

# Plant–rhizobia interactions alter aphid honeydew composition

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Received: 20 November 2013 / Accepted: 19 March 2014 / Published online: 4 April 2014  
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**Abstract** Both above- and below-ground interspecific interactions contribute to ecosystem functioning in terrestrial systems, and the integration of below- and above-ground interactions is crucial for deepening our knowledge of nutrient cycling and community dynamics in terrestrial ecosystems. The present study explored the effects of plant–microbe interactions on aphid honeydew quality and quantity and important factors mediating ant–aphid mutualisms and below-ground nutrient dynamics. Soybean aphids (*Aphis glycines*) were inoculated onto two closely related strains of soybean plants: a nodulating strain that associates with rhizobia and a non-nodulating strain that does not harbor any nitrogen-fixing bacteria. As expected, prior to aphid inoculation, nodulating plants were significantly taller and had more leaves than non-nodulating plants. Aphids feeding on nodulating strains were found to reach slightly larger colony sizes and produce honeydew with significantly different sugar profiles than those feeding

on non-nodulating plants. The honeydew collected from aphid colonies feeding on nodulating plants contained 160 % more total sugars than honeydew collected from colonies feeding on non-nodulating plants, but there was no difference in total amino acid-N content in honeydew from colonies feeding on the different plant strains. We discuss the implications of honeydew composition for nutrient cycling and community dynamics and suggest areas of future research to elucidate the consequences of altered aphid honeydew composition on ecosystem properties.

**Keywords** Above- and below-ground interaction · *Aphis glycines* · Honeydew · Symbiosis · Soybean · Myrmecophily

## Introduction

Terrestrial ecosystems consist of above- and below-ground interspecific interactions, both of which are important in structuring communities and determining ecosystem properties (Wardle et al. 2004). These interactions include positive or negative, direct or indirect, and trophic or non-trophic associations among soil microbes, plants, and insects (Bascompte and Jordano 2007; Ohgushi 2007; Bascompte 2009), and any single interaction may significantly affect others within an interaction web (Moller 2008). Much is known about the below-ground interactions between plants and their symbiotic soil microbes and about the above-ground interactions between plants and insects. Thus, there is increasing appreciation of that the integration of these two interactions is critically important for deepening our knowledge of nutrient cycling between above- and below-ground ecosystem components (van der Putten et al. 2001; Wardle et al. 2004). This importance has

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Handling Editor: Gary Felton.

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incited a recent call for the integration of research on plant–microbe interactions and plant–insect interactions (Pineda et al. 2010). Indeed, a growing body of work has focused on the effects of below-ground symbiotic microbes on above-ground plant–arthropod interactions and arthropod community properties (Gange 2007; Hartley and Gange 2009; Ohgushi 2012) and/or soil nutrient dynamics (Wardle et al. 2004).

Insects are a dominant component of terrestrial ecosystems (Schowalter 2000), and insect excrement (e.g., frass and honeydew) is a key substance in linking above- and below-ground interactions (Hunter 2001). This excrement contains high concentrations of labile carbon (sugars) and nitrogen (ammonium and nitrate) (Wardle 2002) and can influence below-ground nutrient dynamics when added to the soil (Kagata and Ohgushi 2011). Nitrogen-rich frass can accelerate nitrogen mineralization and thus increase the inorganic nitrogen content of the soil, whereas nitrogen-poor frass can slow the nitrogen mineralization by inducing microbial nitrogen immobilization due to increased biomass of below-ground microbes that use inorganic nitrogen from the soil (Kagata and Ohgushi 2011). In addition, some insect excrements, such as aphid honeydew, are important for maintaining mutualistic associations with ants (Völkl et al. 1999). However, little is known about how below-ground mutualisms affect the quality and/or quantity of insect excrements. This study aims to address this research gap by exploring the effects of plant–microbe associations on aphid honeydew quality and quantity and an important factor in determining the intensity of ant–aphid mutualisms and below-ground nutrient dynamics (Stadler et al. 2004).

