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Organic amendments shift the phosphorus-correlated microbial co-occurrence pattern in the peanut rhizosphere network during long-term fertilization regimes

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ABSTRACT

Root-associated microbial communities play important roles in driving the microbial transformation of soil carbon and nitrogen cycling, but evidence of how these communities shape microbial co-occurrence and change phosphorus (P) correlated microbial interactions is still lacking. In this study, a random matrix theory-based network analysis of 16S rRNA genes was used to identify bacterial networks associated with the peanut rhizosphere and bulk in four long-term fertilization schemes. High-throughput sequencing data revealed that with the decrease of rhizosphere bacterial diversity, its positive covariation in the network increased, indicating stronger commensal and mutual assemblage with less functional redundancy in the rhizosphere. P availability is the major variable modulating the peanut rhizosphere microbial community in acidic soil. Two strongly positive P-correlated rhizosphere OTUs (closely related to *Chitinophaga pinensis* and *Nitrospira moscoviensis*), which have the potential to promote carbon-phosphorus and nitrogen-phosphorus synergistic conversion, respectively, were used to investigate the advantages of manure amendment in promoting rhizosphere P cycling in peanut planting agrosystems.

1. Introduction

Soil available phosphorus (P) is crucial for plant growth, but its deficiency is a problem in tropical acid soil, where P is easily converted into poorly soluble Fe and Al phosphates and is thus unavailable to plants (Ziadi et al. 2013). Until recently, traditional nutrient management for preserving high crop productivity has been mainly based on external fertilizer inputs (Pii et al. 2015). East Asia is the area that has contributed the largest cumulative global P surplus due to the low P use efficiency and the largest P fertilizer application (MacDonald et al. 2011). Excessive use of P input poses threats for ecosystem function, as the enrichment can contribute to water eutrophication and biodiversity loss (Aubriot et al. 2011). Therefore, there is substantial interest in improving phosphorus supply utilization in the agro-ecosystem.

Rhizosphere processes associated with microorganisms have an intrinsic biological potential to improve crop nutrient uptake capacity (Pii et al. 2015). Many reports have found that such variable success of carbon and nitrogen uptake are typically attributed to the complexity of microbial communities and their interactions in the zone immediately surrounding the roots (Ai et al. 2015, 2017; Francioli et al. 2016). In the case of phosphorus, another essential element for crop growth, the relationship of its availability and rhizosphere microbial interaction is still unclear. Castrillo et al (2017) demonstrated that the root synthetic bacterial community drives direct integration of phosphate stress in *Arabidopsis thaliana*, implying that plant roots have the potential to assemble specific microbial groups to benefit P cycling. However, there is still little information available regarding rhizosphere effects on P-correlated microbial interactions in agricultural soils.

Advances in culture-independent techniques have provided tools for the study of phosphorus transforming microbial communities in the environment. Since the first probe (ALPS primers) targeting alkaline phosphatase were developed, researchers not only improved new sets of specific primers to target a larger diversity of alkaline phosphatase genes (such as *pho*D), but had various insights into alkaline phosphatase diversity affected by crop management and fertilizers application (Sakurai et al. 2008; Wang et al. 2012; Tan et al. 2013; Fraser et al. 2015). However, some reports noted that ALPS primers likely have an amplification bias, resulting in an overrepresentation of

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Alphaproteobacteria (Tan et al. 2013). These studies focused on alkaline phosphatase but ignored the organic P mineralization from acid phosphatase (EC.3.1.3.2) and inorganic P dissolution. A new approach is required to provide a more comprehensive evaluation of microbial activation of phosphorus.

An ecological network, which is represented by complicated positive (e.g., commensalism and mutualisms) and negative (e.g., predation and competition) interactions (Chow et al. 2014), provides a standardized framework for understanding the interactions among microbial populations in complex systems (Poisot et al. 2012; Toju et al. 2017). Documenting inter-taxa and intra-taxa associations modulated by specific environmental factors across complex microbial communities may help predict the functional roles, habitat affinities and shared physiologies that can guide more focused studies or experimental settings (Cardinale et al. 2015). Jiang et al. (2017b) reported nematode predacious promotion of bacterial diversity and functional (alkaline phosphomonoesterase producing) bacterial abundance through network interaction, indicating this developing approach may forecast the interactions between specific functional microbes (such as P-transforming microorganisms) and the entire microbial community.

Peanuts are the fifth most important oil seed crop and are widely cultivated in tropical and subtropical regions of Asia, Africa and North and South America, areas facing excessive P input but relative low P use efficiency (FAOSTAT 2014). It is important to evaluate the coordinated effects of long-term fertilization schemes and rhizosphere effects on peanut rhizosphere P-correlated specific microbial assemblages. In acidic soil regions, the main fertilization scheme of peanut planting includes (1) traditional chemical fertilization and (2) inorganic (chemical) and organic (straw or manure) combined application. To further develop ecological and circular agriculture, the replacement of chemical fertilizer with manure or straw is the current popular modern eco-agricultural management. We hypothesized that fertilization schemes and rhizosphere effects cooperatively shaped the variation in the rhizosphere microbial community structure and construct the specific network interactions in the millimeter-sized habitat to activate rhizosphere nutrients, such as P. Furthermore, we expected to discover P-correlated microorganisms that are specifically regulated by rhizosphere effects and explore their niche position in the microbial ecological networks.

To verify our speculation, peanut rhizosphere and bulk soil were collected from four long-term fertilization schemes (a no-fertilization control; inorganic nitrogen, phosphorus and potassium, NPK; NPK plus organic manure, NPKM; NPK plus straw, NPKS). The 16S rRNA gene amplicon sequencing coupled with co-occurrence networks analysis were used to present the systematic framework for rhizosphere and bulk microbial diversification and ecological networks. We filtered the P-strongly correlated OTUs through a Pearson-correlation analysis, and we further explored their functional ecological interactions through matching OTUs to the co-occurrence networks and compared their relative abundance in different fertilization schemes.

2. Materials and methods

2.1. Experimental sites and sample collections

A long-term fertilization experiment was initiated in 1988 at the Red Soil Ecological Experimental Station of the Chinese Academy of Sciences located in Yingtan, Jiangxi Province, China (28°15′N, 116°55′E). This region has a subtropical, humid monsoon climate with an annual average temperature and precipitation of 17.6 °C and 1795 mm, respectively. The soil is an acid loamy clay that is derived from quaternary red clay (Udic Ferralsols in Chinese Soil Taxonomy and Ferric Acrisols in the FAO classification system). Four fertilization treatments (triplicate soil blocks for each) were established with a peanut-rape rotation from 1989 to 1995 and peanut cropping since 1996. Treatments consisted of soil without fertilizer (control, CK), fertilizer N, P and K (NPK), fertilizer NPK plus organic manure (NPKM), and fertilizer NPK plus peanut straw (NPKS) in a randomized plot design (5.3 m in width × 6.5 m in length × 1.0 m in depth). For the NPK treatment, fertilizers N, P and K were applied in the form of urea (120 kg N ha^{-1}), superphosphate (75 kg P_2O_5 ha^{-1}) and potassium chloride (75 kg K_2O ha^{-1}), respectively. For NPKM or NPKS treatment, the chemical fertilizer application was 70% of the regular amount of NPK fertilizer (84 kg N ha^{-1} y^{-1}, 52.5 kg P_2O_5 ha^{-1} y^{-1}, and 52.5 kg K_2O ha^{-1} y^{-1}) plus 30% of the same content present in manure or peanut straw (contain 36 kg N ha^{-1} y^{-1}, 22.5 kg P_2O_5 ha^{-1} y^{-1}, and 22.5 kg K_2O ha^{-1} y^{-1}).

