***Title*:** Aphids decelerate litter nitrogen mineralization through changes in litter quality

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***Running title*:** Aphid effects on litter decomposition

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Abstract.

1. Plant-herbivore interactions can be important factors affecting ecosystem functions. Herbivorous insects may have significant impacts on litter decomposition through modification of plant litter quality and quantity. However, our knowledge about the effects of herbivorous insects on the decomposition process is very limited.
2. Here, we conducted experiments to examine how sap-feeding aphids modify plant litter and whether the aphid-induced modification influences litter decomposition processes, using a plant-herbivore system consisting of a soybean (*Glycine max*) and soybean aphids (*Aphis glycines*).
3. First, litter traits produced by aphid-free and aphid-infected plants were compared, and we found that aphids did not affect litter mass and carbon concentration, but significantly decreased nitrogen and increased phenolic concentrations. Such aphid-mediated modification of litter quality may cause deceleration of litter decomposition since litter nitrogen promotes decomposition and litter phenolics inhibit it.
4. Then, we carried out a decomposition experiment to compare decomposition processes of litter between the aphid-free and aphid-infected plants. We found no impacts of aphid herbivory on litter carbon mineralization but negative impacts on nitrogen mineralization. Litter nitrogen mineralization of aphid-infected plants decreased by 40 and 28% compared to that of aphid-free plants one and three months after commencement of the experiment, respectively.
5. Our experimental results clearly showed that aphids decelerated litter nitrogen mineralization by modifying litter quality, suggesting that aphids can play a critical role in nitrogen flux between above- and below-ground ecosystems.

Key words. *Aphis glycines*, decomposition, C/N ratio, ecosystem function, phenolics, trait-mediated interaction, soybean

Introduction

The abundance and species richness of insects are remarkably higher than those of other organisms (Schoonhoven *et al.*, 1998; Schowalter, 2000), and thus insects are one of the dominant components of biodiversity in terrestrial ecosystems. In spite of their great abundance and high diversity, the importance of insects in governing ecosystem functions (e.g., decomposition and/or nutrient cycling) has been long underestimated. One reason is that herbivorous insects generally impose low levels of damage to plants, often less than 20% (Cyr & Pace, 1993), and therefore it has been supposed that the roles of herbivorous insects as resource consumers are not prominent, as argued by the "green world" hypothesis (Hairston *et al.*, 1960). Recently, it has been recognized that herbivorous insects not only consume plant resources, but also provide nutrients to soil via their excrement, and influence the litter decomposition process by modifying plant traits. There is a growing body of evidence that herbivorous insects have significant impacts on the decomposition process and nutrient dynamics in terrestrial systems (Hunter, 2001; Wardle, 2002; Frost & Hunter, 2004; Schweitzer *et al.*, 2005; Chapman *et al.*, 2006; Kay *et al.*, 2008).

Insect excrement, such as frass or honeydew, falls to the soil, where plants absorb them as nutrients after mineralization (Weisser & Siemann, 2004). The effect of insect excrement on soil nutrient dynamics appears quickly within a few days (Lovett & Ruesink, 1995; Hunter, 2001). Since herbivorous insects frequently undergo outbreaks, and sometimes produce large quantities of excrement (Seastedt & Crossley, 1984; Stadler *et al.*, 2004), this source of nutrients could be a major factor in determining the dynamics of nutrient uptake in plants (Frost & Hunter, 2004, 2008). More recently, we are beginning to realize that herbivorous insects can also influence the litter decomposition process through an alternative mechanism, namely, the modification of litter traits (Frost & Hunter, 2008). In biological degradation, soil microbial decomposers (free-living soil bacteria and fungi) mineralize plant litter (Wardle, 1992). The tissue of these microbes has a lower C/N ratio than plant litter (Manzoni &

Porporato, 2007), indicating that the soil microbes require more nitrogen when they consume the litter. Furthermore, litter phenolics inhibit soil microbial activity (Schimel *et al.*, 1998), and reduce decomposition rates (Palm & Sanchez, 1991). Therefore, plant litter with lower C/N ratio and less phenolics is more easily mineralized by microbial decomposers (Manzoni *et al.*, 2008). Plants increase or decrease carbon, nitrogen, and/or phenolics in the tissues in response to herbivory (Karban & Baldwin, 1997; Ohgushi, 2005). Plant traits modified by herbivorous insects often carry over to leaf litter, which in turn influences the decomposition process via litter quality (Hunter, 2001).

