Rice Root-Associated Diazotrophic Community Succession is Driven by Growth Period Combined With Fertilization

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Abstract

Background

Biological nitrogen fixation (BNF) and nitrogenous fertilizers are two crucial ways for nitrogen input in the rice paddy system. Little is known about the effect of nitrogenous fertilizers on root-associated diazotrophs. Here, we investigated the succession of total and active diazotrophs in rhizosphere soil and rice roots under four fertilization treatments (control, NPK, NPK + pig manure, and NPK + rice straw) during three key growth periods (tillering, heading and mature).

Results

The ^{15}N isotope dilution experiment verified that root-associated diazotrophs could supply N for rice under low nitrogen nutrition by BNF (%Ndfa=11.51). Niche differentiation of diazotrophs existed at the rhizosphere soil-root interface. At both DNA and RNA levels, growth period had stronger effects on the community composition of endophytic diazotrophs than rhizosphere diazotrophs. The Chao1 and Shannon indices of total endophytic diazotrophs dramatically increased at the vegetative stage, and underwent relatively minor changes at the reproductive stage. Furthermore, the community structures of total endophytic diazotrophs were more stabilized in the reproductive stage than the vegetative stage. The number of OTUs shared by rhizosphere soil and roots increased during rice growth. Compared with CK, NPK reduced the relative abundances of *Pelobacter* and increased the relative abundances of *Azoarcus*. Pig manure not only improved soil nutrients (OM, TN, TP, AN, AP and $\text{NO}_3\text{-N}$), but reduced the effect of chemical fertilizers on the community composition of natural rhizosphere diazotrophs.

Conclusions

Growth period demonstrated a stronger influence on the root-associated diazotrophs than fertilization practices. The community structures of total endophytic diazotrophs were more stabilized in the reproductive stage than the vegetative stage, and the alpha-diversity indices and complex of network structures of endophytic diazotrophs increased at the vegetative stage. Replacing chemical fertilization with pig manure not only increased soil nutrients, but regulated rhizosphere diazotrophic community structures. Understanding the combined effects of growth period and fertilization on root-associated diazotrophs presents the basis towards the sustainable crop production.

Background

As a critical macronutrient, nitrogen (N) often limits plant yields in the agricultural ecosystem [1]. Over the past several decades, chemical fertilizers increase crop production, however, nitrogen-use efficiency (NUE) in rice is only about 39% [2] and excessive N causes soil acidification and environmental pollution [3]. Alternatively, BNF is an available and eco-friendly way to provide N for plants, which can reduce atmospheric dinitrogen to biologically available ammonium by nitrogen-fixing bacteria and archaea. Numerous Diazotrophs could colonize and reproduce in the bulk, rhizosphere soil and rice tissues [4-7], and increased crop productivity through BNF [8], producing antimicrobials [9], and improving abiotic stress tolerance [10].

Vegetation type [11], plant genotype [12] and soil physicochemical property [13] influenced the abundance, diversity and community composition of diazotrophs. However, effects of these factors on diazotrophic composition and nitrogen-fixing capacity were inconsistent in different ecosystems. For example, the application of N fertilizer or N fertilizer plus pig manure reduced the relative abundant of *Bradyrhizobium* [14-15], but Hu *et al.* [16] indicated that *Bradyrhizobium* was significantly increased by N fertilizer application. However, there are few agricultural experiments comparing fertilization management, especially replacing chemical fertilization by manure, on the rhizosphere and endophytic diazotrophic diversity and community composition in the paddy field.

The establishment of rhizosphere and root microbiota is a dynamic process and plants participate in and regulate this process [17]. The composition of root exudates changes dynamically across developmental stages, and these chemical compounds are used to attract and enhance the root colonization of beneficial microbes [17-19]. For example, legumes secrete signal molecules, like flavonoids [7] and nodule-specific cysteine-rich (NCR) peptides [20], which influence the colonization and terminal differentiation of nitrogen-fixing bacteria. Previous studies had shown that rice root-associated microbiota dramatically varied during the vegetative stage and stabilized during the reproductive stage [21-22]. However, limited researches characterized the community succession of rice root-associated diazotrophs during rice cultivation, especially for the endophytic diazotrophs. Moreover, the existence of diazotrophs didn't mean that they could fix nitrogen actively [23] and their nitrogenase activities could be different [24-25]. Given above, it is noteworthy that the response of nitrogen-fixing microorganisms to fertilization management and growth period at the transcript level.

Here, high-throughput sequencing of the highly conserved *nifH* gene were used to explore the diversity and community structures of the total and active rhizosphere and endophytic diazotrophs under different fertilization treatments (CK, NPK, NPKM and NPKS). We sampled rhizosphere soil and rice roots in a long-term field experiment located in Jintan, China during three key rice growth periods (tillering, heading and mature stages) in 2015 and 2016 (Additional file 1: Table S1). Objectives of our research are as follows: (1) to verify the promotion of diazotrophs in the rice root grinding fluid on rice growth; (2) to compare the community of rhizosphere and endophytic diazotrophs at the DNA and RNA levels in the process of rice cultivation, and select root-enriched diazotrophs; (3) to evaluate effects of growth period and fertilization management on soil physicochemical properties, diversity, and composition of total and active diazotrophs; (4) to study the successional patterns of rhizosphere and endophytic diazotrophs.

Results

BNF plays an important role in N supply during rice growth

The rice yields of three fertilization treatments (NPK, NPKM, NPKS) were significantly higher than CK, while the yields of CK kept consistent since 2015 (Fig. 1a). Non-symbiotic BNF supplied 37.32% crop N for rice [26]. Thus, we inferred that nitrogen-fixing bacteria of CK played an important role to maintain rice production. The ^{15}N isotope dilution method was used to evaluate the contribution of endophytic diazotrophs in the nitrogen nutrition supply for rice growth (Table 1). The root length, plant height and fresh weight of rice of C_{20} and T_{20} had no significant difference but were significantly lower than T_{10} . The N concentration among the three treatments had no significant difference, but $\delta_{15}\text{N}$ of C_{20} ($33.26\% \pm 1.10\%$) and T_{20} ($33.85\% \pm 0.25\%$) were significantly higher than T_{10} ($29.43\% \pm 0.49\%$). It certified that endophytic nitrogen-fixing bacteria could supply N for rice by BNF under low nitrogen nutrition (%Ndfa=11.51).