The rhizosphere and bulk soil samples were collected during the peanut plant flowering stage in June 2015, which is when the rhizosphere effects tend to be pronounced (Shi et al. 2016). Rhizosphere soil was collected by gently shaking the entire plant root system to remove loosely attached soil (Ai et al. 2015). Six soil samples (three from the rhizosphere and the other three from the bulk) from each treatment (in total, 24 samples) were placed in polythene wrappers, chilled on ice following their collection in the field, and immediately transported to the laboratory, where they were sieved to 4 mm to remove visible roots and residue. Each soil sample was then divided into two subsamples and stored at either 4 °C for measurements of their physical and chemical properties or −80 °C for the microbial community analysis.

2.2. Soil physical and chemical properties measurement

The soil pH was determined with a glass electrode in a water-to-soil ratio of 2.5:1 (v/w). Soil total nitrogen and nitrate (NO_3^- − N) and ammonium nitrogen (NH_4^+ − N) were analyzed with a flow injection auto-analyzer (Auto Analyzer 3; Seal Analytical, Norderstedt, Germany). The soil total phosphorus (TP) and available phosphorus (AP) were extracted by sodium carbonate and sodium bicarbonate, respectively; both were quantified using the molybdenum blue method (Olsen et al. 1954). Total potassium (TK) and available potassium (AK) concentrations were determined using flame photometry after their extraction with sodium hydroxide and ammonium acetate, respectively (Kanehiro and Sherman, 1965). Soil organic carbon (SOC) content was measured by wet digestion using the potassium dichromate oxidation method. The soil moisture was determined by drying the soil for 48 h at 105 °C. The cation exchange capacity (CEC) was measured in an ammonium acetate solution at pH 7 (Chapman, 1965).

2.3. 16S rRNA gene amplicons and high-throughput sequencing

The microbial genomic DNA was extracted from 1.0 g of soil for each sample using a Power Soil DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA) according to the manufacturer's instructions. The bacterial 16S rRNA genes were amplified using primers 515F (5′-GTG CCAGCMGCCGCGG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′), which target the V4 hypervariable regions. The PCR was performed in 50 μl reaction volumes containing 25 μl of Premix Taq DNA polymerase, 0.5 μl of forward primer (20 μM), 0.5 μl of reverse primer (20 μM), 23 μl of doubly distilled water (ddH_2O), and 1 μl of DNA template (20 ng of total soil DNA). The thermal cycling conditions were as follows: an initial denaturation at 94 °C for 5 min, followed by 30 cycles at 94 °C for 45 s, 56 °C for 30 s and 72 °C for 45 s, with a final extension at 72 °C for 7 min. Illumina libraries were constructed using the MiSeq Reagent Kit V3 according to manufacturer's instructions. High-throughput, paired-end sequencing was performed on the Illumina MiSeq PE250 platform. Sequencing data were deposited in the European Nucleotide Archive under the accession number PRJEB22659.

2.4. Analysis of sequencing data

Based on the MiSeq Reagent Kit Preparation Guide (Illumina, San

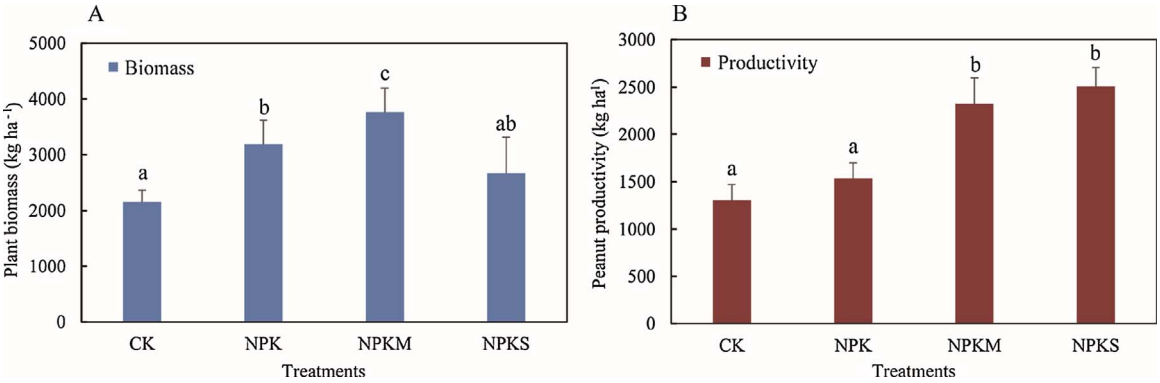

Fig. 1. Variations of plant biomass (A) and peanut productivity (B) in different fertilization schemes. Values are represented as the mean (n = 3) ± the standard deviation of the mean. Values in the same group marked by a superscript lowercase letter indicate a significant difference according to one-way analysis of variance (ANOVA) with Duncan test ($P < 0.05$).

Diego, CA, USA), the purified mixture was diluted and denatured to obtain the 8-pM sample DNA library, which was mixed with an equal volume of 8-pM PhiX (Illumina). Finally, 600 µl of the mixture library was loaded with read 1, read 2 and the index sequencing primers (Caporaso et al. 2012) in a 500-cycle (2 × 250 paired ends) kit and run on a MiSeq apparatus. The high-throughput data were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) package (version 1.9.1). After assigning each sequence to its sample according to its barcode (allowing up to two mismatches), a total of 948,765 reads from both ends were obtained as a partitioned run for the samples. Forward and reverse reads with at least a 50-bp overlap and fewer than 5% mismatches were joined using FLASH (Magoc and Salzberg, 2011). Low-quality sequences (Phred quality score Q < 20 or sequences shorter than 200 bp or sequences with ambiguous bases) were removed, and chimeras were filtered by the UCHIME algorithm (Edgar et al. 2011) using the RDP Gold 16S reference sequences (http://drive5.com/uchime/uchime_download.html). Operational taxonomic units (OTUs) were clustered using the UCLUST program at the 97% similarity level (Edgar, 2010). OTUs were annotated using the RDP Classifier (Wang et al. 2007) with the Greengenes 16S rRNA database (version 13.8). Singleton OTUs were removed, and all samples were rarefied to 10, 947 sequences for downstream analyses.

2.5. Statistical analyses

Data of plant growth and productivity, chemical variables and bacterial diversity were subjected to ANOVA using a Duncan test at $P < 0.05$ (SPSS 18.0). Redundancy analysis (RDA) was used to evaluate the effect of soil chemical variables on the microbial community composition based on a Bray-Curtis distance matrix (Legendre and Legendre, 2012). The statistical significance of environmental variables was obtained by partial Mantel tests (1000 permutations). Variation partitioning analysis (VPA) was used to quantify the contribution of environmental features to bacterial community composition based on partial canonical correspondence analysis (pCCA). The Pearson's correlation test was used to examine the correlation between bacterial abundance and environmental factors (pH, TP and AP). All the above analyses were performed in R (Version 3.2.3, http://www.r-project.org) using the Vegan (Oksanen et al. 2013) and gbmplus packages.

