

# Impacts of root symbiotic associations on interspecific variation in sugar exudation rates and rhizosphere microbial communities: a comparison among four plant families

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## Abstract

**Background and aims** Symbiotic associations between rhizobia and arbuscular mycorrhizal fungi (AMF) influence changes in carbon allocation to plant roots and alter the exudation of signal molecules in their host plants. In this study, we examined the impacts of bacterial and fungal symbiotic associations on the variation in sugar exudation rates from plant roots, and in rhizosphere microbial community structure, by comparing four plant families (e.g., Asteraceae, Brassicaceae, Fabaceae, and Poaceae) with different bacterial and fungal symbiotic associations.

**Methods and results** We determined sugar exudation rates of 23 plant species by regressions of exuded sugars against root mass through periodic samplings of plants grown in pots. Banding patterns from PCR-DGGE analyses of 28 plant species were evaluated by ordination scores relative to their rhizosphere bacterial and fungal community structures. Plant family was the most important source of variation in sugar exudation rates and microbial community structure between plant species. Brassicaceae, which lacks a root symbiotic association, had the lowest sugar exudation rate, the lowest

rhizosphere microbial mass, and a distinct fungal community structure relative to the other plant families. Fabaceae had a different bacterial community structure relative to the other plant families.

**Conclusions** These results demonstrate that root symbiotic associations have great impacts on the rate of sugar exudation and free-living microbial communities that inhabit the rhizosphere.

**Keywords** Bacterial community · Fungal community · Interspecific comparison · Rhizosphere · Root symbiotic association · Sugar exudation rate

## Introduction

Plants exude organic compounds from their roots into the surrounding soil. The amount of exudate normally reaches between 5 and 21 % of photosynthetically fixed carbon (Walker et al. 2003), and between 40 and 70 % of the total carbon belowground (Marschner 2002). The benefit to plants from this carbon loss is the alteration of the rhizosphere environment (Badri and Vivanco 2009; Bais et al. 2006; Morgan et al. 2005). Through the regulation of rhizosphere microbial communities, root exudates play integral roles in alleviating nutrient stress, providing resistance to pathogenic microbes, and promoting tolerance to toxic compounds (Bais et al. 2006; Bakker et al. 2012; Broeckling et al. 2008; Garcia-Pausas and Paterson 2011; Jones et al. 2004; Paterson et al. 2007).

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Increasing evidence has revealed that plant species exude organic compounds from their roots in different compositions and at different rates, which appears to alter rhizosphere microbial community structure among plant species (Berg and Smalla 2009; Bezemer et al. 2006; De Deyn et al. 2011; Lamb et al. 2011; Warembourg et al. 2003). Several studies have revealed that plant species harbor different rhizosphere microbial communities when grown in the same soil (Berg and Smalla 2009; Bezemer et al. 2006; Garbeva et al. 2008; Haichar et al. 2008; Viebahn et al. 2005; Wieland et al. 2001). However, it is poorly understood how the root exudation profile, and rhizosphere microbial community, differs among plant species.

Rhizobia and arbuscular mycorrhizal fungi (AMF) are important symbiotic microbes that interact with plants. The establishment of symbiotic microbes is strongly controlled by chemical crosstalk between plants and microbes at the plant family level (Nadal and Paszkowski 2013; Oldroyd 2013). For example, the plant family Fabaceae establishes two symbiotic associations with rhizobia and AMF, whereas Poaceae associates with only AMF, and Brassicaceae has no known symbiotic association with either. The different root symbiotic associations among these plant families may also influence the root exudation profile and rhizosphere microbial community. Among a wide variety of exuded organic compounds, simple sugars are predominant substances in all plant species (Jones et al. 2004). Because rhizobia and AMF require carbohydrate support from their hosts, root symbiotic associations should increase carbon allocation to the roots, which potentially changes carbon exudation to the rhizosphere. Furthermore, signal molecules, such as flavonoids and strigolactone, determines rhizobia and AMF host range specificity, and may influence the structure of free-living bacterial and fungal communities around the rhizosphere.

In this study, we addressed two questions: (1) whether the sugar exudation rate, and rhizosphere bacterial and fungal community structures, reveals significant differences among plant species, (2) and how many of these variations are attributable to differences in root symbiotic associations at the plant family level. We compared the sugar exudation rates of 23 annual crop species belonging to four plant families (e.g., Asteraceae, Brassicaceae, Fabaceae, and Poaceae) with different root symbiotic associations, as well as rhizosphere microbial community structures of 28 annual crop species belonging to the same plant families.

The root exudation rate generally changes relative to the plant cultivation method, plant age, and plant size (Cheng and Gershenson 2007; Grayston et al. 1997; Haichar et al. 2014; Jones et al. 2004; Phillips et al. 2008). To overcome these problems, we collected exuded sugars from plants grown under pots in silica sand by periodic destructive harvests, which provided an estimate of the exuded sugars under natural conditions (Vranova et al. 2013). Then, we estimated the sugar exudation rate of each plant species by a regression analysis of the exuded sugars against plant mass, which provided a size-independent measure of the sugar exudation rate. Microbial communities vary with the soil used, therefore, we compared rhizosphere microbes between plants grown using the same soil. Bacterial and fungal DNA was amplified from rhizosphere soil using specific primers, and was analyzed by the DNA fingerprinting method, Denaturing Gradient Gel Electrophoresis (DGGE).