Aphids feed by inserting their stylus into the sieve tubes of plant phloem tissue and extracting photosynthate. As phloem feeders, aphids persist on a diet that is low in available nitrogen and rich in carbohydrates. They pass large quantities of phloem through their guts, absorbing nitrogen and excreting carbohydrates in the form of honeydew, an aqueous mixture composed of 90–95 % various sugars, with some amino acids (Auclair 1963). The ecological functions of aphid honeydew have been well studied, and it is recognized to have two prominent roles. First, aphid honeydew causes increased microbial immobilization in soil (Wardle 2002). Because the abundance of free-living soil microbes is often labile carbon limited and honeydew contains abundant sugars, the deposition of honeydew droplets onto the soil increases the abundance of below-ground microbes, resulting in a decrease in inorganic nitrogen in the soil (Dighton 1978; Grier and Vogt 1990; Stadler et al. 2004). This effect likely causes a reduction in nitrogen uptake in plants from the soil (Katayama et al. unpublished data). Second, aphid honeydew is

essential in attracting and maintaining mutualistic associations with ants (Stadler and Dixon 1998, 2005). Many ant species tend aphids to collect the honeydew as food, and in return protect aphids from natural enemies (Katayama and Suzuki 2002, 2003; Stadler and Dixon 2005). Since the ecological importance of ant–aphid mutualisms is well accepted (Wimp and Whitham 2001; Suzuki et al. 2004; Ohgushi et al. 2007), identifying the factors that influence honeydew quality can provide important insight into community organization and ecosystem function.

The vast majority of plants form associations with below-ground microbes such as fungi and bacteria, which provide plants with nutrients—mainly phosphorous and nitrogen—in exchange for photosynthetic carbon. Plants use these nutrients for growth, defense, and reproduction, and thus soil microbes can drastically alter the chemical and nutrient status of plants (Katayama et al. 2010). Leguminous plants are obligately associated with rhizobia, a group of bacteria that live in small growths (nodules) on plant roots and synthesize  $\text{NH}_4^+$  from atmospheric  $\text{N}_2$  (Patriarca et al. 2002). There is a conceptual and applied basis for understanding the bottom-up factors initiated by rhizobia (Katayama et al. 2011a). While several studies have documented the effects of below-ground symbionts on insect performance (Kempel et al. 2009; Katayama et al. 2010), few studies have extended to include the effects of below-ground symbionts on the interspecific interactions among insects that feed and live on the plants (but see Morales and Beal 2006). In this context, Katayama et al. (2011a, b) showed that below-ground rhizobia greatly affect the abundance and richness of above-ground arthropods belonging to several feeding guilds and that symbiotic soil microbes play a prominent role in organizing above-ground insect communities via changes in plant quality.

Given the importance of honeydew in mediating interspecific interactions that structure communities and determine ecosystem functioning, it is surprising that no studies have explored the effects of rhizobia on honeydew quality and quantity. It might be expected that feeding on higher quality plants improves the quality and/or quantity of aphid honeydew, although work by Kempel et al. (2009) and Thamer et al. (2011) have shown that additional plant nitrogen may increase plants' production of defense compounds, thereby counteracting the benefits of increased plant quality to herbivores. While the effects of rhizobia on plant quality are well known, the effect of rhizobia on above-ground food webs is still largely unexplored (Kempel et al. 2009). The present study uses a well-described study system that includes rhizobia, leguminous plants, and aphids, to explore the effects of rhizobia on aphid abundance and honeydew composition. Based on the results, we discuss the implications of honeydew composition for nutrient cycling and community dynamics.

