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Herbivorous insect decreases plant nutrient uptake: the role of soil nutrient availability and association of below-ground symbionts

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Abstract. 1. Plants take nutrients from the rhizosphere via two pathways: (i) by absorbing soil nutrients directly via their roots and (ii) indirectly via symbiotic associations with nutrient-providing microbes. Herbivorous insects can alter these pathways by herbivory, adding their excrement to the soil, and affecting plant–microbe associations.

2. Little is known, however, about the effects of herbivorous insects on plant nutrient uptake. Greenhouse experiments with soybean, aphids, and rhizobia were carried out to examine the effects of aphids on plant nutrient uptake.

3. First, the inorganic soil nitrogen and the sugar in aphid honeydew between aphid-infected and -free plants were compared. It was found that aphid honeydew added 41 g m^{-2} of sugar to the soil, and that aphids decreased the inorganic soil nitrogen by 86%. This decrease may have been caused by microbial immobilisation of soil nitrogen followed by increased microbial abundance as a result of aphid honeydew.

4. Second, nitrogen forms in xylem sap between aphid-infected and -free plants were compared to examine nitrogen uptake. Aphids decreased the nitrogen uptake via both pathways, and strength of the impact on direct uptake via plant roots was greater than indirect uptake via rhizobia. The reduced nitrogen uptake by the direct pathway was as a result of microbial immobilisation, and that by the indirect pathway was probably because of the interaction of microbial immobilisation and carbon stress, which was caused by aphid infection.

5. The present results demonstrate that herbivorous insects can negatively influence the two pathways of plant nutrient uptake and alter their relative importance.

Key words. Honeydew, microbial immobilisation, nitrogen, rhizobia, soybean, xylem sap analysis.

Introduction

Nutrient uptake by plants is a fundamental ecosystem process that determines both the biomass and properties of the plants and thus dictates the dynamics of organisms that utilise them (Wardle, 1992). Plant roots absorb soil nutrients directly from the soil, and plants also take up soil and atmospheric nutrients indirectly via symbiotic interactions with microbes in the rhizosphere (Smith & Read, 1997; Patriarca *et al.*, 2002). These two nutrient uptake pathways have been intensively but separately studied. Recently, researchers have begun to assess the relative importance of these two pathways (Wardle, 2002; Wardle *et al.*, 2004) and interactions between them. For example, nutrient uptake via microbial symbionts may decrease with increasing the amount of soil nutrients (Katayama *et al.*, 2010). Although previous studies have documented the spatial and temporal

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variations in nutrient uptake by plants (Smith & Read, 1997; Dessureault-Rompré *et al.*, 2007), factors affecting nutrient uptake via the two pathways and their relative importance have received little attention. It is critical to examine these factors, to deepen our understanding of plant nutrient dynamics and to gain insight into nutrient cycling between above- and below-ground ecosystem components.

Insects are a dominant component of terrestrial ecosystems, in terms of abundance and biodiversity (Schowalter, 2000), but their importance in controlling ecosystem functions (e.g. decomposition and nutrient cycling) has long been overlooked; recently researchers have begun to pay more attention to the roles that insects play in ecosystem functioning (Weisser & Siemann, 2004). In particular, herbivorous insects affect decomposition processes by influencing the quantity and quality of plant litter, and their excrement adds nutrients to the soil (Hunter, 2001; Wardle, 2002; Frost & Hunter, 2004; Schweitzer *et al.*, 2005; Kay *et al.*, 2008). Herbivorous insects frequently increase the tannin content of plant litter, which inhibits microbial activity and thus indirectly slows the decomposition rate (Chapman *et al.*, 2003; Schweitzer *et al.*, 2005; Kay *et al.*, 2008). Several studies examining how herbivorous insects affect nutrient uptake by plants have focused on the direct absorption of soil nutrients by plant roots (Stadler *et al.*, 1998; Frost & Hunter, 2004). Insect excrement (i.e. frass or honeydew) in the soil also contains nutrients that can be utilised by plants after mineralisation (Weisser & Siemann, 2004). Because insect frass contains high concentrations of labile carbon (sugars) and nitrogen (ammonium and nitrate) (Wardle, 2002), it can either accelerate or decelerate nitrogen mineralisation, depending on the C : N ratio of the frass (Kagata & Ohgushi, 2012). Nitrogen-rich frass can accelerate nitrogen mineralisation and thus increase the inorganic nitrogen content of the soil, whereas nitrogen-poor frass can slow the rate of nitrogen mineralisation by inducing microbial nitrogen immobilisation: an increase in belowground microbial biomass that take up inorganic nitrogen from the soil (Kagata & Ohgushi, 2012). Because the amount of frass added to the soil is often large (Seastedt & Crossley, 1984; Stadler *et al.*, 2004) and its effect on soil nutrient dynamics becomes apparent within a few days (Lovett & Ruesink, 1995; Hunter, 2001), frass can be an important factor determining nutrient uptake dynamics in plants (Frost & Hunter, 2004).