Several studies have reported that plants modified litter quality following leaf herbivory by chewing and gall-making insects, as a result of which litter decomposition was accelerated or decelerated (Chapman *et al.*, 2003, 2006; Kay *et al.*, 2008). However, to date, the effect of sap-feeding aphids on the litter decomposition process by modifying litter quality is poorly understood (but see Schweitzer *et al.*, 2005), despite the fact that aphids affect soil nutrient dynamics by inputting honeydew into the soil (Schmidt *et al.*, 1997; Stadler *et al.*, 1998; Wardle, 2002). The aim of this study is to evaluate how aphids modify plant litter traits, and determine whether the aphid-induced litter modification influences the decomposition process. To doing so, we studied a system consisting of a soybean, *Glycine max* (L.) (Fabaceae), and soybean aphids, *Aphis glycines* Matsumura (Hemiptera: Aphididae).

We expect that aphids influence litter traits, especially litter nitrogen, in several ways. Previous studies reported that sap feeding by aphids could temporarily increase soluble nitrogen content or amino acids in phloem sap and/or leaves (Dorschner *et al.*, 1987; Telang *et al.*, 1999; Sandström *et al.*, 2000; Petersen & Sandström, 2001). The increase in nitrogen levels is likely due to the aphid's manipulation of the nutritional quality of their host plants in their own favor (Sandström *et al.*, 2000). If the increase in nitrogen concentrations is carried over to leaf litter, then litter nitrogen under aphid-infected plants may be increased. On the other hand, we can also expect a counteracting effect, since aphids may also decrease litter nitrogen through their

effects on the decomposition process in the soil. This is because the aphids decrease plant nutrient uptake by affecting soil nutrient dynamics and by changing the association between plants and their nutrient-providing symbiotic microbes (N. Katayama, *et al.*, in preparation). Aphids induce "microbial immobilization", which results in a decrease in inorganic soil nitrogen via the addition of honeydew droplets into soil (Wardle, 2002). The abundance of free-living soil microbes is often labile-carbon limited. Since honeydew contains abundant sugars, the droplets increase abundance of belowground microbes, which result in a decrease in inorganic nitrogen in the soil (Dighton, 1978; Grier & Vogt, 1990; Stadler *et al.*, 2004). Thus, it is likely that plants absorb less nitrogen from the soil in the presence of aphids. In addition, the reduction in nitrogen uptake by plants via symbiotic microbial associations may occur in this system. The plants studied here, soybeans, have a symbiotic association with below-ground rhizobia, and on the symbionts for nitrogen uptake (Katayama *et al.*, 2010). The plants need a large quantity of carbon from photosynthesis to maintain the symbiotic association (Macedo *et al.*, 2003); however, aphids consume the photosynthetic carbon. Hence, aphids may decrease nitrogen uptake from the rhizobia by weakening the plant-rhizobia association. Therefore, we suppose that plants subsequently produce litter with less nitrogen because the aphids decrease nitrogen uptake in the plants.

In this study, we conducted two experiments. The first experiment compared the traits of litter (litter mass, carbon, nitrogen, and phenolic contents) produced by the aphid-free and aphid-infected plants. The second experiment examined whether the aphid-mediated modification of the litter traits influences the decomposition process, focusing in particular on litter nitrogen mineralization. Based on these results, we discussed the effects of aphids on the decomposition process of litter and nutrient dynamics.

Materials and Methods

Materials

Soybean, *Glycine max*, is an annual legume plant native to East Asia. In central Japan, seeds are sown in late June to early July, and the plant flowers in August. Soybeans begin to produce pods in September and pods gradually mature during autumn. Several symbiotic bacteria species, including *Bradyrhizobium japonicum*, *B. elkani*, and *Rhizobium fredii*, form root nodules on soybean roots.

