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Response of symbiotic and asymbiotic nitrogen-fixing microorganisms to nitrogen fertilizer application

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Abstract

Purpose Biological nitrogen fixation (BNF) plays an important role in nitrogen cycling by transferring atmospheric dinitrogen to the soil. BNF is performed by symbiotic and asymbiotic nitrogen-fixing microorganisms. However, the abundance, activity, and community structure of diazotrophs under different nitrogen fertilizer application rates and how root exudates influence diazotrophs remain unclear.

Materials and methods ^{15}N-N_2 and ^{13}C-CO_2 labeling, DNA-based stable isotope probing (SIP), and molecular biological techniques were used in this study. The abundance, activity, and structure of symbiotic and asymbiotic diazotrophs under different nitrogen fertilizer applications in paddy soil were investigated.

Results and discussion We found that the nitrogen fixation capacity in milk vetch (*Astragalus sinicus* L.) and *nifH* gene abundance in the root nodules were significantly higher in the low-nitrogen treatment than in the control (zero) and high-nitrogen treatments. Nitrogen-fixing bacteria were abundant in the soils with a high biodiversity. Soil *nifH* gene sequences were dominated by α-, β-, and δ-*proteobacteria*, as well as by *Cyanobacteria*. The relative abundance of α-*proteobacteria* was lower, and the relative abundance of *Cyanobacteria* was higher under high nitrogen. Incubation of soil with $^{13}CO_2$ and subsequent DNA-SIP analysis demonstrated that OTU65 from α-*proteobacteria* was relatively more abundant in heavy fractions of the ^{13}C-labeled soils than that in the unlabeled soils, indicating that α-*proteobacteria* may prefer rhizodeposition carbon to other organic carbon. However, OTU24 and OTU73 from δ-*proteobacteria* had relatively high abundances in light fractions both in the ^{13}C-labeled and unlabeled samples, indicating that δ-*proteobacteria* may prefer other soil organic carbon to rhizodeposition carbon.

Conclusions The results suggested that soil N availability and rhizodeposition strongly modified the microbial communities of nitrogen-fixing bacteria. Moderate nitrogen application increased the symbiotic biological N fixing activity in the *Astragalus sinicus* L. rhizosphere. The BNF activity in the legume-rhizobia system is regulated by the exchange of organic C and N nutrient between the host plant and N-fixing bacteria.

Keywords ^{13}C-CO_2 labeling · ^{15}N-N_2 labeling · Nitrogen fertilizer · Nitrogen-fixing bacteria · Paddy soil

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1 Introduction

Biological nitrogen fixation (BNF) is the enzymatic reduction of atmospheric dinitrogen to ammonium, which is an important part of the nitrogen cycle that contributes to the soil nitrogen pool. BNF-derived N plays an important role in substituting for chemical N fertilizer use in agricultural systems (Galloway et al. 2004). The amount of N fixed by BNF is estimated to be 2×10^{11} kg N year^{-1}, which accounts for 75% of the N demand for plant growth in the world (FAO 1995).

Growing green manure legumes and recycling their biomass or harvesting residues and adding them to soil generally improve soil fertility, increase the yield of the subsequent rice crop, and reduce the requirement for chemical N fertilizer (Ladha and Reddy 2003; Choudhury and Kennedy 2004). The percentage of N derived from the air (% Ndfa) for green manure legumes is generally more than 80%, and the amount of N fixed by these legumes is as high as 450 kg N ha^{-1} (Ladha and Reddy 2003). Zhu et al. (2014) observed that approximately 82–88% of *Astragalus sinicus* L. N was released within the first 28 days after incorporation, and the released N could meet the N demand for rice plant growth. Many studies have shown that the amount of N provided by green manure alone is equivalent to 30–108% of that provided by inorganic fertilizer (Asagi and Ueno 2009). In addition to symbiotic nitrogen-fixing microorganisms, free-living soil diazotrophs can also contribute N nutrient, especially in paddy fields, which provide an optimal environment for biological nitrogen fixation (Roger and Ladha 1992).

The rate of biological nitrogen fixation is mainly dependent on soil nitrogen availability. Soil nitrogen deficiency can inhibit the synthesis of N-fixing enzymes, and high N fertilizer input decreases biological N fixation (Salvagiotti et al. 2008). Compared to no fertilizer, nodule number and soybean weight were higher when N was applied at low levels but lower in high N treatments (Wahab and AbdAlla 1996). Barbulova et al. (2007) observed inhibition of the symbiotic performance of rhizobia and nitrogen fixation under high N supply. Aeroponic culture experiments suggested that a high ammonium concentration (> 5 mM) inhibited nodulation, whereas a low concentration (0.4 mM) stimulated nodulation in an Acacia species (Weber et al. 2007). The abundance of nitrogen-fixing bacteria and N_2-fixation activity is suppressed when nitrogen is abundant in the environment (Fuentes-Ramirez et al. 1999; Wang et al. 2018). Studies have shown that N fertilizer application significantly decreases the number of culturable diazotrophic bacteria in Indian and Brazilian sugarcanes (Muthukumarasamy et al. 1999; Junior et al. 2000).