Herbivorous insects also influence nutrient uptake by plants through their effects on plant associations with microbial symbionts. Insect herbivory can either positively or negatively affect colonisation and metabolic activity of microbial symbionts on plant roots (Gehring & Whitham, 1994; Nishida *et al.*, 2009) by two mechanisms. First, insect herbivory changes the nutrient status of plants (Karban & Baldwin, 1997; Ohgushi, 2005). When herbivorous insects consume plant tissues, the plants cannot adequately reward symbionts with photosynthetic carbon, thus decreasing symbiont activity. Because symbionts need photosynthetic carbon to be active, insect herbivory may decrease the activity of the symbionts. The second mechanism concerns an effect of nutrients added the soil in insect excrement (Hunter, 2001). The strength of plant–symbiont associations weakens with increasing soil nutrients because the plants are able to obtain enough nutrients directly from the soil (Katayama *et al.*, 2010). As insect frass can increase or decrease inorganic soil nutrients (Kagata & Ohgushi, 2012), we expect that the frass influences the nutrient flow from the symbionts to the host plants.

This study had two objectives. First, we aimed to examine the effects of herbivorous insects on plant nutrient uptake via the two pathways: direct absorption from the soil by plant roots and indirect uptake via associated symbionts. Our second objective was to determine whether and how herbivorous insects changed the relative importance of these two pathways. For this purpose, we conducted greenhouse experiments with a model system consisting of a soybean [*Glycine max* (L.)] and soybean aphids (*Aphis glycines* Matsumura), which is a suitable system for investigating the relative importance of the two hypothesised nutrient uptake pathways for the following reasons (Dixon, 1998; Stadler *et al.*, 2004): (i) the soybean has a mutualistic association with rhizobia that provide nitrogen to the plant, (ii) the aphids are a dominant herbivore on the soybean, and (iii) the aphids excrete sugar-rich honeydew, which may influence the soil nutrient dynamics.

We hypothesise that the honeydew excreted by the aphids decreases the inorganic nitrogen content of the soil: because the abundance of free-living below-ground microbes is often labile-carbon limited, the addition of honeydew to the soil may increase the abundance of such microbes and thus indirectly decrease the soil inorganic nitrogen content to decrease by microbial immobilisation (Dighton, 1978; Grier & Vogt, 1990; Stadler *et al.*, 2004). Thus, the honeydew addition is likely to decrease nitrogen uptake from the soil by plants. In contrast, it may increase the nitrogen uptake via rhizobia, because when soil inorganic nitrogen is low, plants depend more on rhizobia for nitrogen (Katayama *et al.*, 2010). Alternatively, we hypothesise that aphids may decrease plant nitrogen uptake via rhizobia. Photosynthetic carbon is necessary for plants to maintain an association with rhizobia. However, aphids, may produce carbon stress in plants by consuming photosynthetic carbon (Macedo *et al.*, 2003), causing the plants to have difficulty maintaining the rhizobial association. Our system enables us to verify these hypotheses by separately measuring nitrogen derived from rhizobia and soil (Giller, 2001). Soybean xylem sap contains three forms of nitrogen: ureides (allantoin and allantoic acid), amino acids (mainly asparagine and glutamine), and nitrate (Matsumoto *et al.*, 1977). Nitrogen derived from rhizobia is transformed into ureides in root nodules, and is transported through xylem vessels (Streeter, 1979), whereas nitrogen absorbed from the soil is mainly transported in the form of nitrate and amino acids (Thomas & Sodek, 2006).

We carried out two experiments to test these hypotheses. The first experiment quantified the amounts of sugars from honeydew and inorganic nitrogen in soil between treatments with and without aphids on potted soybean plants. The second experiment measured the amounts of nitrogen (N) in the form of ureides (ureide-N, primary form of rhizobia-derived nitrogen) and nitrate and amino acid (nitrate-N and amino acid-N, nitrogen absorbed mainly from the soil) in plants with and without aphids.

