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GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

Interactions of plant growth-promoting rhizobacteria and soil factors in two leguminous plants

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Abstract Although the rhizomicrobiome has been extensively studied, little is known about the interactions between soil properties and the assemblage of plant growth-promoting microbes in the rhizosphere. Herein, we analysed the composition and structure of rhizomicrobiomes associated with soybean and alfalfa plants growing in different soil types using deep Illumina 16S rRNA sequencing. Soil pH, P and K significantly affected the composition of the soybean rhizomicrobiome, whereas soil pH and N had a significant effect on the alfalfa rhizomicrobiome. Plant biomass was influenced by plant species, the composition of the rhizomicrobiome, soil pH, N, P and plant growth stage. The beta diversity of the rhizomicrobiome was the second most influential factor on plant growth (biomass). Rhizomicrobes associated with plant biomass were identified and divided into four groups: (1) positively associated with soybean biomass; (2) negatively associated with soybean biomass; (3) positively associated with alfalfa biomass; and (4) negatively associated with alfalfa biomass. Genera assemblages among the four groups differentially responded to soil properties; Group 1 and Group 2 were significantly correlated with soil pH and P, whereas Group 3 and Group 4 were significantly correlated with soil N, K and C. The influence of soil properties on the relative abundance of plant biomass-associated rhizomicrobes differed between soybean and alfalfa. The results suggest the rhizomicrobiome has a pronounced influence on plant growth, and the rhizomicrobiome assemblage and plant growth-associated microbes are differentially structured by soil properties and leguminous plant species.

Keywords Plant growth-promoting rhizobacteria · Illumina sequencing · Plant-microbial interactions · Rhizosphere · Leguminous plants

Introduction

The rhizosphere, the soil in the immediate vicinity of plant roots, is strongly influenced by both the roots and the surrounding soil (Lundberg et al. 2012) and involves an enormous interactions between the roots, soil and microbes. Plants release up to 40% of their photosynthetically fixed carbon below ground (Hutsch et al. 2002; Staff 2015), of which ~ 11% is retained in rhizodeposition (Jones et al. 2009). The rhizodeposition provides a constant flow of plant-based organic substrates to soil microbes colonising the rhizosphere (Kai et al. 2016). In return, rhizobacteria play a role in promoting or inhibiting plant growth (Kai et al. 2016). Some rhizobacteria, so-called plant growth-promoting rhizobacteria (PGPR), stimulate plant growth indirectly or directly through different mechanisms. For example, they can increase the absorption of mineral nutrients (Vacheron et al. 2013), stimulate the synthesis of phytohormones and secondary metabolites (Gamalero and Glick 2015), suppress phytopathogens and enhance plant tolerance to environmental stress (Anderson and Habiger 2012).

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PGPR comprise a range of species with huge taxonomic diversity, especially within *Firmicutes* and *Proteobacteria* (Vacheron et al. 2013; Vessey 2003). *Bacillus*, *Pseudomonas*, *Escherichia*, *Micrococcus* and *Staphylococcus* have been confirmed as producers of auxins (Ali et al. 2010; Zhang et al. 2007), and *Pseudomonas* is a producer of 2,3-diacetylphloroglucinol, an antimicrobial compound (Picard et al. 2000). *Azospirillum brasilense* can produce nitric oxide (Creus et al. 2005), a signalling molecule modulating plant physiological responses and influencing root growth and developmental processes (Vacheron et al. 2013). *Pseudomonas*, *Bacillus* and *Rhizobium* are able to increase the nutrient absorption of plant roots by dissolving insoluble phosphate (Goldstein 1995; Hariprasad and Niranjana 2009). Additionally, many rhizobacteria can fix N_2 and produce extracellular siderophores that improve plant growth (Cakmakçi et al. 2006; Kloepper et al. 1980). PGPR also are able to enhance plant tolerance to environmental stresses, such as salinity (Tank and Saraf 2010), drought (Dimkpa et al. 2009) and heavy metals (Dell'Amico et al. 2005). Most previous studies on PGPR involved culture-based methods, such as screening bacteria for the production of antibiotics, siderophores and phytohormones (Anderson and Habiger 2012). However, culture-based techniques can only explore a small proportion of rhizomicrobiome species, while most species are overlooked (Anderson and Habiger 2012). By contrast, the Illumina sequencing of 16S rRNA amplicons could help to elucidate the rhizomicrobiome species more comprehensively.

The composition of the rhizomicrobiome is strongly affected by the soil and plants (Lundberg et al. 2012; Peiffer et al. 2013). Significant variations were found in the composition of maize and *Arabidopsis* rhizomicrobiomes in different soil types using a metagenomic approach (Lundberg et al. 2012; Peiffer et al. 2013). Specific soil properties such as pH, nitrogen (N), phosphorus (P), potassium (K) and other mineral nutrients exhibit different effects on the composition of the rhizobacterial community (Berg and Smalla 2009; Edwards et al. 2015; Lundberg et al. 2012; Pii et al. 2016; Schreiter et al. 2014). In three California grasslands, the composition of the wild oat rhizomicrobiome was shown to be significantly correlated with soil pH and K (Nuccio et al. 2016). Soil pH, as well as K, P, cadmium and magnesium levels, was significantly correlated with the relative abundance of *Proteobacteria* in the rhizosphere of Japanese barberry (Coats et al. 2014).

It is believed that specific microbial populations associated with plant roots are selected by their unique root exudates (Berg and Smalla 2009), which provide nutrients for microbes and contain antimicrobial metabolites. Through qualitative and quantitative differences of root exudates, some plant species can selectively modulate the composition of the rhizomicrobiome (Pii et al. 2016). Plant growth stage also influences rhizomicrobiome communities associated with *Arabidopsis*, *Medicago*, maize, pea and wheat by altering both the amount and chemical composition of root exudates (Chaparro et al. 2014). The interplay of all these factors (plant, soil and rhizobacteria) makes the rhizosphere a complex and dynamic microecosystem that requires further exploration. And yet, the effects of soil type, plant species and growth stages on PGPR have not been comprehensively studied.

Herein, we performed a deep Illumina sequencing of 16S rRNA amplicons to reveal the rhizomicrobiome composition of two plant species (soybean and alfalfa) grown in three soil types (chernozem, cinnamon and red soils). In order to investigate the relationships among plants (biomass, growth stage and species), soil properties and the rhizomicrobiome, we tested the following: (1) whether plant biomass is associated with the composition of the rhizomicrobiome; (2) what kind of rhizomicrobes was positively or negatively correlated to plant growth in soils with different properties; and (3) which soil properties determine the rhizomicrobe species correlated with plant growth. This design allowed us to gain a better understanding of the relationship between the rhizomicrobes and plant growth by combining soil parameters, plant species and plant growth stages. The results may assist in further utilising the productive promoting communities in field conditions.

