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Long-term N fertilization altered ^{13}C-labeled fungal community composition but not diversity in wheat rhizosphere of Chinese black soil

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ABSTRACT

Plant root exudates are considered as critical substrates mediating the interaction between rhizosphere microorganisms and plants. However, little is known about how microbial community response to root exudates under nitrogen (N) fertilization in agroecosystems. Here, we applied stable isotope probing to divide fungi in wheat rhizosphere soil (under 37-year N fertilization regimes) into two biological compartments: ^{13}C-labeled and ^{12}C-labeled fungal communities. High-throughput pyrosequencing was followed to characterize the two biological compartments. Long-term N fertilization changed rhizosphere soil physiochemical properties, and increased the quantity of plant root exudates. ^{13}C-labeled fungi had lower diversity than ^{12}C-labeled fungi. The fungal communities were predominantly composed of Ascomycota and Basidiomycota in both the ^{13}C-labeled and ^{12}C-labeled DNA, and the abundance of those two phyla were higher in ^{13}C-labeled than that in ^{12}C-labeled DNA. The Nonmetric Multidimensional Scaling (NMDS) showed that ^{13}C-labeled and ^{12}C-labeled fungal communities were distinct from each other. Long-term N fertilization altered fungal communities in ^{13}C-labeled DNA, with lower abundance of putative fungal pathogens, and higher abundance of Glomeromycota. Although N fertilization significantly decreased ^{12}C-labeled fungal diversity, no significant differences were detected in ^{13}C-labeled fungal diversity, indicating the microbial species responses to root exudates and fertilization both influenced fungal diversity. In addition, ^{13}C-labeled fungal communities were less determined by soil chemical properties than ^{12}C-labeled fungal communities, based on partial Mantel test. Overall, our results revealed that long-term N fertilization, which increased the quantity of plant root exudates, altered ^{13}C-labeled fungal community composition, but not changed ^{13}C-labeled fungal diversity in our studied ecosystem.

1. Introduction

In general, plants transfer about 20–50% of photosynthetic carbon (C) to the roots and about half of this is then released into soil (Kuzyakov and Domanski, 2000). Root exudates are important substrates mediating biological interactions including soil microbe–root interactions (Bais et al., 2006). Many studies have suggested that root exudates have potential to lead to relationships between plants and microorganisms in the rhizosphere (Buée et al., 2009). For example, Ai et al. (2015) reported that soil with high levels of available nutrients will reduce the dependence of rhizosphere bacteria on plant-derived C. Another study reported that root exudates could increase the bacterial diversity in an agricultural soil in China (Guo et al., 2017). However, the interaction between the fungal community and plants has seldom been studied. It has been reported that there are large differences in utilization of root exudates among different fungi groups. For example, Ascomycetes and Glomeromycetes prefer simple root exudates, but Basidiomycetes are attracted to more complex C sources (Hannula et al., 2012). This may result in changes in fungal community–plant interaction as the composition or quantity of root exudates change. In addition, some taxa of soil fungi must acquire energy from plant roots and perform a particular function for its host. For example, *Cortinarius* sp., one of the most important symbiont mycorrhizal fungi around roots of most plant species, was the likely pathway for C-transfer among

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plants (Deslippe et al., 2016). What is more, some chemotactically attracted taxa, such as *Fusarium* sp. (a taxa of plant pathogens), are stimulated by root exudates (Wu et al., 2008; Gkarmiri et al., 2017).

Previous studies have shown that the rhizosphere or endophytic microbiome is a subset of the bulk soil community (Bulgarelli et al., 2012; Lundberg et al., 2012; Mendes et al., 2014; Donn et al., 2015; Wang et al., 2018). However, nitrogen (N) fertilization decreases fungal diversity, reduces biomass, and alters community structure of bulk soil (Zhou et al., 2016); and this can change abundance of fungal species in the rhizosphere (Paungfoolonhienne et al., 2015). In addition, nutrient availability can impact the rhizosphere microbiota indirectly by altering root exudation and root morphology (Rengel and Marschner, 2005). However, most studies have focused on free-living microorganisms in bulk and rhizosphere soil not on the community directly using root exudates; thus, little is known about the N fertilization influence on fungal communities that directly assimilate root exudates. Fungi that actively use root exudate could involve nutrient transfer as well as specific interactions mediated by the release of signaling molecules from plant roots and result in enhanced plant productivity (van Elsas and Salles, 2012; van der Voort et al., 2016). Understanding the influence of long-term N fertilization on such fungal groups could be a key step toward enhancing the health and productivity of agricultural crops.

Soil properties, especially soil pH, are dominant factors affecting the composition of rhizosphere microbial communities (Bulgarelli et al., 2012; Wang et al., 2018). However, whether the main drivers of the core rhizosphere fungal community (directly using root exudates) are soil pH, root exudates or other factors remains largely undetermined. Evidence showed that the relative importance of soil factors affecting the fungal community depends on the proximity of the fungal community to the roots. For example, soil physicochemical factors, such as soil pH, strongly influenced the fungal community in bulk soil, but less affected those communities in soils tightly attached to roots (Zhang et al., 2017). This can be attributed to the microbial community response to soil available resources, because soil microbial species can respond rapidly to available resources in the root proximity (Peiffer et al., 2013). Therefore, understanding the factors that favor the assembly of core rhizosphere microbial communities associations with crops could be leveraged to the stability of agricultural ecosystems.

Limited information is available to explain the relationship between plants and the rhizosphere microbiome including the fungal root pathogens, although the impacts of N fertilization on the soil microbial community have been widely reported (Zhou et al., 2015; Wang et al., 2018). As a result, the aims of this study were to (i) identify the fungal community actively assimilating root exudates, (ii) compare the changes in composition of the fungal community actively assimilating root exudates under different N additions and (iii) assess the plant–-fungi interaction in response to long-term N fertilization. To achieve our aims, we cultivated wheat in soils with three different treatments (i.e. no-fertilization control and low- and high-N fertilization) and labeled with external $^{13}CO_2$. We tracked ^{13}C movement from plants to rhizosphere soil through DNA-SIP (stable isotope probing), and then characterised the fungal community by high-throughput sequencing of ^{13}C- and ^{12}C-labeled DNA. Because N fertilization could result in changes in both the soil fungal community and root exudates, our hypotheses were that (1) the fungal communities in ^{13}C- and ^{12}C-labeled DNA would differ from each other, because of the different ability of fungal groups to utilize root exudates, the root selective effect and fungal pathogens, (2) due to N fertilization changing the quality and quantity of root exudates and environmental factors, the composition of fungal community actively assimilating root exudates would differ for different N fertilization and (3) considering fungal community response to soil available resources and N fertilization could both change fungal composition, the N fertilization may have little influence on the dependence of rhizosphere fungi on plant-derived C.