## Methods

### Materials

Soybean (*Glycine max*) is an annual legume native to East Asia, the roots of which form nodules that house nitrogen-fixing bacteria. The present study used two closely related strains of *G. max*: a nodulating strain (cv. Fujimishiro: R+), the nodules of which are colonized by several species of nitrogen-fixing bacteria (*Rhizobium fredii*, *Bradyrhizobium japonicum*, and *B. elkanii*) and a non-nodulating strain (cv. Touzan No. 90: R−) which does not harbor any nitrogen-fixing microbes. Touzan No. 90 was made by backcrossing to Fujimishiro after crossing between Fujimishiro and T201, another non-nodulating soybean strain. T201 has a mutation in the *rjI* locus that is responsible for root hair curling, such that it does not produce the root nodules necessary to house rhizobia (Williams and Lynch 1954; Mathews et al. 1987; Sukanuma and Satoh 1991). Touzan no. 90 is, therefore, closely related to Fujimishiro except for root nodulation. Seeds were obtained from the Laboratory of Plant Breeding, Faculty of Agriculture, Kyoto University.

Soybean aphids (*Aphis glycines* Matsumura) are common soybean pests, also native to Asia. Like many other aphid species, *A. glycines* excretes honeydew as a byproduct of feeding on phloem sap. This honeydew contains sugars, and thus attracts many species of ants that feed on the honeydew and provide aphids with protection from predators and pathogens. The aphids used in this study were obtained from the Laboratory of Applied Entomology, Faculty of Agriculture, Utsunomiya University, and colonies were reared on soybeans in an environmentally controlled growth chamber at 25 °C and 16L 8D photoperiod.

### Experiments

Experiments were conducted in June–August 2011 at the Kyoto University Center for Ecological Research (34°58′17″N, 135°57′32″E, Otsu, Japan). Two hundred plants each of R+ and R− strains were grown from seed. Seed surfaces were sterilized for 3 min in saturated 1 % sodium hypochlorite solution and germinated on wet cotton. Soil containing *Bradyrhizobium japonicum* (®Konryukin Mame-Zo) was sprinkled on the surfaces of five-day-old germinated seeds, which were then transplanted individually into polyethylene pots (5 cm in diameter and 5 cm in depth) filled with a 1:1 mixture of brunizen and sand, which had been autoclaved-sterilized at 127 °C for 72 min. *Bradyrhizobium japonicum* (®Konryukin Mame-Zo) was provided by the Federation of Tokachi Agricultural Cooperative Association. Two weeks after emergence, 100 healthy

seedlings of each strain were transplanted to larger pots (20 cm in diameter and depth) containing a 1:1 mixture of sand and black soil that had been sterilized by autoclaving for 72 min at 127 °C. Each pot received a fertilizer treatment of 0.3 g of ammonium nitrate, 1.5 g of calcium superphosphate, and 1.5 g of potassium sulfate mixed with the soil mixture. The fertilization treatment was selected based on a previous study (Katayama et al. 2010), which showed that soybean plants grew normally under this regime, but the performance (i.e., egg production) of herbivorous mites feeding on the plants increased in comparison with unfertilized conditions. Mesh cages were constructed around each plant to prevent colonization by insects, and plants were placed outdoors in an experimental field and watered as needed.

Three weeks after transplanting (5 weeks old total), nine plants had died or showed signs of herbivory and so were excluded from the experiment. The remaining 96 R+ plants and 95 R− plants were measured for height and number of leaves. Six R+ plants and five R− plants were selected at random and destructively sampled for checking root nodule formation and leaf chemical analyses. The roots were washed to remove soil, and plants were separated into root nodules and leaves. The root nodules were oven-dried for 48 h at 60 °C, and the dry mass was recorded. The leaves were placed between sheets of paper in a drying room for 1 week at 25 °C and 23 % relative humidity. The dried leaves were ground into a powder, and their carbon and nitrogen content was measured using an elemental analyzer (CHN Corder MT-3, Yanaco, Kyoto, Japan) with 20-mg powder samples. Another 20-mg leaf powder sample was analyzed for plant phenolics, which were extracted with 10 ml of 50 % methanol for 1 h in a 40 °C ultrasonic bath. The concentration ( $\text{mg g}^{-1}$ ) of phenolics was measured using the Folin–Ciocalteu method (Julkunen-Tiitto 1985).