Materials and methods

Sampling strategy and data collection

We collected farmland soil samples representing three major soil types in China: chernozem (CH; Mollisol) from Heilongjiang Province (46° 24′ 10.2″ N, 125° 21′ 59.5″ E), cinnamon soils (CI; Alfisol) from Shaanxi Province (34° 4′ 14.88″ N, 108° 36′ 8.28″ E) and red earth (RE; Acrorthox) from Jiangxi Province (28° 21′ 41.5″ N, 115° 55′ 0.80″ E). Prior to soil sampling, the litter layer was removed. Surface soil samples (0–20 cm) were collected with a sterilised shovel using the five-point sampling method as previously described (Navarrete et al. 2015). Soil samples were transported immediately to the laboratory and stored at 4 °C in the dark. All samples were passed through a sterile 2 mm sieve before use.

Two plant species, soybean (grain legume) and alfalfa (forage legume), were used in this study. After surface sterilisation of seeds (Edwards et al. 2015), soybean (three plants per pot) and alfalfa (eight plants per pot) were grown in pots containing homogenised soil (CI, CH or RE) under a 16-h light (25 °C) and 8-h dark (20 °C) cycles with a relative humidity of 40 to 45%. To ensure sufficient sample for further analysis, rhizosphere soil from two pots were combined as one replicate. A total of 162 pots [two legume species × three soils × three periods × six pots + three soils × three periods × six pots (without plants)] were randomly arranged in a greenhouse. Rhizosphere soil and plant samples were collected from pots at 25 days (Period 1), 40 days (Period 2) and 55 days (Period 3) after sowing as previously described (Xiao et al. 2017). A

total of 54 rhizospheric soil samples (two plant species × three soils × three periods × three replicates), nine bulk soil samples (three soil types × three periods, without plants) and nine original soil samples (three soil types × three replicates) were obtained and stored at − 80 °C until needed.

Each individual sample was subjected to measurement of soil parameters that included pH, total N, total P, total K and organic carbon (C). Soil analyses were performed using the Morgan soil testing system for determination of N, P and K (Lunt et al. 1950). Buffer pH was measured using the modified Mehlich buffer test (Peiffer et al. 2013) and organic matter was discerned by loss on ignition at 550 °C for 5 h (Peiffer et al. 2013).

DNA extraction and 16S rRNA sequencing

DNA was extracted from each rhizosphere and bulk soil sample (0.5 g) using the FastDNA SPIN for soil kit (MP Biomedicals, Solon, USA) according to the manufacturers' instructions. Primers F515 (5′-GTGCCAGCMGCCGCGGTAA-3′) and R926 (5′-CCGYCAATT YMTTTRAGTTT-3′) were used to amplify the hypervariable V4−V5 region of the 16S rRNA gene (Peiffer et al. 2013). All PCRs (50 μL) were carried out in triplicate with 0.5 μL of each primer (50 pmol), 5 μL of 2.5 mM dNTPs, 5 μL of 10× Ex Taq buffer (20 mM Mg^{2+}; TaKaRa Inc., Dalian, China), 0.25 μL of Ex Taq DNA polymerase (TaKaRa) and 1 μL of DNA template. Thermal cycling consisted of an initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 30 s, with a final extension step at 72 °C for 5 min. Amplified products were gel-purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The Quant-iT PicoGreen dsDNA Reagent Kit (Life Technologies, Merelbeke, Belgium) was employed to determine the concentration of each amplicon. Purified PCR amplicons were sequenced by Macrogen (http://www.macrogen.com, Seoul, South Korea) using an Illumina Miseq 250PE platform (Illumina Inc., San Diego, CA, USA).

16S rRNA gene sequence analysis

Sequences were removed if the average quality scores were < 25 or if they contained ambiguous bases or primer mismatches using Quantitative Insights into Microbial Ecology (QIIME) (Peiffer et al. 2013). All sequences were denoised by homopolymer error-correction using Denoiser version 0.91 software (Reeder and Knight 2010). Chimeric sequences were removed using USEARCH (Edgar et al. 2011). Paired-end reads from original DNA fragments were merged using FLASH (Magoc and Salzberg 2011). Sequences were then assigned to different samples according to their barcode using a script derived with the QIIME pipeline (Caporaso et al. 2010). Remaining sequences were binned into operational taxonomic units (OTUs) at 97% sequence similarity (OTU97) using the default QIIME pipeline UCLUST (Edgar 2010). Representative sequences for each OTU were picked. Taxonomy was assigned using the RDP classifier at an 80% confidence threshold (Edwards et al. 2015). In order to gain a deeper understanding of the rhizomicrobiome, functional prediction was performed using Vikodak based on the species abundance data on genus level (Nagpal et al. 2016).

Data analysis

Weighted and unweighted UniFrac distances (WUF and UUF) of all samples were calculated by QIIME (Caporaso et al. 2010). Principal coordinate analysis (PCoA) of UniFrac distances was performed using Ape version 3.4 (Paradis et al. 2004) in the R package. Alpha diversity (Shannon-Wiener index) was calculated using Vegan version 2.3-0 in R version 3.1.1 (Oksanen et al. 2007). The test of homogeneity of multivariate dispersions and the permutational multivariate analysis of variance (PERMANOVA) among soil types were performed using Vegan version 2.3-0 in R version 3.1.1 (Oksanen et al. 2007). Constrained analysis of principal coordinates (CAP) was performed with 999 permutations using Vegan version 2.3-0 in R version 3.1.1 (Oksanen et al. 2007). Wilcoxon rank tests were employed to compare the relative abundance of genera in different samples using Stats version 3.2.4 in the R package, and this was also used to calculate Spearman's correlation coefficients. Visualisation of the correlation matrix was performed using the R package Corrplot version 0.73. One-way analysis of variance (ANOVA) was performed using SPSS Statistics version 18.0 (SPSS Inc., Chicago, IL, USA).

Network analysis was performed on soybean and alfalfa rhizomicrobes based on strong and significant correlations (both positive and negative) between genera and soil properties (non-parametric Spearman's correlation, $P < 0.01$ and absolute value of $r > 0.6$). Genera of low abundance were eliminated from the taxonomy table when less than 0.01% of the total relative abundance across all samples before network analysis. Statistical analysis of the network was carried out in the R environment and the network was visualised using Cytoscape version 3.2.1 (Shannon et al. 2003). Venn diagrams were constructed using VennDiagram version 1.6.0 in the R package. Differences in rhizomicrobiome taxa between different plant species and growth stages were evaluated using a linear discriminant analysis effect size (LEfSe) algorithm (Segata et al. 2011).

Data Accessibility

The sequences of all samples have been submitted to the NCBI's small read archive (SRA) in BioProjectID PRJNA325735, with run number SRR3714933-SRR3714936.

