contained high concentrations of total nitrogen, NO_3^--N, and NH_4^+-N under high fertilizer treatment. In particular, the larvae excreted much more NH_4^+-N than ingested. The relationship between host plant quality and insect frass quality, as well as the potential implications for decomposition and nutrient dynamics, are discussed.

Keywords Ammonium, *Brassica rapa* L., frass, *Maestro brassicae* (L.), nitrate, nitrogen metabolism, nutritional ecology, plant–insect interaction.

Introduction

There is increasing evidence that the consumption of living foliage by herbivorous insects has significant impacts on ecosystem processes (Belovsky & Slade, 2000; Hunter, 2001; Weisser & Siemann, 2004). Deposition of insect frass and honeydew is one of the pathways affecting decomposition and soil nutrient dynamics (Hunter, 2001; Weisser & Siemann, 2004). Insect frass contains more labile carbon than does leaf litter (Lovett *et al*., 2002). Therefore, it can stimulate microbial growth in the soil (Frost & Hunter, 2004), which subsequently affects nitrogen mineralization or immobilization (Lovett & Ruesink, 1995; Frost & Hunter, 2007). Thus, insect herbivores can play important role influencing nitrogen dynamics in soil (i.e. transforming organic nitrogen into inorganic nitrogen and vice versa) through frass decomposition.

The frass of herbivorous insects contains various forms of nitrogen (Cochran, 1985; O'Donnell, 2008). Proteins and amino acids are found in insect frass, and are regarded as the excretion of unabsorbed, excessive amounts of proteins and amino acids derived from the diet (Cochran, 1985). Uric acid and related compounds such as allantoin and allantoic acid are principal end products of nitrogen metabolism in terrestrial insects (Craig, 1960; Cochran, 1985). Nitrogenous excretion by uric acid is an evolutionary adaptation for water conservation in terrestrial animals: uric acid requires less water than ammonia and urea for excretion (Wright, 1995). Nevertheless, ammonia is commonly detected in the frass of terrestrial insects, comprising approximately 9–27% of total nitrogen in the frass (Cochran, 1985; Lovett & Ruesink, 1995; Kuzhivelil & Mohamed, 1998; Lovett *et al*., 2002). This indicates that plant organic nitrogen (proteins and amino acids) ingested by herbivorous insects is transformed to some extent into an inorganic form during insect nitrogen metabolism, before the decomposition of frass deposited to the soil. Hence, understanding the effects of insect herbivores on nitrogen dynamics (mineralization and immobilization) through frass excretion will be incomplete without knowledge of the form of nitrogen in the frass. In addition, the composition of

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such nitrogen compounds in the frass of herbivorous insects may be expected to vary according to diet quality because the nitrogen use efficiency of herbivorous insects is altered by the amount of nitrogen in their host plants (Slansky & Feeny, 1977; Simpson & Raubenheimer, 2001; Giertych *et al*., 2005). The effects of plant quality on the form of nitrogen in the frass of herbivorous insects, which would potentially influence decomposition process and nutrient dynamics in soil, remains, however, poorly understood.

The present study investigated the relationship between the nitrogen status of the host plant and frass of the herbivorous insect, with a particular focus on total nitrogen and inorganic nitrogen concentration, at various levels of fertilizer application. We examined the effects of application of fertilizer on leaf quality of the vegetable crop *Brassica rapa* L. var. *perviridis* Bailey (Brassicaceae), as well as on the consumption, frass excretion and frass quality of its insect pest, cabbage armyworm *Mamestra brassicae* (L.) (Lepidoptera: Noctuidae).