## Materials and methods

### Determination of sugar exudation rate

First, we examined the effects of plant age and plant size on the sugar exudation rate by examining two plant species (e.g., soybean and wheat) grown under different soil nutrient conditions. Seeds were sterilized by soaking in 1 % sodium hydrochloride solution for 10 min, rinsed with sterilized water, and then sown on moist filter paper in sterile petri dishes. The germinated seeds were then transferred to plastic pots (6 cm diameter and 13.5 cm height) containing silica sand (2 to 3 mm in diameter). Each pot contained a single plant. Before use, the silica sand was washed with 0.1 % sulfuric acid and rinsed with tap water. To avoid colonization of green algae on the sand's surface, brown ceramic beads (5 mm diameter) were placed on the surface of the sand. One-hundred milliliters of two different nutrient solutions (N: P<sub>2</sub>O<sub>5</sub>: K<sub>2</sub>O = 5 %: 10 %: 5 %; HANAKOJYO, Sumitomo Co Ltd., Tokyo, Japan) diluted to 1/100 and 1/500 strength were applied twice a week. The plants were watered once or twice a day depending on the plant's size. The plants were grown in a temperature-controlled growth chamber with a day/night temperature of 22/16 °C, a photon flux density of 250 μmol m<sup>-2</sup> s<sup>-1</sup>, a 16-h photoperiod, and 70 % relative humidity.

Destructive samplings were made 12 times during a period of between five and 20 days after transplant to collect exuded sugars from each pot, and to measure shoot and root mass. During each sampling, plants and the sand from each pot, were transferred to a plastic box (14 cm length  $\times$  15.5 cm width  $\times$  7 cm height), and shoots and roots were carefully removed from the sand to measure their dry weights. Sugars were extracted from the sand with 200 mL 80 % ethanol added to the plastic box. The sand was washed several times, and the solution was recovered after filtration. The same extraction procedure was repeated three times, except 100 mL of ethanol was used for the third extraction. A preliminary experiment revealed that three washes were sufficient to recover all sugars from the sand. The extracts were combined into a 500 mL plastic bottle and were concentrated to 5 mL using a rotary evaporator (RE200 Yamato, Tokyo, Japan) set at 60 °C, under reduced pressure. The concentrated solution was then transferred to a 10 mL volumetric flask and 5 mL distilled water was added to reach a final volume of 10 mL.

The extracted solution was poured through a 0.45  $\mu\text{m}$  pore filter, and 20  $\mu\text{L}$  was injected into an HPLC (Lachrom, Hitachi, Tokyo, Japan) using a column (8 mm  $\times$  300 mm; Ionopak KS802, Shodex Co. Ltd., Tokyo, Japan) warmed to 50 °C. Deionized ultrapure water was used as the solvent at a flow rate of 0.5 ml  $\text{min}^{-1}$ , and sugar peaks were measured with a refractive detector. In total, five peaks were observed, and corresponded to the sugars raffinose, sucrose, glucose, sorbitol, and fructose, based on the retention time of standard commercial samples (Wako Chemical Company, Tokyo, Japan).

In the second experiment, we compared 23 crop species belonging to four families. The species studied included six each from Brassicaceae, Fabaceae, and Poaceae, and five from Asteraceae (Table 1). Three seeds were sown per pot, and all but one was removed after germination. Each pot was supplied with 100 mL of the 1/100 (high) strength nutrient solution twice a week. Harvests were made four to ten times depending on plant size. Growth conditions and measurements were the same as those used in the first experiment.

In the experiment using two plant species including nutrient treatments, we applied an analysis of covariance (ANCOVA) using root dry mass as a covariate. The effects of plant species and nutrient levels on sugar exudation rates were determined based on the significance of these interactions with root dry mass. In the

comparison of 23 plant species, the total variation (i.e., sum of squares, SS) of exuded sugars of all individuals ( $n = 166$ ) was partitioned into the four components: (1) variation due to the common slope, (2) variation shared by the regression analyses of each of the four families, (3) variation shared by the regression analyses of species belonging to each family, and (4) the residual error variation according to Sokal and Rohlf (1981). Differences in root exudation rates between the four families, and between species within each family, were tested by heterogeneity of the slopes. The exudation rate of each species was obtained from the slope of the regression. The mean exudation rate of each family was calculated by averaging the slopes of the species belonging to each family. Significant differences in the exudation rates among the four families were determined using the Tukey-Kramer HSD test at a significance level of 0.05.

#### Determination of rhizosphere microbial communities

We used a total of 28 species, seven from each of the four families, to determine the rhizosphere microbial communities relative to each species (Table 1). Seeds were sterilized in 1 % sodium hydrochloride solution, and then germinated on moist filter paper for approximately 1 week. Two germinated seeds of each species were transferred to a pot (7.5 cm diameter  $\times$  6.5 cm height) containing alluvial soil, with a pH of 6.6, and 340 mg N 100  $\text{g}^{-1}$  soil and 14.6 mg  $\text{P}_2\text{O}_5$  100  $\text{g}^{-1}$  soil. Each species was grown in threepots, including the control pots, which did not contain plant material. Plants were grown in a temperature-controlled growth chamber maintained at the same conditions as in the previous experiment. Plants were watered once a day and nutrients (1/100 strength) were applied once a week.