Materials and methods

Materials

Soybean, *G. max*, is an annual leguminous plant native to East Asia. In central Japan, seeds germinate in late June to early July, and the plant flowers in August. Soybean plants begin to produce pods in September, and pods gradually mature during autumn. Several symbiotic bacterial 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, *A. glycines*. The aphid overwinters as eggs and hatches in spring (Wang *et al.*, 1962). It feeds 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 conditions) (A. O. Silva, pers. obs.). The aphid has approximately 15 generations per year in a soybean field, and the population often exceeds 1000 individuals on a single soybean seedling (A. O. Silva, pers. obs.).

To culture the aphids we inoculated one clone of soybean aphids to potted soybeans in an outdoor climate chamber (25 °C; photoperiod, LD 12:12 h). The aphids were provided by the Laboratory of Applied Entomology, Faculty of Agriculture, Utsunomiya University, Tochigi Prefecture, Japan, and maintained for 2 years. To maintain the aphid colony, we sowed at least 100 pre-germinated seeds of soybean individually in polyethylene pots (7 cm in diameter, 7 cm in depth) containing non-sterilised soil (Hana To Yasai No Baiyoudo®; Tachikawa Heiwa Nouen Co., Ltd, Tochigi, Japan) every 2 weeks, and cultivated them in an outdoor climate chamber (25 °C and natural light). After 2 weeks, we prepared eight plastic cages ($30 \times 40 \times 30$ cm^3 deep), and placed 12 potted seedlings in each. We inoculated 10–20 aphids to the soybean seedlings per cage, which was covered with a plastic net to prevent the aphids from escaping and put in an incubator (25 °C; photoperiod, LD 16:8 h). After 2 weeks, we collected aphids from the cages and released 10–20 aphids into each of eight other cages with 12 new seedlings obtained as described above. We repeated this procedure for 2 years.

Plant cultivation

For the following experiments, 200 soybean seeds were sown individually in polyethylene pots (7 cm in diameter, 7 cm in depth) containing non-sterilised soil (Hana To Yasai No Baiyoudo®) each on 22 September 2008 (Experiment 1) and on 23 July 2009 (Experiment 2). After 2 weeks, we transplanted each seedling into a large polyethylene pot (20 cm in diameter, 20 cm in depth, each containing 5 litres of soil). Seedlings were grown in an outside temperature-controlled greenhouse (25 °C and natural light conditions) until the beginning of the experiments.

Experiment 1: effect of aphids on soil inorganic nitrogen

Experiment 1 was designed to evaluate the effect of aphids on the concentrations of total and inorganic nitrogen in the soil of the pots with soybean plants. We also measured the sugar content of the honeydew excreted by aphids during this experiment.

Experimental design. On 29 October 2008, we selected 10 potted plants of a similar size, and inoculated 1000 aphids on each of five plants (aphid-infected treatment), and the remaining five plants were cultivated without aphids as the control (aphid-free) treatment. Each pot was covered with a nylon net (mesh size, 1 mm) to prevent dispersal of the aphids and colonisation by other insects. We inserted 90-cm-long plastic sticks supported by three wire rings into each pot to hold the net. We placed the pots of the two treatments randomly in three rows in an outdoor climate chamber (25 °C and natural light conditions). The rows were spaced 50 cm apart, and the pots within each row were spaced 20 cm apart. All plants were adequately watered every day.

We counted the number of aphids on each plant one every week to maintain a fixed number (1000 individuals), and used a fine-bristle brush to remove any additional aphids, being careful not to drop the aphids. One a week, we randomly rearranged the rows of pots to minimise microhabitat effects.

Two and 4 weeks after the aphid inoculation, we placed a wire ring (20 cm in diameter, 3.14×10^{-4} m^2 in area) covered with an aluminium foil disc 5 cm above the soil surface on each pot for collecting honeydew. After 24 h, we removed the disc and put it in a nylon bag. The nylon bag was stored in the laboratory at room temperature. Within 24 h of the discs with honeydew being collected, each disc was rinsed three times with 5 ml of xylose solution (0.05 μg μl^{-1}) (i.e. a total of 15 ml of xylose solution per sample). The rinsing solution was filtered through a Millipore filter (0.20 μm), and 1.0 ml of the filtered solution was transferred to a 1.5-ml tube. The samples of filtered solution were kept in a freezer at −20 °C until chemical analysis.

One month after the aphid inoculation, we used a vinyl chloride coring tube with a diameter of 2 cm to collect the top 5 cm of soil (about 10 g) in each pot. Each soil sample was put in a nylon bag (14 cm long × 10 cm wide) and stored at −20 °C until they were analysed.