Materials and methods

Culture of B. rapa

Brassica rapa plants (Rakuten, Takii Syubyo Co. Ltd, Japan) were individually grown in 500-mL pots using nutrient-rich compost (Tanemaki-baido, Takii Syubyo Co. Ltd., Kyoto, Japan) as growth medium in a glass-shield greenhouse at 25 °C under natural light conditions. After seeding, plants were watered daily. The plants were fertilized with liquid fertilizer (HYPONeX: N : P : K = 6 : 10 : 5; HYPONeX Japan Co. Ltd, Japan). We used NPK fertilizer instead of nitrogen fertilizer because the aim was to understand the effects of nitrogen addition on host plant quality and insect frass without limitation of other nutrients, such as phosphorus. It is known that the absorption and utilization of one nutrient in plants and insects may be affected by the amount of other nutrients (Sterner & Elser, 2002; Huberty & Denno, 2006). Because our experimental treatments included relatively high fertilizer levels (see below), only nitrogen fertilizer application may have resulted in an insufficient supply of other nutrient elements. Four fertilizer levels were established: (i) high: 30-fold dilution; (ii) medium: 100-fold dilution; (iii) low: 300-fold dilution; and (iv) none (control): water only. This fertilizer is usually used with 500-fold dilution for culture of vegetables. Two weeks after seeding, when the plants reached the four-true-leaf stage, 50 mL of fertilizer solution or 50 mL of water were added to individual pots at 1-week intervals. Plants were grown for 4 weeks, and then used for the analyses of leaf quality and feeding trials.

Leaf quality of B. rapa

Leaf nitrogen analyses were conducted for 12 randomly selected individuals of *B. rapa* for each treatment. Two mature leaves without petiole were collected from each individual. They were oven-dried at 60 °C for 72 h and ground to a fine powder. Leaf total nitrogen concentration was determined using an elemental analyzer (JM 1000CN, J-Science Co. Ltd, Japan). Leaf nitrate-nitrogen (NO_3^--N) and ammonium-nitrogen (NH_4^+-N) were extracted using 1.5 mol/L KCl and concentrations were determined using a continuous flow analyzer (Integral Futura, Alliance Instruments, France).

Consumption, frass excretion and frass quality of M. brassicae

Mamestra brassicae were taken from a laboratory population at the Center for Ecological Research, Kyoto University. Egg clusters were placed individually in Petri dishes (diameter 9 cm) in an environmental chamber under an LD 16 : 8 light/dark photocycle at 25 °C. The hatched larvae were reared together until the third instar, and thereafter five larvae were reared per Petri dish. Before the feeding trials, larvae were provided with artificial diet (Insecta LFS, Nihon Nosan Kogyo Co. Ltd, Japan). Randomly selected sixth (last)-instar larvae were used for the feeding trials. Most (60–80%) of the food consumption of immature stage occurs during the last instar larvae in Lepidoptera (Furuno, 1964; Scriber & Slansky, 1981). Hence, consumption and frass excretion during this larval period are particularly important for host plant and insect frass nitrogen dynamics. The larvae were kept for 12 h without diet before the feeding trials to excrete the frass of artificial diet origin. Each larva was placed in a Petri dish (diameter 14.5 cm) with one or two mature, petiole-removed leaves of *B. rapa* from one each of the four fertilizer treatments described above. The *B. rapa* leaves and *M. brassicae* larvae were weighed before the feeding trials. The larvae were reared for 48 h in the environmental chamber, and then *B. rapa* leaves were removed and the larvae were kept for 12 h without diet to excrete the frass in the gut. Thereafter, larval frass excreted during the feeding trial was collected. *Brassica rapa* leaves, and frass were oven-dried at 60 °C for 72 h to determine dry weight. Twenty replicates were conducted for each treatment. Consumed leaf mass was determined as the difference in leaf dry mass between the start and the end of the experiment. Dry mass of *B. rapa* leaves and *M. brassicae* larvae at the start of the experiment were estimated from their water contents, which were measured using additional samples. The water contents were determined from the difference between the fresh and dry mass, which was measured after being oven-dried at 60 °C for 72 h ($n = 12$ for *B. rapa* leaves for each treatment, and $n = 15$ for *M. brassicae* larvae).

For nitrogen analyses, frass was ground to a fine powder. Total and inorganic frass nitrogen were determined by the same methods as for the analyses of *B. rapa* leaf nitrogen. Frass from ten replicates for each treatment was used for total nitrogen analysis and that from another ten replicates was used for inorganic nitrogen (i.e. NO_3^--N and NH_4^+-N) analysis because one frass sample did not have sufficient mass to measure both total and inorganic nitrogen.

