Biol Fertil Soils (2017) 53:37–48
DOI 10.1007/s00374-016-1155-z

ORIGINAL PAPER

Rice rhizodeposition and its utilization by microbial groups depends on N fertilization

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Received: 18 May 2016 / Revised: 9 October 2016 / Accepted: 14 October 2016 / Published online: 24 October 2016

Abstract Rhizodeposits have received considerable attention, as they play an important role in the regulation of soil carbon (C) sequestration and global C cycling and represent an important C and energy source for soil microorganisms. However, the utilization of rhizodeposits by microbial groups, their role in the turnover of soil organic matter (SOM) pools in rice paddies, and the effects of nitrogen (N) fertilization on rhizodeposition are nearly unknown. Rice (*Oryza sativa* L.) plants were grown in soil at five N fertilization rates (0, 10, 20, 40, or 60 mg N kg^{-1} soil) and continuously labeled in a $^{13}CO_2$ atmosphere for 18 days during tillering. The utilization of root-derived C by microbial groups was assessed by ^{13}C incorporation into phospholipid fatty acids. Rice shoot and root biomass strongly increased with N fertilization. Rhizodeposition increased with N fertilization, whereas the total ^{13}C incorporation into microorganisms, as indicated by the percentage of ^{13}C recovered in microbial biomass, decreased. The contribution of root-derived ^{13}C to SOM formation increased with root biomass. The ratio of ^{13}C in soil pools (SOM and microbial biomass) to ^{13}C in roots decreased with N fertilization showing less incorporation and faster turnover with N. The ^{13}C incorporation into fungi (18:2ω6,9c and 18:1ω9c), arbuscular mycorrhizal fungi (16:1ω5c), and actinomycetes (10Me 16:0 and 10Me 18:0) increased with N fertilization, whereas the ^{13}C incorporation into gram-positive (i14:0, i15:0, a15:0, i16:0, i17:0, and a17:0) and gram-negative (16:1ω7c, 18:1ω7c, cy17:0, and cy19:0) bacteria decreased with N fertilization. Thus, the uptake and microbial processing of root-derived C was affected by N availability in soil. Compared with the unfertilized soil, the contribution of rhizodeposits to SOM and microorganisms increased at low to intermediate N fertilization rates but decreased at the maximum N input. We conclude that belowground C allocation and rhizodeposition by rice, microbial utilization of rhizodeposited C, and its stabilization within SOM pools are strongly affected by N availability: N fertilization adequate to the plant demand increases C incorporation in all these polls, but excessive N fertilization has negative effects not only on environmental pollution but also on C sequestration in soil.

Keywords Nutrient cycling · N fertilization · Stable isotope labeling · Root exudation · Microbial community structure

Electronic supplementary material The online version of this article (doi:10.1007/s00374-016-1155-z) contains supplementary material, which is available to authorized users.

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Introduction

Plants are the primary source of carbon (C) for soil microorganisms and soil organic matter (SOM) (Weintraub et al. 2007; Ge et al. 2015). C is delivered to soil as aboveground plant residues (litter) and belowground plant residues (roots and rhizodeposits). Rhizodeposition is a composite of C lost

via different pathways from the roots and includes (i) organic volatiles; (ii) C transferred to root symbionts (e.g., mycorrhiza); (iii) water-soluble, low molecular weight compounds (e.g., organic acids and cell lysates); (iv) insoluble compounds with high molecular weight (e.g., mucilage); (v) root hairs and border cells sloughed during root elongation; and (vi) whole roots lost by apoptotic and nonapoptotic processes (Kuzyakov and Domanski 2000; Jones et al. 2009).

Rhizodeposited C accounts for, on average, 11 % of net photo-assimilates (Jones et al. 2009). A significant proportion of rhizodeposits, especially root exudates, constitutes a readily available source of C for soil microorganisms and plays a key role in soil C, nutrient cycling, and the replenishment of SOM, strongly influencing C sequestration (Kuzyakov et al. 2003). Increased C sequestration and stabilization are important in paddy soils, because intensively managed rice-based cropping systems contribute 0.52 Gt of CO_2 equivalents (also as CH_4 and partly as N_2O) per year to the atmosphere (FAOSTAT 2013). A better understanding of the emissions of nitrogen (N_2), nitrous oxide (N_2O), ammonia (NH_3), nitric oxide (NO), carbon dioxide (CO_2), and methane (CH_4) from anaerobic paddy soils may aid the development of efficient strategies for mitigating greenhouse gas emissions. Therefore, it is important to investigate the role of rhizosphere C flows in paddy-field C cycles and its ecological implications for soils, as well as the underlying mechanisms of root-derived C utilization by microbial groups and the fate of C within SOM pools of various stability.