Chemical analysis of aphid honeydew. Sugar concentrations in the collected honeydew were analysed by high-performance liquid chromatography, using a Wakosil 5NH_2-MS packed column (4.6×150 mm^2; Wako Pure Chemical, Osaka, Japan) and an 80% acetonitrile mobile phase at room temperature. Area sizes of the different types of sugar present in the honeydew were determined using a refractive index detector (RID; Shimadzu Corp., Kyoto, Japan). The concentrations of the sugars in honeydew were then corrected according to the internal standard (xylose). See Whitaker *et al.* (2014) in detail for the sugar analysis.

Soil chemical analysis. After manual removal of plant litter and macro-organic matters in the collected soil, we added 5 g of each soil sample to a polypropylene bottle (volume, 100 ml) containing 50 ml of KCl solution (1.5 N). The bottle was shaken for 12 h at 200g, and then the solution was filtered through

a No. 2 Whatman filter. The chemical composition was determined using an ammonium and nitrate analyser (FUTURA; Alliance Instruments, Cedex, France) to measure the concentration of inorganic nitrogen (i.e. ammonium-N + nitrate-N) in the soil samples. The remaining soil was freeze-dried for total nitrogen analysis. We sieved the samples through a 1-mm mesh to remove debris and litter, and determined the total nitrogen concentration in 200 mg of dry soil using an elemental analyser (CHN Corder MT-3; Yanaco, Kyoto, Japan).

Experiment 2: effect of aphids on nitrogen uptake via the two pathways

We determined how much of the nitrogen in the soybean plant had been absorbed directly from the soil and how much was obtained via rhizobia by analysing the contents of the different forms (ureide-N, amino acid-N, and nitrate-N; 'ureide assay') of nitrogen in the xylem sap of plants cultivated with and without aphids. Although amino acids are found in xylem sap of nodulated soybeans in nitrogen-free soil, the majority of amino acid-N (more than 80%) in xylem sap is derived from soil (McClure & Israel, 1979). Thus, the 'ureide assay' can evaluate the relative contribution of N-fixation by rhizobia (Giller, 2001; see Introduction).

Experimental design. On 4 August 2009, we selected 20 plants of a similar size. We inoculated 1000 aphids to 10 plants (aphid-infected plants), and used 10 non-inoculated plants as the control (aphid-free plants). The fixed number of aphids (1000 individuals) was maintained as in the same manner mentioned above.

One month after the inoculation with aphids, we clipped the plants at 5 cm above ground level and covered the cut surface of the stem with 1 g of cotton wool wrapped in cellophane film to prevent evaporation of the xylem sap. After 5 h, the cotton wool was removed from each plant and weighed. We collected the xylem sap from the cotton by placing the cotton in a 100-ml centrifuge tube and centrifuging it at $1300 \times \boldsymbol{g}$ force for 1 h. We then determined the xylem sap flow using the following formula:

$$\text{Xylem sap flow } \left(\text{g h}^{-1}\right) = \left[\text{cotton weight after 5 h (g)} - \text{initial cotton weight (g)}\right] / 5\text{h}$$

The collected xylem sap samples were filtered through a Millipore filter (0.20 µm), transferred into 1 ml tubes, and stored in a freezer at −20 °C until chemical analysis. A separate 50-µl subsample of xylem sap was used to determine the amount of each form of nitrogen. Ureide-N, amino acid-N, and nitrate-N were determined using the Young–Conway method (Young & Conway, 1942), the ninhydrin method (Herridge, 1984), and Cataldo's method (Cataldo *et al.*, 1975), respectively. We were unable to analyse the nitrogen composition for one aphid-free and four aphid-infected plants because each of the collected sample volumes was less than 150 µl.

Statistical analysis

Repeated measures ANOVA was used to compare the total amount of sugar on the aluminium foil discs between aphid-free and aphid-infected treatments. To meet parametric assumptions, the values were log(n + 1)-transformed. The concentrations of total soil nitrogen and inorganic soil nitrogen (ammonium-N + nitrate-N), xylem sap flow during the 5 h, and concentrations of soil-derived nitrogen (nitrate-N + amino acid-N) and rhizobia-derived nitrogen (ureide-N) in xylem sap were compared between treatments using *t*-tests. To examine the relative impact of aphids on the direct and indirect pathways of nitrogen uptake, MANOVA was performed using the concentrations of soil-derived nitrogen and rhizobia-derived nitrogen in xylem sap as dependent variables. A significant interaction between 'aphid effect' and 'nitrogen type' means that the aphids influence the relative impacts of the two nutrient uptake pathways.