Plants were harvested 5 weeks after transplant. Plants and soil were removed from each pot, and the plants were shaken carefully to remove soil from their roots. The soil adhering to the roots after shaking was collected and comprised the rhizosphere soil. Root residue and organic matter were removed carefully using tweezers. DNA was extracted from 0.5 g of the rhizosphere soil using a DNA extraction kit (ISOIL for Beads Beating, Nippon Gene Co., Ltd., Japan), according to the manufacturer's protocol.

Microbial mass in the rhizosphere soil was estimated as a function of the absorbance intensity of the extracted DNA solution using a spectrophotometer (NanoDrop

**Table 1** Plant species used in the experiments of root exudation (E1) and rhizosphere microbial communities (E2)

ID	Common name	species name	E1	E2
Brassicaceae				
B1	Rapeseed	<i>Brassica rapa</i> L. var. nippo-oleifera	○	○
B2	Cabbage	<i>Brassica oleracea</i> L. var. capitata	○	○
B3	Broccoli	<i>Brassica oleracea</i> var. italica Brassica	○	○
B4	Chinese cabbage	<i>Brassica rapa</i> L. var. glabra Regal		○
B5	Komatsuna	<i>Brassica campestris</i> var. peruviridis	○	○
B6	Qing geng cai	<i>Brassica chinensis</i> L.	○	○
B7	Radish	<i>Raphanus sativus</i> L.	○	○
Asteraceae				
A1	Burdock	<i>Arctium lappa</i> L.	○	○
A2	Crown daisy	<i>Chrysanthemum coronarium</i> L.	○	○
A3	Zinnia	<i>Zinnia elegans</i> Jacq.	○	○
A4	Safflower	<i>Carthamus trinatorius</i> L.	○	○
A5	Lettuce	<i>Lactuca sativa</i> L.	○	○
A6	Sunflower	<i>Helianthus annuus</i> L.		○
A7	Chrysanthemum	<i>Chrysanthemum morifolium</i> Ramat.		○
Poaceae				
P1	Sweet vernal grass	<i>Anthoxanthum odoratum</i> L.		○
P2	Orchardgrass	<i>Dactylis glomerata</i> L.	○	○
P3	Italian ryegrass	<i>Lolium multiflorum</i> Lam.	○	○
P4	Wheat	<i>Triticum aestivum</i> L.	○	○
P5	Maize	<i>Zea mays</i> L.	○	○
P6	Sorghum	<i>Sorghum bicolor</i> Moench.		○
P7	Oat	<i>Avena sativa</i> L.		○
P8	Barley	<i>Hordeum vulgare</i> L.	○	
P9	Perennial ryegrass	<i>Lolium perenne</i> L.	○	
Fabaceae				
F1	Red clover	<i>Trifolium pratense</i> L.	○	○
F2	White clover	<i>Trifolium repens</i> L.		○
F3	Alfalfa	<i>Medicago sativa</i> L.	○	○
F4	Azuki bean	<i>Vigna angularis</i> Ohwi & Ohashi	○	○
F5	Common bean	<i>Phaseolus vulgaris</i> L.	○	○
F6	Soybean	<i>Glycine max</i> (L.) Merr.	○	○
F7	Chinese milk vetch	<i>Astragalus sinicus</i> L.	○	○

2000, ThermoFisher Scientific, Wilmington, DE, USA). DGGE analysis was performed following the procedure described by Okubo and Sugiyama (2009). Twenty-nine samples, including a control of each replicate, were analyzed in a single gel. Bacterial 16S rDNA fragments were amplified using the primers, 341F-GC [CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG (underlined sequences denote GC clumps)] and 534R (ATT ACC GCG GCT GCT GG). Fungal 18S rDNA

fragments were amplified using the primers, FF390 (CGA TAA CGA ACG AGA CCT) and FR1-GC (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAI CCA TTC AAT CGG TAI T), including nested primers of NS5 (AAC TTA AAG GAA TTG ACG GAA G) and NS8 (TCC GCA GGT TCA CCT ACG GA). DGGE analysis was performed using a universal mutation detection system (Dcode, Bio-Rad Laboratories Inc., Hercules, CA, USA). Bacterial DNA was run on an 8 % acrylamide gel,

containing a 45 %–70 % denaturing gradient, and maintained at a constant voltage of 80 V for 16 h. Fungal DNA was run on a 7 % acrylamide gel, containing a 25–55 % denaturing gradient, and maintained at a constant voltage of 65 V for 13.5 h. After staining with SYBR Green1 (Cambrex Bio Science, Rockland, ME, USA), each gel was captured as a digital image, and the density and location of the bands were determined in each replicate with Quantity One Software (BIO-RAD Laboratories, Inc. Japan).

Characteristic bands from the fungal DGGE analysis were excised, and DNA from the excised bands was eluted for PCR. The purified DNA was then ligated into the plasmid vector, pGEM-T Easy Vector System I (Promega, Madison, WI, USA), following the manufacturer's protocol. Fungal DNA sequences were submitted to the DNA Data Bank of Japan (DDBJ; <http://www.ddbj.nig.ac.jp/>) and the Ribosomal Database Project (<https://rdp.cme.msu.edu/>). Species identification was performed using the BLAST search function within the DDBJ.