One of the dominant insect herbivores on soybean in Japan is the soybean aphid, *Aphis glycines*. Soybean aphids overwinter as eggs, and hatch in spring at 10°C (Wang *et al.*, 1962). They feed on phloem sap from stems and leaves. The developmental time from first instar to adult is 7-10 days in an outdoor climate chamber (25°C, natural light condition) (A. O. Silva, personal observation). The aphid has approximately 15 generations per year in a soybean field (Wang *et al.*, 1962), and populations often exceed 1,000 individuals on a single soybean seedling (A. O. Silva, personal observation).

In March 2008 one soybean aphid clone was provided by Laboratory of Applied Entomology, Faculty of Agriculture, Utsunomiya University, and was reared on potted soybeans in an outdoor climate chamber (25°C and 12L12D photoperiod) for one year. To maintain the aphid clone, at least 12 pre-germinated seeds of soybean were planted in polyethylene pots (7 cm in diameter, 7 cm in depth) with non-sterilized soil (Hana To Yasai No Baiyoudo®, Tachikawa Heiwa Nouen Co., LTD.) per week, and the seedlings were cultivated in an outdoor climate chamber (25°C and natural light conditions) for two weeks. Twelve potted seedlings were placed in one plastic cage (30 cm in wide, 40 cm in length, 30 cm in depth), and 10-20 aphids were released into the cage. The cage was covered by a plastic net to prevent aphids from escaping, and was placed in an incubator (25°C and 16L 8D photoperiod) to maintain the aphid colony. After two weeks, 10-20 aphids were collected from the cage, and were released into another cage with new 12 soybean seedlings as same method mentioned above. Eight cages were prepared, and this procedure was repeated every three weeks.

Experimental design

On 22nd of September 2008, 200 soybean seeds were planted individually into polyethylene pots (7 cm in diameter, 7 cm in depth) with non-sterilized soil (Hana To Yasai No Baiyoudo®). After two weeks, individual seedlings were transplanted into large polyethylene pots (20 cm in diameter, 20 cm in depth, containing 5 L of soil). Seedlings were grown in outside temperature-controlled greenhouses (25°C and natural light conditions) until the beginning of the experiment.

One month after germination, 10 potted plants of similar size were selected. Each pot was covered with a nylon net (mesh-size: 1mm), to prevent dispersal of aphids and colonization of other insects. Plastic sticks of 90 cm length with three wire rings were set into pots to hold the nets. One thousand aphids were inoculated on each of five plants (aphid-infected plants), and the remaining five plants were set as control without aphids (aphid-free plants). These two treatments were placed randomly in the outdoor climate chamber (25°C and natural light condition) in three rows. Rows were spaced at 50 cm intervals and each pot within a row was spaced at 20 cm intervals. These plants were adequately watered everyday.

The number of aphids on each plant was checked every week to maintain the fixed number (1000 individuals), and any additional aphids were removed using a fine brush. After removing surplus aphids, the rows of pots were randomly rearranged to minimize microhabitat effects.

One month after aphid inoculation, all aphids were removed from the plants. The plants were maintained until death (5-6 months from cultivation) to collect naturally senesced litter. The collected litter of each plant was put in paper sacks, and was stored for drying (24°C, 26% RH). When all litter had been collected, it was freeze-dried and weighed.

Chemical analysis of litter

Freeze-dried litter (300 mg per sample) was powdered using an electric blender

(Wonder Blender, Osaka Chemical, Osaka, Japan). Carbon and nitrogen concentrations in 20 mg of litter were determined by an elemental analyzer (CHN Corder MT-3, Yanaco, Kyoto, Japan).

Total phenolics in powdered litter (20 mg) were extracted with 50% methanol: water (5 ml) in a test tube using a sonicator (40°C, 60 min). The test tube was centrifuged (3,500 rpm, 10 min) and the methanol extract was separated from the residue. Phenolics were extracted twice per sample (2 x 5 mL). Total phenol concentration (mg g^{-1}) was calculated using the Folin-Ciocalteu method (Julkunen-Tiitto, 1985), by measuring absorbance of the light at 760 nm with a spectrophotometer (Shimadzu UV-1200).

T-tests were used to compare litter biomass, and C/N ratio and phenolics concentrations in the litter between treatments.