We determined the magnitude of the aphid effect on the amount of each form of nitrogen by calculating the log response ratio (i.e. ln[treatment/control]), which is widely used to compare effect magnitudes in manipulation experiments (Hedges *et al.*, 1999): ln(treatment/control) < 0 means that the effect is negative relative to the control effect, and ln(treatment/control) > 0 means that the effect is positive. Separate bootstrap models were used to calculate mean and 95% CI of the log response ratios of soil- and rhizobia-derived nitrogen from 9999 resampling iterations in each bootstrap model.

Results

Experiment 1: sugar content of honeydew and inorganic nitrogen content of the soil

We identified six forms of sugar on the aluminium foil discs: fructose (mean ± SD, 24.8 ± 4.7% w/w), glucose (17.4 ± 7.4%), sucrose (23.7 ± 5.4%), maltose (6.4 ± 4.9%), trehalose (3.0 ± 3.8%), and melezitose (24.7 ± 6.9%). The total sugar amount differed significantly between treatments, although the sugar amount after 4 weeks was less than after 2 weeks (repeated measures ANOVA: time, $F_{1,8} = 17.00$, $P < 0.001$; aphid, $F_{1,8} = 303.16$, $P < 0.001$; time × aphid, $F_{1,8} = 16.25$, $P = 0.004$; Fig. 1). While the sugar amount in the aphid-free treatment was very low (<0.12 mg) after both 2 and 4 weeks, 20.6 and 6.0 mg of sugar was detected in the aphid-infected treatment after 2 and 4 weeks, respectively. It suggests that the sugars in the aphid-infected treatments were derived from honeydew excreted by the aphids.

The total soil nitrogen concentration did not differ between treatments (*t*-test: $t_8 = 1.24$, $P = 0.251$; Fig. 2a), but the inorganic soil nitrogen (ammonium-N + nitrate-N) concentration in the aphid-free treatment was 7.2 times greater than the aphid-infected treatment (*t*-test: $t_8 = 2.72$, $P = 0.026$; Fig. 2b).

Experiment 2: soybean nitrogen derived from soil and rhizobia

Although aphids did not affect the xylem sap flow during a 5-h collection period (*t*-test: $t_{18} = 1.52$, $P = 0.147$; Fig. 3a),

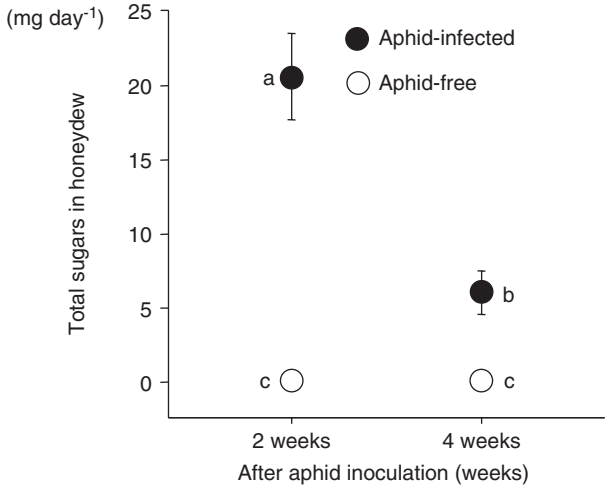

Fig. 1. Total sugar amount on the aluminium foil discs 2 and 4 weeks after aphid inoculation. Different letters indicate a significant difference between treatments (Tukey–Kramer test; $P < 0.05$). Error bars show SE.

we detected a significant aphid effect on nitrogen uptake by the plants (MANOVA: $F_{1,13} = 8.64$, $P = 0.012$; Fig. 3b). Also, there was a significant difference in amount between nitrogen forms (MANOVA: $F_{1,13} = 14.56$, $P < 0.001$). Although the aphids did not affect the nitrate-N concentration [aphid-free: 0.011 ± 0.004 (mean $\pm$ SE) mg ml^{-1}, aphid-infected: 0.019 ± 0.005 mg ml^{-1}; *t*-test: $t_{13} = 1.43$, $P = 0.176$], we detected a significant decrease in the amino acid-N concentrations in xylem sap (aphid-free: 0.47 ± 0.05 mg ml^{-1}, aphid-infected: 0.24 ± 0.05; *t*-test: $t_{13} = 12.13$, $P = 0.004$), indicating a significant decrease in nitrogen uptake from soil (nitrate-N + amino acid-N: *t*-test, $t_{13} = 3.29$, $P = 0.006$; Fig. 3b). Similarly, the aphids decreased rhizobia-derived nitrogen (ureide-N: *t*-test, $t_{13} = 2.69$, $P = 0.019$; Fig. 3b). A significant interaction between 'aphid effect' and 'nitrogen type' suggests that the aphids influenced the relative impacts of the two nutrient uptake pathways (MANOVA: $F_{1,13} = 5.601$, $P = 0.034$; Fig. 3b). The magnitude of the negative effect of aphids on the direct uptake of nitrogen from the soil was 1.4 times greater than uptake via rhizobia ($P < 0.001$, Fig. 3c).