2. Materials and methods

2.1. Soil sampling

Soils for the greenhouse experiment were collected in three different N fertilization treatments from a long-term fertilization site field initiated in 1980 in Harbin, Heilongjiang Province, China (45°40′N, 126°35′E). This region has a temperate continental monsoon climate with an average annual precipitation and temperature of 533 mm and 3.5 °C, respectively. The experimental soil is described as a Phaeozem according to the World Reference Base (FAO) (IUSS Working Group, 2014) and belongs to the pachic Haploborolls subtype of Haploborolls in the Borolls suborder, and is widespread in northeast China (Wen and Liang, 2001). Three different N fertilization treatments with three replicates were (i) no fertilization (CK), (ii) 150 kg N ha^{-1} y^{-1} (N_1) and (iii) 300 kg N ha^{-1} y^{-1} (N_2). The N fertilizer was applied as urea since the beginning of the experiment, and fertilizer treatments were maintained in the same plot location each year. All of the fertilizers were applied once as a basal dressing in the autumn when the crop was harvested. After 37 years of fertilization, soil properties substantially differed under the N fertilization regimes (Table S1) (Zhou et al., 2015; Wang et al., 2018). Soils were sampled in late July 2016, and 10 cores (6 cm in diameter) adjacent to the plants were randomly collected from the plow-layer of soil (0–20 cm) in each replicate plot with a drill, and then mixed uniformly to form one composite sample. To minimize bias, we collected two composite samples in each replicate plot. Thus, a total of 18 composite samples were collected and transported to the laboratory on ice, passed through a 2-mm sieve to remove plant roots and then stored at 4 °C until greenhouse experiments.

2.2. Plant growth under legacies of N fertilization soil and ^{13}C analyses

The plant growth pot and ^{13}C labeling set-up were as described in Ai et al. (2015) with slight changes. Briefly, wheat seeds were grown in rhizoboxes that were separated into three compartments (C1: 3 cm × 12 cm; C2: 4 cm × 12 cm; and C3: 3 cm × 12 cm, respectively) by two layers of a 30-μm nylon mesh holding a 2-mm layer of soil between the nylon membranes (Fig. S1). The rhizoboxes were filled with distinctive soil from the CK, N_1 and N_2 fertilization treatments (no fertilizer was applied during the greenhouse experiments). Wheat seeds were planted into the C2 compartment at a density of eight plants per pot, while C1 and C3 were maintained as root-free bulk soil compartments. Wheat seeds were placed in sterile demineralized water for 24 h at 4 °C. After germination, plants were planted in rhizoboxes and grown in a greenhouse with an average day/night temperature of 23/18 °C and 16-h photoperiod. Soil moisture was maintained at 40–60% of water-holding capacity. The ^{13}C labeling started 40 days later when plants were in an active vegetative growth state. Plants in the rhizobox system were labeled with $^{13}CO_2$ (99 atom % ^{13}C, Shanghai Research Institute of Chemical Industry, Shanghai, China) between 8 a.m. and 4 p.m. (8 h) for seven consecutive days (Lu and Conrad, 2005). The parallel microcosms as control were also constructed under the same conditions, except for using $^{12}CO_2$ rather than $^{13}CO_2$. During the labeling period, the total CO_2 concentration was maintained at 300–400 mg kg^{-1} in the chamber, as elevated CO_2 concentration might affect rhizosphere C flow and associated microbiota (Drigo et al., 2010). At the end of ^{13}C labeling (after 40 days cultivation and 7 days labeling), the rhizosphere and bulk soils were sampled from the rhizoboxes. This labeling period was long enough for the plant to transport photosynthetic ^{13}C to the roots and microorganisms for incorporating ^{13}C into their genomic DNA, but short enough to prevent the label from leaking in significant amounts to the saprotrophic community as dead roots (Sietiö et al., 2018). The ^{13}C-label delivered by wheat roots was determined by isotope ratio mass spectrometry (IsoPrime 100, Cheadle, UK) coupled to an elemental analyzer (Vario MICRO Cube, Elementar, Hanau, Germany) at the Institute of Agricultural Resources and

Regional Planning, CAAS (Beijing, China), based on the method described by Haichar et al. (2008). The $\delta^{13}C$ (‰) value was determined using the equation:

$$\delta^{13}C(‰) = [(R_{sample}/R_{standard}) - 1] \times 1000$$

where $R = {}^{13}C/{}^{12}C$, R_{sample} and $R_{standard}$ was the isotope ratio of the sample and a standard referenced to Pee Dee Belemnite, respectively. The root exudate levels were determined by quantifying ^{13}C enrichment in the wheat rhizosphere soil under $^{13}CO_2$ condition, assuming that ^{13}C increase above natural abundance was derived mainly from root exudates produced by ^{13}C-labeled plants (Guyonnet et al., 2018).

2.3. Soil genomic DNA extraction, gradient fractionation and quantitative real-time PCR

The total soil genomic DNA was extracted from 0.25 g of soil using a Power Soil DNA Isolation Kit (MOBIO Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. Six successive extractions of soil DNA from a replicate soil were combined to minimize the DNA extraction bias (Ding et al., 2016). The DNA was then purified with a DNeasy Tissue kit (Qiagen, Valencia, CA, USA) and checked on 1.0% agarose gel. The extracted DNA was stored at −80 °C until gradient fractionation.

Gradient fractionation was performed as described in Xia et al. (2011). Briefly, 2.0 μg of rhizosphere DNA (mixed well with CsCl stock solution and an initial CsCl BD of 1.725 g ml^{-1}) was fractionated by the CsCl equilibrium density-gradient centrifugation in 5.1-ml Beckman polyallomer ultracentrifuge tubes in a Vti65.2 vertical rotor (Beckman Coulter Inc., Palo Alto, CA, USA). Ultracentrifugation conditions were 177 000 × *g* for 44 h at 20 °C. The centrifuged gradient was fractionated by displacing the gradient medium with sterile water from the top of the ultracentrifuge tube using a NE-1000 single syringe pump (New Era Pump Systems Inc., Farmingdale, NY, USA). Fourteen DNA gradient fractions were generated with equal volume of about 380 μl. The BD of each fraction was determined using an AR200 digital hand-held refractometer (Reichert Inc., Buffalo, NY, USA). Nucleic acids were separated from CsCl solution by precipitation in two volumes of polyethylene glycol (PEG) 6000 solution (30% PEG 6000, 1.6 M NaCl) at 37 °C for 1 h, followed by centrifugation at 13 000 × *g* for 30 min. The fractionated DNA was washed twice with 70% ethanol and dissolved in 30 μl of nuclease-free water.

Quantitative real-time PCR (qPCR) was used to determine abundance of *ITS* gene copy numbers in each fraction according to Zhou et al. (2015) with the *ITS*3 (GCATCGATGAAGAACGCAGC) and *ITS*4 (TCCTCCGCTTATTGATATGC) primers (Gade et al., 2013). The reaction was conducted using an ABI Real-Time 7500 system (Applied Biosystems, Waltham, MA, USA) with the following program: 95 °C for 1 min followed by 40 cycles of 94 °C for 15 s, 55 °C for 34 s and 72 °C for 15 s (Lauber et al., 2013). For measuring *ITS* gene quantity, a clone with the correct insert was ligated into the pMD18-T vector and transformed into *E. coli* JM109 competent cells. The plasmid was obtained from the clone using a Miniprep kit (Qiagen, Germantown, MD, USA). The standard curves were determined by a 10-fold serially diluted standard template, and the R2 of the standard curve was > 0.99. The qPCRs were run in triplicate using DNA extracted from each soil sample. A blank was always run with water as a template instead of soil DNA extract. To minimize bias, the *ITS* gene copies of standard curves and each soil sample were measured at the same time in the same real-time system.