Results

Abiotic soil properties and plant growth

Soil pH, K, N, C and P exhibited significant differences between different soil types (pH, K, N and C; $P < 0.001$; P, $P = 0.001$; Supplementary Table S1). Soil pH varied from 6.81 to 8.18, from weak acid to weak alkali, in the order of RE, CI and CH. Soil K varied from 0.41 to 1.1 g/kg in the order of CH, RE and CI. N (1.40 g/kg) and C (28.20 g/kg) in CH were significantly higher than in RE ($N = 1.30$ g/kg; C = 20.00 g/kg) and CI ($N = 1.20$ g/kg; C = 19.0 g/kg). P (0.67 g/kg) was significantly higher in RE than in CI (0.46 g/kg) and CH (0.45 g/kg; Supplementary Table S1). Plants harvested at the three time points produced biomass (fresh weight/plant) from 2.29 to 5.76 g for soybean, and from 0.10 to 0.96 g for alfalfa (Supplementary Table S2). The heights of soybean and alfalfa plants ranged from 31.50 to 60.12 cm and 5.67 to 12.93 cm, respectively (Supplementary Table S2).

Response of rhizomicrobiome composition to various factors

PCoA based on UUF showed that the composition of rhizomicrobiomes differed among the three soil types (Supplementary Fig. S1). Based on the CAP of WUF, we found that soybean rhizomicrobiomes were significantly affected by soil pH, P and K (53.90%; $P = 0.001$), whereas alfalfa rhizomicrobiomes were significantly affected by soil pH and N (42.03%; $P = 0.001$). Based on the CAP of UUF, we found that soybean rhizomicrobiomes were significantly affected by soil pH and K (26.47%; $P = 0.003$), while alfalfa rhizomicrobiomes were significantly affected by soil pH and N (28.88%; $P = 0.001$; Fig. 1; Supplementary Table S3). There were significant differences in the composition of rhizomicrobiomes among different soil types using PERMANOVA based on WUF with 999 permutations (homogeneity of dispersion among soil types: $P = 0.7856$; PERMANOVA: $R^2 = 0.226$; $P = 0.001$).

LEfSe revealed that 22 bacterial genera were significantly more abundant in the soybean rhizosphere than the alfalfa rhizosphere, of which *Lysobacter*, *Novosphingobium*, *Olivibacter*, *Stenotrophomonas* and *Variovarax* showed the greatest variation. This analysis also identified 12 genera that were more abundant in the alfalfa rhizosphere, of which *Archangium*, *Schlesneria*, *Cellulomonas*, *Bryobacter* and *Nitrospira* showed the greatest variation (Supplementary Fig. S2; Supplementary Table S4). At different periods, the rhizomicrobiomes of soybean exhibited greater differences than those of alfalfa. This phenomenon may be explained by the three periods corresponding to different growth stages of soybean but corresponding to vegetative stages of alfalfa. In the soybean rhizosphere, *Aciditerrimonas*, *Arcticibacter*, *Laceyella*, *Acinetobacter* and *Flavitalea* were more abundant in Period 1 (vegetative stage); *Veillonella*, *Flavihumibacter* and *Sphingmonas* were more abundant in Period 2 (flowering stage); and *Rhizobium*, *Novosphiingobium*, *Ensifer*, *Shinella*, *Legionella* and *Azomonas* were more abundant in Period 3 (podding stage). In the alfalfa rhizosphere, *Isoptericola* and *Bacilli* were more abundant in Period 1 (vegetative stage); *Diaphorobacter* were more abundant in Period 2 (vegetative stage); and *Defluviicoccus*, *Cryptosporangium*, *Treponema* and *Spirochaetia* were more abundant in Period 3 (vegetative stage; Supplementary Fig. S2).

Effects of soil properties on rhizomicrobes

Network analysis was performed on soybean and alfalfa rhizomicrobes and soil properties (Fig. 2). For both soybean and alfalfa, more genera were significantly correlated with soil pH than with soil nutrients (K, P, N and C). For both soybean and alfalfa rhizomicrobiomes, most genera positively correlated with pH belonged to the *Acidobacteria*, *Actinobacteria*, *Bacteroidetes* and *Proteobacteria* phyla, while most genera negatively correlated with pH belonged to *Actinobacteria* and *Proteobacteria* (Fig. 2; Supplementary Fig. S3). Numerous genera (12/27) of the soybean rhizomicrobiome that were positively associated with soil pH overlapped with alfalfa (12/29), including *Terrimonas*, *Steroidobacter*, *Skermanella*, *Rubrobacter*, *Rubellimicrobium*, *Rhodocytophaga* and *Pedobacter*. Similarly, many genera (19/30) of the soybean rhizomicrobiome that were negatively associated with soil pH overlapped with alfalfa (19/37), including *Tumebacillus*, *Sphaerisporangium*, *Sediminibacterium*, *Phycicoccus*, *Phenylobacterium*, *Nonomuraea* and *Methylobacterium* (Supplementary Fig. S4).

In the soybean rhizosphere, most genera positively associated with K were *Proteobacteria* and most negatively associated with K were *Actinobacteria*. Meanwhile, in the alfalfa rhizosphere, most genera positively associated with K were *Proteobacteria*, *Actinobacteria* and *Planctomycetes* and most negatively associated with K were *Actinobacteria* and *Firmicutes* (Supplementary Fig. S3). The genera significantly associated with K, N and C in the soybean rhizosphere did not overlap with those in the alfalfa rhizosphere. Genera significantly associated with K in the soybean rhizosphere were *Afipia*, *Naxibacter*, *Inquilinus*, *Dokdonella*, *Labrys*, *Geodermatophilus*, *Rubrobacter* and *Microlunatus*, compared with *Sphingobium*, *Singulisphaera*, *Actinophytocola*, *Lentzea*, *Llumatobacter*, *Asanoa*, *Domibacillus* and *Thermoactinomyces* in the alfalfa rhizosphere. No significant correlation was detected between soil P and alfalfa rhizomicrobes or between C and soybean rhizomicrobes. Only the genus *Leifsonia* showed a strong correlation with soil N in the soybean rhizosphere (Fig. 2).