Energy and substrate availability are also important factors for biological N fixation. Except for some asymbiotic nitrogen-fixing microorganisms that can derive their energy from photosynthesis and chemoautotrophy, all heterotrophic diazotrophs use organic material as an energy source (Halm et al. 2012). Previous studies have found that nitrogen-fixing efficiency is positively correlated to the available carbon content in the forest soil (Rosch and Bothe 2009). Plant straw application stimulates soil biological nitrogen fixation, and the application of organic material combined with inorganic fertilizer significantly improves soil N fixation efficiency compared to inorganic fertilizer alone (Liao et al. 2018).

Mutualistic cooperation between plants and microorganisms is ubiquitous, especially in symbiotic systems. Generally, leguminous plants provide C substrates to rhizobia and rhizobia supply some N nutrient to the plants. Mutual constraints can also occur in some symbiotic systems. Kiers et al. (2003) found that a decreased O_2 supply from soybean is a possible mechanism to punish "cheating" rhizobia. In soil-plant systems, root exudates are regarded as the main driver of soil microbial communities. However, the effect of root exudates on symbiotic nitrogen fixation is still unclear. Recently, ^{13}C-CO_2 labeling combined with DNA stable isotope probing has been shown to be an effective technique to monitor C flux and plant-soil interactions.

Milk vetch (*Astragalus sinicus* L.) is the most common green manure for paddy soil and is grown prior to rice transplantation. In this study, ^{13}C-CO_2 and ^{15}N-N_2 labelings were conducted to study the response of symbiotic and asymbiotic nitrogen-fixing microorganisms to nitrogen fertilizer application. The main objectives were as follows: (1) determine whether nitrogen application affects biological N fixation and the diazotrophic bacterial community and (2) determine how rhizodeposition affects diazotrophic bacteria.

2 Materials and methods

2.1 Soil and plant material

Paddy soil was collected from the rice field in Taihu region (30°33′N, 116°20′E), Anhui Province, China, in August 2015. The cropping system was rice-green manure rotation. Twenty soil cores (10 cm diameter × 15 cm length) were taken from the plow layer. The soil samples were placed on ice and transported to the lab. Visible roots and stones in the soil were removed, and the soil was thoroughly mixed and sieved through a 2.0-mm mesh. The soil samples were separated into two portions. The first portion was air-dried for chemical analysis except that mineral-N was immediately analyzed. The second portion was stored at 4 °C for the pot experiment. The soil texture was sandy

loam, soil pH was 6.2 (1:5 *w/v*, soil/water), and total C and total N contents were 10.9 g kg^{-1} and 0.81 g kg^{-1}, respectively.

The cultivar of milk vetch (*Astragalus sinicus* L.) used in this study was Yijiangzi. The seeds were sterilized in 10% H_2O_2 for 10 min and then washed exhaustively with sterile water. *A. sinicus* seeds were set at 37 °C for pregermination in the dark.

2.2 ^{13}C-CO_2 labeling experiment

Three treatments were conducted with three levels of nitrogen fertilizer: CK (without nitrogen), NL (nitrogen at 40 mg kg^{-1}), and NH (nitrogen at 100 mg kg^{-1}). Each pot (height 12 cm, diameter 6 cm) with 400 g soil (dry weight) was prepared to plant *A. sinicus*. All treatments were done in triplicate. The nitrogen fertilizer used in this experiment was urea. Monopotassium phosphate was used as P_2O_5 (120 mg kg^{-1} dry soil) and potassium as K_2O (80 mg kg^{-1} dry soil). Similar gemmiparous seeds were chosen to plant in the pots, and 10 seeds were cultivated in each pot. *A. sinicus* was grown in a plant growth chamber, with an average light intensity of 295 UML, a 12.5-h photoperiod, a relative humidity of 60%, and a temperature of 24 °C in the day and 18 °C at night. The incubation conditions were kept consistent during the entire incubation period.

The ^{13}C-CO_2 continuous labeling experiment was carried out after 30 days of *A. sinicus* growth. The pots were incubated in two chambers with normal CO_2 or ^{13}C-CO_2 (99.9 atom %). The total CO_2 content was 350 mL m^{-3}, the flow velocity of air without CO_2 was 10 L min^{-1}, and the flow velocity of CO_2 was 3.8 mL min^{-1}. Destructive sampling was conducted before labeling (30 days) and after 30-day labeling (60 days) from the ^{13}C chamber and unlabeled chamber. The root samples were washed with deionized water, and the nodules were picked using tweezers and quickly frozen in liquid nitrogen and stored at − 80 °C. The shoots and roots were dried to measure the dry weight and nitrogen content. The soil used for DNA extraction was freeze-dried immediately after sampling, and soil samples for other chemical analyses were stored at 4 °C until analysis.

2.3 ^{15}N-N_2 labeling experiment

A ^{15}N-N_2 labeling experiment was conducted after 53-day growth using the pulse labeling method, and the treatments were the same as described above in Section 2.2. The plants were grown in a sealed container (0.086 m^3), and 10%-labeled ^{15}N-N_2 was injected to the container. Another set of plants were grown with ambient air as controls. The plants were labeled for 10 h each day. When 7-day labeling was complete, the nodules were freeze-dried, the abundance of ^{15}N was measured, and the shoots and roots were dried to measure the ^{15}N abundance. Soil with three nitrogen levels without plants was also labeled with ^{15}N-N_2 as a control, and the soil ^{15}N abundance was measured after 7 days of labeling. ^{15}N abundance was determined by a Delta V Advantage isotope ratio mass spectrometer (Thermo Finnigan, Germany).