The phylogenetic molecular ecological networks (pMENs) of the bacteria from the rhizosphere and the bulk were constructed using the random matrix theory (RMT)-based network approach (Zhou et al. 2010; Luo et al. 2007). To reduce rare OTUs in the data set, we removed OTUs with average relative abundances less than 0.1% of the total number of bacterial sequences. To visualize the associations in the network, the co-occurrence network was inferred based on the Spearman correlation matrix ($P < 0.01$). The P-values were adjusted using the Benjamini-Hochberg false discovery rate (FDR) controlling procedure (Benjamini and Hochberg, 1995). To describe the complex pattern of interrelationships of bacterial OTUs, the topological characteristics of the networks were calculated as follow: average path length (APL), average clustering coefficient (*avgCC*) and modularity (M). The topological role of each node was determined based on two properties: the within module connectivity *Zi*, which quantified how well connected a node was to other nodes in their module, and the among-module connectivity *Pi*, which quantified to what extent the node connected to different modules (Guimera and Nunes, 2005, Liang et al.2016). According to the simplified criteria, all species were sorted into four subcategories: peripherals, connectors, module hubs, and network hubs (Olesen et al. 2007). Random networks with equal numbers of nodes and edges as the empirical networks were generated based on a null model by using the Maslov and Sneppen (2002) procedure. Network images were visualized using Gephi software (http://gephi.github.io/).

3. Results

3.1. Effect of long-term fertilization schemes on the rhizosphere, bulk soil chemical variables and plant growth

The variation of the plant biomass was not entirely consistent with the peanut productivity (Fig. 1). As shown in the NPK treatment, although its biomass is 19.47% higher than that of NPKS, its yield is 38.77% lower. Comparatively, manure input both increased the peanut biomass and productivity. The major chemical characteristics in the rhizosphere soil of peanut along with bulk soil in different long-term fertilization schemes are summarized in Table 1. Soil carbon (SOC) and nitrogen (TN, NH_4^+- N and NO_3^-- N) were shown to be more dependent on cropping management, which indicates that they varied with fertilization schemes. Within each treatment, there were no differences between the rhizosphere and the bulk soil. However, AP, AK and pH varied depending on the fertilization and sampling domain: in CK and NPKS treatments, rhizosphere AP and AK were significant higher than that of the bulk, while in NPK treatment, the concentration of rhizosphere AP and AK were lower than the equivalent bulk. The pH value in rhizosphere is higher than that of equivalent bulk soil, except in the NPKM treatment in which rhizosphere pH is slightly lower than that of bulk (Table 1). In general, root secretion of low molecular weight organic acid reduce the rhizosphere pH (such as the phenomenon in NPKM), while when the soil pH < 5 (typical acidic soil), the organic acids existing in the form of anions, which associate with H+, will raise the micro-domain pH(such as the phenomenon in CK, NPK and NPKS). Pairwise Pearson comparisons showed that there were strong correlations among the plant biomass, pH, TP and the AP in the rhizosphere, while the rhizosphere SOC was positively correlated with the TN (Table 2).

Table 1
Chemical properties of long-term fertilization schemes.

Treatments	Fraction	Moisture(%)	SOC(g kg^{-1})	TN(g kg^{-1})	TP(g kg^{-1})	TK(g kg^{-1})	NH_4^+-N(mg kg^{-1})	NO_3^--N(mg kg^{-1})	AP(mg kg^{-1})	AK(mg kg^{-1})	CEC(cmol kg^{-1})	pH
Control	Rhizosphere	27.93 ± 1.48^{a}	13.91 ± 0.32^{b}	0.87 ± 0.09^{ab}	0.48 ± 0.07^{a}	9.75 ± 0.35^{b}	6.67 ± 0.46^{ab}	1.79 ± 0.32^{a}	14.82 ± 1.44^{b}	123.75 ± 2.30^{b}	13.59 ± 0.17^{d}	4.92 ± 0.04^{bc}
	Bulk soil	37.10 ± 2.96^{b}	13.06 ± 0.12^{ab}	0.85 ± 0.08^{ab}	0.42 ± 0.07^{a}	9.65 ± 0.09^{ab}	6.21 ± 0.68^{ab}	2.48 ± 0.72^{ab}	6.76 ± 1.76^{a}	105.83 ± 3.10^{a}	13.66 ± 0.06^{d}	4.77 ± 0.02^{a}
NPK	Rhizosphere	30.92 ± 3.24^{a}	11.96 ± 0.55^{a}	0.78 ± 0.04^{a}	0.71 ± 0.07^{a}	9.00 ± 0.33^{a}	6.57 ± 0.37^{ab}	2.69 ± 0.70^{ab}	28.04 ± 2.11^{d}	143.75 ± 2.37^{c}	12.31 ± 0.90^{ab}	4.97 ± 0.06^{cd}
	Bulk soil	31.29 ± 3.49^{a}	11.80 ± 0.23^{a}	0.79 ± 0.03^{a}	0.62 ± 0.08^{a}	9.2 ± 0.35^{ab}	5.64 ± 0.26^{a}	3.09 ± 0.60^{ab}	35.66 ± 1.88^{e}	160.00 ± 2.67^{d}	12.28 ± 0.21^{ab}	4.90 ± 0.09^{bc}
NPKM	Rhizosphere	27.02 ± 2.34^{a}	15.17 ± 0.43^{b}	1.04 ± 0.08^{c}	1.34 ± 0.03^{b}	9.30 ± 0.24^{ab}	6.22 ± 0.81^{ab}	2.82 ± 0.52^{ab}	114.10 ± 8.63^{f}	176.25 ± 3.76^{e}	13.13 ± 0.50^{abc}	5.05 ± 0.07^{de}
	Bulk soil	29.00 ± 3.60^{a}	14.89 ± 0.85^{b}	1.03 ± 0.03^{c}	1.40 ± 0.15^{b}	9.23 ± 0.28^{ab}	7.00 ± 0.62^{ab}	4.27 ± 0.99^{b}	116.27 ± 4.82^{f}	179.17 ± 4.64^{e}	13.20 ± 0.21^{bc}	5.11 ± 0.01^{e}
NPKS	Rhizosphere	36.67 ± 2.97^{b}	13.40 ± 1.56^{ab}	0.97 ± 0.08^{bc}	0.54 ± 0.04^{a}	9.75 ± 0.14^{b}	7.69 ± 0.58^{b}	3.21 ± 0.93^{ab}	19.26 ± 2.36^{c}	207.5 ± 4.33^{f}	12.21 ± 0.62^{a}	4.90 ± 0.04^{bc}
	Bulk soil	29.24 ± 2.23^{a}	13.45 ± 1.49^{ab}	0.96 ± 0.09^{bc}	0.75 ± 0.27^{a}	9.68 ± 0.75^{ab}	6.62 ± 0.26^{ab}	2.81 ± 1.69^{ab}	14.19 ± 1.01^{b}	159.17 ± 5.27^{d}	12.31 ± 0.35^{ab}	4.82 ± 0.06^{ab}

Values are represented as the mean (n = 3) ± the standard deviation of the mean. Values in the same column marked by a superscript lowercase letter indicate a significant difference according to one-way analysis of variance (ANOVA) with Duncan test ($P < 0.05$).

3.2. Microbial diversity and abundance

To determine whether rhizosphere process is as important as fertilization in controlling microbial community diversity, we compared the bulk and rhizosphere microbe diversity in four fertilized soil samples (Fig. 2). The bulk soil showed higher microbial diversity (richness and Shannon index) compared with the equivalent rhizosphere, except in NPKS (Fig. 2). The bulk of NPKM possessed the highest microbial diversity, whereas the rhizosphere of NPKM showed the lowest.

In total, the 870,295 sequences that were classified as bacteria belonged to 34 phyla, 131 classes, 264 orders, 429 families and 816 genera. The bacterial communities were dominated by Actinobacteria (13.31%), Acidobacteria (12.49%), Chloroflexi (11.80%), Alphaproteobacteria (10.75%) and Betaproteobacteria (9.32%) (Fig. S1, Supporting information). The relative abundances of Planctomycetes, and Gemmatimonadetes in the rhizosphere were increased in the bulk of organic fertilization, whereas Firmicutes were decreased in the rhizosphere in CK, NPK and NPKS (Fig. 3). Betaproteobacteria showed a decrease in the rhizopshere of fertilization schemes. Deltaproteobacteria and Chloroflexi varied between the rhizosphere and bulk only within NPK treatment (Fig. 3). The phyla of Acidobacteria, Actinobacteria, Alphaproteobacteria, Armatimonadetes, Cyanobacteria, Gammaproteobacteria and Verrucomicrobia varied among the different fertilization schemes, but no differences were found between the rhizosphere and bulk within treatments (Fig. S2, Supporting information).