2.4. High-throughput sequencing

The DNA was purified using a DNeasy Tissue kit (Qiagen) and checked on a 1.0% agarose gel. Partial *ITS* amplicons were produced using the primer set *ITS*3–*ITS*4 (Gade et al., 2013), which is the universal DNA barcode marker for the molecular identification of fungi (Zhang et al., 2017). The volumes of PCR reactions were 30 μl with 12.5 μl of 2 × Taq PCR MasterMix, 3 μl of bovine serum albumin, 1 μl of forward (5 μM) and reverse primers (5 μM) and 30 ng of template DNA. The condition of amplification of the *ITS* gene was as follows: 5 min at 95 °C, 35 cycles of 45 s at 95 °C, 50 s at 58 °C and 45 s at 72 °C, followed by a final elongation step of 10 min at 72 °C. Amplicons were gel purified using the PureLink® Quick Gel Extraction Kit (Invitrogen, Carlsbad, CA, USA). After purification, the PCR products were quantified using the quantitative DNA-binding method (QuantiFluor™-ST, Promega, Madison, USA), normalized in equimolar concentrations and then sequenced on the Illumina HiSeq PE250 platform. Sequence data were deposited in the NCBI Sequence Read Archive under the accession number: PRJNA506578.

2.5. ITS sequence analyses

The reads were assigned to samples according to sample-specific barcodes, and then analyzed using QIIME (Version 1.8 http://qiime.org/). The primer sequences and barcodes were cut off, after which, low-quality sequences were filtered under several specific filtering conditions according to Bokulich et al. (2013). The paired-end reads were merged by the FLASH analysis tool based on the read overlaps from the opposite ends of the same DNA fragment (Mago and Salzberg, 2011). Chimeras were detected by comparing the sequences with those in the reference database using the UCHIME algorithm (Edgar et al., 2011) and then removed. The effective tag sequences were analyzed using Uparse software (Edgar, 2013), with a similarity of ≥97% being considered as the same OTU. One sequence was then selected from each group as a representative sequence for annotation base on the UNITE + INSDC fungal *ITS* database (Abarenkov et al., 2010). Non-fungal OTUs and singletons were removed, and all samples were normalized according to the sample with the least number of sequences. All subsequent analyses were performed according to the normalized data.

2.6. Statistical analyses

The indices of Chao1, OTUs and phylogenetic diversity (a biodiversity index that identifies sets of taxa that maximize the accumulation of 'feature diversity' (Forest et al., 2007) were calculated using QIIME (Version 1.8 http://qiime.org/) software to assess fungal alpha diversity. The $\delta^{13}C$ value, relative abundance of phyla or family, and alpha diversity among different N fertilization regimes were analyzed by one-way analysis of variance (ANOVA), and significant differences in group means were compared by Tukey's procedure. An independent *t*-test was used to test the difference of alpha diversity differences, relative abundance of phyla or family between ^{13}C- and ^{12}C-labeled DNA. The one-way ANOVA and independent *t*-test were performed using SPPSS 19.1 statistical software (SPSS, Chicago, IL, USA) (Ahn et al., 2012), and $P < 0.05$ was considered significant. A partial Mantel test (9999 permutations) was used to examine the influence of soil properties on ^{13}C- and ^{12}C-labeled DNA fungal community composition (Horner-Devine et al., 2004). FUNGuild was used to annotate the plant pathogenic fungi (Nguyen et al., 2016), and only the guild confidence ranking assigned to 'probable' and 'highly probable' was accepted. Nonmetric Multidimensional Scaling (NMDS) analyses were used to determine the fungal community β-diversity base on the Bray–Curtis distance between samples. Redundancy analysis (RDA) was performed to visualize the relationship between environmental variables (pH, TN, NO_3^-, NH_4^+, AP, AK, SOM and root exudate value) and fungal communities. The NMDS and RDA were conducted using the CANOCO 5.0.

3. Results

3.1. Long-term N addition increased wheat ^{13}C-rhizodeposits

The ^{13}C-label delivered by wheat roots was only detected in the

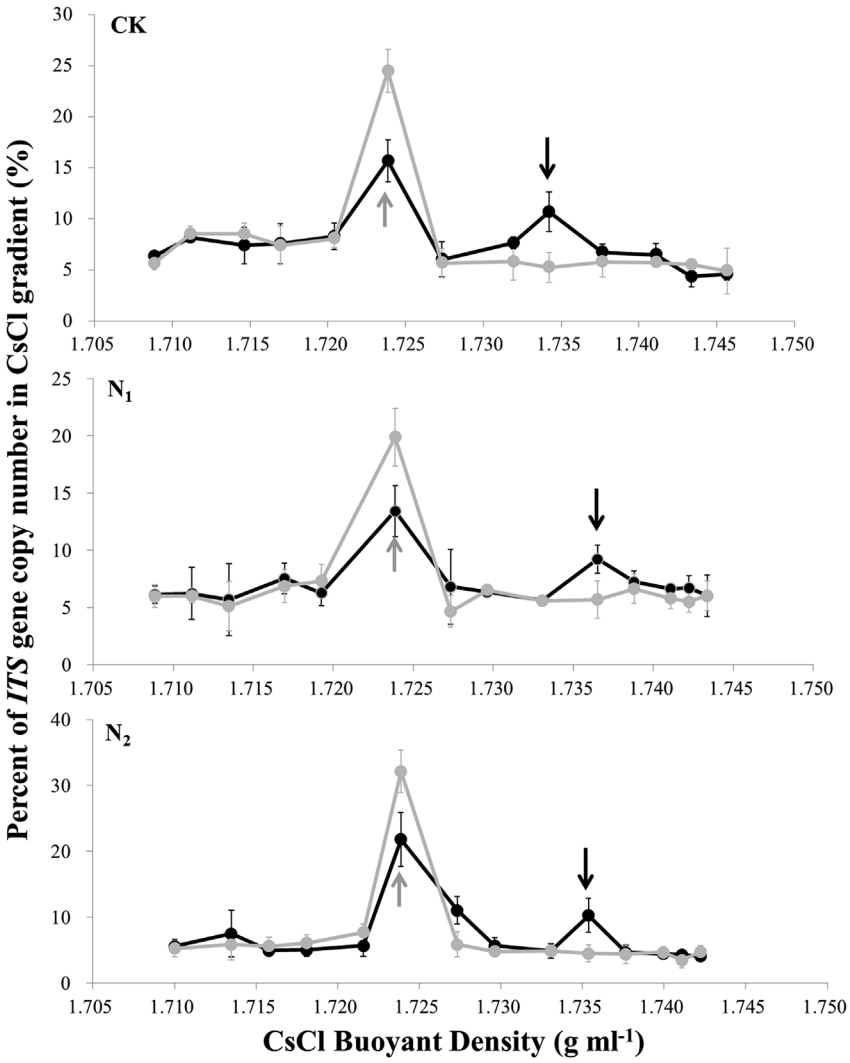

Fig. 1. Quantitative distribution of fungal *ITS* sequence copy numbers across the entire buoyant density gradient of the fractionated DNA from the rhizosphere incubated with either $^{13}CO_2$ (black line) or $^{12}CO_2$ (gray line) after incubation for 7 days under the no-fertilization, N_1 and N_2 treatments, respectively. The normalized data are the ratio of the gene copy number in each DNA gradient fraction to the maximum quantities from each treatment. The values given are the means ± standard deviation of three separate treatments. The black and gray arrows indicate the locations of ^{13}C- and ^{12}C-labeled rhizosphere DNA, respectively, which were used to construct amplicon libraries for high-throughput pyrosequencing.