Rice (*Oryza sativa* L.) is the basic food for nearly half of the human population worldwide, and N fertilization is the major management practice in modern agriculture for increasing rice yields (Cassman et al. 1998). However, many South-East Asian farmers use excessive amounts of N fertilizers, a practice that has resulted in the N contamination of groundwater due to nitrate leaching, surface runoff, ecosystem eutrophication, ammonia (NH_3) volatilization, and nitrous oxide (N_2O) emissions (Leip et al. 2014). In China, the consumption of chemical N fertilizers in 2007 reached 32.6 million tons, which was 191 % higher than that in 1980 and accounted for 30 % of the global agricultural N consumption (Guo et al. 2010; National Bureau of Statistics of China 1949–2010). Agricultural activities are the main source of N pollution (Vitousek et al. 2009) that leads to serious eutrophication in water systems (Wang et al. 2004; Qiao et al. 2012). Therefore, the environmental impact of N fertilizers in paddy soils has attracted considerable attention in recent years.

N fertilization affects the above/belowground distribution of plant C and the fate of plant-assimilated C in soils (Kuzyakov et al. 2002; Ge et al. 2015). Johansson (1992) showed that in barley, the incorporation of photosynthesis-derived C into SOM decreases with the increasing N fertilization. In paddy ecosystems, N fertilization stimulates rice C assimilation, root and shoot biomass (Ge et al. 2015), grain yield, and soil C sequestration (Liu et al. 2007), suggesting that the distribution and turnover of photosynthetic C and rhizodeposition in the soil are affected by N fertilization.

N fertilization has direct and indirect effects on microbial activity, community composition, and community functioning (Ramirez et al. 2012). Changes in N availability are indirectly associated with shifts in microbial physiology and microbial community composition (Ramirez et al. 2012) because N fertilization alters the amount and composition of C inputs into the soil, which, in turn, affect microbial activity (Meier and Bowman 2008).

Phospholipid fatty acids (PLFAs) have been used as biomarkers for microbial groups (Frostegård et al. 1993) and reflect the general composition of soil microbial communities (Lu et al. 2007). ^{13}C incorporation into individual PLFAs allows assessment of the function and composition of microbial communities. The measurement of ^{13}C-PLFAs is an efficient approach to trace the fate of root-derived C and to evaluate the importance of rhizodeposition for the functions and activity of microbial groups (Paterson et al. 2007; Yao et al. 2015).

The incorporation of C derived from rice roots into microbial groups has been previously studied (Lu et al. 2007; Yao et al. 2012; Tian et al. 2013a), with a focus on rice growth stages (Lu et al. 2007) or soil water conditions (Yao et al. 2012; Tian et al. 2013a). Although pulse ^{13}C labeling is commonly used to study the effects of N fertilization on the incorporation of root C into microbial groups (Lu et al. 2002a; Tian et al. 2013a), continuous ^{13}C labeling has not been used. The ^{13}C enrichment from pulse labeling typically increases to a maximum, and then falls back toward the prelabeling natural ^{13}C abundance (Lu et al. 2002a; Pausch et al. 2013; Ge et al. 2015). Further, pulse labeling does not label all plant C pools to the same degree (it is inhomogeneous; De Visser et al. 1997). In contrast, continuous labeling provides an integrated picture of root C distribution and utilization. The ^{13}C enrichment of soil C pools, including PLFAs, gradually increases from natural ^{13}C levels toward a new steady state (Yao et al. 2012). Continuous $^{13}CO_2$ labeling can be used to determine the relative turnover of each C pool in soil (Paterson et al. 2011).

We performed $^{13}CO_2$ continuous labeling (18 days) of rice plants grown in a paddy soil under a range of N fertilization rates during the vegetative growth period (including the entire tillering stage). Our objectives were to (1) quantify C input into soil by rice rhizodeposition depending on N fertilization rates, (2) assess the microbial community composition in the rice rhizosphere and identify microbial groups that preferentially capture root C depending on N fertilization, and (3) investigate the incorporation of rhizodeposited C into SOM pools. We hypothesized that (1) N fertilization increases total plant biomass and, consequently, belowground C allocation; (2) the preferential utilization of rice rhizodeposits (^{13}C labeled) by microbial groups depends on the N fertilization

level; and (3) the proportion of rhizodeposited C incorporated into fungi increases with N availability, relative to that incorporated into bacteria.