Twenty-five isolates isolated from surface-sterilized rice roots including Proteobacteria (80%), Firmicutes (12%) and Actinobacteria (8%) (Additional file 2: Table S2). The *nifH* gene fragments were amplified from five isolates, then their nitrogenase activities were measured by ARA (Additional file 3: Table S3). The nitrogenase activity of strain BV6 (*Burkholderia*) was highest ($25.71 \mu\text{mol/h/ml}$).

The diversity of total rhizosphere and endophytic diazotrophs were shaped by growth period, year and fertilization

Illumina sequencing was performed to investigate the community compositions of rhizosphere and endophytic diazotrophs and their response to different fertilization managements in different growth periods. 318 samples collected from the field (120 rhizosphere soil DNA samples, 119 root DNA samples, 48 rhizosphere soil RNA samples and 31 root RNA samples) were sequenced, and after normalization, each sample had 22552 sequences. These sequences were clustered into 706 operational taxonomic units (OTUs) at 90% identity.

For alpha diversity analyses, richness index (Chao1), evenness index (Shannon) and phylogenetic diversity index (PD) of rhizosphere diazotrophs were significantly higher than endophytic diazotrophs at the DNA level (Additional file 4: Table S4), which was consistent with the results of rarefaction curves (Additional file 5: Figure S1). Interestingly, total rhizosphere diazotrophs had the highest α -diversity at the tillering stage, while total endophytic diazotrophs had the lowest α -diversity at this stage (Additional file 4: Table S4). The effects of growth period on all tested α -diversity indices were statistically significant, but fertilization only had significant effects on the evenness index (Table 2). For soil and root DNA samples, almost all α -diversity indices of diazotrophs among four fertilization treatments (CK, NPK, NPKM and NPKS) had no significant difference (Additional file 6: Table S5, Additional file 7: Table S6).

The phylogenetic trees of 50 dominant OTUs based on the sequences of *nifH* genes were generated (Fig. 2, Additional file 8: Figure S2). The relative abundances of dominant OTUs of total rhizosphere diazotrophs were notably different from total endophytic diazotrophs. Moreover, the whole community structure of total rhizosphere diazotrophs was notably different from and more stable than total endophytic diazotrophs (Additional file 9: Table S7, Fig. 1b). We visualized and quantified the differences between diazotrophic communities (β -diversity) using non-metric multidimensional scaling (NMDS) and permutational multivariate analysis of variance (PERMANOVA). The growth period was a primary driver of the total rhizosphere ($R^2=0.22$, $P<0.001$) and total endophytic diazotrophic β -diversity ($R^2=0.33$, $P<0.001$) (Fig. 1c-d, Additional file 10: Figure S3a-b, Table 2), revealing a stronger temporal effect on total endophytic diazotrophs than total rhizosphere diazotrophs. However, total rhizosphere diazotrophs were more sensitive to the year and fertilization management ($R^2=0.19$ and 0.11 , $P<0.001$) than total endophytic diazotrophs ($R^2=0.06$ and 0.05 , $P<0.001$). Year, growth period and fertilization management showed significant interactions in pairs.

Taxonomic composition and fertilizer-sensitive taxa

Fourteen phyla were determined by pooling the *nifH* sequences, and Proteobacteria was the predominant phylum, contributed approximately 57.0% and 76.1% of soil and root samples, respectively (Additional file 11: Figure S4). The top three classes of soil samples were Alphaproteobacteria (25.29%), Deltaproteobacteria (20.65%) and Betaproteobacteria (8.83%) (Fig. 1e, Additional file 12: Figure S5a). However, Betaproteobacteria (34.95%), Gammaproteobacteria (18.51%) and Alphaproteobacteria (15.37%) were the three dominant classes of root samples. At the genus level, *Bradyrhizobium* (24.80%), *Geobacter* (7.72%) and *Burkholderia* (5.71%) were the top three genera of soil samples (Additional file 12: Figure S5b-c), but for root samples, they were *Burkholderia* (26.74%), *Thiorhodospira* (8.88%) and *Bradyrhizobium* (8.25%).

In these four cluster trees (Fig. 1e, Additional file 12: Figure S5), soil DNA samples and root DNA samples were divided into two clusters, and samples from the same growth period were frequently clustered together except for soil samples in 2016. However, PERMANOVA and ANOSIM suggested that the growth period significantly affected the total rhizosphere diazotrophic communities in 2016 (data not shown). Generally, the communities of either total rhizosphere or endophytic diazotrophs of NPK and NPKS treatments were clustered together, and they were separated from CK and NPKM treatments (Fig. 1e, Additional file 12: Figure S5a). Furthermore, the soil samples of CK were quite different from the other three fertilization treatments, and differences between CK and NPKM were smaller than CK and NPK or CK and NPKS (Additional file 13: Table S8). We noted that the relative abundances of *Pelobacter* of NPKM were lower than that of CK and higher than that of NPK, but *Azoarcus* was the opposite (Additional file 14: Figure S6). Furthermore, for *Bradyrhizobium*, *Geobacter*, *Heliobacterium*, *Rubrivivax* and *Desulfomonile*, their abundance of NPKM were between that of CK and NPK at most sampling points.

The rhizosphere soil physicochemical indices OM, TN, TK, AN, AP, AK, pH, NO₃-N and NH₄-N were tested (Additional file 15: Table S9). Soil pH value of CK was highest among four fertilization treatments. NPKM increased the content of organic matter (OM), total N (TN), total P (TP), available N (AN) and available P (AP) and NO₃-N. Fertilization management, year and period had significant effects on all tested physicochemical parameters ($P<0.01$, Additional file 16: Table S10), except that the period had no significant effects on TN and NH₄-N ($P>0.05$). Most rhizosphere and endophytic

diazotrophic α -diversity indices had negative correlation with TP and AN at six sampling points (Additional file 17: Table S11). The Mantel test showed that AK, TK and $\text{NH}_4\text{-N}$ were significantly negatively correlated with the community structures of total rhizosphere and endophytic diazotrophs (Additional file 18: Table S12). Correlation analysis showed that pH had a significant positive correlation with *Pelobacter*, but had a significant negative correlation with *Thermodesulfovibrio* in rhizosphere soil (Additional file 19: Figure S7).

The community structures of total endophytic diazotrophs were more stabilized in the reproductive stage than the vegetative stage

The similarity distances (1-Bray dissimilarity) of diazotrophic community structures among three rice growth periods were calculated at each fertilization treatment (Fig. 3a-b). For root samples, the similarity distances between the tillering and heading stages (red nodes) were higher than the tillering and mature stages (green nodes), but lower than the heading and mature stages (blue nodes). However, this trend was not observed in soil samples.