Sixty plants of each strain were selected at random and were inoculated with 10 alate (winged females) soybean aphids, simulating immigration by dispersing individuals. Aphid population growth rates are strongly influenced by environmental conditions such as temperature, but doubling time during the summer months typically ranges from 2 to 3 days under favorable conditions (Hirano et al. 1996; McCornack et al. 2004). Aphid abundance was counted weekly for 3 weeks, thus allowing sufficient time for multiple generations to be produced.

After aphid colonies grew for 3 weeks, we placed a wire ring (20 cm in diameter,  $3.14 \times 10^{-4} \text{ m}^2$  in area) above the soil surface in each pot, and honeydew collection disks made from aluminum foil sheets were mounted to the rings. Disks were removed after 24 h, placed in individual bags, and frozen at −20 °C to await chemical analysis. Disks were then thawed and rinsed three times in 5 ml of

xylose solution ( $0.05 \mu\text{g } \mu\text{L}^{-1}$ , for a total of 15 ml), and the collected solution was filtered through a Millipore filter ( $0.20 \mu\text{m}$ ), and 1.0 mL of the filtered solution was transferred to a 1.5-mL tube. Samples were stored in a  $-20 \text{ }^\circ\text{C}$  freezer until chemical analysis. Sugar concentration of honeydew samples was analyzed using high-performance liquid chromatography (HPLC), with a Wakosil 5NH<sub>2</sub>-MS packed column ( $4.6 \times 150 \text{ mm}$ ; Wako Pure Chemical, Osaka, Japan) on 80 % acetonitrile mobile phase at room temperature and flow rate of  $1 \text{ mL min}^{-1}$ . Peak sizes for different types of sugars were determined by a refractive index detector (RID, Shimadzu Corp., Kyoto, Japan). Honeydew samples were optimized using seven sugar standards (xylose, fructose, glucose, sucrose, maltose, trehalose, melezitose), and the composition of each sample was tentatively determined by the comparison of retention times with those from a standard sample measured within the same day. The concentrations of the sugar in honeydew were corrected according to the internal standard (xylose). Total amino acid-N (nitrogen) in honeydew was determined by the ninhydrin method (Herridge 1984) using 1.0 mL of the filtered solution.

#### Statistical analyses

All statistical analyses were carried out in R (R Development Core Team 2013). Data were visually checked for normality of residuals and, if necessary,  $\log(x + 1)$ -transformed prior analysis. Plant traits (foliar C:N and foliar phenolics) of nodulating and non-nodulating plants prior to aphid inoculation were compared using *t* tests (for normally distributed data) or Wilcoxon's signed-rank tests (if transformation was not effective).