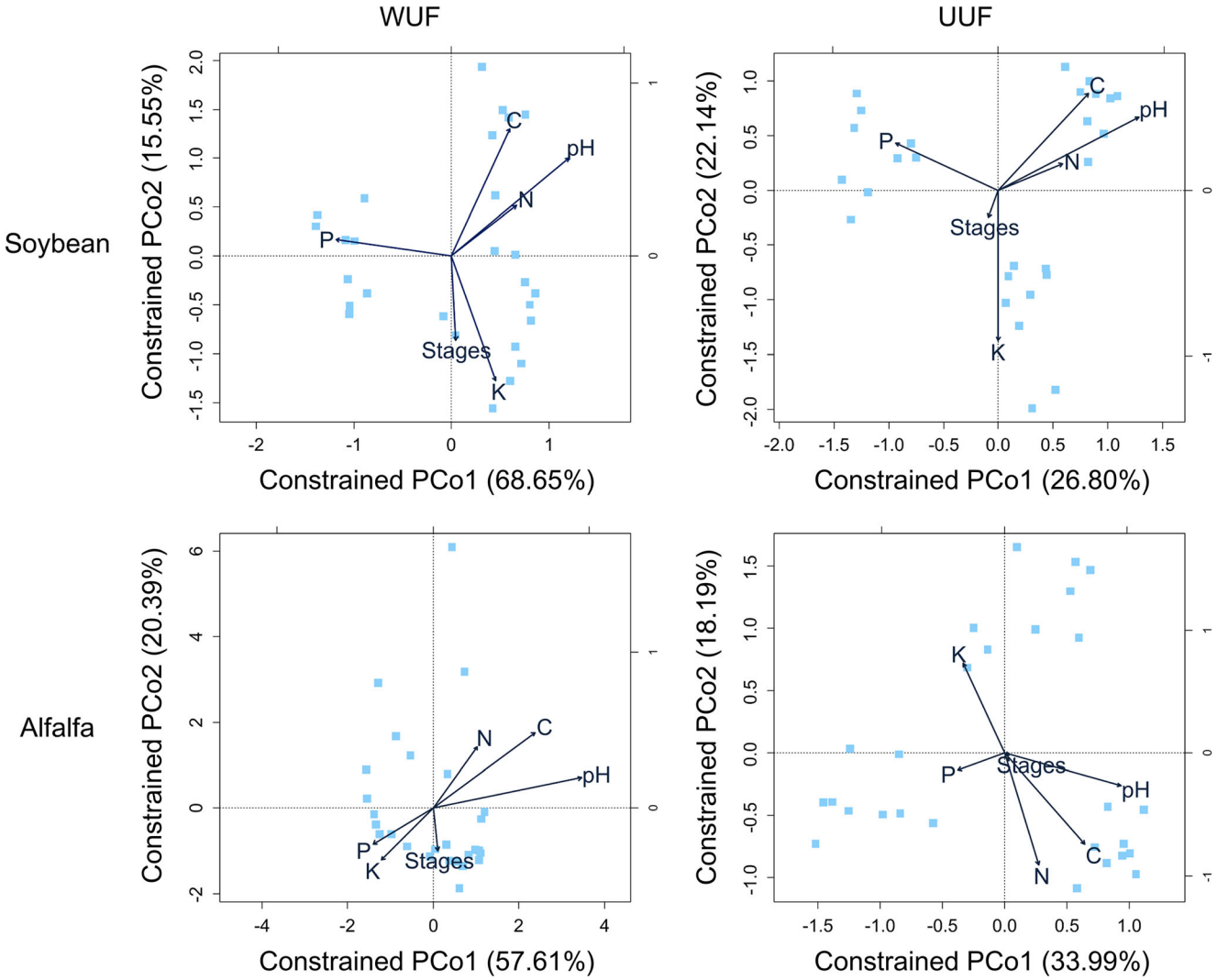

Fig. 1 Dot plot of the constrained analysis of principal coordinates for the rhizomicrobial communities of soybean and alfalfa using weighted UniFrac distance (WUF) and unweighted UniFrac distance (UUF) metrics

Correlations between plant biomass and various factors

The first axis of PCoA based on WUF (Supplementary Fig. S1) was used as an indicator of the beta diversity of rhizomicrobiomes in subsequent analyses. CAP revealed that plant species, alpha and beta diversities of the rhizosphere microbial community, soil pH, N, P and periods cumulatively accounted for 92.37% of the variation in plant biomass (P = 0.001). Plant species was the largest contributor, followed by beta diversity of the rhizomicrobiome, then soil properties and growth stages (Fig. 3; Supplementary Table S5).

To further explore the relationship between the rhizomicrobiome and plant biomass, we compared the predicted metabolic functions of the rhizosphere microbiota and the bulk soil microbiota (Fig. 4). Major metabolic activities, such as carbohydrate metabolism, amino acid metabolism, lipid metabolism, energy metabolism, metabolism of cofactors and vitamins, biosynthesis of other secondary metabolites and nucleotide metabolism, were significantly higher in the rhizosphere microbiota than in the bulk soil microbiota for both soybean and alfalfa. Correlation coefficients of the main functions and plant biomass showed that the metabolism of terpenoids and polyketides was strongly positively correlated with plant biomass, as was the biosynthesis of other secondary metabolites (Fig. 4).

Relationships between rhizomicrobes and plant biomass

Spearman's correlation coefficients of the major rhizomicrobes (the mean of relative abundance > 0.001) and plant biomass were calculated at the genus level (Table 1). Soybean biomass was positively correlated with *Massilia*, *Flavisolibacter*, *Burkholderiaceae_Other*, *Sphingomonadaceae_Other*, *Luteimonas* and *Burkholderia* (Group 1) and negatively