Tracking diazotrophic community changes at the class level revealed that many classes showed distinct temporal dynamics in root samples (Fig. 3c-d). For example, Alphaproteobacteria and Deltaproteobacteria enriched as time went on, but Betaproteobacteria decreased. The endophytic diazotrophic abundance of “others” (mainly unclassified taxa) remarkably increased, and was close to rhizosphere diazotrophs at the mature stage. The abundance of shared OTUs between soil and root samples in soil samples (Pink flows) apparently increased during rice cultivation, but slightly decreased in root samples.

The linear discriminate analysis (LDA) effect size (LEfSe) was used to identify the distinguishing diazotrophs during different growth periods in paddy soil and rice roots [27]. The numbers of the discriminating taxa of rhizosphere diazotrophs, whose LDA scores between different growth periods greater than 2, were 172 and 96 in 2015 and 2016, and of the endophytic diazotrophs were 149 and 97 (data not shown). The discriminating genus significantly increasing at the same sampling period over two years were filtered and heatmaps were drawn to demonstrate more particular information of the responses of these genera to the growth period (Fig. 3e-f). For the rhizosphere soil samples, the relative abundances of *Treponema*, *Chloroherpeton*, *Paludibacter*, *Coralimargarita* and *Desulfobulbus* were significantly higher at the tillering stage than at the heading and mature stage (Fig. 3e), and they were correlated positively with AK (Additional file 19: Figure S7a-b); the relative abundances of *Oscillatoria*, *Cyanothece* and *Coleofasciculus* were significantly higher at the heading stage, and *Oscillatoria* was correlated negatively with $\text{NO}_3\text{-N}$; the relative abundances of *Heliobacterium*, *Sinorhizobium* and *Frankia* were significantly higher at the mature stage, and they were correlated positively with $\text{NO}_3\text{-N}$. For root samples, the relative abundance of *Tolomonas* was significantly higher at the tillering stage than at the other stages (Fig. 3f); the relative abundances of *Thiorhodospira*, *Sulfurospirillum*, *Pelosinus* and *Selenomonas* were significantly higher at the heading stage, and *Thiorhodospira* was correlated negatively with pH and $\text{NO}_3\text{-N}$; the relative abundance of *Rhizobium*, *Desulfovibrio*, *Geobacter*, *Frankia* and *Acetobacterium* were significantly higher at the mature stage, and they were correlated negatively with AK (Additional file 19: Figure S7c-d).

Diversity and community structures of active nitrogen-fixing bacteria

Consistent with the total root-associated diazotrophs, the community structures of active rhizosphere diazotrophs were notably different from active endophytic diazotrophs (Additional file 9: Table S7); growth period was a primary driver of the active rhizosphere ($R^2=0.15$, $P<0.001$) and active endophytic diazotrophic β -diversity ($R^2=0.17$, $P<0.001$); fertilization management had a higher effect on the β -diversity of active rhizosphere diazotrophic ($R^2=0.14$, $P<0.001$) than endophytic diazotrophs ($R^2=0.10$, $P>0.05$) (Additional file 20: Table S13, Additional file 10: Figure S3c-d). The rhizosphere diazotrophic community structures of CK and NPKM had no significant difference, and both of them had significant differences with the rhizosphere diazotrophic community structures of NPK and NPKS. (Additional file 13: Table S8). The relative abundances of *Geobacter* of NPKM were higher than that of CK and lower than that of NPK in soil RNA samples

(Additional file 14: Figure S6). Furthermore, TP, and AN, and had negative correlation with α -diversity indices of active rhizosphere and endophytic diazotrophs, and $\text{NH}_4\text{-N}$ and $\text{NO}_3\text{-N}$ had significant negative correlation with active rhizosphere diazotrophs (Additional file 17: Table S11). The Mantel test showed that TK, AN, AP, pH and $\text{NO}_3\text{-N}$ were significantly correlated with the community structures of active rhizosphere diazotrophs, and $\text{NO}_3\text{-N}$ had a significant correlation with the community structures of active endophytic diazotrophs (Additional file 18: Table S12).

Richness, Evenness and PD indices of all DNA samples were higher than corresponding RNA samples (Additional file 4: Table S4). The β -diversity of the total and active diazotrophic communities was investigated as well (Additional file 10: Figure S3e). The soil DNA samples clustered together, whereas the soil RNA samples, root DNA samples and root RNA samples were more dispersed. To compare their aggregation difference, the similarities (Z scores) in between-group diazotrophic community composition were visualized in bar plots. Z scores of the soil RNA samples, root DNA samples and root RNA samples had not significant differences but they were significantly lower than the soil DNA samples (Fig. 4a).

The rhizosphere DNA and RNA samples separated on the cluster tree (Fig. 4c), but root DNA samples and their corresponding RNA samples frequently clustered together (Fig. 4d). The correlation coefficient of the total and active diazotrophic communities in soil (Spearman correlation: $r = 0.244$, $P < 0.05$) was slightly smaller than that in roots at the OTU level (Spearman correlation: $r = 0.271$, $P < 0.05$). In Fig. 4b, Z scores of the soil DNA and RNA samples at the heading stage were highest, but there was no significant differences between Z scores of the soil DNA and RNA samples at tillering and mature stages, and Z scores of the root DNA and RNA samples at heading and mature stages.

The abundance changes of diazotrophs at the RNA and DNA levels were calculated in the rhizosphere soil and rice roots (Additional file 21: Figure S8a-b). *Burkholderia*, *Anaeromyxobacter*, *Bradyrhizobium* and *Pelobacter* predominated in the total and active rhizosphere diazotrophic communities. *nifH* gene expressions in *Burkholderia* and *Anaeromyxobacter* were somewhat higher than that of the total rhizosphere diazotrophic community, whereas *nifH* gene expressions in *Bradyrhizobium* and *Pelobacter* were opposite. *Burkholderia*, *Azoarcus*, *Thiorhodospira* and *Bradyrhizobium* predominated in the total and active endophytic diazotrophic communities. Growth period and fertilization appreciably affected the *nifH* gene expressions of endophytic diazotrophs. For instance, the abundance changes of *Azoarcus* at the RNA and DNA levels at the heading and mature stage were the opposite. The abundance changes of *Burkholderia*, *Thiorhodospira* and *Bradyrhizobium* at the RNA and DNA levels of CK and the other three fertilization managements were the opposite. Due to high between-sample variations, which might be caused by environmental differences, there was a few or no statistically significant difference of diazotrophic abundance at the RNA and DNA level in the soil or roots.