rhizosphere soil under $^{13}CO_2$ condition ($RS^{13}C$) (Fig. S2). In detail, the soil $\delta^{13}C$ values were significantly higher in $RS^{13}C$ (> 126‰) than in the bulk soil under $^{13}CO_2$ condition ($BS^{13}C$) (−17‰), the rhizosphere under the ambient $^{12}CO_2$ condition ($RS^{12}C$) (−19‰) and bulk soils under ambient $^{12}CO_2$ condition ($BS^{12}C$) (−21‰). However, there was no significant difference in soil $\delta^{13}C$ values among $BS^{13}C$, $RS^{12}C$ and $BS^{12}C$. These results indicated successful incorporation of wheat ^{13}C-rhizodeposits into rhizosphere soil. In $RS^{13}C$, the $\delta^{13}C$ values steadily increased with increasing N-fertilizer inputs from 126‰ to 408‰, and there was a higher $\delta^{13}C$ value for high than low N-fertilizer treatment, indicating that N fertilization could promote plant to secrete more root exudates into rhizosphere soil in our study.

3.2. *^{13}C enrichment and distributions of nucleic acids in centrifugation gradients*

The *ITS* abundance in $^{12}CO_2$ microcosms showed a unique peak in the light fractions around a BD of 1.720–1.725 g ml^{-1} (Fig. 1). In ^{13}C microcosms, two peaks were observed in *ITS* abundance. A clear shift to heavy fractions (1.730–1.740 g ml^{-1}) but not light fractions (1.720–1.725 g ml^{-1}) was shown in fungal communities. Those results implied incorporation of $^{13}CO_2$ into the rhizosphere genomic DNA of fungal communities. Similar BD distributions were obtained for the *ITS* gene from rhizosphere soil under different N-fertilizer regimes (Fig. 1). Based on the above results, DNA with a BD of approximately 1.725 g ml^{-1} in the light fraction and 1.735 g ml^{-1} in the heavy fraction was considered to be ^{12}C-labeled and ^{13}C-labeled DNA, respectively (Fig. 1). The ^{12}C-labeled and ^{13}C-labeled DNA in ^{13}C microcosms under CK, N_1 and N_2 treatments were further sequenced by high-throughput pyrosequencing.

3.3. *^{13}C- and ^{12}C-labeled fungal species diversity*

In total, 1 426 120 high-quality sequences were obtained in the fungal community analysis of 36 soil samples (639 343 from ^{13}C-DNA and 786 777 from ^{12}C-DNA). The Good's coverage values all exceeded 99% with a 97% similarity cutoff, indicating that the current numbers of sequence reads were sufficient to capture the fungal diversity and composition of the ^{13}C- and ^{12}C-labeled fungi. Based on Mothur clustering, the numbers of OTUs in rhizosphere soil were in the range of (242 ± 54)–(501 ± 83), which revealed lower fungal OTUs in the ^{13}C-labeled ((242 ± 54)–(324 ± 36)) than ^{12}C-DNA-labeled fungal community ((329 ± 49)–(501 ± 83)).

Fungal alpha diversity, as evaluated by Chao1, OTUs and phylogenetic diversity is shown in Table 1 and S1. The ^{13}C-labeled alpha fungal diversity was significantly lower than that of the ^{12}C-labeled fungal community ($P < 0.05$). For the ^{12}C-labeled fungal community, the Chao1, OTU and phylogenetic diversity indices were all decreased by N fertilization. In the case of ^{13}C-DNA labeled fungal community, although it was slightly higher in the N_1 treatment (150 kg N ha^{-1} y^{-1}) and lower in the N_2 treatment (300 kg N ha^{-1} y^{-1}) than in the CK, it was not significantly ($P > 0.05$) changed by N addition (Table S2).

Table 1
Comparison of alpha diversity indices between ^{13}C- and ^{12}C-labeled DNA.

	^{13}C-labeled	^{12}C-labeled	*P*
Chao1	341 ± 87	437 ± 68	**0.001**
Phylogenetic diversity	58 ± 13	72 ± 22	**0.015**
OTUs	273 ± 59	407 ± 93	< **0.001**

Values are mean ± standard deviation (n = 6). Bold values indicate significance at $P < 0.05$ (paired-sample *t*-test).

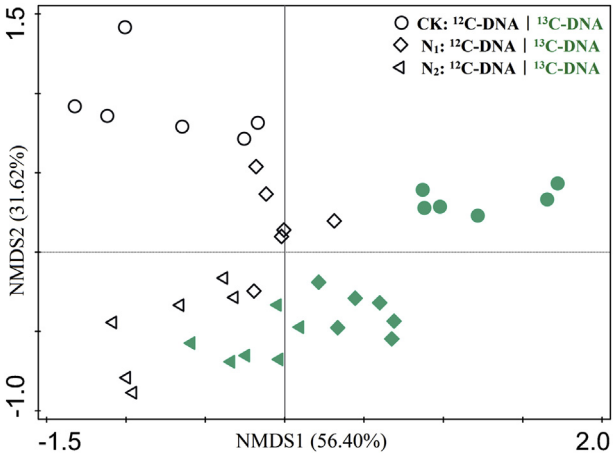

Fig. 2. Beta-diversity analysis based on non-metric multidimensional scaling (NMDS) analyses derived from the Bray–Curtis distances of fungal communities found in ^{12}C- or ^{13}C-labeled DNA in the rhizosphere of wheat for different N additions.

Nonmetric Multidimensional Scaling (NMDS) analyses were used to analyze the variations in fungal communities caused by N addition and root exudation. The first two axes represented 56.40% (NMDS1) and 31.62% (NMDS2) of the variation in fungal communities, respectively (Fig. 2). There were obvious separate groups in each of the samples, indicating that the fungal communities were affected by root exudates and long-term N addition, and the members of fungal community responded to root exudates were different in the different fertilizer treatments. Furthermore, the NMDS1 generally distributed the fungal communities along with the ^{13}C- and ^{12}C-labeled DNA, although differences in fungal communities among samples from plants cultivated under distinct N-addition doses were detected in the NMDS2. These results indicated that the root exudate was responsible for more of the variation in the rhizosphere fungal community than the N fertilization.

3.4. ^{13}C- and ^{12}C-labeled fungal communities

To investigate the impact of nutrients and/or signaling compounds produced by wheat under long-term N addition on the fungal community, two DNA fractions of ^{13}C- and ^{12}C-labeled rhizosphere DNA separated by cesium chloride (CsCl) density-gradient centrifugation were analyzed by high-throughput sequencing. Paired-sample *t*-test identified the relative abundance of phyla Ascomycota and Basidiomycota as significantly more abundant in the ^{13}C- than in ^{12}C-labeled samples by 28.81% and 77.86%, respectively; while Zygomycota and Chytridiomycota were significantly less abundant in ^{13}C- compared to ^{12}C-labeled samples by 65.57% and 144.44%, respectively (Table 2). At the family level, the relative abundance of 10 families significantly ($P < 0.05$) differed between the ^{13}C- and ^{12}C-labeled samples (Table 3). The Pleosporaceae, Trichocomaceae, Davidiellaceae, Malasseziaceae and Typhulaceae were more abundant in the ^{13}C- than ^{12}C-labeled samples. The Chaetomiaceae, Lasiosphaeriaceae, Mortierellaceae, Chytridiaceae and Microascaceae were dominant families which were mostly depleted in ^{13}C- compared with ^{12}C-labeled

Table 2
Comparison on relative abundance of phyla between ^{13}C- and ^{12}C-labeled DNA.