Litterbag experiment

On 9th of June 2009, 300 mg of each litter sample was placed in an 8 cm x 8 cm polyethylene fabric fine-meshed litterbag. Two samples of each replicate of the aphid-inoculated and aphid-free (control) treatments were prepared, for a total of 20 litterbags (2 treatments x 5 replications x 2 collections). Thirty plastic pots (12 cm in diameter and 10 cm in depth) were filled with 150 g of non-sterilized soil (Hana To Yasai No Baiyoudo®), and each litterbag was placed on the soil surface in a plastic pot. The pots were weighted to check initial weight. The pots were watered once a week to keep the initial pot weight and soil humidity constant (60% on average). Then, the pots were placed in an incubator (25°C, in a dark condition) and randomly arranged to remove any effects of spatial arrangements of pots. After one and three months of incubation, one litterbag from each replicate was collected. The litter in the bag was freeze-dried, and dry mass was measured as a decomposition index. Then, the litter was powdered for measurements of carbon and nitrogen content. Carbon and nitrogen concentration in 20 mg of dry litter was determined using an elemental analyzer (CHN Corder MT-3, Yanaco, Kyoto, Japan).

A relative amount of litter mass (RLM, %) was calculated using following formula:

$$RLM\ (\%) = LM_t / LM_0 \times 100$$

LM_0 and LM_t represent the initial litter mass (300 mg) and the litter mass of the t-th collection, respectively. In addition, the relative amounts of carbon mass (RCM, %) and of nitrogen mass (RNM, %) were calculated using following formula:

$$RCM\ (\%) \text{ or } RNM\ (\%) = [\text{Concentration}]_t \times LM_t / [\text{Concentration}]_0 \times LM_0 \times 100$$

$[\text{Concentration}]_0$ represents the initial concentration of carbon or nitrogen in litter, and $[\text{Concentration}]_t$ represents the concentration of the t-th collection.

Since one litterbag in the aphid-free treatment was accidentally lost during the experiment, it was removed from statistical analyses. Repeated measures ANOVAs were used to compare the effect of aphids on RLM (%), nitrogen concentration, and RNM (%) through decomposition time and among treatments. Tukey-Kramer tests were conducted to examine significant differences between treatments for carbon and nitrogen concentrations and RNM (%) as a post-hoc test.

Results

Litter quality and quantity

Although aphids did not affect litter quantity ($t_8 = 1.32$, $P = 0.225$), litter quality was altered after aphid herbivory (Fig. 1). While there was no significant difference in carbon concentration between treatments ($t_8 = 0.33$, $P = 0.747$), nitrogen concentration was lower under aphid-infected plants than under aphid-free plants ($t_8 = 2.56$, $P = 0.034$). As a result, the litter

C/N ratio was greater under aphid-infected plants (t_8 = 2.90, P = 0.020). In addition, litter phenolic concentration was greater under aphid-infected plants than under aphid-free plants (t_8 = 2.75, P = 0.025).

Litter decomposition process

Although litter mass decreased to less than 50% of the initial mass in both treatments three months after the commencement of the experiment, there was no significant difference between treatments (time: $F_{1,7}$ = 47.01, P < 0.001, treatment: $F_{1,7}$ = 3.30, P = 0.112, time x treatment: $F_{1,7}$ = 0.12, P = 0.739; Fig. 2).

There was a significant difference in litter carbon concentration between treatments, and we found a significant time x treatment interaction (time: $F_{1,7}$ = 1.51, P = 0.294, treatment: $F_{1,7}$ = 37.15, P = < 0.001, time x treatment: $F_{1,7}$ = 8.88, P = 0.016; Fig. 3a). Although little change in litter carbon was observed in the aphid-infected plants, the carbon concentration of litter in the aphid-free plants decreased after one month. However, there was no significant difference in relative carbon mass between treatments, and we found no significant time x treatment interaction (time: $F_{1,7}$ = 37.0, P < 0.001, treatment: $F_{1,7}$ = 0.15, P = 0.711, time x treatment: $F_{1,7}$ = 1.32, P = 0.288; Fig. 3b).