3.3. Relationship between microbial composition and environmental variables

Redundancy analysis (RDA) showed the relationships between the response and explanatory variables in the bulk and rhizosphere, respectively (Fig. 4). All explanatory variables were tested for significance by Spearman's rank correlation of the Mantel test before RDA analysis (Table S1, Supporting information). Axes 1 and 2 explained 79.60% of total variability in the bulk soil physiochemical data. The bacterial communities were clearly clustered into 4 groups (Fig. 4A). AK was the significant variable which was positively correlated to the bulk of manure addition (NPKMB), while CEC was the other variable positively correlated to the control. Variables SOC, TN, TP, NO_3^--N, pH and AP were synergistically correlated with NPKMB ($P > 0.05$) (Table S1, Supporting information).

RDA explained 67.4% of the total variation in the rhizosphere soil. The manure addition (NPKMR) was separate from the control (CKR) and chemical addition (NPKR) along RDA 1, which accounted for 51.5% of the variability (Fig. 4B). The straw application (NPKSR) clustered close to the NPKR but distinctly separated from the control. Among all environmental variables, TP and AP were the significant variables showing positive correlations to the rhizosphere bacterial communities in organic fertilization schemes. TN, NO_3^--N and NH_4^+ -N were positively related to manure amendment, whereas AK, SOC and pH were correlated to straw addition ($P > 0.05$) (Table S1, Supporting information).

3.4. Co-occurrence patterns of rhizosphere and bulk bacterial networks

We further explored the bacterial co-occurrence patterns in the rhizosphere and bulk based on fMEN analysis. The values of average path length (APL), average clustering coefficient (*avgCC*), and modularity in the empirical networks were higher than those of their respective, identically sized random networks (Table 3). Although there was no significant difference of the average connectivity and modularity between the rhizosphere and the bulk, the percentage of positive correlations in the rhizosphere was higher than the bulk with the lower network size (lower number of nodes and edges) (Table 3).

Based on the network topology and their module memberships, the

Table 2
Pairwise Pearson correlations (r values) to major soil variables.

Rhizospheresoil variable	MOS	NO3	NH4	pH	SOC	TN	TP	TK	AP	AK	CEC
Biomass	−0.171	0.398	−0.381	0.588*	0.505	0.570	0.834***	−0.590*	0.794**	0.161	−0.013
MOS	1	0.108	0.266	−0.241	−0.202	0.046	−0.334	−0.023	−0.300	0.514	−0.277
NO3		1	0.045	0.254	−0.019	0.121	0.223	−0.190	0.215	0.327	−0.498
NH4			1	−0.315	0.072	−0.046	−0.347	0.6283*	−0.264	0.294	−0.246
pH				1	0.151	0.064	0.821**	−0.454	0.794**	0.045	0.314
SOC					1	0.839***	0.422	0.194	0.531	0.143	0.243
TN						1	0.460	0.070	0.533	0.546	0.019
TP							1	−0.400	0.971***	0.220	0.180
TK								1	−0.314	0.143	0.188
AP									1	0.234	0.147
AK										1	−0.316
CEC											1

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

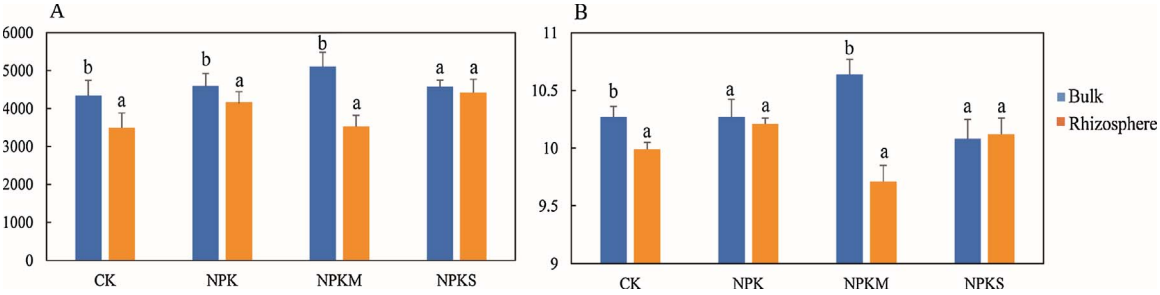

Fig. 2. α-Diversity of different long-term fertilization schemes (A: richness index; B: Shannon index). Values are represented as the mean (n = 3) ± the standard deviation of the mean. Values in the same group marked by a superscript lowercase letter indicate a significant difference according to one-way analysis of variance (ANOVA) with Duncan test ($P < 0.05$).

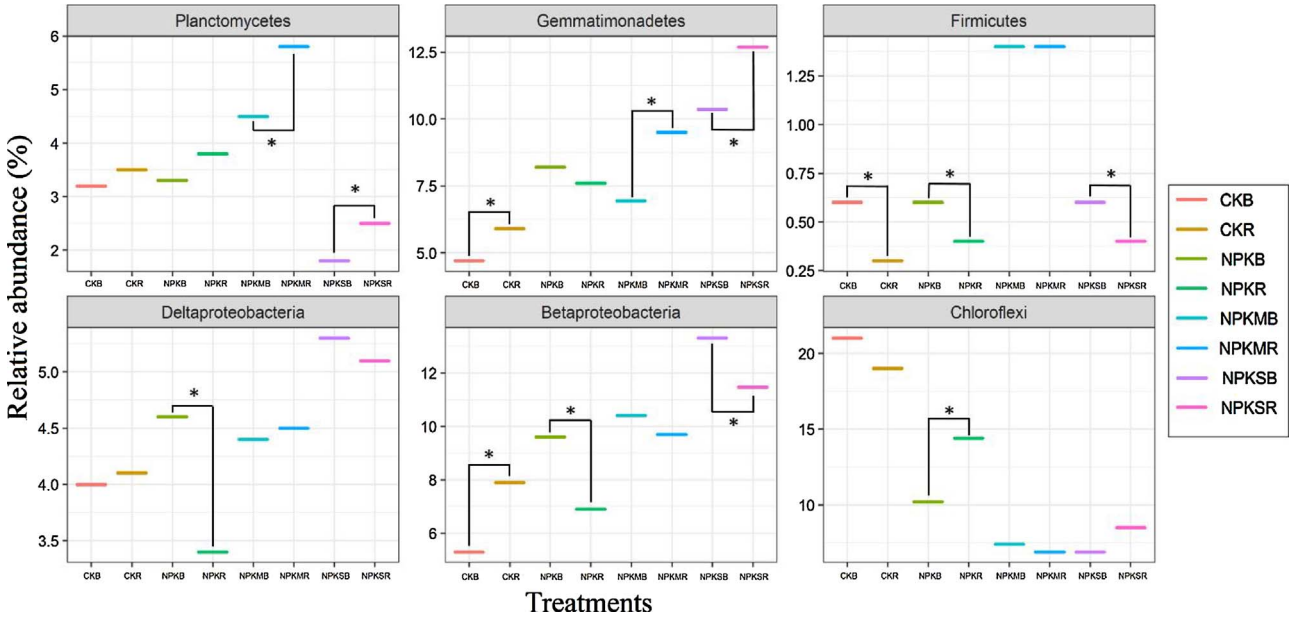

Fig. 3. Distributions of dominant phyla which showed differences in the bulk and the rhizospehre in the treatments. A dash with "*" between two treatments indicate significant differences ($P < 0.05$) according to one-way analysis of variance (ANOVA) with Duncan test.