Root-enriched nitrogen-fixing bacteria

To investigate the distribution of nitrogen-fixing bacteria in rice roots and rhizosphere soil, the relative abundances of 39 dominant genera in these two niches were compared by the STAMP software. The size of the bubbles in the chart (Fig. 5) represents the relative abundance of 39 dominant genera in rice root samples, and the color of the bubbles represents the difference between the square root of the relative abundances of these genera in the roots and soil.

At the DNA and RNA levels, the relative abundances of *Thiorhodospira*, *Rhizobium* and *Burkholderia* in all root samples at three growth periods were almost significantly higher than that in corresponding soil samples ($P < 0.05$); the relative abundances of *Paludibacter*, *Magnetospirillum*, *Clostridium* and *Rhodospirillum* in root samples at the heading and mature stages were significantly higher than that in corresponding soil samples; the relative abundances of *Sulfurospirillum* at the heading stage, *Azoarcus* and *Frankia* at the mature stage in root samples were significantly higher than that in corresponding soil samples. However, *Bradyrhizobium*, *Pelobacter*, *Heliobacterium*, *Geobacter* and *Anaeromyxobacter* had significantly higher relative abundance in the rhizosphere soil than that in rice roots.

Co-occurrence patterns of total and active root-associated diazotrophs

Co-occurrence networks were built to explore potential interactions and niche-sharing of diazotrophs in paddy soil and rice roots during three key growth periods (Fig. 6, Additional file 22: Figure S9). In each of these networks, nodes represent OTUs (relative abundance>0.01%) and edges represent significant co-occurrence relationships (Spearman $|r|>0.65$ and $P<0.01$). Topological properties of co-occurring network were calculated (Additional file 23: Table. S14). Overall, the ecological networks of rhizosphere and endophytic diazotrophs had different network sizes and degree of connectivity, and showed distinct time related successions at three key rice growth periods. Consistent with the α -diversity analyses (Additional file 4: Table. S4), networks of rhizosphere diazotrophs were more complex in comparison with endophytic diazotrophs. For instance, the numbers of nodes (237.5 ± 51.64) and edges (1444 ± 840.69) in soil DNA samples were greater than the numbers of nodes (117.67 ± 48.55) and edges (201.83 ± 136.28) in root DNA samples. Moreover, soil RNA samples had greater numbers of nodes (224.67 ± 43.04) than root RNA samples (150 ± 36.77), and had greater numbers of edges (704.67 ± 306.95) than root RNA samples (485 ± 417.19).

Compared with networks at the tillering stage, the rhizosphere and endophytic diazotrophic assemblages formed larger and more complex networks at the heading and mature stages. Networks of total rhizosphere and endophytic diazotrophs at the heading and mature periods exhibited similar network topological structures (nodes, links, average path distance, average clustering coefficient, average degree and modularity) in 2016. However, for active diazotrophs, soil RNA network at the mature stage was most complex, and root RNA network at the heading stage was more intricate. The percent of positive correlations in total rhizosphere diazotrophic networks ($57.31\%\pm3.63\%$) was much less than that in total endophytic diazotrophic networks ($83.05\%\pm7.52\%$), active rhizosphere diazotrophic networks ($88.81\%\pm9.22\%$) total endophytic diazotrophic networks ($88.43\%\pm15.64\%$).

Discussion

Rice is the dominant crop in the middle and lower reaches of the Yangtze River Basin in China and produces more than 65% rice yield of the total country. Rice productivity is usually limited by nitrogen [28]. Nitrogenous fertilizer and BNF to a large extent raise rice productivity and make up for the depletion of soil nitrogen nutrient stocks caused by crop uptake [26]. Fertilization management [29-31] and growth period [32-33] varied the diazotrophic community compositions in the agricultural ecosystem. However, the combined effects of fertilization and growth period on diazotrophic communities in the paddy field still remain largely unknown. In this study, we investigated the combined effects of fertilization and growth period on the community composition of rhizosphere and endophytic diazotrophs in the paddy ecosystem at the DNA and RNA levels using amplicon sequencing. The strength of this experiment is that revealing niche differentiation of diazotrophs and including the effects of fertilization and temporal changes into the experimental design.

Different responses of rhizosphere and endophytic diazotrophs

Rice root exudates induce a chemotactic response for rhizosphere [18] and endophytic bacteria [34], and defense responses of plants limit bacterial invasion [35]. Thus, specific bacteria can penetrate root tissues and colonize in plants [36]. The OTU richness indices of endophytic diazotrophs were more variable than rhizosphere diazotrophs, as depicted by the rarefaction curves (Additional file 5: Figure S1). It is probably caused by sporadic and non-uniform colonization of endophytic diazotrophs in the roots [37]. The community composition of rhizosphere and endophytic microbiome were different in various plants [36,38-40]. In this present study, the community compositions of rice rhizosphere diazotrophs were significantly different from rice endophytic diazotrophs at the DNA and RNA levels (Additional file 9: Table S7), suggesting that rice roots represent a unique niche for diazotrophic communities. Phylogenetic analysis showed that Alphaproteobacteria (25.29%) was the dominant class and *Bradyrhizobium* (24.8%) was the dominant genus in rice rhizosphere soil. Similar observations were reported in rice bulk soil [16] [41]. For root samples, Betaproteobacteria (34.95%) was the dominant class in rice roots [42-43]. The previous study has reported endophytic diazotrophs were

dominated by Gammaproteobacteria (66–98%) before flooding, whereas after flooding Betaproteobacteria was the dominant class (26–34%) [44].

The rhizosphere diazotrophic communities were more diverse than the endophytic diazotrophic communities at the DNA and RNA levels (Additional file 4: Table S4) [36,39], and the community structures of rhizosphere diazotrophs were notably different from endophytic diazotrophs (Additional file 9: Table S7) [40,45]. Moreover, compare with year and fertilization management, growth period (temporal variations) was the dominant factor affecting the community composition of rhizosphere and endophytic diazotrophs (Table 2, Additional file 20: Table S13). Previous studies had reported that the diversity of free-living diazotrophic and total bacterial populations were more sensitive to temporal and seasonal effects than fertilization management in agricultural soil [46]. Notably, growth period had a higher significant effect of the endophytic diazotrophic communities than that of the rhizosphere diazotrophic communities, but fertilization management had a stronger effect on the community composition of rhizosphere diazotrophs than endophytic diazotrophs. Taken together, growth period, year, and fertilization affect soil and root diazotrophic communities differently.