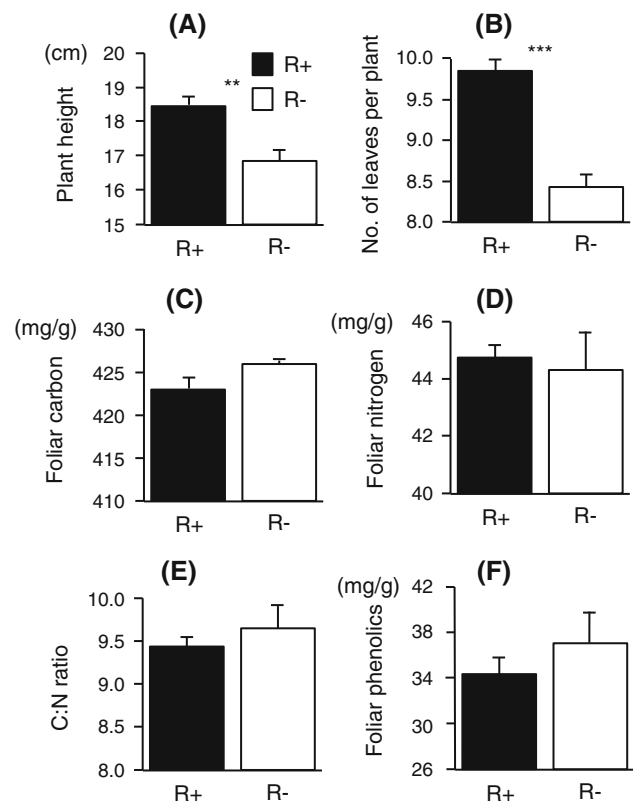
The effect of rhizobia on aphid colony size was measured using ANOVA with repeated measures. Non-metric multidimensional scaling (NMDS) and analysis of similarity (ANOSIM) were used to compare overall sugar composition between aphid colonies feeding on nodulating and non-nodulating plants. Similarity percentage analysis (SIMPER) was used to examine the contributions of individual sugars to the dissimilarities in honeydew sugar composition between nodulating and non-nodulating plants. These analyses were carried out using the *vegan* package in R, and the ANOSIM and SIMPER analyses employed Bray–Curtis dissimilarities with 999 permutations. For analyses of individual sugars, total nitrogen, and amino acid content, *t* tests were used to compare the composition of honeydew from aphid colonies feeding on the different plants strains. In cases where transformation did not restore normality, Wilcoxon's signed-rank tests were used instead for these comparisons. Honeydew composition was also analyzed per individual aphid (versus whole colony), for which the amounts of sugars and amino

acids were divided by the size of each aphid colony during the final week.

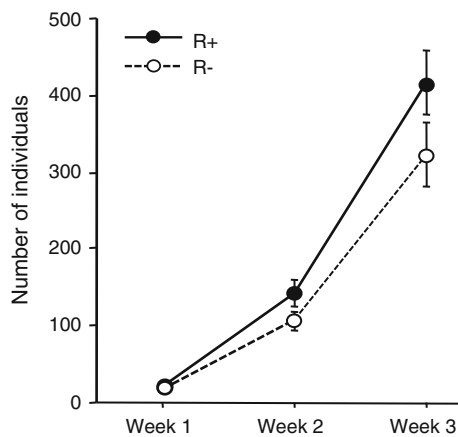
## Results

### Plant traits

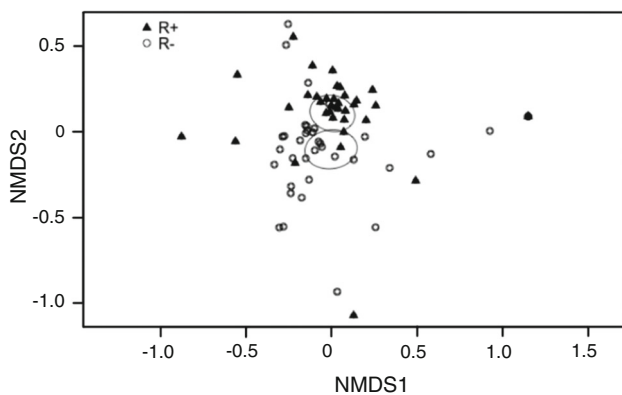
Nodulating plants were formed root nodules (mean  $\pm$  SD:  $12.95 \pm 15.08 \text{ mg}$  in dry weight), but non-nodulating plants were confirmed to have no root nodules, and are therefore considered to be free of nutrient-fixing bacteria. As expected, before aphid inoculation, nodulating plants were significantly taller ( $t = -3.929$ ,  $P < 0.001$ ; Fig. 1a) and had more leaves ( $W = 2007$ ,  $P < 0.001$ ; Fig. 1b) than non-nodulating plants. Non-nodulating plants had marginally significantly more foliar carbon than nodulating plants ( $t = 2.236$ ,  $P = 0.059$ ), though there were no differences in total N, C:N ratio, or phenolics in leaves between strains prior to aphid inoculation (N:  $t = -0.322$ ,  $P = 0.76$ ; C:N ratio:  $t = 0.645$ ,  $P = 0.55$ ; phenolics:  $t = 0.919$ ,  $P = 0.39$ ; Fig. 1c–f).