There was no significant difference in litter nitrogen concentration between treatments, but we found a significant time x treatment interaction (time: $F_{1,7}$ = 111.58, P < 0.001, treatment: $F_{1,7}$ = 0.82, P = 0.396, time x treatment: $F_{1,7}$ = 58.18, P < 0.001; Fig. 4a). The initial nitrogen concentration of litter from aphid-infected plants was significantly lower than that from aphid-free plants. The nitrogen concentration of litter under aphid-infected plants increased after one month, although little change in litter nitrogen was observed under the aphid-free plants. In addition, the relative litter nitrogen mass under aphid-infected plants was significantly greater than that under aphid-free plants one and three months later (time: $F_{1,7}$ = 2.06, P = 0.194, treatment: $F_{1,7}$ = 36.14, P < 0.001, time x treatment: $F_{1,7}$ = 0.71, P = 0.427; Fig. 4b). This

suggests that nitrogen mineralization of litter was decelerated under soybeans infected by aphids.

Discussion

There is a growing body of evidence that herbivorous insects can have significant consequences on decomposition processes through modification of plant litter (Chapman *et al.*, 2003, 2006; Schweitzer *et al.*, 2005; Kay *et al.*, 2008). However, our knowledge of the effects of herbivorous insects on the decomposition process is limited, and the effects of most insects have been poorly explored. The present study clearly demonstrated that aphid-infected soybeans produced litter with low nitrogen and high phenolic content, and nitrogen was retained longer in such litter than in litter from aphid-free plants. These results suggest that aphids decelerated nitrogen mineralization by decreasing litter quality.

Effect of aphids on litter nitrogen

Aphids are small sap-feeding insects belonging to a group of Aphididae. They are distributed worldwide, and are one of the most successful insects especially in temperate regions (Dixon, 1998). Nymphs and adults feed on phloem sap, which is rich in sugar but typically lacking in essential amino acids (Douglas, 1998). To compensate for the nitrogen shortage, aphids ingest large amount of phloem sap, and excrete surplus sugars as honeydew after absorbing nitrogen (Dixon, 1998; Douglas, 1998). The functions of this sugar-rich honeydew have been well studied, and are recognized to have prominent roles in attracting mutualistic ants (Stadler & Dixon, 1998, 2005), and/or in causing microbial immobilization in soil (Wardle, 2002).

The ability of aphid feeding to induce changes in the traits of their host plants is well documented (Moran & Whitham, 1990; Petersen & Sandström, 2001; Ando & Ohgushi, 2008). Several studies reported that aphid feeding could temporarily increase soluble nitrogen content

or amino acids in phloem sap and/or leaves (Dorschner *et al.*, 1987; Telang *et al.*, 1999; Sandström *et al.*, 2000; Petersen & Sandström, 2001). If changes in nitrogen content persist until leaf senescence, then nitrogen contents in litter produced by aphid-infected plants may increase. However, our results showed an opposite trend, i.e. litter nitrogen decreased as a result of the aphid infection (Fig. 1c).

This result may be due to the effects of aphids decreasing nitrogen uptake in the plants (N. Katayama *et al.*, in preparation). In our previous study, we determined that a soybean seedling infected by 1000 individuals of soybean aphids added an average of 2.6 mg L^{-1} d^{-1} of sugars to potting soil. This amount would be enough to induce microbial immobilization, which results in a decrease in inorganic soil nitrogen (Dighton, 1978; Schmidt *et al.*, 1997). Actually, the amount of inorganic soil nitrogen was 7-fold less in the presence of aphids, and the plants decreased nitrogen absorption from the soil. Furthermore, the aphids can also decrease nitrogen uptake in plants through an alternative path, i.e., by weakening the association of nitrogen-providing rhizobia with the plants. Aphid-infected plants had nitrogen uptake decreased up to 67%, compared to plants without aphids (N. Katayama *et al.*, in preparation). Decreased nitrogen uptake may result in aphid-infected plants producing litter with a lower nitrogen concentration (Fig. 1c).