We also found some root-enriched nitrogen-fixing bacteria whose abundances in roots were significantly higher than that in soil (Fig. 5). *Thiorhodospira*, *Rhizobium* and *Burkholderia* were root-enriched diazotrophs at three growth periods, and *Paludibacter*, *Magnetospirillum*, *Clostridium* and *Rhodospirillum* were root-enriched diazotrophs at the heading and mature stages. They occupied high relative abundance in rice roots at the DNA ($46.29\pm14.93\%$) and RNA ($47.77\pm13.01\%$) levels. *Burkholderia* and *Rhizobium* were common plant endophytic diazotrophs [47–49], and some strains of these genera have been used as plant growth-promoting bacteria (PGPB) and biocontrol bacteria [50–52]. Besides, *Burkholderia* sp. BV6, *Burkholderia* sp. CJ42 and *Rhizobium* sp. CJ54 were isolated from surface-sterilized rice roots in this work (Additional file 2: Table S2). *Sulfurospirillum* is a reductively dechlorinating bacterium [53]. *Thiorhodospira* is a purple sulfur bacterium, but its nitrogenase activity has not been tested [54]. It is widely admitted that endophytic diazotrophs are capable to fix N more efficiently than the rhizosphere or rhizoplane diazotrophs [55], so we speculate that these root-enriched diazotrophs play an important role for rice growth and development in the whole life cycle of rice.

Shifts of the total community composition of rhizosphere and endophytic diazotrophs during rice growth

Previous work showed that temporal variations in the abundance and diazotrophic community composition, but these studies were carried out either with low resolution detection methods or ignoring endophytic diazotrophs [32–33,56–57]. We studied the structural variability in the root-associated diazotrophs during a rice growing season. Our research demonstrated that the community structures of total endophytic diazotrophs varied dramatically during the vegetative stages and stabilized at the reproductive stage (Fig. 3b), which was consistent with the results of rice root microbiota by 16S rRNA gene amplicons sequencing [21–22]. Additionally, the richness, evenness and PD indices of total endophytic diazotrophs significantly increased at the vegetative stage, but the α -diversity indices of total rhizosphere diazotrophs had no significant changes (Additional file 4: Table S4). Thus, we deduced that abundant diazotrophs recruited from the paddy soil colonize and reproduce in rice roots at the vegetative stage, which caused large changes in diazotrophic community structure. We see support for this idea as the proportion of nitrogen-fixing bacteria shared by soil and root samples increased clearly over time, and the percentage of unclassified diazotrophs in roots was close to that in soil (Fig. 3e). Considering that root exudation increased with rice growth until panicle initiation and decreased from the flowering period to the maturing stage [58], the community succession of rice endophytic diazotrophs is probably driven by root exudation. However, further studies are needed to investigate the regulation mechanism of root exudates on the endophytic diazotrophic community. Moreover, when applying nitrogen-fixing bacteria as bio-fertilizers, it should be taken into account whether they could colonize efficiently in the roots at a particular time.

Pig manure application maintained the community composition of natural rhizosphere diazotrophs

Nitrogen deficiency promotes root growth, but decreases shoot biomass, shoot and root nitrogen content [59-60]. Furthermore, the efficiency of BNF is promoted with low N concentration [61], but rapidly reduced or even inhibited under a high concentration of ammonium [62]. The ^{15}N isotope dilution experiment verified that low nitrogen nutrition promoted elongation of rice roots, and rice inoculated with a mixture of endophytic diazotrophs obtain a higher level of BNF contribution with a low N content (Table 1).

Previous researches showed that applying chemical or organic fertilizers decreased the diversity of dominant diazotrophs [31], but we found that fertilization management was inconsequential to the α -diversity indices of total diazotrophs in rhizosphere soil and rice roots (Additional file 6: Table S5, Additional file 7: Table S6), which was consistent with Wakelin and Ogilvie's observations [63-64]. According to the correlated analysis, AN and TP had significantly negative correlation with α -diversity indices of total rhizosphere diazotrophs (Additional file 17: Table S11).

Given that the β -diversity of rhizosphere diazotrophs were more affected by fertilization than that of endophytic diazotrophs (Table 2, Additional file 20: Table S13), we focused on the effect of fertilization on rhizosphere diazotrophs. Compared with NPK and NPK + rice straw (NPKS), the rhizosphere diazotrophic community of NPK + pig manure (NPKM) and control (CK) had higher similarities at the DNA and RNA levels (Additional file 13: Table S8). Thus, the application of pig manure not only increased soil nutrients (OM, TN, TP, AN, AP and $\text{NO}_3\text{-N}$) (Additional file 15: Table S9) [65], but reduced the effect of chemical fertilization on the natural rhizosphere diazotrophic community structure.

Active rhizosphere diazotrophic communities are less diversity but more divergent than total rhizosphere diazotrophic communities

We used RNA-derived *nifH* gene Illumina sequencing to elucidate the accurate diazotrophic community structure and characterize the *nifH* expression profile of rice root-associated diazotrophs. The α -diversity indices of rhizosphere and endophytic diazotrophs in the DNA samples were less than their corresponding RNA samples (Additional file 4: Table S4). The community structures of total and active diazotrophs in soil or roots were had evident deviations [66], and the community structures of active rhizosphere diazotrophs were more volatile than that of total rhizosphere diazotrophs (Fig. 4a, Additional file 10: Figure S3e). This is consistent with previous observations that seasonal variation and management practices had more effect on the active diazotrophs than total diazotrophs in bulk and rhizosphere soil [29,67-68]. Our results suggest that AN, TP, $\text{NH}_4\text{-N}$ and $\text{NO}_3\text{-N}$ had significant negative correlation with α -diversity indices of active rhizosphere diazotrophs (Additional file 17: Table S11). Our work extend previous studies by specifically characterizing the distribution and transcriptional activity of active root-associated diazotrophs in the paddy field.