Statistical analysis

All comparisons were tested by one-way analysis of variance (ANOVA) with the Tukey–Kramer honestly significant difference test ($P < 0.05$). Percentage data were arcsine-square

root transformed before analysis. The excretion efficiency for consumed biomass, total nitrogen, NO_3^--N, and NH_4^+-N was shown by regression plots with treatment averages, and the relationships between ingested and excreted mass were tested by regression analysis. All analyses were conducted using JMP, version 6 (SAS Institute Japan, Japan).

Results

Leaf quality of B. rapa

The water content of *B. rapa* leaves was not affected by fertilizer treatment (ANOVA: $F_{3,47} = 1.31$, $P = 0.28$), and the mean ± SE percentage of leaf water was 87.4 ± 0.3%. Fertilizer treatment significantly affected total nitrogen, NO_3^--N, and NH_4^+-N concentration in leaves (ANOVA: $F_{3,47} = 19.3$, $P < 0.0001$ for total nitrogen; $F_{3,47} = 13.3$, $P < 0.0001$ for NO_3^--N; $F_{3,47} = 16.6$, $P < 0.0001$ for NH_4^+-N; Fig. 1a–c). Total nitrogen concentration was greatest in the high fertilizer treatment, followed by the medium fertilizer treatment. Plants in the low and no fertilizer treatments had the lowest percentages of total nitrogen, and there was no significant difference between these two treatments (Fig. 1a). NO_3^--N concentration was greatest in the high fertilizer treatment followed by the medium fertilizer treatment. NO_3^--N concentration did not differ between the low and no fertilizer treatments (Fig. 1b). Plants in the high fertilizer treatment had a significantly greater percentage of NH_4^+-N compared with the other three treatments. NH_4^+-N concentration did not significantly differ among the medium, low and no fertilizer treatments (Fig. 1c).

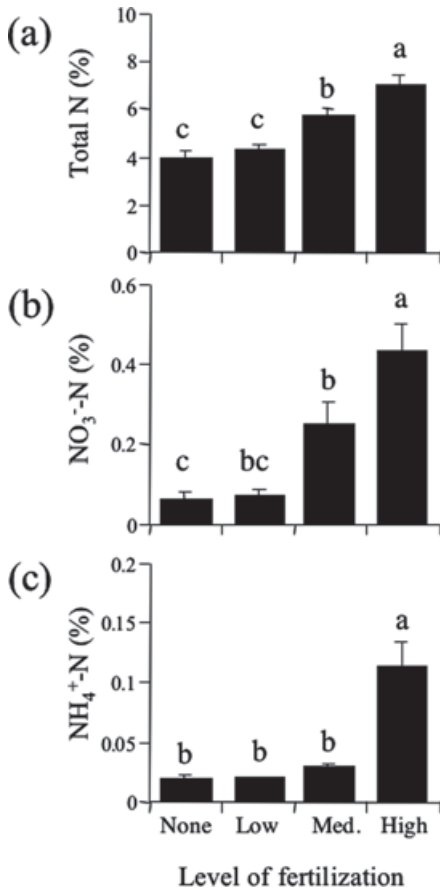

Figure 1 (a) Total nitrogen concentration, (b) nitrate-nitrogen (NO_3^--N) concentration and (c) ammonium-nitrogen (NH_4^+-N) concentration of the *Brassica rapa* leaves under different fertilizer treatments. Data are presented as the mean ± SE. Different letters above bars indicate a statistically significant difference ($P < 0.05$).

Consumption, excretion and frass quality of M. brassicae

The larval dry mass at the start of the experiment did not differ significantly among the four treatments (ANOVA: $F_{3,79} = 1.05$, $P = 0.38$, overall mean ± SE = 86.1 ± 1.0 mg). At most, the larvae consumed 70% of the biomass of the leaves provided for the feeding trials, indicating that food shortage did not occur during the experiment. The dry mass of *B. rapa* leaves consumed by *M. brassicae* did not significantly differ among the treatments (ANOVA: $F_{3,79} = 0.79$, $P = 0.50$; Fig. 2a). The