Fungal taxa	^{13}C-labeled abundance (%)	^{12}C-labeled abundance (%)	*P*-value
Ascomycota	38.93 ± 21.56	30.70 ± 15.23	**0.045**
Basidiomycota	4.82 ± 4.11	2.71 ± 1.24	**0.037**
Zygomycota	1.22 ± 0.73	2.02 ± 1.15	**0.005**
Glomeromycota	1.01 ± 0.87	1.29 ± 1.24	0.367
Chytridiomycota	0.54 ± 0.60	1.32 ± 1.69	**0.030**
Fungi pathogen	7.29 ± 5.73	5.59 ± 3.11	0.193

Values are mean ± standard deviation (n = 6). Bold values indicate significance at $P < 0.05$ (paired-sample *t*-test).

Table 3
Comparison on relative abundance of family between ^{13}C- and ^{12}C-labeled DNA.

Fungal taxa	^{13}C-labeled abundance (%)	^{12}C-labeled abundance (%)	*P*-value
Pleosporaceae	11.00 ± 15.20	1.41 ± 0.75	**0.015**
Nectriaceae	5.13 ± 4.41	5.50 ± 3.28	0.740
Chaetomiaceae	2.73 ± 1.56	5.86 ± 1.72	< **0.001**
Lasiosphaeriaceae	2.18 ± 2.99	6.36 ± 7.3	**0.015**
Trichocomaceae	3.67 ± 4.78	1.07 ± 0.62	**0.038**
Incertae_sedis	2.45 ± 1.58	1.97 ± 1.25	0.126
Phaeosphaeriaceae	3.26 ± 11.30	0.33 ± 0.46	0.290
Mortierellaceae	1.22 ± 0.73	2.02 ± 1.15	**0.005**
Davidiellaceae	1.59 ± 2.20	0.31 ± 0.15	**0.026**
Chytridiaceae	0.39 ± 0.57	1.25 ± 1.73	**0.013**
Polyporaceae	1.25 ± 4.49	0.14 ± 0.26	0.314
Bionectriaceae	0.48 ± 0.86	0.70 ± 0.86	0.095
Myxotrichaceae	0.43 ± 0.60	0.51 ± 0.27	0.597
Pyronemataceae	0.49 ± 1.25	0.44 ± 0.37	0.851
Malasseziaceae	0.81 ± 0.96	0.05 ± 0.05	**0.003**
Paraglomeraceae	0.31 ± 0.41	0.52 ± 0.68	0.232
Herpotrichiellaceae	0.30 ± 0.53	0.37 ± 0.21	0.602
Leptosphaeriaceae	0.45 ± 1.13	0.20 ± 0.25	0.306
Microascaceae	0.12 ± 0.13	0.51 ± 0.25	< **0.001**
Coniochaetaceae	0.45 ± 1.11	0.13 ± 0.11	0.243
Typhulaceae	0.47 ± 0.68	0.08 ± 0.07	**0.024**
Stephanosporaceae	0.24 ± 0.38	0.22 ± 0.29	0.724

Values are mean ± standard deviation (n = 6). Bold values indicate significance at $P < 0.05$ (paired-sample *t*-test).

samples. We further annotated the fungi to function guilds using FUNGuild. The relative abundance of ^{13}C-labeled pathogenic fungi was higher than ^{12}C-labeled fungi in the wheat rhizosphere (Table 2).

The ^{13}C-labeled fungal communities under different doses of N fertilization differed from each other (Fig. 3 and S2). At the phylum level, Ascomycota was significantly less abundant in N_2 (17.8% ± 8.2%) and N_1 (39.2% ± 15.8%) treatments than in CK (59.8% ± 14.3%). Phylum Anthopthyta was significantly more abundant in N_2 (31.8% ± 7.2%) and N_1 (24.5% ± 8.7%) treatments than in CK (12.9% ± 8.0%). Glomeromycota was especially less abundant in CK (0.3% ± 0.5%) than in N_1 (1.5% ± 0.9%) and N_2 (1.2% ± 0.8%) treatments. We assessed the effect of N addition on the relative abundance of ^{13}C-labeled fungal pathogens using FUNGuild, which showed they were significantly more abundant in CK (10.9% ± 7.2%) and N_1 (8.2% ± 3.9%) treatments than in N_2 (2.6% ± 1.4%).

At family and genus levels, the relative abundance of Pleosporaceae in the ^{13}C-labeled community was significantly influenced by N fertilization; it was more abundant in CK (24.5% ± 20.8%) than in N_1 (5.5% ± 2.4%) and N_2 (3.0% ± 3.6%) treatments (Fig. 4). Furthermore, a total of 11 OTUs from Pleosporaceae were detected and, of these, nine OTUs were further classified as *Alternaria*, *Edenia*, *Curvularia*, *Bipolaris*, *Drechslera* and *Dendryphia*.

A total of 10 OTUs of the Zygomycota from family Mortierellaceae were detected in the ^{13}C-labeled community, and all these OTUs were identified as genus *Mortierella*. Mortierellaceae was more abundant in