The temporal dynamics of root-associated diazotrophic networks

Habitat heterogeneity influenced the bacterial [69] and fungal [70] network interactions. In our work, rhizosphere diazotrophs formed larger and more complex networks than endophytic diazotrophs at the DNA and RNA levels. The strong host-specific screening effect is imposed by plants on soil microbes, resulting in that only specific microorganism can colonize roots [71]. Previous alpha diversity analyses showed that the richness and evenness indices of rhizosphere diazotrophs were higher than endophytic diazotrophs (Additional file 4: Table S4), meaning number of OTUs in soil was greater than that in roots. The percent of positive correlations in total rhizosphere diazotrophic networks were lowest in all networks, meaning that total rhizosphere diazotrophs had more competitive relationships than total endophytic diazotrophs, active rhizosphere diazotrophs and endophytic diazotrophs (Fig. 6).

Conclusions

This study provides a comprehensive understanding of the combined effects of growth periods and fertilization management on the diversity, composition and co-occurrence network of root-associated diazotrophs in the paddy field

during the whole growth period. We proved that the structural variability of rhizosphere diazotrophs is much lower than that of the endophytic diazotrophs, and growth period and fertilization management are undeniably important factors affecting nitrogen-fixing bacteria community. Specific diazotrophs appear to be recruited from the paddy soil to colonize and reproduce in rice roots during rice growth. Furthermore, the community structures of total endophytic diazotrophs were more stabilized in the reproductive stage than the vegetative stage. Follow-up studies need to investigate the effects of plant age, developmental progression, climatic variations and cropping management on diazotrophs in the paddy system, reveal the colonization process of diazotrophs, and further deepen our knowledge of root-rhizosphere soil-diazotrophs interactions.

Methods

Field experimental design

The field experiment is located in Jintan City, Jiangsu Province, China (31°39'N, 119°28'E). The soil type is classified as typical Wushan soil, with a long-term annual rotation of winter wheat (*Triticum aestivum* L.) and summer rice (*Oryza sativa* L.). The fertilization experiment started in 2010 using a random block design (Additional file 1: Table S1). Here, we chose four fertilization treatments including no fertilization (CK), 100% chemical fertilizers (NPK), 50% chemical fertilizers plus 6000 kg/ha pig manure (NPKM), 100% chemical fertilizers plus 8000 kg/ha crop straw (NPKS). 100% chemical fertilizers contained urea: 300 kg/ha, P₂O₅: 120 kg/ha and K₂O: 100 kg/ha. All P, K, manure fertilizers and crop straw were applied as basal fertilizers before planting, whereas N fertilizers were used as basal fertilizers and supplementary fertilizers (basal fertilizers: tillering supplementary fertilizers: panicle supplementary fertilizers = 4 : 3 : 3).

Hydroponic experiment

Fresh rice roots were sampled from the experimental field, and surface-sterilized with 75% ethanol for 1 min and 1% NaClO for 4 min. Then the roots were ground with a mortar, and made into the rice root grinding fluid. *Oryza sativa* seeds were sterilized with 5% sodium hypochlorite for 1 h and then germinated. After 16 days, roots of rice seedlings were sampled and surface-sterilized, then rice root DNA was extracted. The endophytic diazotrophs was undetected using polF/polR primers [72]. The remaining 36 rice seedling (three-leaf stage) were divided into three groups. Thereinto, the T₁₀ and T₂₀ treatments were inoculated by soaking roots in the rice root grinding fluid for 12 h at room temperature, and the excess inoculum was washed off with tap water. Seedlings of the control group (C₂₀) were soaked using autoclaved root grinding fluid. Next, all rice seedlings were transferred into the 5L-beakers and each beaker had two seedlings. They were cultured in the Yoshida's culture solution [73] with some modification. The concentration of ¹⁵N-labeled (NH₄)₂SO₄ (50 atom% excess) of C₂₀ and T₂₀ treatments was 20 ppm, while of T₁₀ was 10 ppm. The nutrient solution was changed every five days and pH remained 5.5-6.5 throughout the experiment.

After planting for 62 days, fresh weight, root length and plant height were measured. Destructive sampling of the whole plant was undertaken for determination of total N content and ¹⁵N concentration. The 12 seedlings were divided into three groups, and every group was dried in forced ventilation oven at 60°C for 6-8 h, grinded and sieved (0.25 mm). Total N of the dry matter was determined using Kjeldahl digestion. ¹⁵N concentration was measured by Finnigan-MAT-251 mass spectrometer (Bremen, Germany) in the institute of Soil Science, Chinese Academy of Sciences, and the precision is ± 0.20‰. The percentage of N derived from the atmosphere by BNF (% *Ndfa*) was calculated according to the equation: % *Ndfa* = [1 - R_t / R_c] × 100. R_t is the ¹⁵N/ total N ratio of the treatment, and R_s is the ¹⁵N/ total N ratio of control.

Endophytic diazotrophs isolated from rice roots

Ten-fold serial dilutions of the rice root grinding fluid mentioned above were plated on YMA or Döbereiner's N-free media. After 3-5 d of incubation, isolates with different morphology were transferred and streaked on corresponding agar plates.

25 strains were purified and their genome DNA were extracted with the TIANamp Bacteria DNA kit (TIANGEN) following the protocol. Their 16S rRNA and *nifH* gene were amplified by PCR using 27F/1492R and polF/polR primers. PCR products were ligated into pMD19-T-Simple Vector (TaKaRa, Dalian, China), sequenced, and identified their closest phylogenetic relatives [74]. Nitrogen fixation activity was measured using the modified ARA [75]. The isolates were cultivated in semisolid Döbereiner's media in sterilized test tubes, and a final concentration of 1% acetylene was added after growth for 2 days at 30°C. The concentration of ethylene was detected 24 h later. Non-inoculated test tubes were used as negative controls and every isolate had three replicates. Ethylene was measured with a gas chromatograph (Shimadzu GC-2010, Kyoto, Japan) equipped with a Porapak N packed column and a flame ionization detector (FID). The injector, detector and oven temperatures were 180°C, 220°C and 100°C, respectively. The carrier gas was N₂ (highest available purity) at a flow rate of 10 ml/min, and the supply of H₂ and air for the FID were 30 and 300 ml/min, respectively. The retention time of ethylene was 5.23 min, and the retention time of acetylene was 7.15 min. Isolates were cultured in YMA medium with or without 0.5 g/l Trp for 24 h. After centrifugation, the IAA production of the supernatant was determined by Salkowski reagent (12 g FeCl₃ per liter in 7.9 M H₂SO₄) [76]. CAS solution was used for the quantitation of siderophore production of supernatants [77]. Isolations were inoculated on the solid National Botanical Research Institute's phosphate growth medium (NBRIP) and their soluble phosphorus circles were observed.