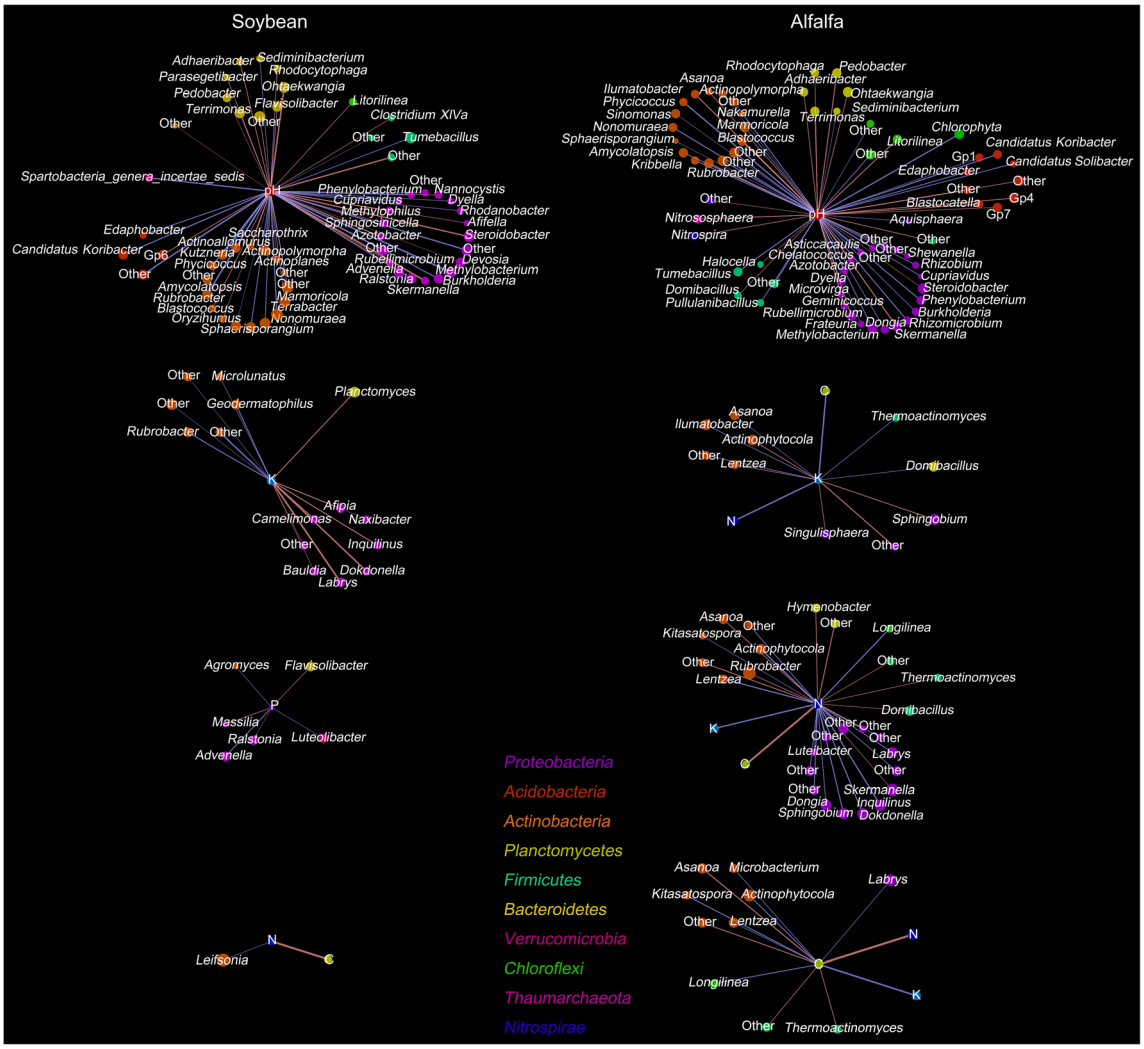

Fig. 2 Network analysis of the rhizomicrobiomes of soybean and alfalfa and soil properties based on correlation analysis. Only strong (Spearman's $r > 0.6$ or $r < -0.6$) and significant ($P < 0.01$) correlations are shown. Each node represents an operational taxonomic unit (OTU). Different node colours represent OTUs belonging to different phyla. The width of each line is proportional to the correlation coefficients of the connections. Red lines represent the positive correlations and blue lines represent negative correlations

correlated with *Pirellula*, *Nitrososphaera*, *Adhaeribacter*, *Blastococcus*, *Sphingobacterium*, *Paenibacillaceae_Other*, *Ohtaekwangia*, *Promicromonospora*, *Skermanella*, *Aeromicrobium* and *Planctomycetaceae_Other* (Group 2). The total relative abundance of all positive and negative genera was summed and linear regression was performed on the total relative abundance and soybean biomass (Fig. 5). The results showed that soybean biomass increased with the total abundance of positive genera ($R^2 = 0.173$; $P = 0.030$) and decreased with the total abundance of negative genera ($R^2 = 0.305$; $P = 0.003$). Meanwhile, alfalfa plant biomass was positively correlated with *Ensifer*, *Micrococcaceae_Other* and *Geodermatophilaceae_Other* (Group 3) and negatively correlated with *Flavobacteriaceae_Other*, *Chitinophaga*, *Olivibacter*, *Stenotrophomonas*, *Alcaligenaceae_Other*, *Inquilinus*, *Phyllobacterium*, *Pseudoxanthomonas* and unknown genera in *Rhodospirillales* and *Proteobacteria* (Group 4). The results of linear regression exhibited that alfalfa biomass increased with the total abundance of positive genera ($R^2 = 0.398$; $P < 0.001$; Fig. 5) and decreased with the total abundance of negative genera ($R^2 = 0.228$; $P = 0.010$; Fig. 5).

Spearman's correlation coefficients ($P < 0.05$) for different factors (soil properties and periods) and the relative abundance of the four groups are shown in Supplementary Fig. S5. For soybean, the total abundance of Group 1 showed a negative relationship with soil pH and a positive relationship with soil P. By contrast, the total abundance of Group 2 was positively correlated with soil pH and negatively correlated with soil P. For alfalfa, the total abundance of Group 3 showed a positive relationship with soil N and C and a negative relationship with soil K. By contrast, the total abundance of Group 4 was negatively correlated with soil N and C and positively correlated with K.

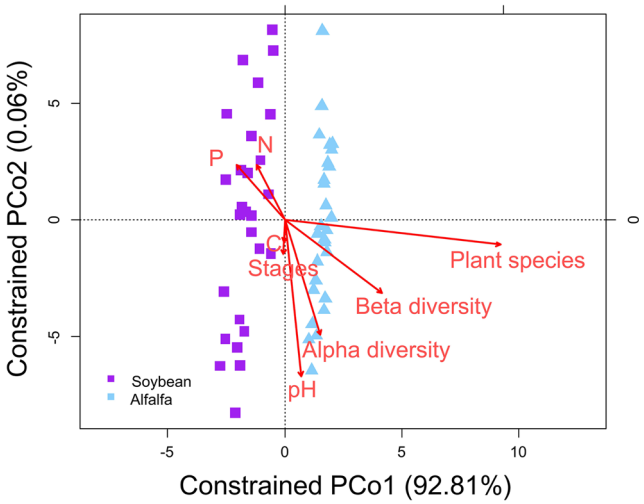

Fig. 3 Dot plot of the constrained analysis of principal coordinates for plant biomass based on Bray-Curtis distance

Discussion

The composition and structure of rhizospheric microbial community is known to vary across different soil types and plant species (Berg and Smalla 2009; Lundberg et al. 2012; Peiffer et al. 2013). Previous studies on rhizomicrobiomes have mainly focused on how biotic and abiotic factors influence their structure, composition and function (Edwards et al. 2015; Lundberg et al. 2012; Mendes et al. 2014; Peiffer et al. 2013; Pii et al. 2016). In the present study, we evaluated the relationship between plant biomass and the rhizomicrobiome of two distinct leguminous plants grown in soils with different properties. We found that plant biomass was mainly influenced by plant species, but the diversity of the rhizomicrobiome, soil pH, N, K and growth stage all contributed and beta diversity played an important role.

Effects of rhizomicrobes on plant biomass

The rhizosphere supports the activity and development of a variety of microbes, including PGPR (Berg 2009). In the present study, possible reasons for the positive correlation between rhizomicrobes and plant (soybean and alfalfa) biomass were the following: (1) these microbes were potential PGPR for these plant species and (2) plant growth (increased biomass) selectively stimulated the accumulation of these microbes in the rhizosphere by providing nutrients or signal substances. All microbes positively correlated with plant biomass for both soybean and alfalfa (*Massilia*, *Burkholderia*, *Ensifer*, *Flavisolibacter*, *Luteimonas*, *Burkholderiaceae*, *Sphingomonadaceae*, *Micrococcaceae* and *Geodermatophilaceae*) were divided into three classes based on the existing literature and are discussed below.