2.4 Soil and nodule DNA extraction

Soil DNA was extracted using the FastDNA spin kit for soil (MP Biomedicals, USA) following the manufacturer's instructions. Nodule DNA was extracted using CTAB according to the method of Kim et al. (1999). A NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific, USA) was applied to assess DNA concentration and quality. All DNA samples were stored at − 80 °C for subsequent use.

2.5 Soil and nodule *nifH* gene abundance determination

The abundance of *nifH* was assessed in triplicate using a real-time quantitative PCR (qPCR) detection system (LightCycler 480, Roche, USA), with the primer sets PloF (5′-TGCGAYCCSAARGCBGACTC-3′) and PloR (5′-ATSGCCATCATYTCRCCGGA-3′) (Poly et al. 2001). Each reaction mixture contained 10 μL SYBR 2 Premix Ex Taq (Takara Shuzo, Shiga, Japan), 0.25 μM of each primer, and 2 μL of 10-fold diluted template DNA and made up to 20 μL with ddH_2O. All standard curves were generated from 10-fold stepwise dilutions of plasmid DNA with the correct target genes. Negative controls were included, using water to replace the template DNA, in each plate. The quantitative amplification conditions were 95 °C for 4 min, 40 cycles of 95 °C for 45 s, 55 °C for 30 s, and 72 °C for 40 s. Quantitative PCR efficiencies between 90% and 110% were employed in this study.

2.6 DNA SIP fractionation

Both ^{13}C-labeled and unlabeled soil DNA were used for gradient fractionation. The method was the same as described by Neufeld et al. (2007). Approximately 2-μg DNA was mixed with the CsCl solution to achieve the initial buoyant density gradient of 1.691 g mL^{-1} according to the refractive index (1.3999) measured by an AR200 hand-held refractometer (Reichert, Inc., Buffalo, NY). The speed of the ultracentrifugation was 45,000 rpm at 20 °C for 40 h (Beckman Coulter, German). LSP01-1A

single-channel syringe pumps (Longer Precision Pump Co., Ltd., China) were used to fractionate the DNA samples; each sample was evenly separated to 16 layers. The collected DNA was purified and dissolved in 30 μL of sterile water. The copy number of the *nifH* gene in the fractionated DNA was measured by qPCR as described in Section 2.5.

2.7 Cloning and sequencing analyses

The whole soil sample DNA and the fractionated DNA (including ^{13}C-labeled and unlabeled fractions) were amplified for cloning and sequencing. The obtained PCR products were gel-purified using the universal DNA purification kit (Tiangen Biotech, Beijing, China), and the purified products were ligated into pMD19-T vectors. For each of the three replicates, 80 positive clones were randomly selected and sequenced (240 clones for each treatment) (Shanghai Majorbio Bio-Pharm Technology Co., Shanghai, China). Chromas LITE software (version 2.01, Technelysium Pty Ltd., Australia) was used to check the sequence quality. All sequences were analyzed by the BLAST program in the NCBI GenBank database. Reference sequences from the GenBank database and the respective OTUs (98% similarity) sequences were selected to construct the phylogenetic tree (Long et al. 2018).

The gene sequences retrieved in this study were uploaded to the National Center for Biotechnology Information. The access numbers of the whole soil samples are KY011303-KY011835. The accession numbers of the unlabeled soils are KY046404-KY046914 and KY046916-KY047104, and the accession numbers of the ^{13}C-labeled soils are KY121112-KY121811.

2.8 Statistical analysis

Quantitative PCR data were log-transformed before further analysis. To compare the abundances of *nifH* genes among all treatments, data were analyzed using ANOVA with SPSS 19.0 software (IBM, USA). The nucleic acid sequences were translated to protein sequences with MEGA software (version 6.0), and the operational taxonomic units (OTUs) of the protein sequences were classified with 95% similarity by DNAMAN (version 6.0). The representatives of each OTU and the reference sequences were aligned using the Clustal W program with MEGA software, and the phylogenetic tree was constructed using the neighbor-joining method. The confidence values of the tree nodes were estimated by a bootstrap analysis with 1000 replicates. Heat map plotting was performed in R 3.5.0 with the pheatmap package.

3 Results

3.1 Symbiotic nitrogen-fixing activity and microorganisms

Nitrogen fertilizer had a significant influence on plant biomass as well as the amount of total and labeled N. The shoot and root dry weights with the NH treatment were 2.18 and 0.86 g, respectively, which were much higher than those with the CK and NL treatments. The total N amounts in the shoots and roots with the NH treatment were 40.4% and 61.1% higher than CK, respectively, and 28.3% and 14.0% higher than the NL treatment, respectively (Table 1). However, the dry weight and total N of nodules with the NL treatment were the highest among the three treatments, and there was no significant difference between CK and NH treatments with respect to nodule dry weight and N content. The labeled N content of the shoots, roots, and nodules was significantly higher in the NL-treated plants, and there was no significant difference between the CK and NH treatments. Although the dry weight and total N content of nodules were much lower than the root, the labeled N content in these two parts was similar. The amount of labeled ^{15}N in the soil was 0.14 ± 0.06, 0.11 ± 0.04, and 0.09 ± 0.04 mg in the CK, NL, and NH treatments, respectively, but there was no significant difference among the three treatments.