Sample collection of the field experiment

Rice samples were sampled on 8 July (tillering stage), 1 September (heading stage) and 17 October (mature stage) 2015 and 27 July (tillering stage), 12 September (heading stage) and 25 October (mature stage) 2016. In 2015, every fertilization treatment had three plots, and two plants were randomly collected from each plot, then totally six samples were obtained. In 2016, every fertilization treatment had four plots, and three plants were collected and mixed as one sample in each plot. The plants were uprooted and immediately transported to the laboratory on ice. After separated from roots, the rhizosphere soil was thoroughly homogenized and sieved (2 mm). Then the soil was divided into two parts: part one was stored at -80°C for DNA and RNA extraction; part two was air-dried and sent to the Qiyang Red Soil Experimental Station (26°45'N, 111°52'E, Hunan Province, China) for soil physical and chemical analysis [78]. Soil nitrate-nitrogen (NO₃-N) and ammonium-nitrogen (NH₄-N) were analyzed using a continuous flow analytical system [79]. Before DNA and RNA extraction, roots were sterilized with 75% ethanol for 1 min and 1% NaClO for 4 min. The last washing solution was coated in TSA solid medium to detect whether the roots were completely disinfected.

DNA extraction and *nifH* Illumina sequencing

Soil DNA and RNA were extracted using FastDNATM SPIN Kit for Soil (MP Biomedical, Irvine, CA, USA) and RNA PowerSoil® Total RNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. Rice root DNA and RNA were extracted by PowerPlant® DNA Isolation kit (Mo Bio) and RNApure Plant Kit (PD biotech, Shanghai, China). The extracted DNA and RNA were evaluated on a 1% agarose gel. The reverse transcription was performed using PrimeScriptTM RT reagent Kit with gDNA Eraser (Takara, Dalian, China).

The primers *nifH*1 (5'-barcode-TGYGAYCCNAARGCNGA-3') and *nifH*2 (5'-ADNGCCATCATYTCNCC-3') were used to amplify a 359-bp sequence of *nifH* gene [80]. Barcode is an eight-base sequence unique to each sample. PCR reactions were performed in triplicate to minimize PCR bias which contained 4 µL of 5×FastPfu Buffer, 2 µL of 2.5 mM dNTPs, 0.8 µL of each primer (5 µM), 0.4 µL of FastPfu Polymerase, and 10 ng of template DNA or complementary DNA [81]. Purified PCR products were pooled in equimolar and paired-end sequenced (2×250 bp) on the Illumina MiSeq platform (Biozeron, Shanghai, China) according to the standard protocols.

Sequence processing and statistical analyses

Raw fastq files were quality-filtered with the following criteria: (1) Bases of each read whose quality scores < 20 were discarded. Only the reads with perfectly matched barcodes, primers < 2 nucleotides mismatch and containing no ambiguous character were kept. (2) Then the barcodes and primers were deleted. (3) The remaining forward and reverse reads with at least 10-bp overlap were combined into a single sequence using FLASH. Combined sequences of less than 250-bp were discarded. The quantified reads were translated into protein sequences and corrected potential frameshifts using FrameBot program (<http://fungene.cme.msu.edu/FunGenePipeline/>) and the corresponding FunGene database (<http://fungene.cme.msu.edu/>) (min length = 100 amino acids, hmm = 50%) as a reference [43,82-83]. Reads having in-frame stop codon (s) were manually removed. Then the protein reads were removed singleton, dereplicated and clustered into OTUs using the UPARSE pipeline at 90% identity [84-86]. Chimeric sequences were then identified and removed using UPARSE in de novo mode. Finally, OTUs that contained one sequence were removed. To assign putative taxonomy, representative sequences were aligned to a closest-match sequence of the FunGene database by BLASTp [87-88].

Chao1, Shannon and PD indices were calculated by Mothur software after sequences were normalized. Rarefaction curves, bar plots, cluster trees, heatmaps and bubble plots were drawn using R (Version 3.4.0). The Mental tests, PERMANOVA and ANOSIM analysis were conducted using the `mental()`, `adonis()` and `anosim()` functions from the Vegan package in R, respectively. NMDS was conducted on the Bray-Curtis distance matrix using the vegan R package. Spearman correlation analysis between diazotrophic taxa and soil physicochemical indices was performed using the `corrplot` library in R. To identify the phylogenetic affiliation of *nifH* sequences, neighbor-joining (NJ) trees were constructed using the molecular evolutionary genetics analysis (MEGA) software for the 50 most abundant OTUs together with selected reference sequences. To track the dynamics of individual OTUs among different samples, Sankey plots were constructed based on D3.js (v.5.14.2) (d3js.org) [89]. Bacterial taxonomic flow at the class level was drawn based on the OTU table of all samples, and for OTUs shared by soil and root samples, the OTU table was further filtered to OTUs whose abundance is less than 0.05%. The extended error bar plots were carried by STAMP software (Statistical Analysis of Metagenomic Profiles; <https://beikolab.cs.dal.ca/software/STAMP>) to filter fertilization-sensitive and root-enriched biomarkers. The online LEfSe program (http://huttenhower.sph.harvard.edu/galaxy/root?tool_id=PICRUSt_normalize) was performed to find biomarker whose relative abundance significantly changed during rice growth. The co-occurrence network was constructed with the 'psych' package by using the Spearman correlation, and the correlations with a Spearman coefficient of less than 0.65 and an adjusted P values of more than 0.01, were removed. The network images were generated and the network properties were calculated with Gephi (<https://gephi.org/>). The other statistical analyses were conducted using SPSS 19.0. (SPSS Inc., USA).

Abbreviations

BNF: biological nitrogen fixation; NUE: nitrogen-use efficiency; ARA: acetylene reduction activity; NCR: nodule-specific cysteine-rich; OTUs: operational taxonomic units; OM: organic matter; TN: total N; TP: total P; TK: total K; AN: available N; AP: available P; AK: available K; NO₃-N: nitrate-nitrogen; NH₄-N: ammonium-nitrogen; PGPB: plant growth-promoting bacteria.

Declarations

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Author information

Author notes

Xue Luo and Xianfeng Ye contributed equally to this work.