Materials and methods

Soil

Hydragric Anthrosol (Eutric, Siltic, Gleyic; Gong et al. 2007) developed from a granite parent material after very long, intensive subtropical weathering was collected from a rice field (113° 19′ 52″ E, 28° 33′ 04″ N, 80 m above the sea level) located at the Changsha Research Station for Agricultural and Environmental Monitoring, Hunan Province, China. The study site has a mean annual temperature of 17.5 °C, mean annual rainfall of 1300 mm, sunshine of 1663 h per year, and a frost-free period of 274 days per year (Shen et al. 2014). Soil samples were collected from the Ap horizon (0–20 cm; 15 % water content) and sieved (<4 mm) to remove coarse plant residues. The soil texture was 7.5 % clay, 68.4 % silt, and 24.1 % sand. The soil bulk density was 1.31 g cm^{-3} and soil contained 18.1 g kg^{-1} organic C, 1.8 g kg^{-1} total N, and 0.4 g kg^{-1} total P, and had a pH of 5.6 (1:2.5, soil to water ratio).

Experimental layout

Rice plants were grown at five N fertilization rates: N-free (N0), 10 mg N kg^{-1} soil (N10), 20 mg N kg^{-1} soil (N20), 40 mg N kg^{-1} soil (N40), and 60 mg N kg^{-1} soil (N60). These N fertilization rates are equivalent to 0, 22.5, 45, 90, and 135 kg N ha^{-1}, respectively. N fertilizer (as $(NH_4)_2SO_4$) was mixed with soil (2.0 kg on an oven-dry basis), and the mixture was placed in a plastic pot (17.2 cm diameter and 16.7 cm height). Six pots were prepared for each fertilization regime. All pots were sprayed with NaH_2PO_4 (20 mg P kg^{-1} soil) and KCl (80 mg K kg^{-1} soil) using a 10-ml pipette (Eppendorf Reference® 2, Germany) and submerged in deionized water. The water level was maintained at 3–5 cm above the soil surface throughout the rice-growing season. Three pots were used for continuous ^{13}C labeling (see below) and three as unlabeled controls. The unlabeled controls were used as references for determining natural ^{13}C abundance and to calculate the ^{13}C-atom-%-excess. Unlabeled controls were placed outside, 10–15 m away from labeled chambers. The soil properties and the $\delta^{13}C$ signature of soil and plants did not change in the control soil over the course of the experiment (data not shown).

Rice growth conditions and $^{13}CO_2$ continuous labeling

We used the experimental protocol described by Ge et al. (2012, 2015), with some modifications. Briefly, three 30-day-old rice seedlings (*O. sativa* L. 'Zhongzao 39') with an average dry matter weight of 0.10 g were transplanted to each pot on April 28, 2013. Deionized water was used for irrigation through a nylon tube (inner diameter, 5 mm) connected to each pot, and a 3–5-cm water layer was maintained above the soil surface throughout the growing season. Weeds were removed manually.

Labeled rice plants were subjected to $^{13}CO_2$ continuous labeling for 18 days (May 14–31, 2013) during the vegetative growth period, including the entire tillering stage. During the labeling period, plants were transferred to an automatically controlled gas-tight growth chamber system (area 110 cm × 250 cm, height 180 cm). Growth chambers were placed in a rice field so that the environmental conditions would be identical for the labeled plants and unlabeled controls. The surface of each plastic pot (17.2 cm diameter and 16.7 cm height) was covered with a black plastic sheet to prevent algal photosynthesis in the floodwater, ensuring that only the rice shoots were exposed to $^{13}CO_2$. No specific precautions, such as leak checks, were taken to ensure that shoots and roots were isolated, because the downward diffusion of $^{13}CO_2$ into acid floodwater (pH < 7.0) is considered negligible (Lu et al. 2002a).

The CO_2 concentration in the growth chamber was measured using an infrared analyzer (Shsen-QZD; Qingdao, China) and maintained at 360–380 μl l^{-1}. When CO_2 concentration in the chamber was less than 360 μl l^{-1}, CO_2 that was generated by the reaction of $NaH^{13}CO_3$ (99 atom% ^{13}C; Cambridge Isotope Laboratories, Tewksbury, MA, USA) with H_2SO_4 (0.5 M) was introduced into the chamber. When CO_2 concentration in the chamber was higher than 380 μl l^{-1}, a switch diverted the gas flow to pass through CO_2 traps (NaOH solution). One temperature and humidity sensor (SNT-96S; Qingdao, China) was installed inside the chamber and another outside the chamber. An air-conditioning system was used to control the temperature inside the chamber within 1 °C of the ambient temperature in the rice field. Two fans continuously circulated the air in the growth chamber.