**Fig. 1** Plant traits prior to aphid inoculation. **a** Plant height, **b** number of leaves per plant, **c** foliar carbon, **d** foliar nitrogen, **e** C:N ratio, and **f** foliar phenolics. \*\* $P < 0.001$ , \*\*\* $P < 0.0001$ . Error bars show SE



**Fig. 2** Aphid colony growth on nodulating (R+) plants and non-nodulating (R-) plants (plant strain:  $P = 0.076$ , time:  $P < 0.001$ , plant strain\*time:  $P = 0.153$ ). Error bars show SE



**Fig. 3** Non-metric multidimensional scaling of honeydew sugar composition. Circles and triangles represent aphid colonies feeding on nodulating (R+) and non-nodulating (R-) plants, respectively, with 95 % confidence ellipse for each plant strain

## Aphids

The average number of aphids per plant increased over the course of 3 weeks (Time:  $F_{2,116} = 121.3$ ,  $P < 0.001$ ), with mean final abundances on nodulating plants 1.3-fold greater than on non-nodulating plants (mean  $\pm$  SE: R+:  $323 \pm 39$ , R-:  $416 \pm 37$ ) (Fig. 2). During this time (1–2 weeks after aphid inoculation), aphid abundance on nodulating plants was also 1.29–1.35-fold greater than that on non-nodulating plants, although this difference was not significant (Rhizobia:  $F_{1,111} = 2.956$ ,  $P = 0.076$ ). There was no interaction between plant strain and time ( $F_{2,116} = 1.909$ ,  $P = 0.153$ ).

The results of NMDS and ANOSIM indicate that the overall sugar composition of honeydew from aphid colonies feeding on the two plant strains was significantly

different (one-way ANOSIM: global  $R = 0.034$ ,  $P = 0.04$ ) (Fig. 3). The honeydew collected from aphid colonies feeding on nodulating plants contained 270 % more sucrose ( $t = 3.599$ ,  $P < 0.001$ ), 470 % more trehalose ( $W = 1,270$ ,  $P < 0.001$ ), 160 % more melezitose ( $W = 1,085$ ,  $P = 0.022$ ), and 160 % more total sugars ( $t = 2.079$ ,  $P = 0.041$ ) than honeydew collected from colonies feeding on non-nodulating plants (Fig. 4a). Results from SIMPER indicate that sucrose and fructose are two of the most important sugars, which explain 24 and 12 % of the overall dissimilarity in honeydew sugar composition between nodulating and non-nodulating plants, respectively. The remaining sugars did not significantly differ between the plant strains (Fig. 4a). There was no difference in total amino acid-N content in honeydew from colonies feeding on nodulating versus non-nodulating plants (Fig. 4b).