DGGE banding patterns were converted to binary data (i.e., presence/absence), and principal coordinate (PCO) analysis was applied based on Jaccard similarity indices in each replicate using the software package CANOCO (ver.4.45 Biometrics; Plant Research International). Ordination scores from the PCO analysis, calculated from each replicate, were tested using a one-way analysis of variance (ANOVA) from each of the bacterial and fungal communities. The total SS among all individuals was partitioned into (1) family variation, (2) species variation within each family, and (3) residual error variation. The Tukey–Kramer HSD test was used to detect significant differences between the family means.

## Results

### Sugar exudation rate

Five simple sugars (glucose, fructose, sucrose, sorbitol, and raffinose) were identified in the root exudates. The relative proportions of the five sugars averaged over all harvests in each family are depicted in Fig. 1. There were significant differences in the proportions of the four sugars, except for glucose, among the four families. More than 70 % of the exudates consisted of monosaccharides (e.g., glucose, fructose, and sorbitol), whereas less than 5 % consisted of trisaccharides (e.g., raffinose).

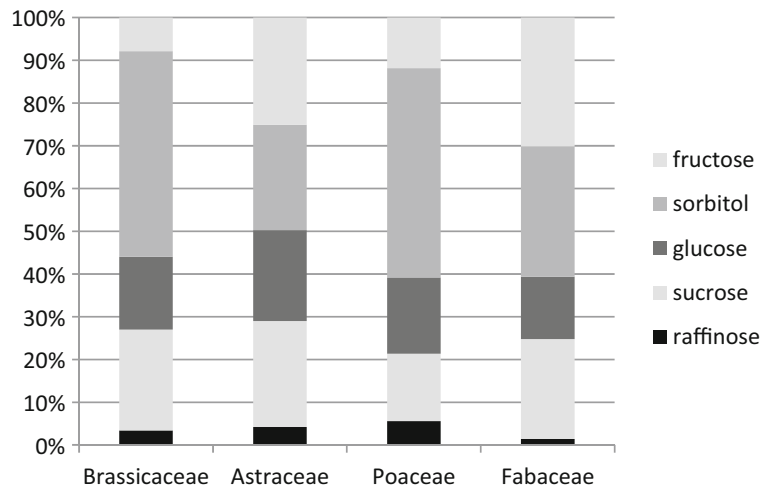
Asteraceae and Fabaceae had a higher proportion of fructose, and a lower proportion of sorbitol, than Brassicaceae and Poaceae.

Changes in the total exuded sugars, by plant age for wheat and soybean under the two nutrient treatments, are depicted in Fig. 2a. The total exuded sugars did not increase until 9 days after the transplant under the low nutrient treatment, and 13 days under the high nutrient treatment, when they significantly increased in both species. Linear regressions against plant age, after log (ln) transformation, explained 59.3 and 19.3 % of the low and high nutrients of wheat, respectively, and 59.8 and 66.2 % of the low and high nutrients of soybean, respectively. In contrast, exuded sugars linearly increased with root mass. Linear regressions against root dry mass explained 82.3 and 81.1 % of the low and high nutrients of wheat, respectively, and 81.7 and 84.9 % of the low and high nutrients of soybean, respectively (Fig. 2b). An analysis of covariance (ANCOVA) of the exuded sugars with root dry mass showed significant interactions between root dry mass and plant species, and between root dry mass and nutrient treatment, indicating that slopes of the regressions significantly differed between plant species and nutrient treatments (Table 2). Alternately, there were no significant interactions in regressions with plant age. The results indicate that regressions against root dry mass are more appropriate for measuring root exudation rate, compared with regressions against plant age. Soybean had a significantly steeper slope than wheat, whereas the low nutrient treatment resulted in significantly steeper slopes than the high nutrient treatment (Fig. 2b).

The relationship between the exuded sugars and the root mass among 166 individual plants of the 23 species is depicted in Fig. 3a. Partitioning of the SS in the exuded sugars revealed that a single common regression accounted for only 3.8 % of the variation, whereas plant family accounted for 23.1 %, and species differences within each family accounted for 14.9 % of the total variation (Table 3). In addition, the six species in Poaceae accounted for 5.3 % of the variation, the five species in Asteraceae accounted for 9.0 %, and Brassicaceae and Fabaceae did not show significant differences between species within these families. Our results demonstrate that variation at the family level explained most of the variation among the 23 species.

The sugar exudation rates, represented by the slopes of regressions against root mass, differed among plant species: from 4.2 mg g<sup>-1</sup> root (*Brassica campestris*) to

**Fig. 1** Mean composition (%) of exuded sugars from four plant families. Values were averaged over all harvests and for all species in each family