Effect of aphids on litter phenolics

Many plants increase the concentration of secondary metabolic substances in their tissues in response to insect herbivory (Karban & Baldwin, 1997). The metabolic substances are sometimes retained in litter (Schweitzer *et al.*, 2005; Kay *et al.*, 2008). For example, Schweitzer *et al.* (2005) found that *Populus* trees produced leaf litter with 39-47% higher phenolics concentration in response to herbivory of gall-making aphids. Our result (i.e., litter phenolics of aphid-infected plants increased by 20%; Fig. 1e) is similar to these findings. However, the observed increase in litter phenolics can be explained by an alternative hypothesis based the

trade-off between nitrogen and phenolics content (Jones & Hartley, 1999). Since there is a negative correlation between phenolics and nitrogen in plant tissues in general (Bryant *et al.*, 1983; Jones & Hartley, 1999), we suspect that decreased nitrogen uptake might cause an increase in leaf phenolics. Although this study did not examine which mechanisms are responsible for the increase in litter phenolics, regardless of mechanism, aphid herbivory increased the litter phenolics (Fig. 1e).

Effect of aphids on litter decomposition

Litter quality is likely to influence litter mineralization in soil, and in general, litter nitrogen generally promotes the mineralization process (Belyea, 1996; Bridgham & Richardson, 2003; Bragazza *et al.*, 2007; Manzoni *et al.*, 2008), whereas litter phenolics inhibit it (Palm & Sanchez, 1991; Schimel *et al.*, 1998). The decomposition experiment in this study illustrated that the aphid-induced litter modification did not affect the temporal change in relative litter mass (Fig. 2). This is because the mineralization of litter carbon, which was the majority of litter contents, was not affected by the aphid-induced modification (Fig. 3b). In contrast, the aphid-induced modification affected nitrogen mineralization in the litter (Fig. 4b). As compared with initial nitrogen content, nitrogen decreased by 50% in the litter of aphid-free plants over the course of one month. On the other hand, 70% of nitrogen was retained in the litter produced by aphid-infected plants after one month, and even after three months, 64% of nitrogen remained. These results suggest that aphids decelerated litter nitrogen mineralization by decreasing litter quality.

Aphids are dominant herbivores in natural systems, and are also an important pest in agricultural systems. Many researchers have reported that aphids cause plant damage, which results in low growth rates and even death, and a decrease in the ecosystem productivity or vegetable yields (reviewed by Dixon, 1998). In addition, aphids decrease nutrient uptake by plants, which influences ecosystem productivity and dictates dynamics of organisms utilizing

the plants (N. Katayama *et al.*, in preparation). However, we know little about the effects of aphids on ecosystem functioning. In this study, we demonstrated that aphids could significantly influence litter decomposition process via the modification of litter quality. These findings indicate that aphids negatively affect nitrogen flux in an ecosystem.

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Contribution of authors

N. Katayama and A. O. Silva originally formulated the experimental design, and performed the experiments. O. Kishida and T. Ohgushi developed the methodology. N. Katayama analyzed the data and wrote the manuscript.

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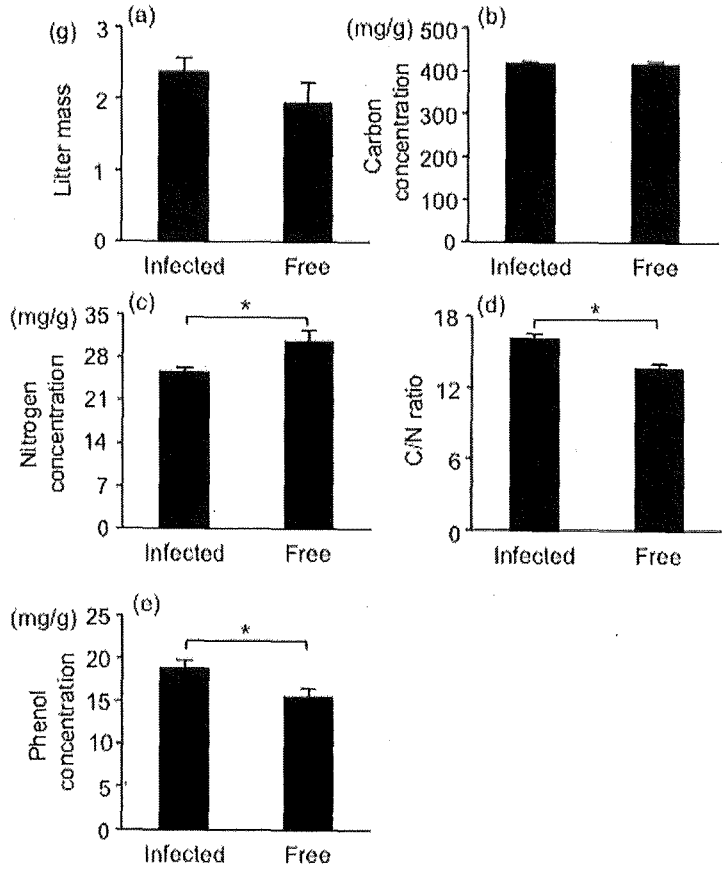
Figure legends