The abundance of the *nifH* gene in the nodule was 3.7×10^{10} copies g^{-1} (nodule dry weight) in plants under the CK treatment after 30 days of growth, and there was no significant difference between the two N fertilizer treatments. However, the *nifH* gene abundance in the NL treatment was 6.0×10^{10} copies g^{-1} dry nodule after 60 days of growth, which was significantly higher than that in the CK and NH treatments (Fig. 1).

A phylogenetic tree of the *nifH* gene of the nodule samples showed that the symbiotic nitrogen-fixing bacteria only included two gene types. More than 99% of the *nifH* genes belonged to *Mesorhizobium*, and less than 1% *nifH* genes belonged to *Bradyrhizobium*. All the *Bradyrhizobium* species were from the NH treatment.

3.2 Asymbiotic nitrogen-fixing activity and microorganisms

Compared to symbiotic nitrogen-fixing bacteria, the amount of nitrogen fixation by asymbiotic microorganisms was much lower. In the ^{15}N-N_2-labeled soil without plants, the labeled N content was only 0.05 ± 0.01 mg, which accounted for 5.1% of the labeled N fixed by symbiotic nitrogen-fixing organisms. The abundances of the *nifH* gene in these soils were 1.3×10^7 and 1.2×10^7 copies g^{-1} dry soil in the CK and NL treatments after 30 days of growth, whereas the *nifH* gene abundance in the NH treatment was much lower, at only $0.9 \times$

Table 1 Plant dry weight, total, and labeled N amount of *Astragalus sinicus* L. after the ^{15}N-N_2 labeling

Item	Treatments	Shoot	Root	Nodule
Dry weight (g)	CK	1.71 ± 0.10^{b}	0.55 ± 0.18^{c}	0.094 ± 0.001^{ab}
	NL	2.05 ± 0.34^{a}	0.64 ± 0.09^{b}	0.097 ± 0.002^{a}
	NH	2.18 ± 0.13^{a}	0.86 ± 0.11^{a}	0.092 ± 0.001^{b}
Total N amount (mg)	CK	69.0 ± 6.0^{b}	16.2 ± 2.1^{b}	4.99 ± 0.2^{a}
	NL	75.5 ± 5.1^{b}	22.9 ± 2.4^{a}	5.34 ± 0.2^{a}
	NH	96.9 ± 2.2^{a}	26.1 ± 1.0^{a}	4.97 ± 0.1^{a}
Labeled N amount (mg)	CK	0.59 ± 0.03^{b}	0.13 ± 0.01^{b}	0.14 ± 0.01^{b}
	NL	0.67 ± 0.05^{a}	0.18 ± 0.01^{a}	0.20 ± 0.01^{a}
	NH	0.57 ± 0.02^{b}	0.15 ± 0.02^{b}	0.14 ± 0.01^{b}

Different letters indicate a significant difference at $P < 0.05$

10^7 copies g^{-1} dry soil. The *nifH* gene abundance in soils significantly decreased to 6.9 to 8.8×10^6 copies g^{-1} dry soil after 60 days of growth among the three treatments (Fig. 2). These results suggested that the abundance of the *nifH* gene in the soil decreased with the application of N fertilizer.

Nitrogen-fixing bacteria were abundant in the soils with a high species biodiversity. In total, 107 OTUs were identified at 95% similarity using DNAMAN software. The nitrogen-fixing bacteria were mainly distributed among α-, β-, and δ-*proteobacteria*, as well as *Cyanobacteria*, which occupied more than 98% of the total sequences (Fig. 3). Compared to the CK treatment, N fertilizer application increased the relative abundance of δ-*proteobacteria*, which accounted for 25.0%, 27.2%, and 29.0% of CK, NL, and NH treatments, respectively. However, high-level N application significantly decreased the relative abundance of α-*proteobacteria.* The relative abundances of *Cyanobacteria* were 16.5%, 16.1%, and 21.3% in the CK, NL, and NH treatments, respectively, indicating that high nitrogen conditions favor C*yanobacteria* growth. More specifically, the relative abundance of *Burkholderiales* was much higher with the CK treatment than that with the N application treatments, whereas the relative abundance of *Desulfovibrionales* was higher with N addition.