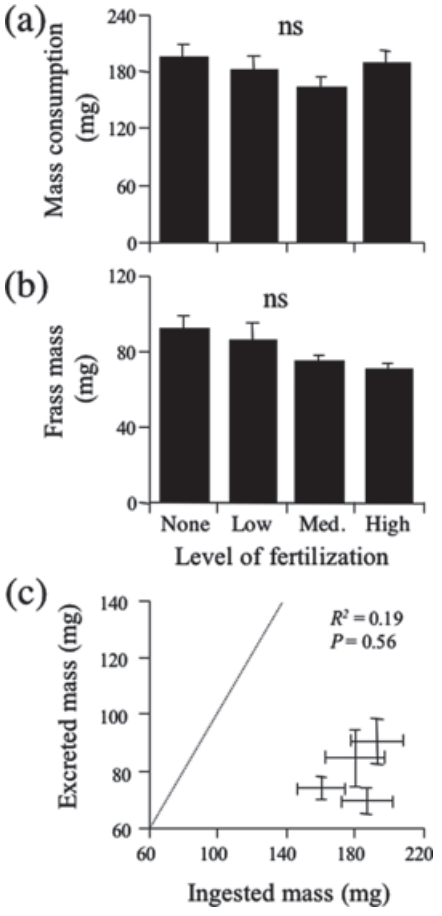

Figure 2 (a) Mass consumption and (b) frass mass excreted by *Mamestra brassicae* during the 48-h feeding trials. Data are presented as the mean ± SE. ns, no significant difference ($P > 0.05$). (c) Efficiency of frass excretion, shown by the relationship between ingested and excreted mass. Each point represents the treatment mean ± SE. Dotted line shows $y = x$. The area above the line indicates that excreted mass is greater than ingested mass, and vice versa.

amount of frass excreted by *M. brassicae* did not differ among the treatments (ANOVA: $F_{3,79} = 1.85$, $P = 0.14$; Fig. 2b). There was no relationship between ingested and excreted mass (Fig. 2c) and, on average, $50.2 \pm 3.4\%$ of ingested food was excreted as frass.

Total nitrogen concentration in frass differed significantly among the treatments ($F_{3,39} = 14.2$, $P < 0.0001$; Fig. 3a). Frass excreted by the larvae in the high fertilizer treatment had the greatest concentration of nitrogen, followed by that from larvae in the medium fertilizer treatment. Frass nitrogen in the no fertilizer treatment was the lowest. NO_3^--N and NH_4^+-N concentrations in frass were also affected by the fertilizer treatment (ANOVA: $F_{3,39} = 6.16$, $P = 0.0017$ for NO_3^--N; $F_{3,39} = 10.2$. $P < 0.0001$ for NH_4^+-N; Fig. 3b, c). Frass in the high fertilizer treatment had the greatest concentrations of NO_3^--N and NH_4^+-N, and frass in the low and no fertilizer treatments had the lowest concentrations.

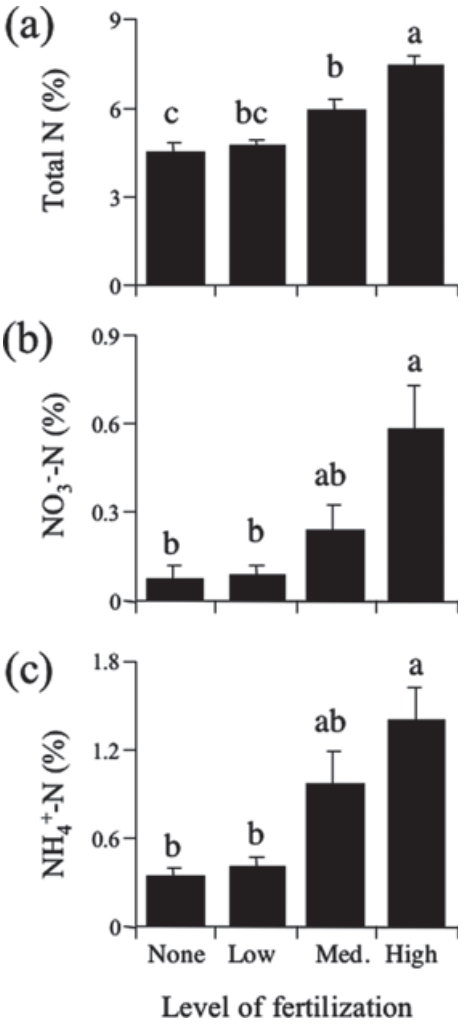

Figure 3 (a) Total nitrogen concentration, (b) nitrate-nitrogen (NO_3^--N) concentration and (c) ammonium-nitrogen (NH_4^+-N) concentration in the frass of *Mamestra brassicae*. Data are presented as the mean ± SE. Different letters above bars indicate a statistically significant difference ($P < 0.05$).