keystone populations that play key roles in the overall networks were defined as module hubs (highly connected to numerous OTUs in their own modules, $Pi > 6.2$), connectors (highly linked to some modules, $Zi > 2.5$) and network hubs (acting as both module hubs and connectors, $Pi > 6.2$ and $Zi > 2.5$) (Guimera and Nunes, 2005; Liang et al. 2016; Jiang et al. 2017b). In total, 17 OTUs derived from Proteobacteria (5), Acidobacteria (2), Gemmatimonadetes (1) and Nitrospirae (1) in the bulk, and Actinobacteria (2), Acidobacteria (1), Gemmatimonadetes (2), Armatimonadetes (1), Proteobacteria (1), Verrucomicrobia (1) in the rhizosphere were categorized as module hubs that were strongly interdependent with nodes (≥5) in their own modules (Table 4 and Fig. 5). Additionally, only 1 OTU (derived from Acidobacteria) in the bulk and 5 OTUs assigned to Proteobacteria (3), Gemmatimonadetes (1), and Firmicutes (1) were categorized as the connectors that were highly connected to several modules (Table 4, and Fig. S3 in Supporting information).

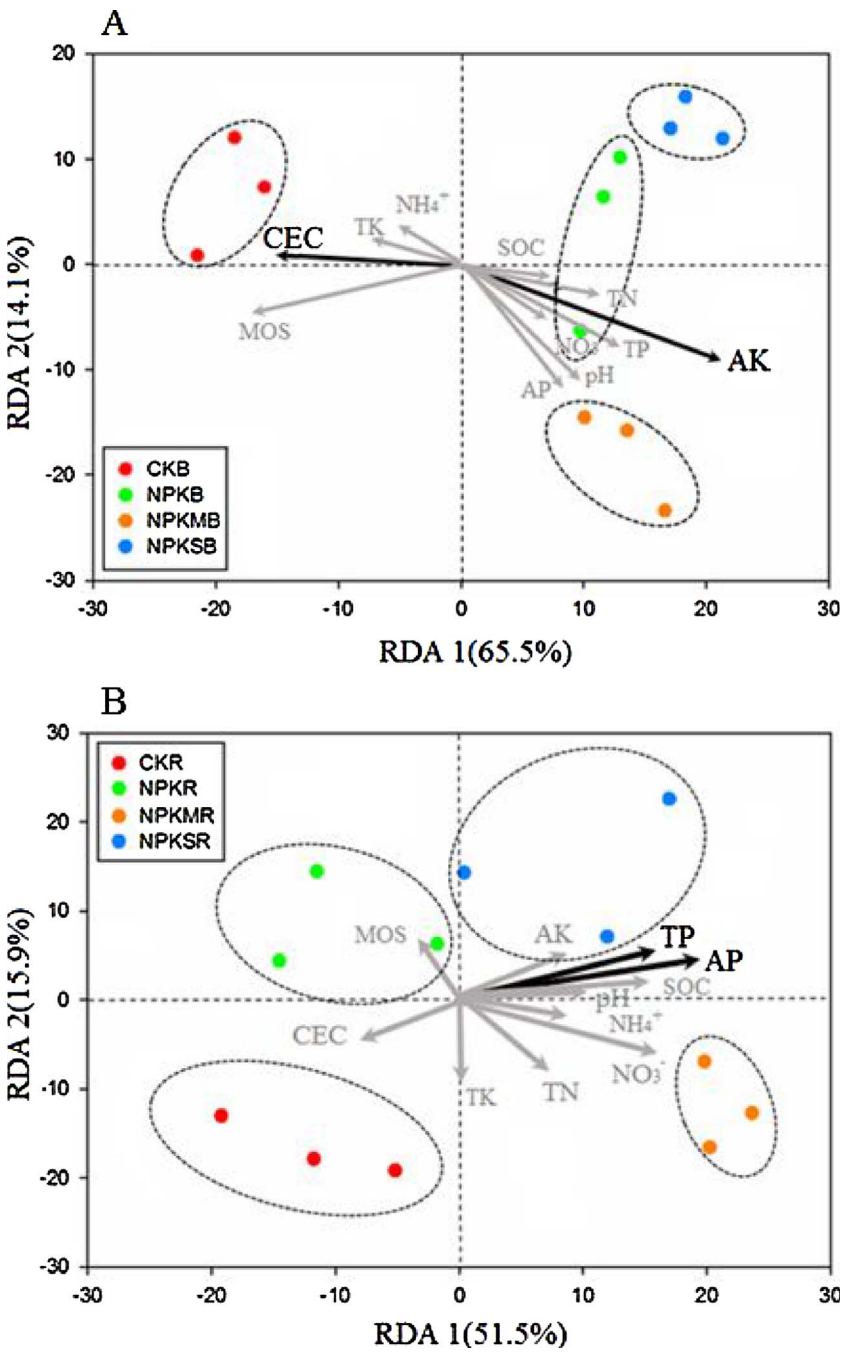

Fig. 4. Redundancy analysis (RDA) plot depicts the Bray-Curtis distance of bacterial communities in the bulk (A) and the rhizosphere (B) under the four treatment regimes. Environmental factors denoted with a black arrow indicate the significant correlations to microbial communities based on spearman's rank correlation of Mental test ($P < 0.05$).

3.5. P-correlated OTUs and their co-occurrence pattern in the networks

As pH is strongly correlated with AP and TP (Table 2), to avoid the bias of P-correlated OTU screening caused by pH synergistic correlation, a Pearson correlation analysis was performed between OTU relative abundances and environmental factors (pH, TP and AP) for selected P specific correlated microbiota. We identified the "P-correlated microbes" that only significantly correlated to phosphorus ($P < 0.05$) among bacterial communities sampled from the rhizosphere and the bulk. In general, 61 bacterial OTUs (23 OTUs in the rhizosphere, 36 OTUs in the bulk, and 2 in the both) belonging to 20 phyla and 43 orders showed strong correlation with P (Table S2, Supporting information). Generally, the relative abundance of P-correlated OTU in NPKM were higher than that of equivalent domains of treatments. P-correlated OTU belonging to Alphaproteobacteria, Acidobacteria and Betaprobacteria are common in all samples, but the relative abundance varied by the fertilizations and rhizosphere effect: P-correlated OTU closing to Alphaproteobacteria and Betaprobacteria were decreased in the fertilized rhizosphere. P-correlated OTUs belonging to Acidobacteria showed the similar decreasing tendency in NPKM rhizosphere, but the opposite trend in the rhizosphere of NPK and NPKS. P-correlated OTU belonging to Actinobacteria were less sensitive to rhizopshere process, as its abundance only varied between fertilizations. It is noteworthy that the relative abundance of OTUs belonging to Bacteroidete and Nitrospirae increased in the rhizosphere of NPKM and were significantly higher than that of other fertilizations ($P < 0.05$). (Fig. 6).

Among the 61 P-correlated OTUs, only 3 (OTU39813, OTU54432, OTU48774) could match the rhizosphere network nodes (Fig. 5). Surprisingly, no P-correlated OTU was found in the bulk bacterial network. Further structural analysis of the 3 OTUs' connectivity demonstrated that their co-occurrence patterns were different (Fig. 7A–C). OTU54432

Table 3
Topological properties of the empirical molecular ecological networks (MENs) of the microbial community in bulk and rhizosphere soils under different fertilization schemes and their associated random MENs.