Both labeled and unlabeled soil DNA samples were distributed in 16 layers, and the buoyant density ranged from 1.578 to 1.745 g mL^{-1}. A slight shift in the buoyant density was observed for the heavy layers, which indicated that ^{13}C was incorporated into diazotrophic organism genomes. The abundances of the *nifH* gene in the 16 fractions were determined by qPCR, and the peak value was in layer 6 (Fig. 4). The *nifH* gene diversity was analyzed, comparing the lighter fractions (7–9 layers) and the heavy fractions (4–6 layers). All *nifH* sequences were translated to protein sequences and clustered at 95% similarity. As with the whole soil samples, α-, β-, δ-*proteobacteria*, and *Cyanobacteria* were the four most abundant phyla in both the light and heavy fractions. γ-*proteobacteria*, *Actinobacteria*, and *Verrucomicrobia* had low relative abundances, and each occupied less than 1.0% of the total sequences. The relative abundances of some genotypes/OTUs in the light and heavy fractions were shifted

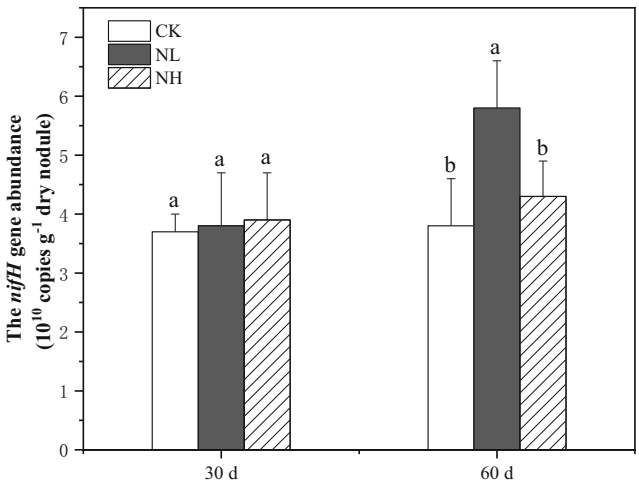

Fig. 1 Abundance of *nifH* gene in nodules under three different N fertilizer application rates. Error bars denote standard error of the mean. Different letters indicate a significant difference at $P < 0.05$

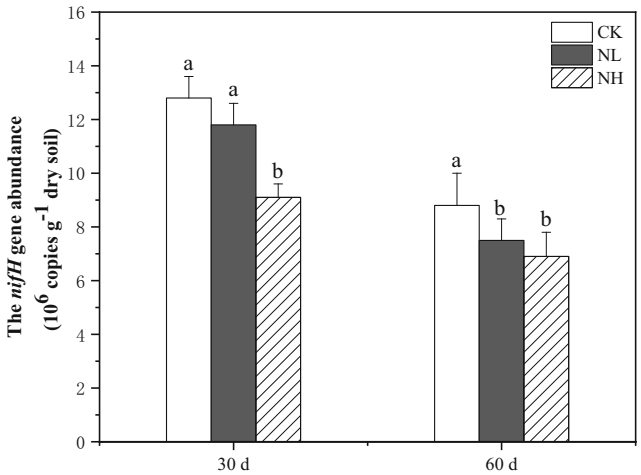

Fig. 2 Abundance of the *nifH* gene in soil under three different N fertilizer application rates. Error bars denote standard error of the mean. Different letters indicate a significant difference at $P < 0.05$

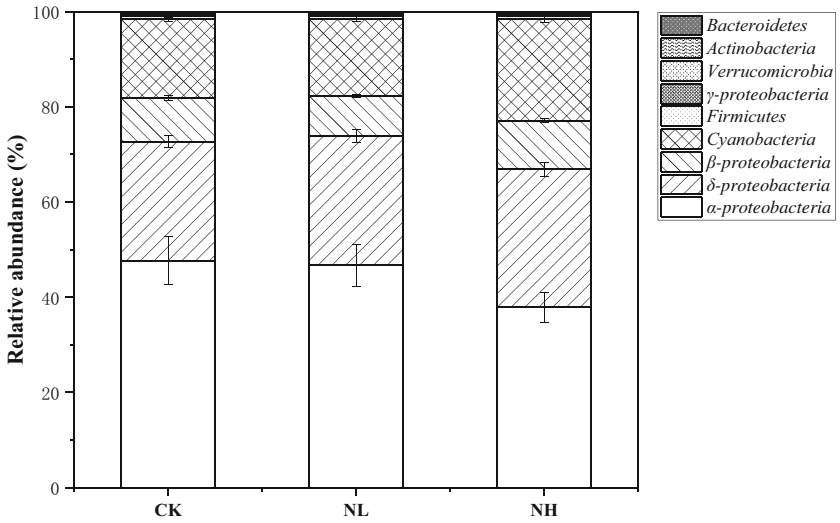

Fig. 3 Soil diazotrophic community compositions at the phylum level under three different N fertilizer application rates

after ^{13}C labeling. The heat map showed that OTU65, which belongs to *α-proteobacteria*, had a higher relative abundance in heavy fractions of the ^{13}C-labeled soils than the unlabeled soils, which indicated that *α-proteobacteria* may prefer rhizodeposition carbon to other organic carbon. However, OTU24 and OTU73, which belong to the *δ-proteobacteria*, had relatively high abundances in the light fractions both in the ^{13}C-labeled and unlabeled samples, which indicated that *δ-proteobacteria* may prefer other soil organic carbon to rhizodeposition carbon (Fig. 5).