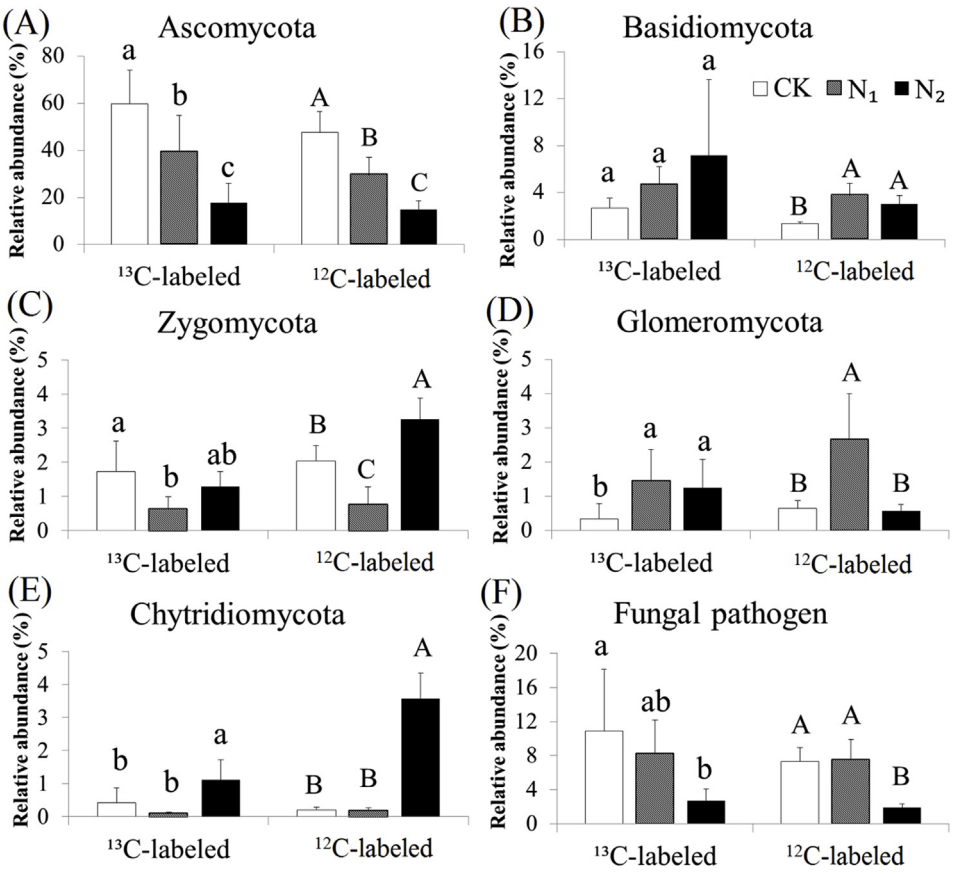

Fig. 3. Relative abundance of the seven most abundant phyla in ^{13}C- and ^{12}C-labeled DNA under different long-term N fertilizer treatments. Error bars indicate the standard deviation of relative abundance between six replicate samples. Same letters above columns denote no significant difference (lowercase and capital letters for ^{13}C- and ^{12}C-labeled DNA, respectively) ($P < 0.05$, Tukey's test).

CK (1.7% ± 0.9%) than in N_1 (0.6% ± 0.3%) and N_2 (1.2% ± 0.5%) treatments (Fig. 4).

Two OTUs were classified in family Davidiellaceae; of these, one OTU was further identified as genus *Cladosporium*. Davidiellaceae was more abundant in CK (3.2% ± 2.1%) than in N_1 (0.2% ± 0.15%) and N_2 (1.4% ± 2.5%) treatments (Fig. 4).

Two OTUs were categorized to family Chytridiaceae, which was especially more abundant in N_2 (1.05% ± 0.57%) than in N_1 (0.07% ± 0.03%) and CK (0.06% ± 0.05%) treatments (Fig. 4).

3.5. Factors correlating with fungal community structure in ^{12}C-labeled and ^{13}C-labeled DNA

Redundancy analysis (RDA) was performed to establish the linkages of soil properties with fungal community structures in the ^{12}C- and ^{13}C-labeled DNA (Fig. 5). In ^{12}C-labeled DNA, the soil properties together explained 63.53% of the variation in fungal community structure, but in ^{13}C-labeled DNA all soil properties only explained 44.53%. This result indicated that the ^{13}C-labeled fungi community was less influenced by soil environment factors caused by N fertilization than the ^{12}C-labeled fungi community. The fungal community of the N_2 treatment in both ^{12}C-labeled and ^{13}C-labeled DNA was positively correlated with soil nutrients [i.e. available phosphorus (AP), total nitrogen (TN), SOM, ammonium (NH_4^+), nitrate (NO_3^-) and root exudate level], with root exudate level the most influential factor in the model.

Partial Mantel tests were used to examine the relationships between fungal community structures and soil variables (Table 4). Fungal community structure was significantly ($P < 0.05$) correlated with the selected soil variables. The root exudate level and soil pH were the two most influential factors correlated with fungal community structures in both ^{12}C-labeled and ^{13}C-labeled DNA, but the correlations were lower for ^{13}C-labeled than in ^{12}C-labeled DNA.

4. Discussion

4.1. The fungal communities in ^{13}C- and ^{12}C-labeled DNA differ from each other

We first hypothesized that the fungal communities in ^{13}C- and ^{12}C-labeled DNA differed from each other, because of the different ability of fungal groups to utilize root exudates, the root selective effect and fungal pathogens. In our study, ^{13}C-labeled fungal community composition significantly differed from that for ^{12}C-labeled (Fig. 2), which partly supported this hypothesis. Similar results were found in numerous previous studies. For example, Hannula et al. (2012) compared ^{13}C- and ^{12}C-labeled fungal communities in the potato rhizosphere and found that phyla Ascomycetes, Glomeromycetes and Basidiomycetes were the main groups receiving C from the plant. Distinct fungal communities in ^{13}C- and ^{12}C-labeled DNA were also found in oilseed rape (Gkarmiri et al., 2017) and *Betula nana* (Deslippe et al., 2016) rhizosphere soil.

Differences in fungal community composition between ^{13}C- and ^{12}C-labeled DNA can be attributed to the response of difference fungal species (k- or r-strategists) to root exudates (Sietiö et al., 2018). Ascomycota and Basidiomycota, which were the dominant groups in wheat rhizosphere soil, were the two groups receiving C from the plant (Table 2), consistent with some previous studies (Hannula et al., 2012; Gkarmiri et al., 2017). This may be due to the DNA-based approach might favor fast-growing fungi, such as saprotrophs and molds, at the expense of slower growing fungi, as this approach allows the detection of only those fungi which use the photosynthetic ^{13}C to repair or duplicate their DNA (Sietiö et al., 2018). Ascomycota are particularly vulnerable to high nutrient levels (Zhou et al., 2016), and some groups in this phylum such as Dothideomycetes have been implicated in assimilating C derived from plants (Freedman et al., 2015). The relative abundance of families Pleosporaceae, Trichocomaceae and