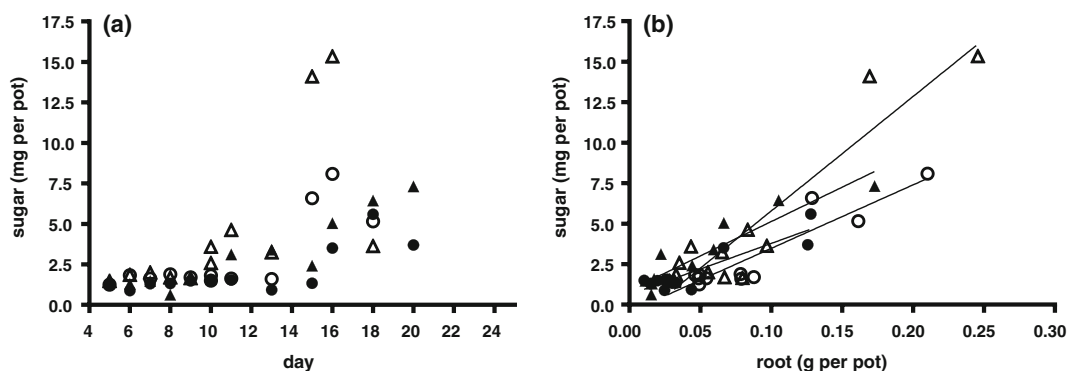
16.9 mg g<sup>-1</sup> root (*Brassica oleracea*) in Brassicaceae; from 19.7 mg g<sup>-1</sup> root (*Lolium perenne*) to 185.4 mg g<sup>-1</sup> root (*Zea mays*) in Poaceae; from 6.2 mg g<sup>-1</sup> root (*Lactuca sativa*) to 86.4 mg g<sup>-1</sup> root (*Chrysanthemum coronarium*) in Asteraceae; and from 23.2 mg g<sup>-1</sup> root (*Medicago sativa*) to 93.2 mg g<sup>-1</sup> root (*Phaseolus vulgaris*) in Fabaceae (Supporting Information Table S1). The mean sugar exudation rate of each species in the four families is depicted in Fig. 3b. Poaceae had the largest mean exudation rate (63.8 mg g<sup>-1</sup> root), whereas Brassicaceae had the lowest exudation rate (7.7 mg g<sup>-1</sup> root). Fabaceae and Asteraceae also had larger rates (59.6 mg g<sup>-1</sup> root and 40.6 mg g<sup>-1</sup> root, respectively) than Brassicaceae.

#### Community structure of rhizosphere microbes

In total, 49 bands of bacteria, and 63 bands of fungi, were identified in our DGGE analysis. The mean band

number in each sample was 45.5 for bacteria and 31.3 for fungi (Supporting Information Fig. S1). Fungi were richer in total band number, and were more heterogeneous in band pattern between samples, than were bacteria. Eleven bands in the fungal community showed a significant difference among the four families (Supporting Information Fig. S1). There were no family-specific bands of bacteria, whereas fungi had one family-specific band that was absent in all species of Brassicaceae. Sequence analysis of this band identified this species as an AMF (*Glomus* sp.; Supporting Information Table S2). We excluded this band from the analysis to clarify patterns of free-living fungal communities.

Initial PCO scores of fungal community structure were significantly different among the 28 species studied ( $F_{27,56} = 3.46$ ,  $P < 0.001$ ), as well as among the four families ( $F_{3,56} = 23.4$ ,  $P < 0.001$ ), but there were no significant differences in subsequent PCO scores. The



**Fig. 2** Changes in exuded sugar quantity against plant age after transplantation (a) and against root dry mass (b). Open and closed symbols correspond to low and high nutrient treatments, respectively. Circles denote wheat, and triangles denote soybeans

**Table 2** Analysis of covariance of total exuded sugar against growing days (a) and root mass (b). Total sugar was log (ln) transformed in the regression against growing days

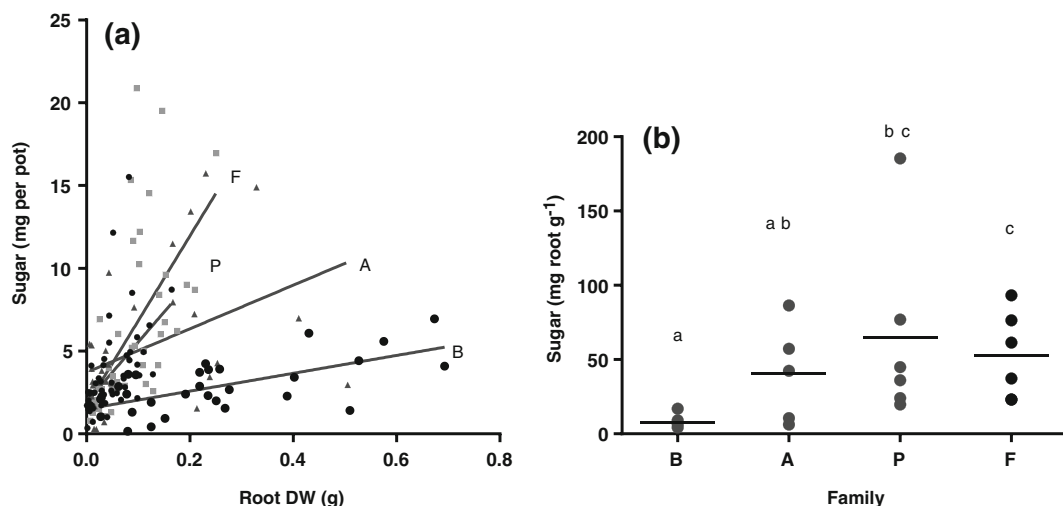
Factor	df	SS	F	p
<b>(a)</b>				
days (D)	1	10.544	36.80	< 0.0001
Species (Sp)	1	3.010	10.51	0.002
Nutrient (N)	1	0.798	2.79	0.103
Sp × D	1	0.925	3.23	0.080
N × D	1	0.022	0.08	0.783
Sp × N	1	0.105	0.37	0.549
Error	42	12.033		
<b>(b)</b>				
Root mass (R)	1	232.461	165.59	< 0.0001
Species (Sp)	1	1.465	1.04	0.313
Nutrient (N)	1	18.197	12.96	0.001
Sp × R	1	10.007	7.13	0.011
N × R	1	17.610	12.54	0.001
Sp × N	1	0.176	0.13	0.725
Error	42	58.982		