Contributions

ZC, HC and WR conceived the study. XL and WW performed data analyses. XL, XY, WW, ZL, and YQ collected samples and performed the DNA and RNA extractions. YC, CD, and YZ performed parts of the experiment. XL, XY, WW, and ZC edited the manuscript. YH, MH, XF and HJ revised the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

The datasets generated and analyzed during the current study are available in the NCBI Sequence Read Archive (SRA) repository under the BioProject with accession codes PRJNA666552 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA666552>).

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that there are no competing interests.

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Tables

Table 1 Effect of inoculation of root grinding fluid on rice root, plant height and biomass under different N concentrations in the hydroponic experiment.

	C ₂₀	T ₂₀	T ₁₀
fresh weight/g	2.25±0.41 b	2.36±0.46 b	3.26±0.54 a
root length/cm	13.46±1.19 b	13.53±0.91 b	19.51±2.03 a
plant height/cm	37.04±1.68 b	36.35±1.85 b	39.08±1.47 a
N concentration/%	2.84±0.06 a	2.77±0.04 a	2.83±0.3 a
δ ¹⁵ N/atom%	33.26±1.1 a	33.85±0.25 a	29.43±0.49 b

Note: T₂₀ and T₁₀ were inoculated with rice root grinding fluid, and C₂₀ was uninoculated. C₂₀, T₂₀ and T₁₀ were cultured at 20, 20 and 10 ppm ¹⁵N-labeled (NH₄)₂SO₄ (50 atom% excess), respectively. Twelve seedlings were cultured per treatment, and four seedlings were taken as a sample when measuring N concentration and δ¹⁵N. Different lowercase letters indicate significant differences between treatments (P<0.05, Tukey's honestly significant difference (HSD) test).

Table 2 Effects of experimental variables on total rhizosphere and endophytic diazotrophic α-diversity and β-diversity.

	Total rhizosphere diazotrophs				Total endophytic diazotrophs			
	β diversity	Richness (Chao1)	Evenness (Shannon)	PD	β diversity	Richness (Chao1)	Evenness (Shannon)	PD
year	0.19***	0	0.22***	0	0.06***	0.05***	0.05***	0.03**
period	0.22***	0.08*	0.18***	0.26***	0.33***	0.51***	0.51***	0.53***
fertilizer	0.11***	0.05	0.06**	0.02	0.05***	0.02	0.04**	0.01
year:period	0.11***	0.04	0.05**	0.01	0.08***	0.04**	0.09***	0.04***
year:fertilizer	0.02**	0.02	0	0.01	0.02*	0	0.01	0.02
period:fertilizer	0.04**	0.05	0.04	0.03	0.09***	0.02	0.05**	0.05*
year:period:fertilizer	0.03	0.03	0.03	0.07	0.04***	0.01	0.03*	0.03

Note: The effects of experimental factors and their interactions were assessed by a non-parametric PERMANOVA analysis. Significance level: $P < 0.05$, *; $P < 0.01$, **; $P < 0.001$, ***. The PERMANOVA analysis of β -diversity was performed at the OTU level.

Figures

Figure 1

Rice yields in different fertilization treatments (a). NMDS analysis of total diazotrophic community structure of all DNA samples in 2015 and 2016 (b). NMDS analyses of total diazotrophic community structure in rhizosphere soil (c) and rice roots (d) in 2016. Diazotrophic community composition of the rhizosphere soil and rice roots DNA samples in 2016 at the class level (e). Note: Different lowercase letters indicate significant differences between different fertilization treatments ($P < 0.05$, Tukey's HSD test). NMDS analysis and cluster tree were based on Bray-Curtis similarities at the OTU level.

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Figure 2

Neighbor-joining tree of the 50 dominant OTUs of all DNA samples in 2016 with their aligned *nifH* sequences from the FunGene database (bootstrap replications = 1000). Note: The inner phylogenetic tree depicts the evolutionary relationship of the 50 dominant OTUs, and the outer little rectangles represent the variances of the relative abundances of these dominant OTUs during rice growth. Different colors of the inner lines represent the classes of the OTUs and their aligned *nifH* sequences. The color of each outer little rectangle represents the percentage of each sample in respect of the total. S1: soil DNA samples at the tillering stage; S2: soil DNA samples at the heading stage; S3: soil DNA samples at the mature stage; R1: root DNA samples at the tillering stage; R2: root DNA samples at the heading stage; R3: root DNA samples at the mature stage.

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Figure 3

Scatter plots of the mean pairwise z-scores for similarity, computed as (1-Bray Curtis dissimilarity), between growth periods in rhizosphere soil (a) and rice roots (b) for the 2015 and 2016 DNA samples. Sankey plots of associated diazotrophic flow at the class level during rice growth in 2016 (c) and 2015 (d). Color bars indicate different classes, and the heights of bars indicate the relative number of classes. Pink flows indicate variations in abundance of shared OTUs between soil and root samples at the same sampling point. Heatmap plots showing changes in the relative abundances of discriminating genera throughout rice growth in rhizosphere soil (e) and rice roots (f).

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Figure 4

Similarities in the between-community variation of diazotrophs in the soil DNA, root DNA, soil RNA and root RNA libraries (a) in 2016. Similarities of the diazotrophic communities between the DNA samples and their corresponding RNA samples among three growth periods in 2016 (b). Total and active diazotrophic community composition of the rhizosphere soil (c) and rice root (d) samples in 2016 at the class level. Note: The cluster tree was based on Bray-Curtis similarities at the OTU level. Different lowercase letters indicate significant differences between libraries ($P < 0.05$, Tukey's HSD test).

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Figure 5

Bubble plot showing the relative abundance of the dominant genera (relative abundance > 0.1%) in root DNA and RNA samples during three growth periods. Note: The cluster tree was based on the differences of the relative abundance in root and soil samples at the genus level using the complete linkage method. The size of each bubble represents the square root of the relative abundance of each genus in the root sample. The color of each bubble represents the difference between the square root of relative abundance of each genus in the root and in the rice sample. Red bubbles indicate genera which are abundant in the roots, and blue bubbles indicate genera which are abundant in the rhizosphere soil. Insignificant differences are not shown ($P > 0.05$).

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Figure 6

The co-occurrence networks of total root-associated diazotrophs in 2016. Points (nodes) represent different OTUs. Note: Red solid lines show strong (Spearman's correlation coefficient $r \geq 0.65$) and significant ($P < 0.01$) positive correlations. Blue solid lines show strong (Spearman's correlation coefficient $r < -0.65$) and significant ($P < 0.01$) negative correlations. The size of each point is the node's weighted degree.

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