Network metrics	Niches	
	Bulk	Rhizosphere
Empirical networksSimilarity threshold (*St*)	0.87	0.84
Number of nodes	348	292
Number of edges	450	374
Percentage of positive correlations	29.56%	45.19%
Percentage of negative correlations	70.44%	54.81%
Average path length(APL)	7.402	6.675
Average connectivity (*avgK*)	3.58	3.56
Average clustering coefficient (*avgCC*)	0.126	0.136
Number of modules	8	8
Modularity	0.831	0.791
Random networks		
APL ± SD	5.505 ± 0.113	5.474 ± 0.127
avgCC ± SD	0.007 ± 0.005	0.007 ± 0.005
Modularity ± SD	0.693 ± 0.008	0.691 ± 0.008

Table 4
Information of the nodes identified as module hubs or connectors of bacterial networks among the rhizosphere and the bulk.

OTU ID	Role	Node degree	Classification (Phylum)	*Z* value	*P* value
Bulk					
OTU1480	Module hub	8	Gemmatimonadetes	3.29	0
OTU5829	Module hub	10	Acidobacteria	2.53	0.46
OTU14709	Module hub	8	Nitrospirae	2.63	0.41
OTU20807	Module hub	8	Alphaproteobacteria	2.53	0.38
OTU25941	Module hub	9	Deltaproteobacteria	2.65	0
OTU60504	Module hub	11	Alphaproteobacteria	2.94	0.17
OTU66450	Module hub	6	Betaproteobacteria	2.65	0
OTU73852	Module hub	6	Acidobacteria	2.87	0
OTU74808	Module hub	13	Betaproteobacteria	2.94	0.38
OTU18048	Connector	4	Acidobacteria	-0.27	0.63
Rhizosphere					
OTU8450	Module hub	5	Actinobacteria	2.63	0
OTU16233	Module hub	9	Gemmatimonadets	3.00	0.37
OTU20692	Module hub	8	Armatimonadetes	2.83	0
OTU21586	Module hub	11	Deltaproteobacteria	2.95	0.30
OTU37939	Module hub	14	Verrucomicrobia	3.53	0.37
OTU47362	Module hub	8	Actinobacteria	2.79	0
OTU83497	Module hub	9	Gemmatimonadetes	2.91	0.20
OTU88272	Module hub	7	Acidobacteria	3.11	0
OTU58393	Connector	4	Betaprotepbacteria	-.0224	0.63
OTU58905	Connector	4	Betaproteobacteria	-0.331	0.63
OTU2438	Connector	4	Betaproteobacteria	-0.49	0.63
OTU35029	Connector	4	Firmicutes	-0.40	0.63
OTU90016	Connector	5	Alphaproteobacteria	-0.21	0.64

showed complicated positive interregional interactions with 9 nodes, while OTU39813 negatively and OTU48774 positively correlated to 5 and 3 first neighbors, respectively (Fig. 7A–C). The results from the relative abundance of the three P-correlated OTUs in the rhizosphere indicated that manure addition increases the abundance of OTU54432 and OTU39813, whereas fertilization significantly reduced the abundance of OTU48744 (Fig. 7a-c).

4. Discussion

4.1. Overall pattern of plant growth and microbial communities

Plant rhizosphere processes associated with microorganisms play pivotal roles in plant growth and productivity (Li et al. 2007). However, under the pressure of land use intensification, most studies focus on the response of microbial communities and functions to fertilizers input, and the synchronous studies concerning the rhizobacterial composition and network interactions in various fertilization schemes are still rare. In addition to straw treatment, the bacterial diversities in the rhizosphere are lower than those of the bulk, indicating that even under intensified anthropogenic activities, the root still plays roles in selecting specific species that adapt to niche utilization. Similar diversity decreases have also been observed in the rhizospheres of blueberries, blackberries and wild oak (Jiang et al. 2017a; Shi et al. 2016). Straw application homogenized microbial diversity in rhizosphere and bulk soil. This finding may be attributed to the attachment of straw in the root surface, providing an interface for rhizosphere microorganism dispersal. This result is consistent with the hierarchical cluster tree showing that bacterial communities in the rhizosphere (except NPKS treatments) clearly clustered into one group separated from the bulk (Fig. S2, Supporting information). The assembly of the rhizosphere-inhabiting microbial community was based on the niche-based processes resulting from the selection power of the plant and soil environmental factors (Mendes et al. 2014). We observed some phyla to be consistently enriched in the rhizosphere (Fig. 3); for example, *Gemmatimonadetes* and *Planctomycetes* in particular, have been shown previously to be enriched in maize cultivars (Li et al. 2014; Correa-Galeote et al. 2016). In contrast, the proportion of Firmicutes in the rhizosphere of peanut decreased in nearly all treatments. Similar results were seen in field-grown lettuce (Schreiter et al. 2014). It has long been assumed that microbial community assemblage is tightly linked to the root filtering, a kind of environment variable, as species have properties that allow them to exploit unique niches available (Mendes et al. 2014). In the present study, changes in certain phyla abundances were consistent with the niche-based theory in the rhizosphere. Additionally, Deltaproteobacteria and Chloroflexi varied in the rhizosphere and non-rhizosphere in NPK treatments indicating these two phyla were sensitive to the chemical application.

4.2. Relationships between microbial community and environmental variations

Microbial diversity is influenced by physical and chemical properties and reflects resource distribution (Fierer et al. 2007). Major environmental factors modulating bacterial communities in the bulk and the rhizosphere shift from CEC and AK to TP and AP, indicating the enhancement of rhizosphere P availability (Fig. 4). As Hinsinger, (2001) and Devau et al. (2009) proposed that soil pH controls phosphorus efficiency, our pairwise comparisons analysis results suggest that there were strong correlations among pH, TP and AP (Table 2). Recent work addressing the association between pH and microbial diversity using 16S rRNA gene microarray supports the notion that the spatial scaling of microbial diversity is related to the pH (Lauber et al. 2009). However, how much phosphorus contributes to the rhizobacterial community remains poorly characterized. In this study, Variation Partitioning Analysis (VPA) showed that soil P availability explained 16.39% variations in the community composition of rhizosphere, higher than pH contribution (Fig. S4, Supporting information), indicating that in an acidic agro-ecosystem, pH as well as P are driving factors shaping the plant rhizosphere microbial community.

4.3. Microbial co-occurrence patterns in networks

The network analysis of taxonomic co-occurrence patterns allows us to obtain insights into the bacterial community assembly rules responding to rhizosphere processes (Layeghifard et al. 2017). Generally, the rhizosphere and bulk networks were clearly nonrandom and had typical module structures, with much higher average path length (APL), average clustering coefficient (*avgCC*) and modularity (M) than the random network and the available modularity values (>0.4) (Table 5, Newman, 2006). The “scale-free, small-world” characteristics in biological systems made the network stronger than the random distribution.