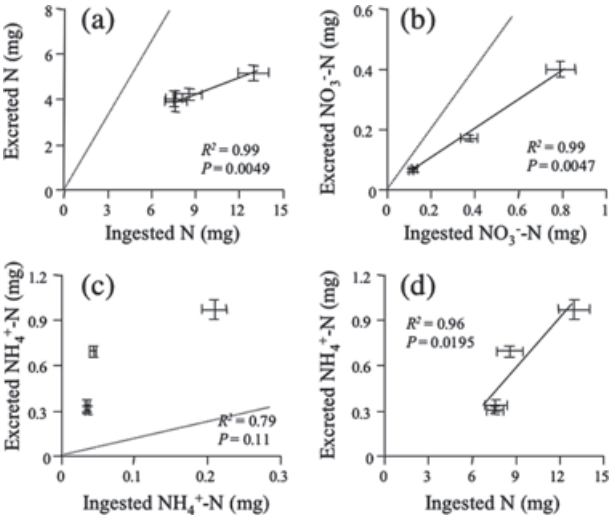

Figure 4 (a) Efficiency of total nitrogen excretion, (b) efficiency of leaf nitrate-nitrogen (NO_3^--N) excretion, (c) efficiency of ammonium-nitrogen (NH_4^+-N) excretion, and (d) relationship between ingested nitrogen and excreted NH_4^+-N. Efficiency of excretion is shown by the relationship between ingested and excreted mass. Each point represents the treatment mean ± SE. Dotted line shows $y = x$. The area above the line indicates that excreted mass is greater than ingested mass, and vice versa.

Excreted mass of nitrogen and NO_3^--N increased linearly in response to the amount of each ingested (Fig. 4a, b). Overall, $54.6 \pm 3.8\%$ and $60.4 \pm 4.4\%$ of the ingested nitrogen and NO_3^--N was excreted as frass, respectively. Although there was no significant relationship between ingested and excreted NH_4^+-N, the larvae excreted more NH_4^+-N than they ingested (Fig. 4c). There was, however, a significant relationship between ingested nitrogen and excreted NH_4^+-N, and excreted NH_4^+-N increased in response to ingested nitrogen (Fig. 4d).

Discussion

Accumulation of inorganic nitrogen in B. rapa *leaves*

The present study clearly showed that the application of fertilizer to *B. rapa* not only increased total nitrogen, but also the accumulated amount of inorganic nitrogen (i.e. NO_3^--N and NH_4^+-N) into their leaves. The process of nitrogen assimilation in plants has been well documented (Huppe & Turpin, 1994; Crawford, 1995). In brief, plants can use both nitrate and ammonia as nitrogen resource. Although ammonia is directly utilized for synthesis of amino acids, nitrate is first reduced to nitrite by nitrate reductase. Nitrite is then reduced to ammonia by nitrite reductase. Thereafter, ammonia is fixed into glutamate to produce glutamine by the action of glutamine synthetase. When plants are provided with excess nitrate, the nitrate is stored in vacuoles by regulation of nitrate reduction (Martinoia *et al*., 1981). The high level of accumulation of nitrate in *B. rapa* leaves observed in the high fertilizer treatment indicates that the fertilizer level in the present study was excessive beyond the level of nitrate that *B. rapa* is able to assimilate. Yorifuji *et al*. (2005) reported that *B. rapa* plants in

Japanese markets have on average 4060 p.p.m. nitrate ($n = 197$, range 128–9460 p.p.m.) in fresh weight. This indicates that many of *B. rapa* in Japanese markets contained higher concentrations of nitrate than the maximum level in vegetables (4500 p.p.m.), which was established by European Commission Regulation (European Commission, 2005). When the NO_3^--N concentration observed in the present study was converted to the nitrate concentration in fresh weight, the highest concentration was 5337 p.p.m. for the leaves in the high fertilizer treatment. This value is within a range of the concentration of *B. rapa* in Japanese markets. Hence, the fertilizer level in the present study appears to be within the range of the fertilizer level used in *B. rapa* culture in Japan. In addition to the nitrate accumulation, the results obtained in the present study showed that ammonium was also accumulated in the leaves under high fertilizer treatment, although the concentration was lower than that of nitrate. Because the fertilizer used in the present study contained both nitrate and ammonium, the fertilizer level in the present study would be also excessive beyond the levels of ammonium that *B. rapa* is able to synthesize glutamine.