28 species accounted for 62.2 % of the total variation observed in the initial analysis. Of the variation among the 28 species, 76.2 % was explained by family, and the rest (23.8 %) was explained by species within each family (Table 4). Our results demonstrate that plant

family explained the variation in fungal rhizosphere communities between these 28 species. Initial PCO scores for Brassicaceae were significantly different from those of the other families (Fig. 4,  $P < 0.0001$ ), and the scores of Asteraceae were significantly different from those of Fabaceae ( $P = 0.037$ ).

Initial PCO scores for bacterial communities did not differ significantly among the 28 species studied ( $F_{27,56} = 1.1$ ,  $P = 0.40$ ), whereas there were significant differences among the four families ( $F_{3,56} = 3.54$ ,  $P = 0.018$ ; Table 4). The initial PCO scores for Fabaceae were significantly different from those of the other families ( $P = 0.003$ ; Fig. 4). The proportion of the variation explained by plant family was much smaller (15.7 %) in bacterial communities, than fungal communities. The second PCO score for bacterial communities did not show any significant differences between species or families.

Rhizosphere soil had a significantly greater quantity of DNA ( $63.0 \mu\text{g g}^{-1}$  soil) than control soil ( $36.2 \mu\text{g g}^{-1}$  soil,  $F_{1,84} = 11.59$ ,  $P < 0.001$ ), indicating a greater microbial biomass in the rhizosphere than in control soil. The quantity of DNA from the rhizosphere soil was significantly different among the 28 species ( $F_{27,48} = 3.48$ ,  $P < 0.001$ ), and four families ( $F_{3,48} = 16.78$ ,  $P < 0.001$ ; Table 4). The 28 species accounted for 66.2 % of the total variation (SS), and 61.6 % of this variation was explained by differences



**Fig. 3** The relationships between exuded sugars and root dry mass among all individuals (a). Each line represents a regression analysis of one of the four families; B: Brassicaceae, A: Asteraceae, P: Poaceae, and F: Fabaceae. Mean sugar exudation rates of each family (b). Small closed circles represent the

exudation rate of each plant species obtained by a slope of regression for each species listed in Fig. 3(a). Line represents the mean of each family. Different letters indicate a significant difference according to the Tukey-Kramer HSD test

**Table 3** Analysis of variance for heterogeneity of slopes in regression analyses of exuded sugars against root mass. The total variation (sum of squares, SS) was partitioned into variation between the four families, and variation between each species and residual error. The differences among the regression lines were tested by heterogeneity of slopes (*F* value)

Factor	df	SS	R <sup>2</sup> (%)	Heterogeneity of slopes (F)
Total	165	2255.3		
Between species	22	938.4	41.6	10.2***
Common slope		86.0	3.8	
Between families	3	521.0	23.1	11.24***
Within family				
Brassicaceae	5	12.9	0.6	0.6
Asteraceae	4	202.0	9.0	3.5*
Poaceae	5	118.4	5.3	3.6**
Fabaceae	5	0.0	0.0	16.6***
Error	144	1316.9	58.4	0.3

\*, \*\*, and \*\*\* represent significant differences at alpha values of 0.05, 0.01, and 0.001 %, respectively

between the four plant families (Table 4). Poaceae had the greatest quantity of DNA, whereas Brassicaceae had the lowest quantity of DNA.

## Discussion

Our results demonstrate that plant species explained 41.6, 62.2, and 34.7 % of the total variation in the sugar exudation rate, the fungal community structure, and bacterial rhizosphere community structure, respectively.

**Table 4** Analysis of variance for the initial PCO analysis scores for bacterial and fungal communities, and the quantity of DNA in rhizosphere soil. The variation (sum of squares, SS) among 28

Factors	df	Fungi PCO1		Bacteria PCO1		DNA quantity	
		SS	%	SS	%	SS	%
Between species	27	6.76***	62.2	6.11	34.9	186.8***	66.2
Between families	3	5.15***	23.4	2.75**	15.7	115.2***	40.8
Within Brassicaceae	6	0.06	0.1	0.10	0.6	4.3	1.5
Within Asteraceae	6	0.19	0.4	1.21	6.9	17.5	6.2
Within Poaceae	6	1.02	2.3*	0.55	3.1	34.0**	12.0
Within Fabaceae	6	0.34	0.8	1.51	8.6	15.9	5.6
Error	56	4.11		11.42		95.5	

\*, \*\* and \*\*\* indicate significant differences at alpha values of 0.05, 0.01 and 0.001 %, respectively

Furthermore, more than half of the interspecific variations in the sugar exudation rate, and the fungal community structure, were ascribed to the four plant families with different root symbiotic associations. These results support the hypothesis that root symbiotic associations have great impacts on variations in sugar exudation rates, and rhizosphere microbial community structures, among different plant species.