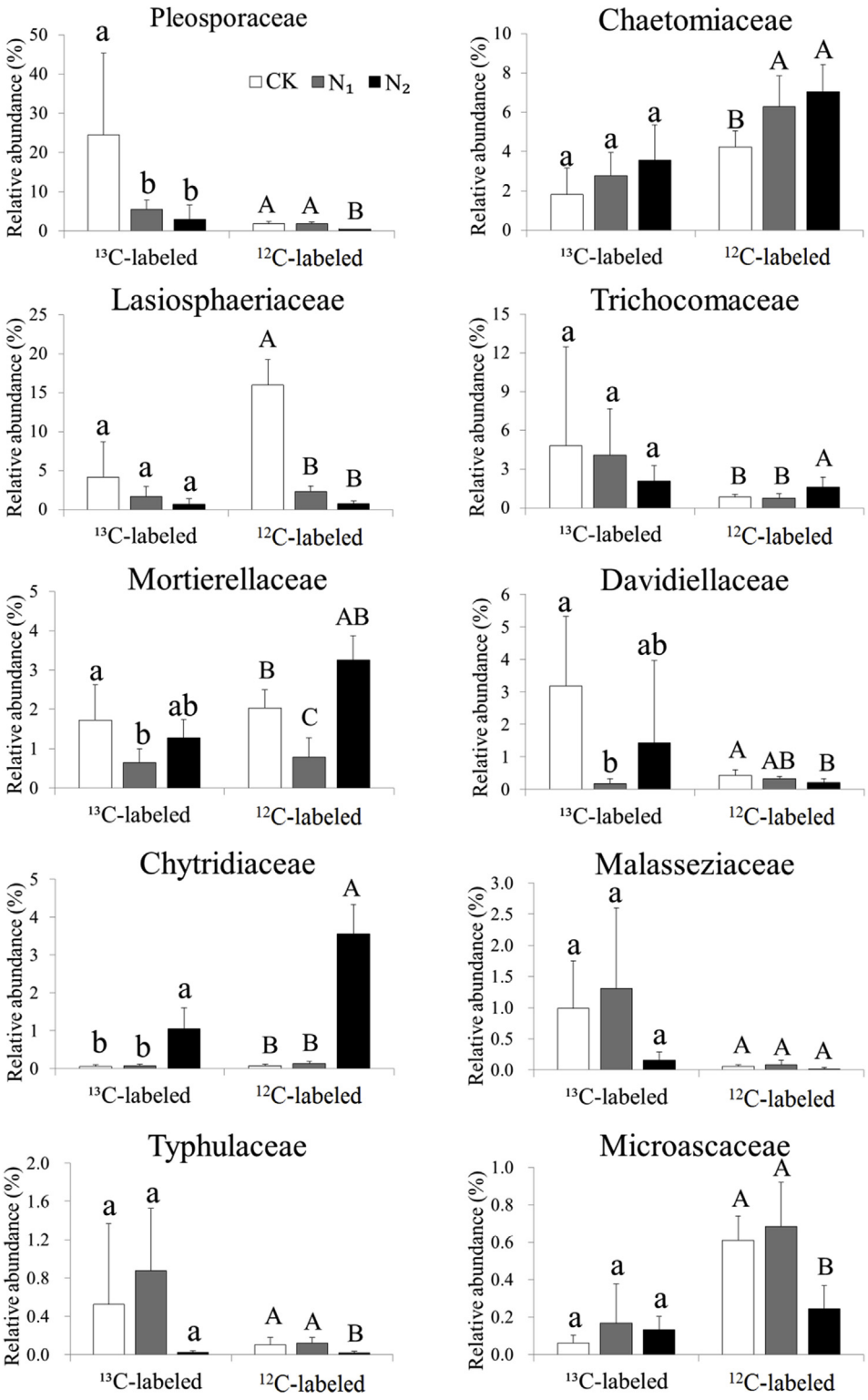

Fig. 4. Relative abundance of the 10 different families (significantly different between ^{13}C- and ^{12}C-labeled DNA) in ^{13}C- and ^{12}C-labeled DNA under different long-term N fertilizer treatments. Error bars indicate the standard deviation of relative abundance between six replicate samples. Same letters above columns denote no significant difference (lowercase and capital letters for ^{13}C- and ^{12}C-labeled DNA, respectively) ($P < 0.05$, Tukey's test).

Davidiellaceae in this phylum was significantly higher in ^{13}C- than in ^{12}C-labeled DNA (Table 3), suggesting that those fungi were active in incorporating recently assimilated carbon from wheat. For example, the family Pleosporaceae was well-known for their plant opportunistic pathogens, and some genera, such as *Dendryphion*, which was efficient in using ^{13}C-labeled root exudates (Gkarmiri et al., 2017), could cause root rot of potato (Lenc et al., 2012). Basidiomycota was another phylum that was efficient in using root exudate in the rhizosphere. This is consistent with previous observation that Basidiomycota can benefit from plant root exudates carbon after labeling and probably better adapted to high nutrient levels, especially more recalcitrant compounds (Hannula et al., 2012). The relative abundance of some families of this

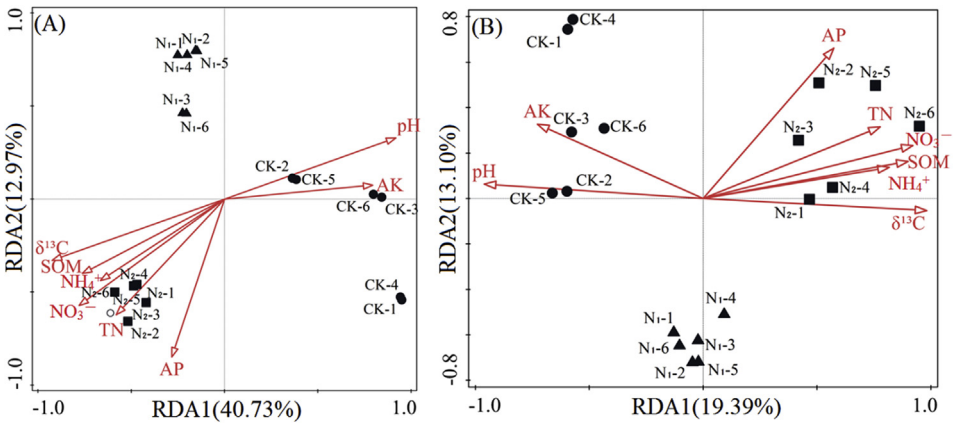

Fig. 5. Redundancy analysis (RDA) of the linkages of soil properties with fungal community structures in the ^{12}C- (A) and ^{13}C-labeled DNA (B). Soil factors indicated in red text include AK (available potassium), AP (available phosphorus), pH, δ^{13}C, NH_4^+ (soil concentration of ammonium), NO_3^- (soil concentration of nitrate), TN (total nitrogen) and SOM (soil organic matter). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 4
The correlations (r) and significance (P) determined by Mantel test between the fungal community composition and environmental variables in13C- and12C-labeled DNA.

Group	^{13}C-labeled	^{12}C-labeled
pH	0.466(0.001)	0.855(0.001)
AP	0.200(0.026)	0.514(0.001)
AK	0.218(0.015)	0.457(0.002)
TN	0.234(0.018)	0.541(0.001)
NH_4^+	0.219(0.026)	0.490(0.001)
NO_3^-	0.334(0.002)	0.771(0.001)
SOM	0.250(0.007)	0.608(0.001)
δ^{13}C (root exudate)	0.485(0.001)	0.871(0.001)

Soil factors indicated include AK (available potassium), AP (available phosphorus), pH, δ^{13}C, NH_4^+ (soil concentration of ammonium), NO_3^- (soil concentration of nitrate), TN (total nitrogen) and SOM (soil organic matter).

phylum (e.g. Malasseziaceae and Typhulaceae) was also higher in ^{13}C- than in ^{12}C-labeled DNA (Table 3). These results indicated that groups generally considered to be r-strategists, and fast-growing fungi whose populations fluctuate opportunistically, were able to respond quickly to soil available resources. Plant-derived C sources may play a role not only in recruiting certain microbes, but also in depleting certain microbes because of competition. In this study, two phyla Zygomycota and Chytridiomycota were mainly detected in ^{12}C-labeled rhizosphere soil, consistent with a study in *Brassica napus* (Gkarmiri et al., 2017). The genus *Mortierella*, which belongs to Zygomycota was more abundance in ^{12}C-labeled than in ^{13}C-labeled DNA, has been found primarily assimilated unlabeled carbon, possibly from older structural pools (Gkarmiri et al., 2017). This result indicated that these two groups were k-strategists and slow to respond to soil available resources in the rhizosphere (Akob and Küsel, 2011).