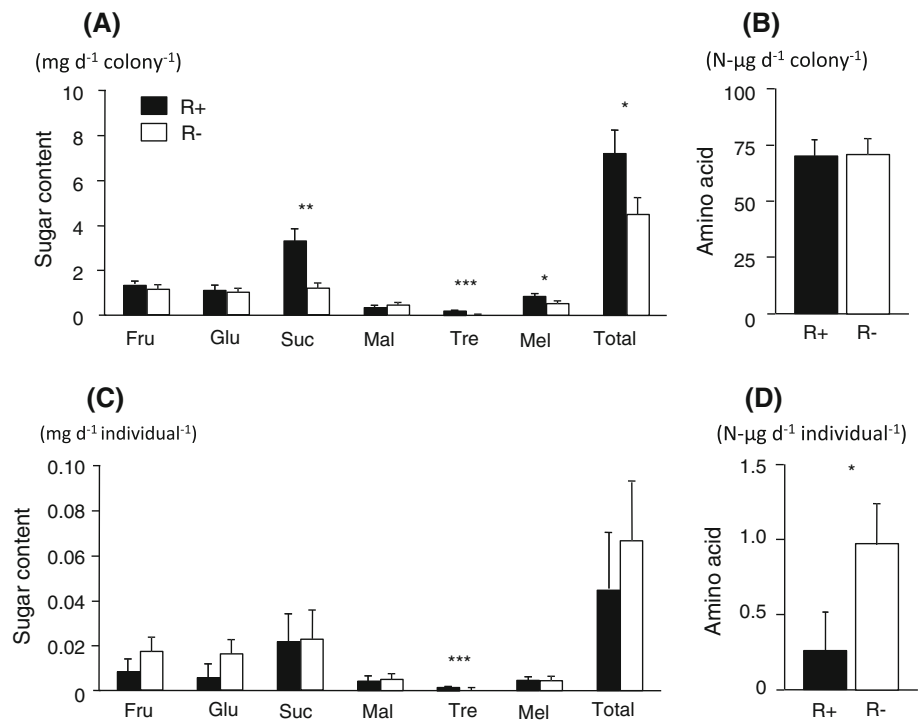
For analyses of honeydew excreted per individual aphid, rather than per colony, results were very different. Per aphid sugar excretion was not affected by rhizobia, with the exception of trehalose, which was found in greater quantities from aphids feeding on nodulating plants ( $W = 956$ ,  $P < 0.001$ ) (Fig. 4c). However, individual aphids feeding on non-nodulating plants excreted greater amounts of amino acids than individuals feeding on nodulating plants ( $W = 891$ ,  $P = 0.02$ ) (Fig. 4d).

## Discussion

Soybean plants were significantly taller and had more leaves in the presence of rhizobia, yet we did not observe the expected differences in foliar nitrogen content between plant strains. However, previous research in this same system confirmed that foliar nitrogen in nodulating plants was 50 % higher than in non-nodulating plants (Katayama et al. 2010), thus we suspect that the plant growth period may have been too short—5 weeks in the present study (vs 3 months in Katayama et al. 2010)—to allow for changes in plant chemistry to be observed. Even in this situation, the present study provides significant results suggesting that rhizobia have profound effects on honeydew excretion by aphids.

Aphids reached higher population densities on nodulating plants than on non-nodulating plants, though this difference was marginally significant. The composition of honeydew produced by aphids feeding on each strain was significantly different, such that amounts of melezitose, sucrose, trehalose, and total sugars excreted by aphid colonies increased in the presence of rhizobia while our analysis of per individual honeydew composition indicates that the sugars excreted by individual aphids were not affected by rhizobia. These results suggest that the amount of total

**Fig. 4** Honeydew composition. **a** Composition of sugars per colony. **b** Amino acid-N per colony. **c** Composition of sugars per individual. **d** Amino acid-N per individual \* $P < 0.05$ , \*\* $P < 0.001$ , \*\*\* $P < 0.0001$ . Error bars show SE



sugars excreted in aphid honeydew may increase with aphid colony size, even though we did not detect significant size differences between colonies feeding on nodulating and non-nodulating plants. Conversely, we detected significant differences in the amounts of amino acids excreted by individual aphids. Given that aphid colony sizes were larger when feeding on nodulating plants, this result may provide interesting insights into the efficiency of nitrogen assimilation in aphids. It is well known that nitrogen forms derived from soil differ from those provided by rhizobia (Giller 2001; Thomas and Sodek 2006) and that the availability of nitrogen to herbivores may differ depending on its form (Wilson and Stinner 1984; Katayama et al. 2010; Thamer et al. 2011). It is possible that aphids feeding on nodulating plants may be better able to assimilate dietary nitrogen into their tissues and therefore excrete less nitrogen in their honeydew than those feeding on non-nodulating plants. Regardless of the mechanisms underlying the observed differences in honeydew composition in the presence of rhizobia, these differences may have important consequences for community and ecosystem properties, particularly soil nutrient cycling and/or aphid interactions.