4 Discussion

Nitrogen fertilizer application significantly altered symbiotic nitrogen fixation activity and N-fixing bacterial abundance in nodules. The ^{15}N-N_2 labeling experiment showed that the BNF activity in the moderate N application treatment was higher than that in the CK and the high N input treatment (NH). In the legume-rhizobia system, there is a symbiotic relationship of mutual cooperation and restraint between the host plant and N-fixing bacteria. The host legume can impose stimulation or sanction on rhizobia by increasing or decreasing the supply of a required resource for growth (Denison 2000; Kiers et al. 2003). Soil N content is one of the key constraints for this symbiotic system (Thrall et al. 2011). The moderate N application stimulated plant growth and increased the carbon and energy supply for nitrogen-fixing bacteria, which contributed to the BNF activity, because the N-fixing process is very energy-consuming (West et al. 2002; Kiers et al. 2003). When soil nitrogen availability is abundant, the host plant is more likely to use soil N directly rather than investing in symbiotic nitrogen fixation (Simms and Taylor 2002; Perez-Fernandez and Lamont 2016). Consequently, the BNF declines with increasing soil N availability, and high levels of N fertilizer can result in a decrease in nodulation rate and N-fixing efficiency (Caetanoanolles and Gresshoff 1991; Arias et al. 1999; Thomas et al. 2000). The symbiotic BNF activity in the legume-rhizobia system is regulated by the exchange of organic C and N nutrient between the host plant and N-fixing bacteria.

Like the BNF activity, the *nifH* gene abundance in nodules of the NL-treated plants was significantly higher than with the CK and NH treatments. However, no significant effect was found for the *nifH* gene types. The correlation between the N-fixing activities and *nifH* gene abundance suggests that the N-fixing efficiency of rhizobia is regulated by microbial abundance rather than microbial diversity. The increased substrate and energy supply from the legume to the rhizobia promoted the growth and reproduction of N-fixing bacteria. Not only microbial community abundance but also community diversity can be affected by substrate availability (Yao et al. 2011). The similar microbial genotype across the three treatments was consistent with the narrow host specificity of rhizobia (Yang et al. 2010). Some signaling molecules, including surface polysaccharides and secreted proteins, are regarded as the possible mechanisms for host specificity (Fauvart and Michiels 2008). In our study, the growth period of milk vetch was only 60 days, so this cannot be ruled out as a reason for the similar rhizobia genotypes.

The qPCR results suggested that nitrogen fertilization resulted in a decrease in soil *nifH* gene abundance, as previously reported for several agricultural soils (Coelho et al. 2009; Lindsay et al. 2010). Field experiments have also shown this suppressive effect of N fertilization and a significant negative relationship between inorganic N and *nifH* gene abundance (Lindsay et al. 2010; Silva et al. 2013). The *nifH* gene abundance in the N-related treatments was 3 to 4 times lower than that in the control treatment in long-term field experiments (Wang et al. 2017;