Fig. 1. Quantity and quality of litter produced by aphid-infected and aphid-free plants. (a) Dry mass, (b) carbon concentration, (c) nitrogen concentration, (d) C/N ratio, and (e) total phenolics concentration. Asterisks represent significant differences between treatments (*$P < 0.05$). Error show SE.

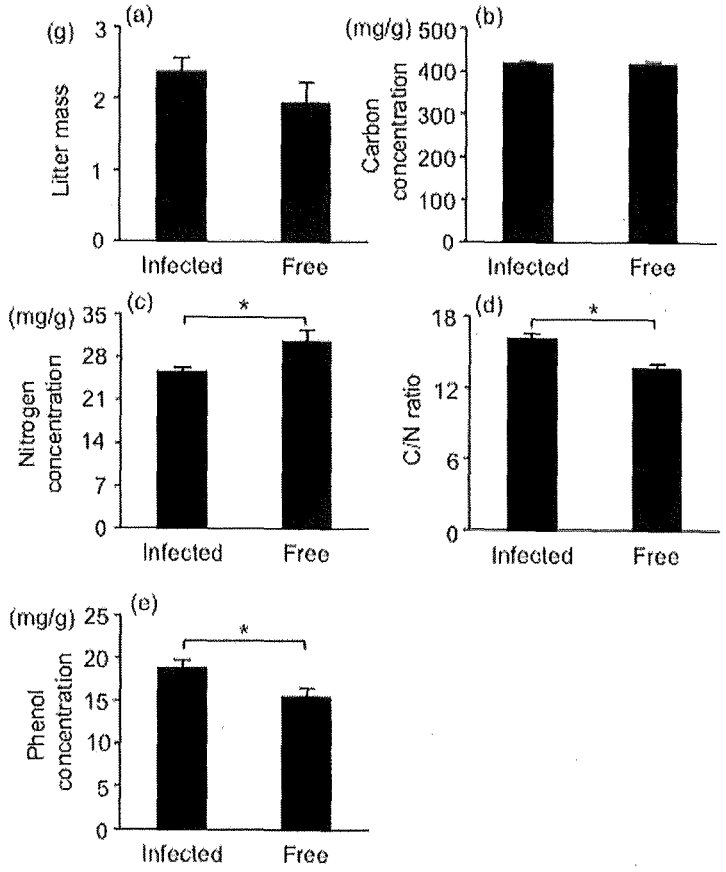
Fig. 2. Relative litter mass (RLM, %). Open diamonds with dotted lines and solid diamonds with solid lines indicate litter from aphid-free and aphid-infected plants, respectively. Error bars show SE.

Fig. 3. (a) Carbon concentration and (b) relative carbon mass (RCM, %) of litter. Open diamonds with dotted lines and solid diamonds with solid lines indicate litter produced by aphid-free and aphid-infected plants, respectively. Different letters represent significant differences among treatments ($P < 0.05$). Error bars show SE.

Fig. 4. (a) Nitrogen concentration and (b) relative nitrogen mass (RNM, %) of litter. Open diamonds with dotted lines and solid diamonds with solid lines indicate litter produced by the aphid-free and aphid-infected plants, respectively. Different letters represent significant differences among treatments ($P < 0.05$). Error bars show SE.