Discussion

Plant roots can absorb soil nutrients directly from underground, and plants also take up soil and atmospheric nutrients indirectly via microbial symbionts in a rhizosphere. In this context, we know little about how insect herbivory affects nutrient uptake via these two pathways. We demonstrated (i) inorganic soil nitrogen and nitrogen uptake by the plants from the soil were decreased in the presence of aphids, (ii) nitrogen uptake via rhizobia was decreased in the aphid-infected plants, and (iii) the negative impact of the aphids on soil nitrogen uptake was greater than uptake via rhizobia. Our results clearly illustrated that herbivorous insects negatively influence both pathways of nutrient uptake in plants, directly from the soil and indirectly via symbiotic microbes, and that the negative effect of insect herbivory on the direct pathway from soil is greater than the indirect pathway via symbionts.

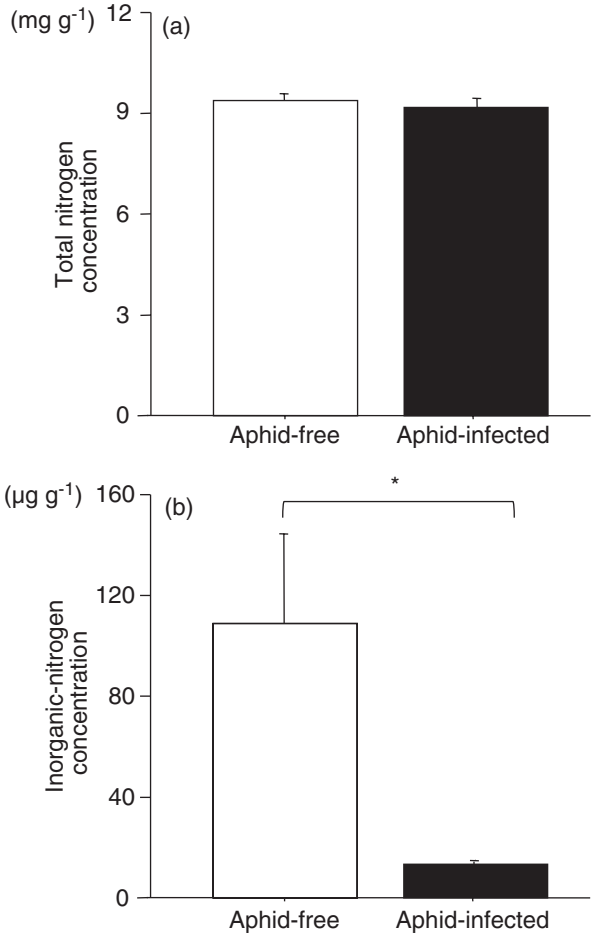

Fig. 2. (a) Total nitrogen and (b) inorganic nitrogen (ammonium-N + nitrate-N) concentrations in soil in the aphid-free and aphid-infected treatments. *$P < 0.05$, *t*-test. Error bars show SE.

Aphid effects on soil nitrogen and soil nitrogen uptake

In our experiment, aphids reduced the inorganic soil nitrogen sevenfold (Fig. 2b). As total soil nitrogen did not differ between the aphid-free and aphid-infected treatments (Fig. 2a), the aphids would not have added (or released) nitrogen to the soil. Instead, the decrease in inorganic soil nitrogen may be as a result of microbial nitrogen immobilisation in soil, through the addition of a large amount of aphid honeydew (Schmidt *et al.*, 1997). The aphid-infected treatment showed that on average, 13 mg of sugars from honeydew was added to 3.14×10^{-4} m^2 soil area every day (equivalent to 41 g m^{-2} per day, Fig. 1). This amount is large enough to induce microbial nitrogen immobilisation, although it may be underestimated owing to the potential presence of undetected macromolecular oligosaccharides in honeydew. For example, Dighton (1978) experimentally estimated the impact of honeydew on free-living soil microbes using