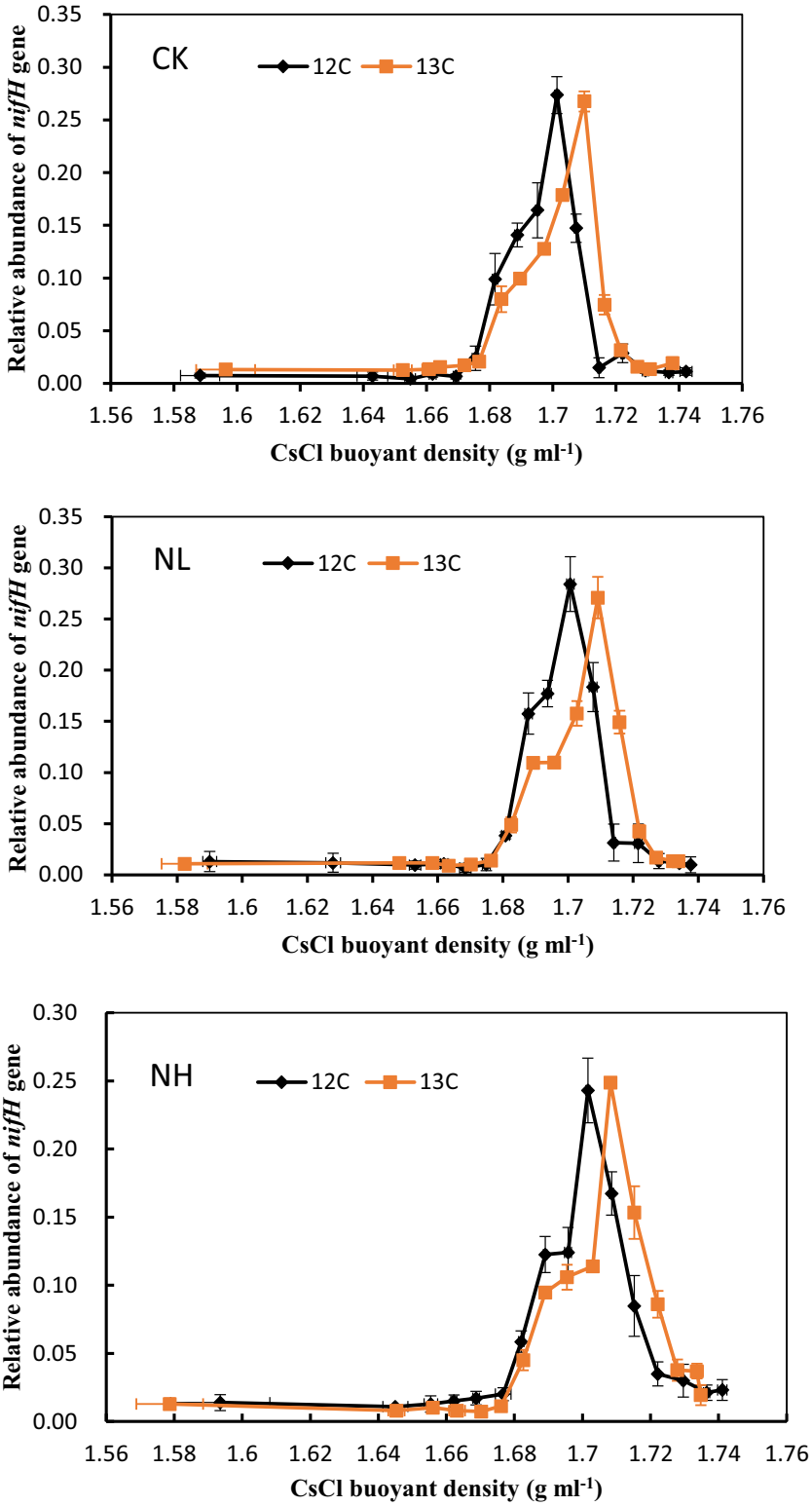

Fig. 4 Quantitative distribution of *nifH* gene copy numbers for the ^{13}C-CO_2 and ^{12}C-CO_2 treatments. Y-axis error bars indicate standard errors of the mean relative gene abundance, and X-axis error bars indicate the standard errors of the mean buoyant density

Wang et al. 2018). Soil acidification and high inorganic N content were considered as two major impact factors (Feng et al. 2018). Asymbiotic N-fixing bacteria usually preferentially use soil available N instead of fixing it (Barron et al. 2009), due to the high energy demand of the latter process. However, some studies have reported the converse results of higher *nifH* gene abundance with N fertilization (Mergel et al. 2001; Liao et al. 2018). This may be due to the indirect effects of the N fertilizer on N-fixing bacteria because soil N availability can regulate the soil microbial