Katayama et al. Fig. 1



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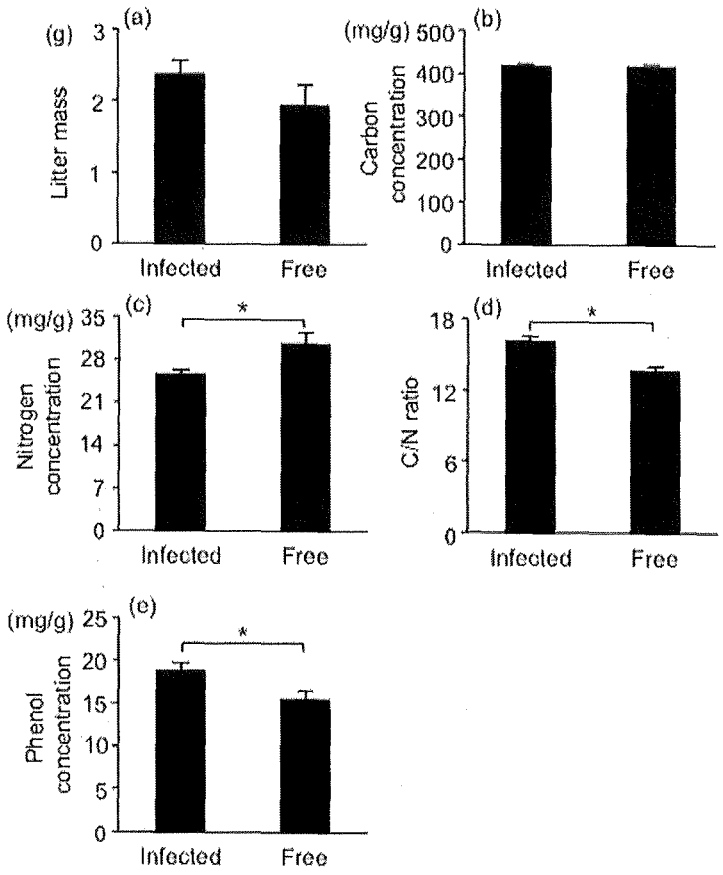

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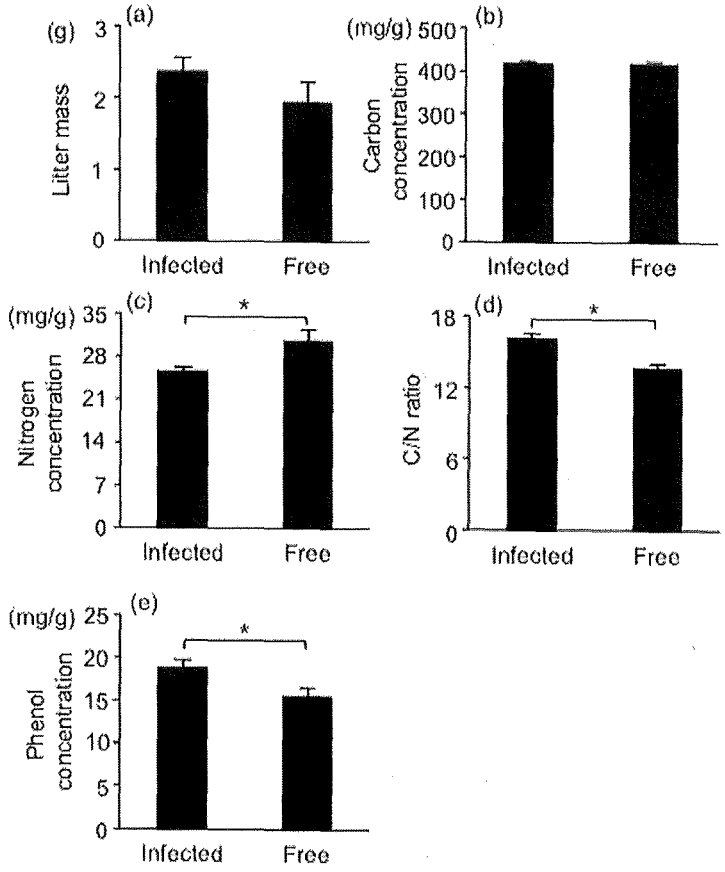

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