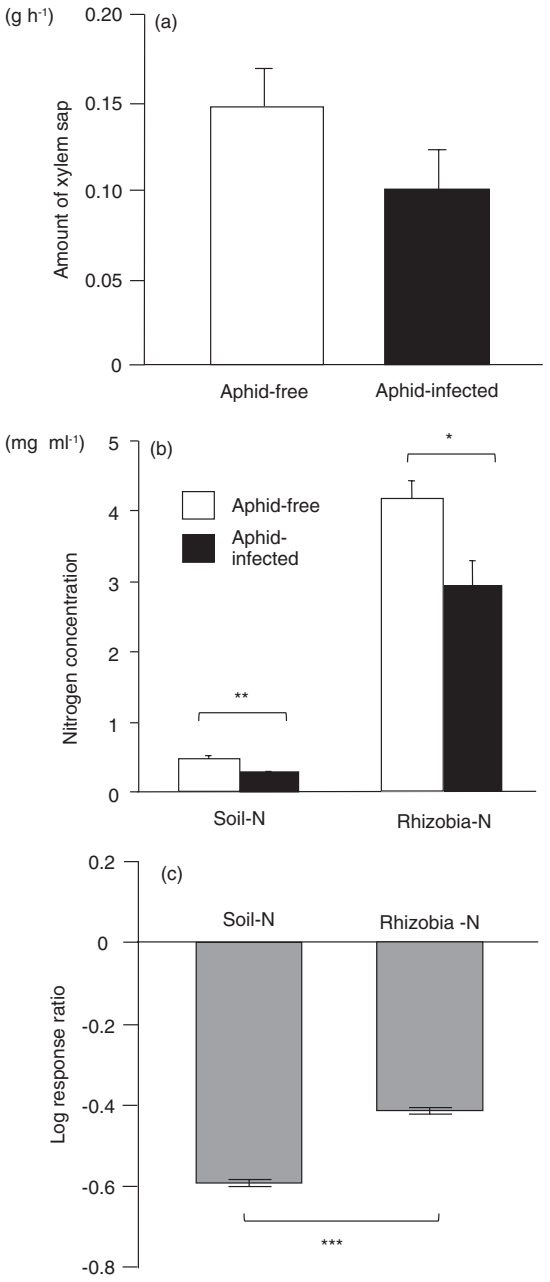

Fig. 3. (a) Xylem sap flow and (b) concentrations of nitrogen derived from soil (nitrate-N + amino acid-N) and from rhizobia (ureide-N) in xylem sap. $*P < 0.025$, $**P < 0.001$, *t*-test. Error bars show SE. (c) Magnitude of the aphid effect, expressed as the log response ratio, on the relative amounts of nitrogen derived from soil and nitrogen from rhizobia. $***P < 0.001$, *t*-test. Error bars show 95% CI.

a sugar solution that mimicked aphid honeydew. The addition of 50 g m^{-2} of artificial honeydew per day caused a 30% increase in soil fungal biomass and a 300% increase in bacterial biomass. In contrast, Schmidt *et al.* (1997) reported that the addition of 0.8–1.6 mg day^{-1} of glucose into soil induced microbial immobilisation and decreased the inorganic nitrogen content by 50% (the average amount of glucose added to the soil as honeydew in our experiment was 2.3 mg day^{-1}). Although other mechanisms, such as root exudation, might decrease inorganic soil nitrogen (Paterson, 2003), the available evidence from previous studies supports our finding that the addition of honeydew reduced the inorganic soil nitrogen content by promoting microbial immobilisation.

Decreased available soil nitrogen owing to microbial immobilisation may subsequently influence nitrogen uptake from the soil by the host plants. The xylem sap analysis showed that the flow of xylem sap did not differ between the treatments (Fig. 3a), but the nitrate and amino acid contents of the sap were significantly lower in the aphid-infected treatment than the aphid-free treatment (Fig. 3b). This indicates that the plants were able to equally absorb water from the soil, and thus the nitrate and amino acid contents in the xylem sap are likely to reflect the amount of soil inorganic nitrogen. Therefore, we conclude that the aphids reduced the amount of soil inorganic nitrogen but they did not affect the plant's ability to absorb water.