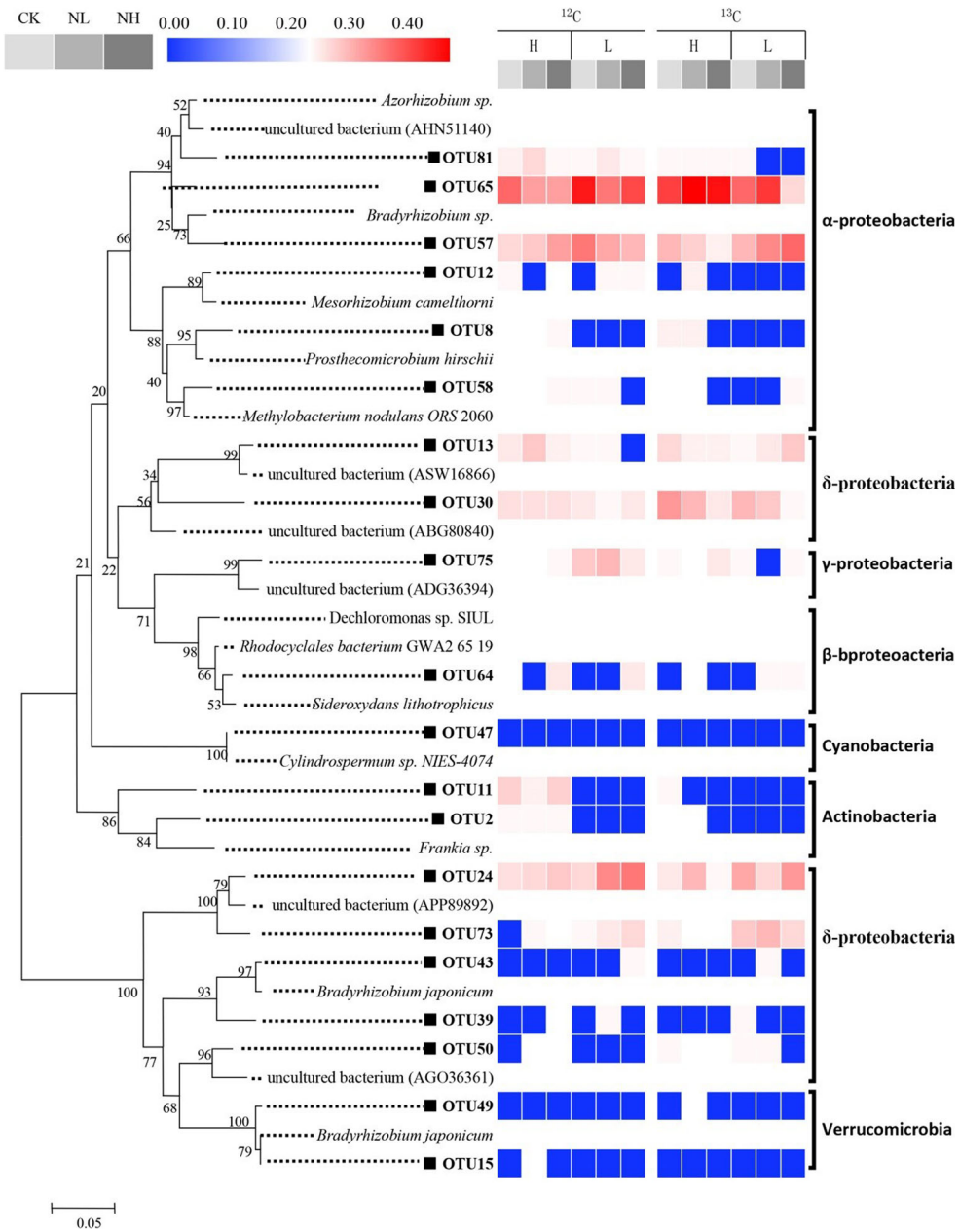

Fig. 5 Phylogenetic tree of soil *nifH* gene sequences retrieved from selected density fractions of the ^{13}C-labeled and ^{12}C-unlabeled treatments (protein sequences, 95% similarity). Their relative abundances are shown as a heat map distribution. The heat map colors represent the relative percentages of the OTU in different samples

community, plant growth, and root exudation patterns (Reis et al. 2000; Meng et al. 2012; Liao et al. 2018).

According to the soil *nifH* clone library, *nifH* genes are mainly distributed into α-, β-, and δ-*proteobacteria*, and *Cyanobacteria.* The results were consistent with previous studies (Ogilvie et al. 2008; Liao et al. 2018), which found a similar microbial distribution. Because different diazotrophic bacteria species have different sensitivities to N levels available in the soil (Harke et al. 2016), N fertilizer input altered the relative abundances of various diazotrophic bacteria. Wang et al. (2018) found that N input significantly increased the relative abundances of α-*proteobacteria* and *Cyanobacteria*, but decreased the relative abundance of δ-*proteobacteria*. The higher *nifH* gene abundance in the *Cyanobacteria* phylum in this study may suggest that photoautotrophic species require more nitrogen than other diazotrophic bacteria.

Desulfovibrio is an anaerobic sulfate-reducing bacterium. The high relative abundance of *Desulfovibrionales* indicated that the long-term flooding of the paddy field provides a suitable habitat for it. Soil N cycling is generally coupled with sulfur and iron cycling, and high N availability can increase the iron- or sulfur-related genes (Bao et al. 2014; Minamisawa et al. 2016). Here, we found that the relative abundance of *Desulfovibrionales* increased with N fertilizer input. This result was consistent with those of Sim et al. (2012), who reported that N limitation inhibited the growth of sulfate-reducing bacteria.

The genus *Burkholderia* represents a versatile group of organisms (Yabuuchi et al. 1992; Salles et al. 2006) and has been exploited for various purposes including N-fixation, plant growth promotion, and biological control (Coenye and Vandamme 2003). In this study, the relative abundance of *Burkholderiales* decreased with N fertilizer application based on the *nifH* gene analysis. The results suggest that the N-fixing function of *Burkholderiales* can be inhibited by high soil N (Kumar et al. 2018). The decrease in the relative abundance of *Burkholderiales* may be due to the direct inhibition by inorganic N of nitrogenase activity or through indirect effects on other microbial groups (Yoch and Whiting 1986).

The rhizosphere is the major soil ecological environment for plant-microorganism interactions (Meena et al. 2017; Li et al. 2018). Root exudates provide nutrients and energy sources to soil microorganisms (Bertin et al. 2003; Landi et al. 2006; Wang et al. 2016). The rhizospheric microbial communities are deeply affected by root exudates. On the other hand, rhizospheric microorganisms are the drivers of nutrient turnover and are beneficial for plant growth (Landi et al. 2006; Meena et al. 2017). Because root exudates are only a minor component of the total organic carbon sources in the soil (Nguyen 2003; Yao et al. 2012), understanding the effect of rhizodeposition on the rhizosphere microbial community should include consideration of other soil carbon pools (Dennis et al. 2010). ^{13}C steady-state labeling is an efficient approach to understand the relative importance of rhizodeposition in determining microbial community diversity (Paterson et al. 2007; Li et al. 2016). Several studies (Wang et al. 2016; Liao et al. 2018) have confirmed that fungi and gram-negative bacteria are the main consumers of root exudates based on phospholipid fatty acid analysis. In this study, the soil microbial community was not only affected by N fertilizer but also root exudates. Continuous labeling results showed that *α-proteobacteria* were the main microbes incorporating native soil organic carbon. The findings suggested that asymbiotic nitrogen-fixing bacterial community diversity is strongly modified by rhizodeposition.

5 Conclusions

The results of this study demonstrated that low-nitrogen application increases the dry weight, nitrogen-fixation, and the abundance of the *nifH* gene in the root nodules of milk vetch (*Astragalus sinicus* L.). Additionally, the asymbiotic nitrogen-fixing bacterial community diversity is strongly influenced by root exudates and N fertilizer input. Further work is required to confirm the findings using field experiments and to understand the subtle changes in N-fixing bacterial species in the legume-rhizobia system.

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