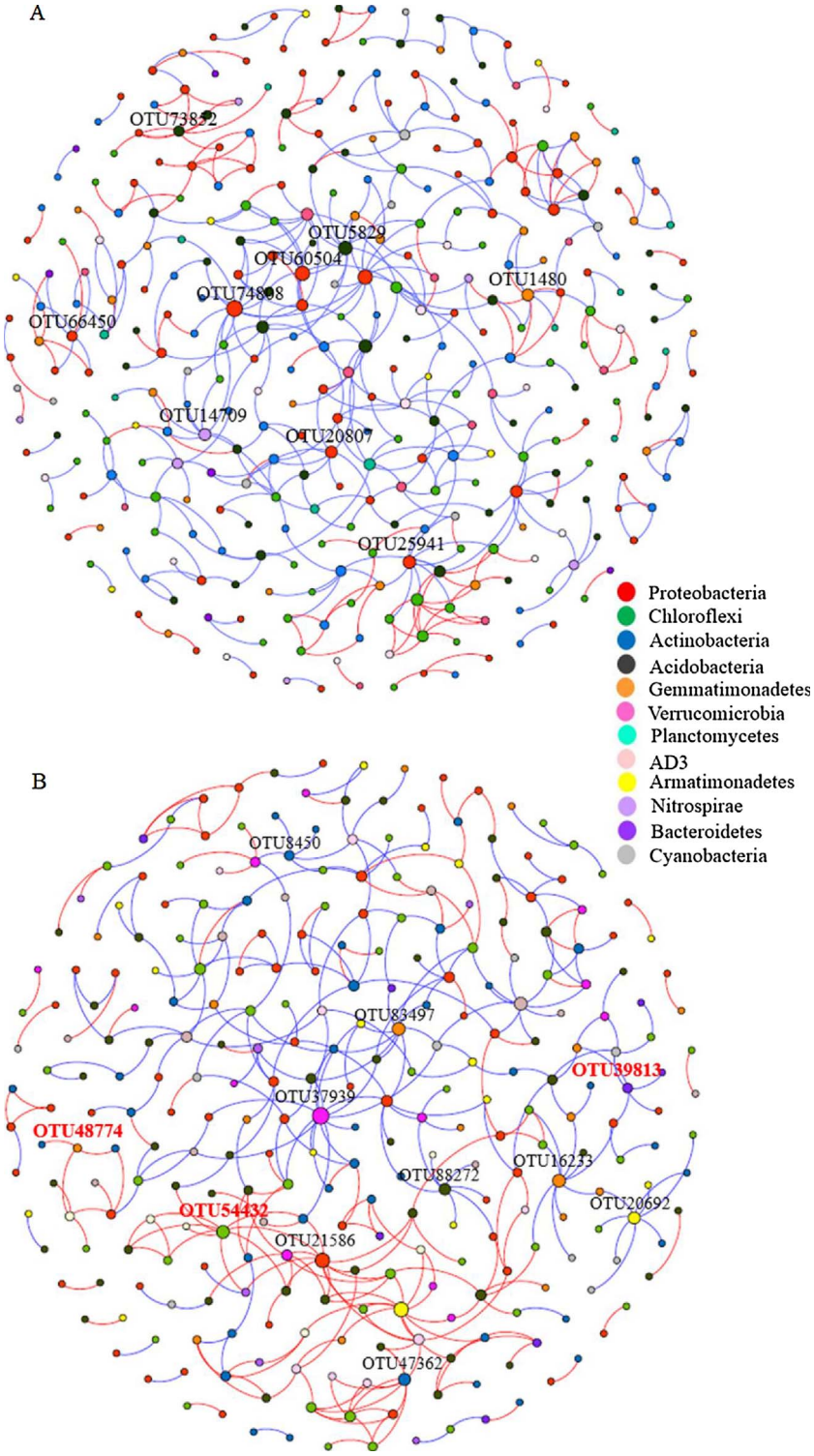

Fig. 5. Networks of co-occurring bacterial OTUs in the bulk (A) and in the rhizosphere (B) based on correlation analysis. A connection stands for a strong (Spearman's $\rho > 0.6$) and significant ($P < 0.01$) correlation. The co-occurring networks are colored by phylum. For each panel, the size of each node is proportional to the node degrees. A blue edge indicates a negative interaction between two individual nodes, while a red edge indicates a positive interaction. Nodes marked with OTU ID above were the Module hubs (black) and P-correlated OTUs (red) in the network.

These highly modularized structures provide the potential for quicker and easier comparisons between the rhizosphere and the bulk to explore whether and how the traits of rhizosphere process influenced the assembly of soil microbial community (Jiang et al. 2017a, b).

Multiple mechanisms were used to explain the increased size, connectivity and complexity of rhizosphere network, which developed over time as the plant grew (Shi et al. 2016). However, in this study, there were no significant differences of network connectivity and complexity between the bulk and rhizosphere (Table 4). Compared with an *Avena fatua* seedling experiment (Shi et al. 2016) whose bulk samples did not include fertilizer application, the bulk soil in this study had been applied chemical or organic materials. Long-term exogenous nutrient addition alters the soil environment by changing pH, carbon and nitrogen, which in turn alter ecological networks (Barberan et al. 2012). Thus, the construction of a non-rhizosphere network is the result of long-term field management.

Based on the fMEN analysis, 17 module hubs (9 in the bulk and 8 from the rhizosphere) and 6 connectors (1 in the bulk and 5 in the rhizosphere) with no overlap, were discovered in the two networks (Fig. 5, Table 4). Moreover, the percentage of positive correlations

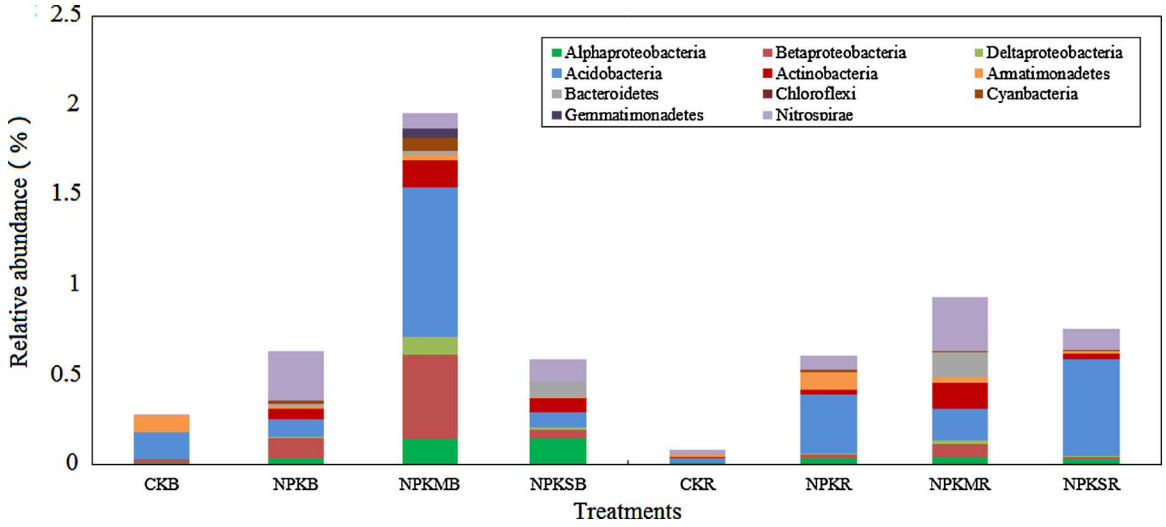

Fig. 6. The relative abundance of P-correlated OTUs in the bulk and rhizosphere in four fertilization schemes. CKB, the bulk of Control; NPKB, the bulk of chemical fertilization; NPKMB, the bulk of chemical plus manure fertilization; NPKSB, the bulk of chemical plus straw fertilization; CKR, the rhizopsphere of Control; NPKR, the rhizosphere of chemical fertilization; NPKMR, the rhizosphere of chemical plus manure fertilization; NPKSR, the rhizosphere of chemical plus straw fertilization.

increase in the rhizosphere microhabitat network. We interpret such a fundamental difference as increased community organization, which is combination of both increased cooperative interaction and the development of shred guilds or niches. These findings are consistent with the results from the root acting as a strong environmental filter for rhizosphere microbial assembly (Nuccio et al. 2016). Compared with the bulk, rhizosphere microbial diversity was lower (Table 2). Roots under different anthropogenic disturbances (fertilization schemes) promote the development of niches adopted dominant taxa, which may yield decreased the diversity but change the ways of interaction. The increasing positive characteristic of the bacterial co-occurrence in the rhizosphere is consistent with cooperative or syntrophic interactions, and suggest the potential for extensive mutualistic interactions among rhizosphere bacterial assemblages (Rasmann and Turlings, 2016).