In our study, the fungal alpha diversity indices (Chao1, OTUs and phylogenetic diversity) were all lower in ^{13}C- than in ^{12}C-labeled DNA (Table 1). This may be due to the selective effect of roots. The root selective effect was exemplified in our study with a total of 407 and 273 fungal taxa in ^{12}C- and ^{13}C-labeled DNA, respectively (Table 1). This difference caused by root exudates and possibly organic acids, sugars or phytohormones may involve molecular signals. For example, Rudrappa et al. (2008) showed that *Arabidopsis* could secrete large amounts of malic acid as molecular signals for attracting *Bacillus subtilis* in the rhizosphere to inhibit pathogens. Previous studies have shown that plant roots select for specific microorganisms to colonize the roots to help plant improve nutrient acquisition and combat pathogenic taxa (Berendsen et al., 2012; Dennis et al., 2010). In our study, ^{13}C-labeled DNA had a greater relative abundance of Trichocomaceae (Table 3), which has potential to use of root exudates such as organic forms of P (Daynes et al., 2008), and therefore could be involved in providing mineral nutrients for plants. However, other studies have shown that root exudates could select microorganisms including soil-borne pathogens. For example, Wu et al. (2008) reported that roots exuding phenolic compounds (e.g. vanillic acids) benefited the growth and development of fungal pathogens. In our study, FUNGuild analysis also showed that the relative abundance of pathotrophic fungi was higher in ^{13}C- and ^{12}C-labeled DNA (Table 2). In addition, the family Pleosporaceae was significantly more abundant in ^{13}C- than in ^{12}C-labeled DNA. Some taxa of this family, such as *Cochliobolus sativus*, can colonize roots and cause root rot (Manamgoda et al., 2011; Lenc et al., 2012).

4.2. The long-term N fertilization changed fungal community in ^{13}C-labeled DNA

In our study, the composition of the fungal community actively assimilating root exudates differed for different N fertilization. This may be because N fertilization changed the quality and quantity of root exudates and environmental factors, because such changes both have potential to change the microbial C use efficiency (Fisk and Fahey, 2001). The ^{13}C-labeled fungal community composition in our study revealed significant differences among different dosages of N fertilizer (Fig. 2). We found that the amount of ^{13}C in rhizosphere soil was significantly increased by N addition after 7 days of labeling. This is in accordance with earlier findings of increasing N rate enhancing the root exudate quantity (Zhu et al., 2016). This increased root exudate quantity may have a significant role in shaping the rhizosphere soil fungal community. Although the relative abundance of Ascomycota was higher in ^{13}C- than in ^{12}C-labeled DNA, these fungal taxa including Pleosporaceae, Lasiosphaeriaceae, Davidiellaceae and Microascaceae decreased with the increasing dosage of N (Fig. 3A). Wang et al. (2015) reported that N was harmful to members of this phylum in a biological soil crust, as N fertilization decreased the soil pH and lower pH was stressful for most taxa of this phylum. For example, the relative abundance of Microascaceae significantly decreased as the soil pH value fell (Zhou et al., 2016). In addition, some studies have showed that fungal abundance, especial Ascomycota, was positively correlated with soil C:N (Zhou et al., 2016), indicating that Ascomycota in N-fertilized soils need more C that is taken from the root exudates for growing. The changes in their abundance due to N addition may dramatically affect the rhizosphere nutrient cycle, as Ascomycota are the key decomposers in agricultural soils (Zhou et al., 2016). In contrast to Ascomycota, the other group using ^{13}C-labeled rhizodeposits, the Basidiomycota, was increased by N addition (Fig. 3B). Some Basidiomycota species, such as *Russula ochroleuca* and *Thelephora terrestris*, responded positively to increasing N or environments with high levels of soil nutrients (Cox et al., 2010). This result also confirmed previous observations that Basidiomycota are better adapted to high nutrient levels (Hannula et al., 2012). Thus, it was shown that N fertilization was also an important factor that changed ^{13}C labeled fungal communities in wheat rhizosphere soil.

It has been demonstrated that long-term N fertilization promotes the fungal community with known pathogenic traits (Zhou et al., 2016). Our results suggest that long-term N fertilization not only decreased the ^{12}C-labeled fungal pathogens, but also the ^{13}C-labeled fungal pathogens; however, these patterns need to be investigated further as it is difficult to infer whether the pathogens actually cause disease in specific plants in a specific ecosystem (Nguyen et al., 2016; Voort et al., 2016). We also found that relative abundance of ^{13}C-labeled Glomeromycota was enhanced by long-term N addition. This could be highly associated with Glomeromycota receiving their C supply from their host plants and compensating the plant through enhanced nutrient acquisition (Karasawa et al., 2012), and Williams et al. (2017) also found that as N fertilization increased, C allocation to Glomeromycota increased. This result indicated that N fertilization increased the prevalence of symbionts in this soil.

4.3. The N fertilization has little influence on the dependence of rhizosphere fungi on plant-derived C

Although N addition significantly decreased the ^{12}C-labeled DNA fungal alpha diversity, it did not significantly change for ^{13}C-labeled (Table S2). This is consistent with a recent finding that fungal community composition in rhizosphere soil tightly attached to roots was weakly determined by environmental factors (Zhang et al., 2017). In our study, we also found that the soil environmental factors changed by long-term N addition (e.g. pH, TN and NO_3^-) were more correlated with ^{12}C- than ^{13}C-labeled DNA fungal composition (Table 4). This might be attributed to the fungal taxa, which rapidly used fresh root exudates, were considered to be r-strategists and more prone to be affected by labile carbon sources than soil environmental factors (Fierer et al., 2007; Peiffer et al., 2013; Zhang et al., 2017). These results indicated that the core rhizosphere fungi (^{13}C-labeled fungi) were mainly determined by root exudate, and weakly by environmental factors. This may be due to the plant could regulate their rhizosphere fungi (especially the fungi that directly assimilated the root exudates) by changing the quantity or quality of the root exudates (Broeckling et al., 2008), as the environmental factors changed. Some other studies also found similar results. For example, Tedersoo et al. (2013) found that host plants were the strongest predictor of root-associated fungal species richness and community composition. In another study, analysis of the community of different plant root-associated fungi suggested that different plants provided different ecological niches in their roots that some root-associated fungi preferred, and the fungi most abundant in the total root community samples were also utilizing photosynthetic ^{13}C the most intensively (Sietiö et al., 2018). These results suggested that rhizosphere fungi that were dependent on plant-derived C were not altered, but ^{12}C-labeled fungi were significantly decreased by applying N. These findings implied that long-term N fertilization had little influence on the dependence of rhizosphere fungi on plant-derived C and did not significantly change the complex of core fungi in wheat rhizosphere soil in our study, results in a relative stable association between wheat and its rhizosphere fungal communities. This is a striking result, because previous studies have shown that the diversity of microbial community in agricultural ecosystems is critical to maintaining soil quality, productivity and ecological balance in cropland areas (Li et al., 2014; Zhou et al., 2016). This stability of core rhizosphere fungal diversity under different N fertilization regimes may result in a relative stable agroecosystem and further lead to sustainable crop production.

In conclusion, our results suggest that the ^{13}C-labeled root exudates are dominantly assimilated by Pleosporaceae, Trichocamaceae and Davidiellaceae the three dominant families in wheat rhizosphere soil. The microbial community responsible on plant fresh root exudates (^{13}C-labeled fungal communities) is different to that mainly responsible on SOM (^{12}C-labeled fungal communities), and long-term N fertilization change the composition but not diversity of the microbial community responsible on plant fresh root exudates. Our results also provide evidence that the complex of ^{13}C-labeled fungi in wheat rhizosphere soil was not significantly changed by long-term N fertilization. The increases in the quantity of plant root exudates probably maintain the association between crop and fungi stable under long-term N fertilization in our studied ecosystem. These findings emphasize the need to study the mechanism of how plant root exudates – both quantity and quality – affect the fungal assembly in the rhizosphere.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2019.04.009.

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