Consumption, excretion and frass quality of M. brassicae

Although a number of studies have dealt with the effects of host plant quality on feeding behaviour, nutrient utilization and growth of herbivorous insects (Slansky & Feeny, 1977; Fischer & Fiedler, 2000; Simpson & Raubenheimer, 2001; Giertych *et al*., 2005; Chen *et al*., 2007; Hwang *et al*., 2008; Staley *et al*., 2009), the effects of host plant quality on the frass quality, in particular inorganic nitrogen, have received less attention.

The present study showed that *B. rapa* leaf quality altered by fertilizer treatment did not affect the amount of leaf consumption and excreted frass of *M. brassicae* larvae. One reason why plant quality did not affect the feeding and excretion behaviour of the insect would be the short length of the feeding trials. Although *M. brassicae* larvae takes approximately 1 month until pupation under our rearing conditions (H. Kagata, personal observation), the feeding trial was conducted for only 48 h with final-instar larvae. Therefore, our experimental design would not be sufficient to detect the effects of host plant quality on such behaviour.

The present study, however, clearly demonstrated that the quality of *M. brassicae* frass was influenced by host plant quality, and that the frass had high concentrations of total nitrogen, NO_3^--N, and NH_4^+-N as a result of the altered quality of the host plants in the high fertilizer treatment. Inorganic nitrogen in the frass mostly comprised the ammonium form; from 9% (in no fertilizer) to 27% (in high fertilizer) of total nitrogen in the frass (Fig. 3). The remaining nitrogen would have been organic nitrogen such as amino acids and proteins, which were unabsorbed in the insect gut, and uric acid and related compounds as end products of nitrogen metabolism (Cochran, 1985; Lovett *et al*., 2002). NO_3^--N in the frass is probably of diet origin, rather than a nitrogen metabolism product of the herbivorous insect, because the amount of excreted NO_3^--N was less than that ingested, and was well explained by amount of ingested NO_3^--N. In addition, there is no report, to our knowledge, that nitrate was produced as a consequence of nitrogen metabolism in herbivorous insects. By contrast, NH_4^+-N in the frass would originate from nitrogen metabolism by the insect, rather than of diet origin. The amount of excreted NH_4^+-N was from five- to 17-fold greater than that ingested (Fig. 4c), and the amount of excreted NH_4^+-N in the frass was explained by the amount of ingested nitrogen rather than by ingested NH_4^+-N (Fig. 4c, d). These amounts indicate that high levels of nitrogen in the host plants would promote nitrogen metabolism and subsequently accelerate ammonium excretion of the herbivorous insect.

Thus, we concluded that nitrogen in the host plant largely influenced insect frass quality, especially the ammonium concentration, by enhancing the metabolic process of the herbivorous insect. Such frass characteristics altered by their host plants are likely to influence soil decomposition and nutrient dynamics. In general, nitrogen is one of the factors that control the decomposition rate, and substrates with high nitrogen are more rapidly decomposed (Hättenschwiler *et al*., 2005). Nitrate and ammonium in the frass may also have impacts on nutrient dynamics because they are in the form that plants can directly utilize without the time lag necessary for nitrogen mineralization in the normal decomposition process. In addition, frass quality differs according to insect species, even when fed on the same host plant (Madritch *et al*., 2007). Therefore, the effects of insect frass on decomposition and nutrient dynamics may be more variable than expected from frass decomposition experiments using a single plant–insect interaction (Lovett & Ruesink, 1995; Christenson *et al*., 2002; Frost & Hunter, 2004, 2007). Further studies aiming to clarify the effects of insect frass quality on decomposition and nutrient dynamics will contribute to an understanding of how herbivores can influence ecosystem processes.

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