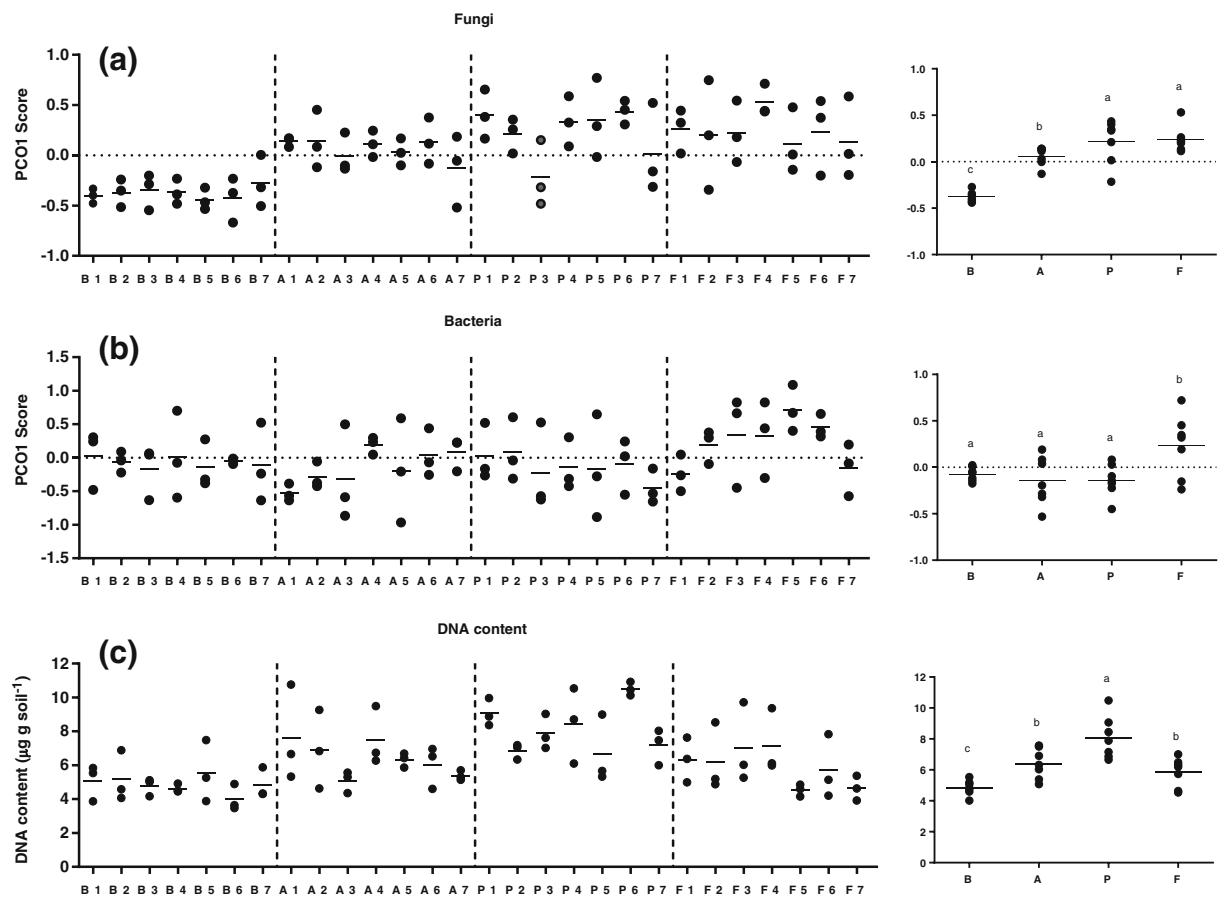
## Sugar exudation rates

It is well known that plants exude large quantities of organic compounds from their roots; however, quantification of these exuded compounds has rarely been conducted because of the difficulty in establishing reliable methods (Cheng and Gershenson 2007). Whereas cultivation under non-sterile soil underestimates the exudation rate, due to microbial consumption, the absence of microbes in sterile soil significantly reduces carbon efflux from the roots (Grayston et al. 1997; Marschner 2002). Nutrient solution culture leads to a considerable underestimation of the quantity of root exudates, because of the lack of mechanical impedance to the roots (Cheng and Gershenson 2007; Marschner 2002). Thus, there are no perfect cultivation methods to measure the root exudation rate. Although our methods appeared to underestimate the sugar exudation rate resulting from microbial consumption, the high coefficient of determination (i.e., R<sup>2</sup>) of the regression analyses should provide a reliable estimate of the sugar exudation rate of each species.

The sugar exudation rate varied widely among plant species, ranging from 4.2 mg g<sup>-1</sup> root (*Brassica*

species was partitioned into the between-families variation, and between-species variation, within each family. The sum of squares and the relative contribution (%) of each term are displayed





**Fig. 4** Initial ordination scores for fungal (a) and bacterial rhizosphere communities (b) based on the banding pattern of DGGE analysis and microbial mass estimated from DNA in rhizosphere soil (c). The ordination scores are presented for each species (left

and for each family (right). Each species is denoted by the species code (1 to 7) given in Table 1. Lines represent means of each species or each family. Different lower case letters indicate significant differences according to the Tukey-Kramer HSD test