The effects of insect excrement on nutrient cycling in the soil are well documented (Hunter 2001; Stadler et al. 2004), and a recent study showed that soil nutrient dynamics are affected by C:N ratio of insect excrement (Kagata and Ohgushi 2012). For example, the deposition of nitrogen-rich frass can accelerate nitrogen mineralization in the soil, wherein organic nitrogen is converted into inorganic forms that are usable by plants. Conversely, the

addition of nitrogen-poor frass can induce nitrogen immobilization, in which the plant-accessible inorganic forms of nitrogen are converted to organic forms.

This study demonstrated that honeydew from aphid colonies feeding on nodulating plants contained significantly greater amounts of sugars than aphid colonies on non-nodulating plants, but the honeydew from these colonies did not differ in terms of total nitrogen content. As such, the excrement of aphids feeding on nodulating plants became more carbon based, suggesting that deposition of honeydew from aphids feeding on nodulating plants may strongly induce nitrogen immobilization in the soil. If this is the case, one might expect to see a decrease in inorganic nitrogen associated with aphid herbivory on nodulating plants. This raises the possibility of a positive feedback in which aphids feeding on nodulating plants indirectly decrease levels of available nitrogen in the soil, and thereby increase their host plants' dependence on rhizobia as a source of inorganic nitrogen. Alternatively, aphid herbivory may increase carbon stress in host plants, resulting in less photosynthetic carbon available for maintaining plant–rhizobia associations. Recently, we tested whether inducing microbial immobilization reinforces soybean plant–rhizobia associations and found that aphid herbivory actually decreased the concentration of rhizobia-synthesized nitrogen in xylem sap, suggesting that carbon stress experienced by the host plants actually weakens the plant–rhizobia associations in this system (Katayama et al. unpublished data).

Aphid honeydew is also known to play a crucial role in maintaining mutualistic associations between ants and

aphids (Yao and Akimoto 2002; Zhou et al. 2013), and both quantitative and qualitative differences in honeydew production can affect ant attendance (Völkl et al. 1999). Aphids likely compete for the protective and hygienic services provided by ants, and their attractiveness to ants is at least partly determined by honeydew quality (Cushman 1991; Völkl et al. 1999). Ants may respond to individual sugars in aphid honeydew, and preferences for these sugars can be species specific (Blüthgen and Fiedler 2004; Heil et al. 2005): some ants are respond most strongly to honeydew that contains high quantities of melezitose (Völkl et al. 1999), while others prefer sucrose to melezitose (Blüthgen and Fiedler 2004; Katayama et al. 2013). Our study demonstrated that aphid colonies feeding on nodulating plants produced honeydew with significantly higher levels of melezitose and sucrose, suggesting that the plant–rhizobia interaction could intensify ant–aphid association in this system, even given species-specific differences in ants’ sugar preferences.

## Conclusions

The present study demonstrates that below-ground interactions between plants and rhizobia significantly affect the composition of aphid honeydew and suggest further research areas to elucidate the consequences of these changes on nutrient cycling and arthropod community dynamics. Aphid honeydew is an important factor linking above- and below-ground interactions among plants, soil microbes, and insects. Further work is needed to elucidate the consequences of rhizobia-mediated changes in honeydew composition for ecosystem functioning and/or ant–aphid interactions. Even so, this is the first work to explore the possibility that a “foundational” mutualism between plants and symbiotic soil microbes may exert bottom-up effects on positive species interactions among free-living taxa at different trophic levels.

**Acknowledgments** Sincere gratitude is extended to Koya Hashimoto and Masami Kimura for their support and assistance. Early versions of the manuscript were greatly improved by comments from Heather Dwyer. This work was funded by a Japan Society for the Promotion of Science and National Science Foundation EAPSI award (OISE-1108405) award to MRLW.

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