The first class included *Massilia*, *Burkholderia* and *Ensifer*, which have been reported as PGPR. Some species of *Massilia* promote plant growth through indole acetic acid production, siderophore production, and antagonism towards *Phytophthora infestans* (Ofek et al. 2012; Poupin et al. 2013). *Burkholderia* have also been detected as root endophytic PGPB in potato, tomato, sugarcane and grapevine (Paungfoo-Lonhienne et al. 2014; Trda et al. 2014). It has been speculated that the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase produced by *Burkholderia* promotes plant growth by reducing the plant ethylene hormone (Poupin et al. 2013). Additionally, some species of *Burkholderia* in the maize rhizosphere can solubilise phosphate and show antagonism against pathogenic fungi (Zhao et al. 2014). In addition to symbiosis with legumes to facilitate nitrogen fixation, members of *Ensifer* can solubilise inorganic phosphate, secrete indole acetic acid, produce siderophores, induce systemic resistance and display ACC deaminase activity (Sorty et al. 2016; Tian et al. 2014). Intriguingly, *Ensifer* can also promote the growth of non-leguminous plants (Galleguillos et al. 2000). These bacteria might therefore promote the growth of soybean and alfalfa in the present study.

The second class included *Burkholderiaceae*, *Sphingomonadaceae* and *Micrococcaceae* families that include genera with plant growth-promoting activity (Chapelle et al. 2016). The *Pandoraea* genus of *Burkholderiaceae* possesses ACC deaminase activity that could increase the root length of canola (Anandham et al. 2008). *Pandoraea* sp. OXJ-11 is capable of suppressing *Sclerotinia sclerotiorum* infection in *Brassica napus* (Jin et al. 2007). The *Sphingomonas* and *Novosphingobium* genera of *Sphingomonadaceae* can produce the phytohormones salicylic acid, gibberellins, indole-3-acetic acid and abscisic acid (Yang et al. 2014) and induce systemic resistance (Hahm et al. 2012) to promote plant growth. The *Rothia* genus of the *Micrococcaceae* family can alleviate *Spodoptera litura* infestation and increase the biomass and yield of tomato (Bano and Muqarab 2017). The *Arthrobacter* genus of *Micrococcaceae* can inhibit the growth of phytopathogenic fungi and enhance salt tolerance in plants (Velazquez-Becerra et al. 2013). The *Sinomonas* and *Micrococcus* genera of *Micrococcaceae* promote plant growth via phosphate solubilisation, biological control activity, auxin production, ACC deaminase activity and siderophore production (Adhikari et al. 2017; Dastager et al. 2010). Not all genera of *Burkholderiaceae*, *Sphingomonadaceae* and *Micrococcaceae* have been reported as PGPR; therefore, their potential for promoting soybean and alfalfa growth needs further exploration.

The third class includes *Flavisolibacter*, *Luteimonas* and *Geodermatophilaceae*, none of which has been reported as PGPR previously. Species of *Flavisolibacter* have mainly been isolated from or detected in soil (Huang et al. 2016; Joo et al. 2015; Lee et al. 2016; Yoon and Im 2007). Species of *Luteimonas* have been isolated from various environments,

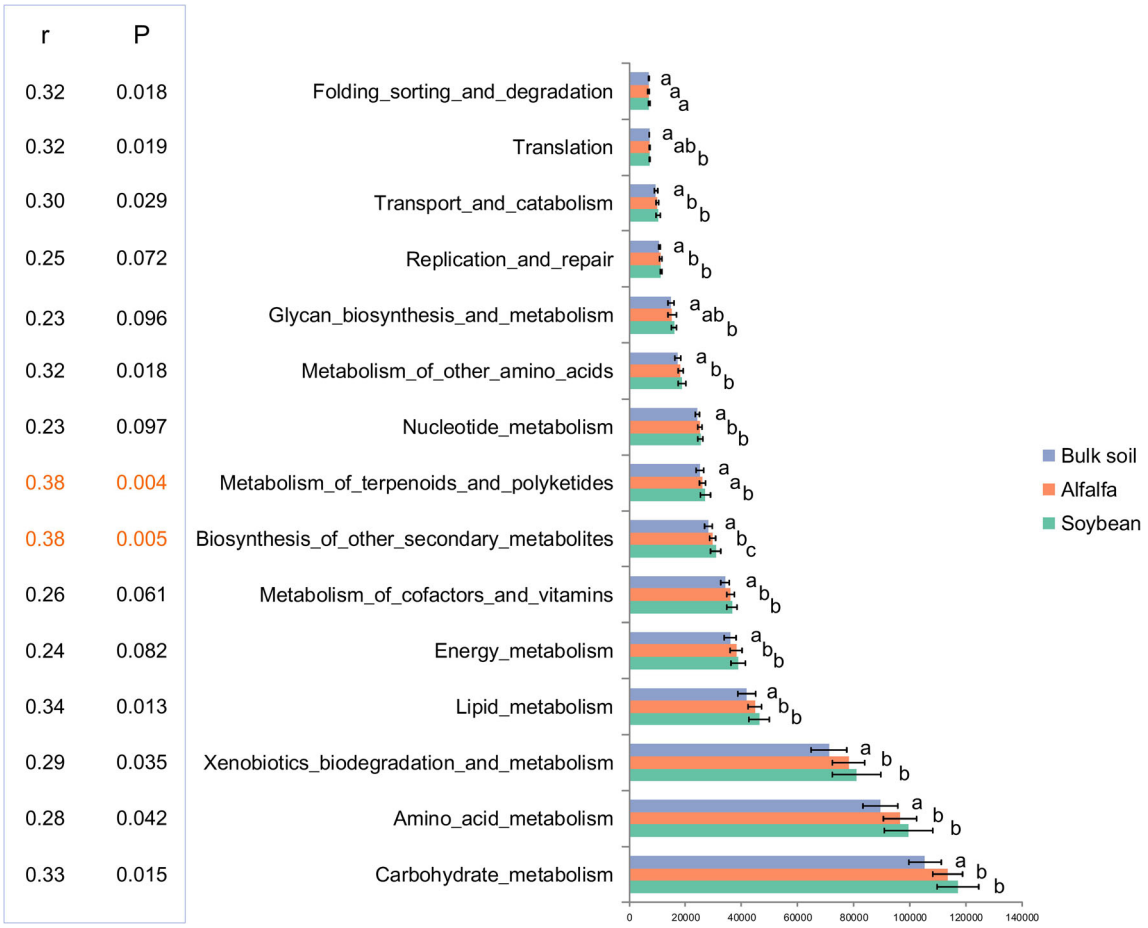

Fig. 4 Predicted functions of microbiomes ($P < 0.05$) associated with the soybean rhizosphere, the alfalfa rhizosphere and bulk soil. Bars with different letters are significantly different ($P < 0.05$; ANOVA Duncan's test) from one another. The table on the left shows correlation coefficients between predicted functions and plant biomass. Biosynthesis of other secondary metabolites and metabolism of terpenoids and polyketides are strongly correlated with plant biomass

including soil, water, seashore sediment, tidal flat sediment, deep sea sediment, cucumber leaf, food waste and biofilters (Cheng et al. 2015; Fan et al. 2014). Members of *Geodermatophilaceae* have also been found in various environments, including soil samples, soil crusts, seafloor sediments, stone habitats, dry-hot valleys and deserts (Sun et al. 2015; Zhang et al. 2011). There is no direct evidence indicating the ability of *Flavisolibacter*, *Luteimonas* and *Geodermatophilaceae* species to promote plant growth but they may include PGPR species and/or stimulate plant growth in this study.