Aphid effects on nitrogen-fixing bacteria

There is a growing body of evidence that below-ground symbiotic microbes can influence above-ground plant–insect interactions (see Hartley & Gange, 2009 for a review). In this context, several studies have reported the positive effects of nitrogen-fixing bacteria on above-ground herbivorous insects (Kempel *et al.*, 2009; Katayama *et al.*, 2011a,b). For example, Kempel *et al.* (2009) demonstrated that the rhizobia increased the body weight of lepidopteran caterpillars and the colony size of aphids on clover (*Trifolium repens* L.). However, effects of above-ground insect herbivory on plant–rhizobia associations have not been explored. In this study, we hypothesised that aphids would affect plant–rhizobia associations by two, but not mutually exclusive, mechanisms: (i) decreasing the inorganic nitrogen concentration by inducing microbial immobilisation would reinforce the plant–rhizobia association, and (ii) carbon stress in the host plants would weaken the plant–rhizobia association. Our results showed that aphid herbivory decreased the concentration of ureides in xylem sap (Fig. 3b), suggesting that the later mechanism worked more strongly in this system. Leguminous plants need a large amount of photosynthetic carbon, i.e. 6–14% of the photosynthetic production of the plants, to maintain their mutualistic association with rhizobia (Tate, 2000), whereas aphids consume large amounts of photosynthetic carbon. As photosynthetic carbon is essential for nitrogen fixation by rhizobia (Rawsthorne *et al.*, 1980), it is likely that the carbon stress caused by aphids has a greater negative effect on the rhizobia–plant association than the positive effect of microbial immobilisation. Thus, the nitrogen uptake of plants via rhizobia may have decreased in the presence of aphids.

Relative impacts of aphids on nitrogen derived from soil and rhizobia

We found that although aphids negatively affected nitrogen uptake via both pathways, the strength of the impact on direct

uptake via plant roots was significantly greater than indirect uptake via rhizobia (Fig. 3c). The mechanism of the aphid effect on the nitrogen uptake from the soil may be because of induced microbial nitrogen immobilisation by adding aphid honeydew, resulting in a decrease in available soil nitrogen. In contrast, aphids may affect nitrogen uptake via rhizobia in a more complicated way, reflecting both microbial immobilisation and carbon stress on the plant–rhizobia association. Because microbial immobilisation positively affects the association but carbon stress negatively does, the net effect of aphids depends on the relative importance of these two mechanisms. Our results suggest offset of the negative effect of carbon stress by the positive effect of microbial immobilisation.

The availability of nitrogen to herbivores differs, depending on its form (Wilson & Stinner, 1984; Katayama *et al.*, 2010; Thamer *et al.*, 2011). Ureides, the major form of nitrogen taken up via the rhizobia, are enzymatically degraded by allantoinase in above-ground plant tissues, and the nitrogen is used for amino acid synthesis (Matsumoto *et al.*, 1977). However, insects lack allantoinase unless they harbour endosymbionts that can synthesise it (Cochran, 1975). As a result, plant tissues with a higher proportion of ureides may have less nitrogen available to herbivorous insects (Wilson & Stinner, 1984). In addition, a lack of plant nitrogen leads to a decrease in the abundance, species richness, and diversity of herbivorous insects (Katayama *et al.*, 2011a) as herbivorous insects use nitrogen in plant tissue for survival and reproduction (Mattson & Scriber, 1987). If aphids simultaneously decrease the total nitrogen in plant tissue and increase the proportion of ureides-N, the aphids on the plants may negatively influence other insect herbivores.

Ecosystem function of aphids

Insect excrement can drive soil nutrient dynamics (Weisser & Siemann, 2004), and different C ; N ratios of the excrement influence differently plant growth (Kagata & Ohgushi, 2012). Honeydew is extremely carbon rich but nitrogen poor because the sugars in honeydew are 100 times more than the amino acid concentration (Dixon, 1998; Katayama *et al.*, 2013b). Aphids are a dominant component in some ecosystems, and their production of honeydew may have a prominent ecosystem function, negatively affecting the availability of inorganic soil nitrogen to plants, and suppressing rhizobial activity and thus decreasing plant nitrogen uptake via rhizobia. These negative impacts of aphids on nitrogen uptake could influence other ecosystem processes such as primary plant productivity and litter decomposition. The reduction in nitrogen uptake by aphid-infected plants would inhibit plant growth, and decrease seed production. In addition, this reduction could also produce litter with low nitrogen content. Litter nitrogen content importantly affects decomposition rates as litter with a high C : N ratio is more refractorily mineralised by microbial decomposers (Manzoni *et al.*, 2008). Recently, Katayama *et al.* (2013a) demonstrated that nitrogen mineralisation in the litter produced by aphid-infected plants would occur more slowly. These findings suggest that aphids can negatively affect ecosystem nitrogen fluxes. Future work is needed to compare the effects of aphids and other herbivorous insects on plant nutrient uptake in plant–rhizobia symbiotic systems, which will provide a critical insight into how insects shape below- and above-ground nutrient dynamics (van der Putten *et al.*, 2001; Wardle *et al.*, 2004).

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