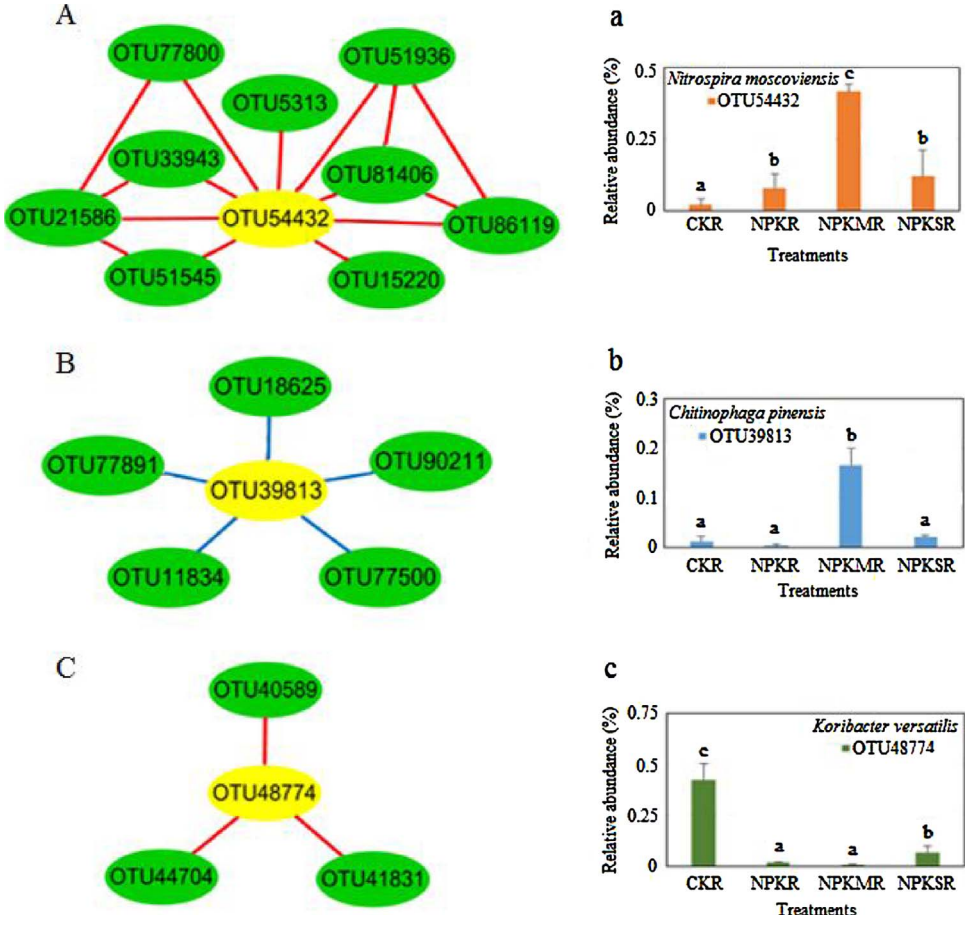

Fig. 7. Connection (a–c) of specific P-correlated OTUs in the rhizosphere network and their relative abundance (a–c) in different fertilization schemes. A blue edge indicates a negative interaction between two individual nodes, while a red edge indicates a positive interaction. Bars with different lowercase letters indicate significant differences ($P < 0.05$) among the treatments.

4.4. P-correlated rhizosphere OTUs in networks

Interestingly, although soil P is the main variable regulating the rhizosphere microbiome (Fig. 4), neither keystone populations (module hubs and connectors) had a significant Pearson's correlation to soil P ($P > 0.05$). Networks represent coordinated variability, where the members' abundances co-vary in response to interactions among the members (Deng et al. 2012). Keystone populations, which are extensively connected with other nodes, showed a stronger capacity for nutrient exchange and mutual coexistence, indicating their stronger resistance to environmental limiting factors. We further filtered the specific microbial OTUs that strongly correlated to P in the rhizosphere and/or bulk, and tried to match them with the nodes in the networks. Surprisingly, no P-correlated OTU matched as a node in the bulk network. Bulk soil organisms are thought to occupy heterogeneously (Torsvik et al. 2002). The high percentage of negative co-occurrence with module implied the interaction encompass antagonistic by resource composition. When P was not the limiting factor, the interaction of bulk habitats disconnected. In the rhizosphere, three OTUs positively correlated with P (OTU39813, OTU48774 and OTU54432) were found in the rhizosphere network (Fig. 5, Table S2 in the Supporting information). OTU39813 belonging to Bacteroidetes, which is most closely related to *Chitinophaga pinensis* and has its highest relative abundance in NPKM, is closely related to gram-negative bacteria that are isolated from soil and contain a large variety of catabolic carbohydrate-active enzymes (CAZymes) to degrade carbohydrates (McKee and Brumer, 2015; Rawat et al. 2012). When phosphate is in limited supply, the bacteria are able to break the stable carbon-phosphorus (C-P) bond to maintain their growth (Seweryn et al., 2015). This finding is an example for proving microbial carbon-phosphorus coupling metabolism to promote C and P cycling. By contrast, OTU48774 belonging to Acidobacteria (most closely related to *Koribacter versatilis*) showed the highest relative abundance in the rhizosphere in the control samples but declined in fertilized soil. The species belongs to the phylum Acidobacteria, a typical oligotrophic slow-growing microbiota (Fierer et al. 2007). Although OTU48774 showed a positive correlation to AP, its function may be weakened with decreasing abundance under fertilizers disturbance.

Among the three OTUs, OTU54432 (Nitrospirae) which is closely related to *Nitrospira moscoviensis*, showed the most complex positive interactions with other nodes. It also showed a higher relative abundance in the rhizosphere of manure fertilization (NPKM). It is well-known that Nitrospira is a diverse group of nitrite-oxidizing bacteria (NOB) and among the environmentally most widespread nitrifiers (Koch et al., 2015). Koch et al (2014) reported that *Nitrospira moscoviensis,* the slow-growing species has the capability of utilizing hydrogen (H_2) as the sole electron donor to fix CO_2 and form HCO_3^-, indicating the release of H^+ to the niche zone has the potential to ligand-exchange coprecipitate P ions (such as Fe and Al in red soil). OTU54432 could be defined as a highly connected node, which rendering the network more robust to change (Montoya et al. 2006; Liang et al. 2016). The loss of OTU54432 may dramatically change the network structure. Its metabolic versatility, including the participation in nitrogen cycling process and the synergistic phosphorus cycling process, may be the key factors for the construction of the rhizosphere network.

5. Conclusions

It was shown that over long-term fertilization, the rhizosphere still has profound effects on root microbial assembly with the coordination of different fertilization schemes in agricultural soil. Increases in positive covariations occurring within modules were concurrent with decreases in bacterial diversity, which emphasize the importance of community organization characterization in addition to microbial diversity quantification. In acidic soil regions, phosphorus availability is the main environmental factor controlling the peanut rhizosphere microbial community. P-correlated OTU39813 and OTU54432, which related to *Chitinophaga pinensis* and *Nitrospira moscoviensis,* showed higher relative abundance in the NPKM rhizosphere; their positive response to available phosphorus may be attributed to carbo N-P hosphorus and nitroge N-P hosphorus synergistic metabolisms, respectively.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.apsoil.2017.11.023

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