The plant growth-promoting ability of rhizobacteria mainly includes biofertilisation, biostimulation and biocontrol activities (Gaiero et al. 2013). Using functional prediction (Fig. 3), a strong correlation was observed between the metabolism of terpenoids and polyketides and the biosynthesis of other secondary metabolites with plant biomass, implying that these are key functions in the plant growth-promoting process. Terpenes are a large class of chemicals that are used to build plant growth-promoting substances, such as phytohormones (gibberellins and abscisic acid), membrane-related sterols and defence-related compounds (volatiles and sesqui- and di-terpenic phytoalexins) (Piccoli and Bottini 2013). Researchers have described the production of polyketides with antibiotic activity by rhizobacteria of wheat, sugar beet, cotton and tobacco (Brodhagen et al. 2004). In addition to terpenes and polyketides, many other classes of secondary metabolites with diverse structural groups, such as steroids, xanthones, chinones, phenols, isocoumarins, benzopyranones, tetralones, cytochalasines and enniatines (Schulz et al. 2002), possess antimicrobial activity that in many cases protect plants (tobacco, wheat, maize, tomato, sugar beet, potato and alfalfa) against pests and phytopathogens (Ludwig-Müller 2015; Lugtenberg and Kamilova 2009).

Influence of soil factors on rhizomicrobes

The influence of soil properties differed between the four groups of rhizobacteria and between the entire rhizomicrobiomes (Supplementary Table S3; Supplementary Fig. S5). Soil properties had an opposite influence on Group 1 vs. Group 2 and on

Table 1 Significant relationships ($P < 0.01$) between the relative abundance of rhizomicrobes and plant (soybean and alfalfa) biomass at the genus level calculated by Spearman's correlation coefficient

Soybean	*r*	*P*	Group
Bacteria; *Proteobacteria*; *Betaproteobacteria*; *Burkholderiales*; *Oxalobacteraceae*; *Massilia*	0.568	0.002	Group1
Bacteria; *Bacteroidetes*; *Sphingobacteriia*; *Sphingobacteriales*; *Chitinophagaceae*; *Flavisolibacter*	0.506	0.007	
Bacteria; *Proteobacteria*; *Betaproteobacteria*; *Burkholderiales*; *Burkholderiaceae*; Other	0.452	0.018	
Bacteria; *Proteobacteria*; *Alphaproteobacteria*; *Sphingomonadales*; *Sphingomonadaceae*; Other	0.451	0.018	
Bacteria; *Proteobacteria*; *Gammaproteobacteria*; *Xanthomonadales*; *Xanthomonadaceae*; *Luteimonas*	0.443	0.021	
Bacteria; *Proteobacteria*; *Betaproteobacteria*; *Burkholderiales*; *Burkholderiaceae*; *Burkholderia*	0.415	0.032	
Bacteria; *Planctomycetes*; *Planctomycetia*; *Planctomycetales*; *Planctomycetaceae*; *Pirellula*	− 0.559	0.002	Group 2
Archaea; *Thaumarchaeota*; *Nitrososphaerales*; *Nitrososphaerales*; *Nitrososphaeraceae*; *Nitrososphaera*	− 0.474	0.013	
Bacteria; *Bacteroidetes*; *Cytophagia*; *Cytophagales*; *Cytophagaceae*; *Adhaeribacter*	− 0.467	0.014	
Bacteria; *Actinobacteria*; *Actinobacteria*; *Actinomycetales*; *Geodermatophilaceae*; *Blastococcus*	− 0.449	0.019	
Bacteria; *Bacteroidetes*; *Sphingobacteriia*; *Sphingobacteriales*; *Sphingobacteriaceae*; *Sphingobacterium*	− 0.435	0.023	
Bacteria; *Firmicutes*; *Bacilli*; *Bacillales*; *Paenibacillaceae* 1; Other	− 0.426	0.027	
Bacteria; *Bacteroidetes*; *Bacteroidetes_incertae_sedis*; *Ohtaekwangia*; *Ohtaekwangia*; *Ohtaekwangia*	− 0.410	0.034	
Bacteria; *Actinobacteria*; *Actinobacteria*; *Actinomycetales*; *Promicromonosporaceae*; *Promicromonospora*	− 0.402	0.037	
Bacteria; *Proteobacteria*; *Alphaproteobacteria*; *Rhodospirillales*; *Rhodospirillaceae*; *Skermanella*	− 0.398	0.040	
Bacteria; *Actinobacteria*; *Actinobacteria*; *Actinomycetales*; *Nocardioidaceae*; *Aeromicrobium*	− 0.391	0.044	
Bacteria; *Planctomycetes*; *Planctomycetia*; *Planctomycetales*; *Planctomycetaceae*; Other	− 0.385	0.047	
Bacteria; Other; Other; Other; Other; Other	− 0.384	0.048	
Alfalfa	*r*	*P*	Group
Bacteria; *Proteobacteria*; *Alphaproteobacteria*; *Rhizobiales*; *Rhizobiaceae*; *Ensifer*	0.488	0.010	Group 3
Bacteria; *Actinobacteria*; *Actinobacteria*; *Actinomycetales*; *Micrococcaceae*; Other	0.484	0.011	
Bacteria; *Actinobacteria*; *Actinobacteria*; *Actinomycetales*; *Geodermatophilaceae*; Other	0.426	0.027	
Bacteria; *Bacteroidetes*; *Flavobacteriia*; *Flavobacteriales*; *Flavobacteriaceae*; Other	− 0.632	0.000	Group 4
Bacteria; *Proteobacteria*; Other; Other; Other; Other	− 0.588	0.001	
Bacteria; *Proteobacteria*; *Alphaproteobacteria*; *Rhodospirillales*; Other; Other	− 0.502	0.008	
Bacteria; *Bacteroidetes*; *Sphingobacteriia*; *Sphingobacteriales*; *Chitinophagaceae*; *Chitinophaga*	− 0.498	0.008	
Bacteria; *Bacteroidetes*; *Sphingobacteriia*; *Sphingobacteriales*; *Sphingobacteriaceae*; *Olivibacter*	− 0.469	0.013	
Bacteria; *Proteobacteria*; *Gammaproteobacteria*; *Xanthomonadales*; *Xanthomonadaceae*; *Stenotrophomonas*	− 0.452	0.018	
Bacteria; *Proteobacteria*; *Betaproteobacteria*; *Burkholderiales*; *Alcaligenaceae*; Other	− 0.443	0.021	
Bacteria; *Proteobacteria*; *Alphaproteobacteria*; *Rhodospirillales*; *Rhodospirillaceae*; *Inquilinus*	− 0.430	0.025	
Bacteria; *Proteobacteria*; *Alphaproteobacteria*; *Rhizobiales*; *Phyllobacteriaceae*; *Phyllobacterium*	− 0.394	0.042	
Bacteria; *Proteobacteria*; *Betaproteobacteria*; Other; Other; Other	− 0.388	0.045	
Bacteria; *Proteobacteria*; *Gammaproteobacteria*; *Xanthomonadales*; *Xanthomonadaceae*; *Pseudoxanthomonas*	− 0.388	0.046	