*campestris*) to 185.4 mg g<sup>-1</sup> root (*Phaseolus vulgaris*). The exudation of uncharged compounds, such as sugars, is likely to occur by passive diffusion between roots and soil, whose rate is determined by the membrane permeability of root tissues, and the concentration gradient between roots and soil (Badri and Vivanco 2009; Jones et al. 2004). The allocation rate appears to be more important than membrane permeability, because increased membrane permeability in root tissue does not necessarily lead to increased exudation due to the flow back of exuded sugars from soil to the roots (Darrah 1998). Previous research has shown that plants grown under low nutrient conditions, which generally increases carbon allocation to the root relative to the shoot, have higher carbon exudation rates from their roots than plants grown under high nutrient conditions (Badri and Vivanco 2009; Bais et al. 2006; Carvalhais et al. 2011; Marschner 2002). We obtained the same result,

suggesting that higher carbon allocation to roots induces a greater sugar exudation rate from the roots under low nutrient conditions.

Because the carbon cost of the rhizobia-AMF association is estimated to range from 4 to 25 % of the total fixed carbon (Lambers et al. 2002), plant species with a rhizobia-AMF association would be expected to allocate more sugars to their roots than plant species lacking this association (Marschner 2002), resulting in a greater concentration gradient between the roots and soil. The sugar exudation rate of the four plant families studied showed a consistent, ordered pattern relative to the root symbiotic association: Brassicaceae had the lowest rate, Fabaceae and Poaceae had the highest rates, and Asteraceae had an intermediate rate. Because no nodules were found in roots of Fabaceous plant species, differences in sugar exudation rates among the plant families represents

inherent plant traits rather than direct effects of rhizobia infection.

### Rhizosphere microbial community

Deep sequence technology allows for a direct measure of the microbial community. Rosenzweig et al. (2013) has shown that inbred maize lines grown under field conditions, reveals significant heritable variation in rhizosphere bacterial community structure. Wehner et al. (2014) also showed that the phylogenetic relationship of the host plant is the most important predictor of root-associated fungal communities within Asteraceae in a semi-arid grassland. These results demonstrate that rhizosphere bacterial and fungal communities are not random assemblages of the soil microbial pool, but are inherent communities selected by plant species or genotypes.

PCR-DGGE is an indirect method for detecting differences in rhizosphere microbial communities, and allows differences between dominant members in the microbe community to be identified. The number of distinct bands identified was 63 for fungi and 49 for bacteria, which revealed a much lower diversity than the operational taxonomic units found in a pyrosequencing analysis (Wehner et al. 2014). Furthermore, ordination scores, based on the DGGE banding pattern, are exposed to various stochastic error variations produced by the processes of plant cultivation, DNA extraction, PCR, and electrophoresis. Nevertheless, the ordination scores revealed a consistent pattern between plant species, which indicates that the dominant members of the rhizosphere microbial community clearly differed between plant species.

In our study, plant families with different root symbiotic associations accounted for a considerable proportion of the variation in fungal rhizosphere community structure. Brassicaceae had distinctive fungal community structures from those of the other families, and because we analyzed fungal community structures after excluding an AMF-specific band, the distinctive fungal community in Brassicaceae did not reflect the effects of an AMF association. Brassicas exude specific compounds toxic to fungi, such as glucosinolate and isothiocyanate, from their roots (Bressan et al. 2009; Fahey and Talalay 2001; Ponce et al. 2004; Rumberger 2003). These compounds are known to have inhibitory effects on AMF infection and on free-living fungal communities in the rhizosphere (Bressan et al. 2009; Vierheilig

et al. 2000). Thus, the distinctive fungal rhizosphere community structure in Brassicaceae appears to be caused by these toxic compounds. Additionally, Fabaceae had a significantly different bacterial community structure from those of the other three families. Fabaceous plants exude isoflavonoid as a signaling molecule to initiate colonization by rhizobia (Peters and Long 1986), a compound which also has antibiotic effects on bacteria (Dakora and Phillips 1996). Therefore, molecules involved in the infection of rhizobia might also contribute to specific rhizosphere bacterial communities from soil microbial pools, resulting in the distinct bacterial communities we observed in Fabaceae.

The four plant families also showed significant differences in the quantities of DNA in the rhizosphere soil, an indirect representation of the rhizosphere microbial mass. Brassicaceae had the lowest quantity of DNA, whereas Poaceae had the highest. Differences in DNA quantity between the plant families was consistent with the differences in sugar exudation rates, suggesting a close link between exuded sugar from the roots and the rhizosphere microbial mass. Exuded sugars are an important energy source for microbial growth; therefore, a large sugar supply in Poaceae may permit rapid growth of microbial populations and enhance microbial activity around the rhizosphere. In contrast, the reduced microbial mass of brassicas, with the lowest sugar supply, may result in reduced microbial activity around the rhizosphere, resulting in slow decomposition and nutrient cycling in the soil.

Previous research has shown that plant species can control soil functions through aboveground plant traits, such as litter quality (Bezemer et al. 2006); whereas less is known regarding how belowground traits regulate microbe communities and soil functions. This study demonstrated that changes in sugar exudation rates, through root symbiotic associations, contributed to the alteration of rhizosphere microbial communities among plant families. However, rhizosphere microbial communities also appear to be influenced by other factors, such as plant phylogenetic history and functional traits. A further detailed analysis of the root exudation profile and microbial communities will clarify how plants influence rhizosphere microbial communities through root exudation.

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