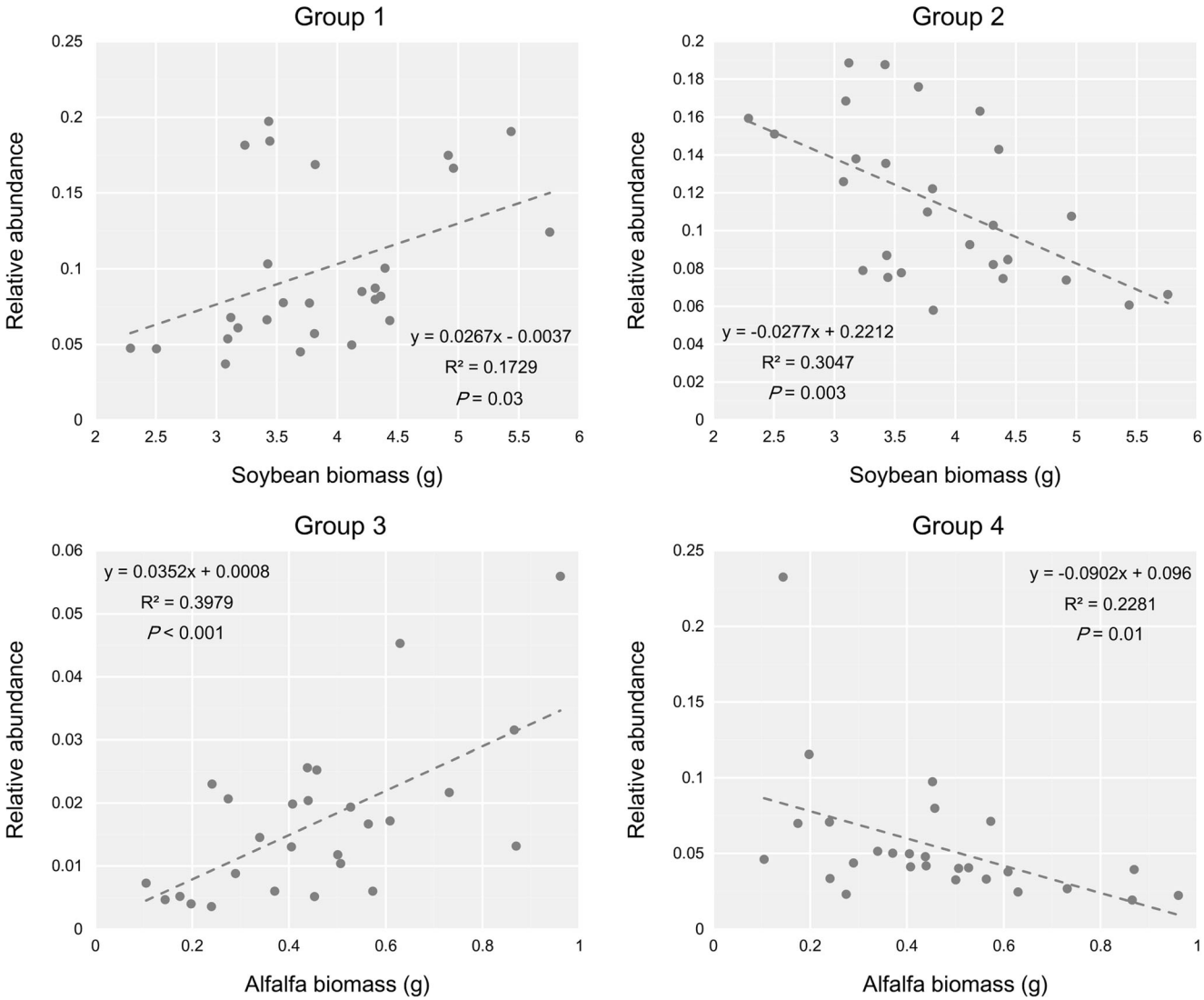

Fig. 5 Relationships between soybean and alfalfa biomass productivity and the relative abundance of aggregate genera in each category (positive/negative correlations with soybean or alfalfa biomass) indicated by linear regression

Group 3 vs. Group 4. This might be because bacteria in groups 1 and 3 inhibit the growth of bacteria in groups 2 and 4, either directly by competition in the narrow niches and secreting specific secondary metabolites or indirectly by promoting plant growth (Vacheron et al. 2013). Plant roots can also secrete root exudates to modulate soil abiotic properties and rhizomicrobial assemblage (Dennis et al. 2010). The quantity and composition of root exudates vary with plant species, plant growth and external factors such as biotic and abiotic stressors (Badri and Vivanco 2009; Chaparro et al. 2014). To counteract infection and confer tissue-specific resistance, plants release biologically active compounds into the rhizosphere. Root exudates are known to perform a multitude of functions by acting not only as signalling molecules, attractants and stimulants but also as inhibitors or repellents, which may have a profound effect on PGPR and pathogens (Baetz and Martinoia 2014). This may explain the opposite response to soil properties in Group 1 vs. Group 2 and in Group 3 vs. Group 4.

This study demonstrated that leguminous plant biomass was influenced by plant species, rhizomicrobiome diversity, soil properties and growth stage, among which rhizomicrobiome diversity played an essential role. A positive correlation was revealed between leguminous plant growth and some rhizobacteria, including *Flavisolibacter*, *Luteimonas* and *Geodermatophilaceae_Other*, none of which has been identified as PGPR previously. The PGPR and rhizomicrobiome assemblies were modulated by plant species, growth stage and soil properties, presumably for specific functions. Overall, our findings suggest that plants, rhizomicrobiomes and soils are in constant communication through the exchange of nutrients and signals.

Using a deep Illumina sequencing of 16S rRNA amplicons, we explored the diversity of rhizomicrobiome and PGPR

more comprehensively than using culture-based methods. And the variations of PGPR among different environmental conditions (plant species, growth stages and soil properties) were further analysed. This ecological knowledge on PGPR populations might advance the development of more accurate and effective PGPR inoculants adapting to different environmental conditions. This might be a prerequisite to develop more practical management strategies for sustainable agriculture.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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