Sampling and harvesting

After 18 days of continuous ^{13}C labeling, the rice plants were harvested, and soil samples were collected from each pot. At harvest, the shoots were cut off at the stem base, allowing the separation of the root, shoot, and soil components. The separation of roots and soil was performed in two steps as described by Lu et al. (2002a) with minor modifications. First, the roots were separated from the soil by washing through a 2-mm sieve with 1.0 l distilled water. Root debris >2 mm size

were collected and combined with the root samples. Then, soil slurries, consisting of soil and wash water, were mixed well and centrifuged at 13,000×*g* for 20 min. Low-density fine roots were removed together with the supernatants. To assess the effectiveness of this method of removing most of the fine living roots from the soil, ten 15-g soil samples (two for each labeling event) were examined under a dissecting microscope. Very few fine roots were found in two of the samples and no roots were found in the other eight samples. We compared the $\delta^{13}C$ values of the two soil samples that contained fine roots before and after the removal of fine roots using the fumigation-extraction method (Wu et al. 1990), but we did not identify any differences. Therefore, we considered that sieving with subsequent centrifugation effectively removed most of the fine living roots and was comparable to visual separation under a microscope. However, some very fine root materials might have remained in the soil samples, leading to a minor overestimation of plant C input. Shoot and root compartments were dried in an oven at 70 °C for 72 h, weighed, and pulverized. Fresh soil samples were stored at 4 °C and −70 °C for microbial biomass C and PLFA analysis, respectively. The remaining portion of each soil sample was mixed thoroughly, air-dried, ball-milled, and stored at 4 °C until analysis.

Measurement of C_{tot} and $\delta^{13}C$

The total C content of soils, shoots, and roots was analyzed using an automated C/N analyzer (Vario MAX; Elementar Analysensysteme, Hanau, Germany). Soil microbial biomass C (MBC) was measured using the fumigation-extraction method (Wu et al. 1990). Briefly, samples of wet soil (equivalent to about 40 g of oven-dried soil) were divided into two equal subsamples: fumigated and nonfumigated. Fumigated soil samples of wet soil (equivalent to about 20 g of oven-dried soil) were amended with 2 ml alcohol-free $CHCl_3$ from the surface of the slurries. Samples were then additionally fumigated by exposing the soil to alcohol-free $CHCl_3$ vapor for 24 h in a vacuum desiccator (Wu et al. 1990). Residual $CHCl_3$ was removed by vacuuming 5–10 times, for about 5 min each time. Then, the wet fumigated and nonfumigated soils were extracted with 80 ml of 0.05 M K_2SO_4 by shaking at 250 rpm for 30 min. The resulting suspensions were filtered through Whatman No. 42 filter papers. Organic C in the K_2SO_4 extracts was analyzed by an automated procedure using a total carbon analyzer (Phoenix 8000, USA). For isotope measurement, the extracts were freeze-dried and the dried salts were ground to a fine powder using a mortar and pestle. Root residues were carefully removed using tweezers before the fumigation and extraction procedures.

The stable C isotope ratios ($^{13}C/^{12}C$) of plants, soils, and ground salt extracts were measured using an isotope ratio mass spectrometer (MAT253) coupled with an elemental analyzer (FLASH 2000; Thermo-Fisher Scientific, Waltham, MA, USA). The natural ^{13}C abundance was expressed as parts per thousand relative to an international standard, Pee Dee Belemnite (PDB), using delta units (δ‰).

PLFA extraction and analysis

PLFAs were extracted, fractionated, and purified as described by Ge et al. (2013) with minor modifications. Briefly, approximately 2 g freeze-dried soil was extracted twice using a 22.8-ml single-phase mixture of chloroform/methanol/citrate buffer (1:2:0.8 *v*/*v*/*v*, 0.15 M, pH 4.0). Phospholipids were then separated from neutral lipids and glycolipids using silica acid columns (Supelco, Bellefonte, PA, USA). Following the methylation of phospholipids, the PLFA methyl esters (PAMES) were separated and identified using a gas chromatograph (GC; N6890; Agilent, Santa Clara, CA, USA) and fitted using the MIDI Sherlock microbial identification system 4.5 (MIDI, Newark, DE, USA). Methyl nonadecanoate fatty acid (19:0) was added before derivatization as an internal concentration standard to quantify phospholipids. The $\delta^{13}C$ of individual PLFAs was analyzed by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) using a Trace GC Ultra gas chromatograph with a combustion column attached via a GC Combustion III to a Delta V Advantage isotope ratio mass spectrometer (Thermo-Fisher Scientific), as described by Thornton et al. (2011). Iso- and anteiso-branched fatty acids (except for 10Me-branched PLFAs) were used as indicators for gram-positive (G^+) bacteria, whereas monounsaturated and cyclopropyl fatty acids were used as indicators for gram-negative (G^-) bacteria. Saturated straight chain fatty acids were considered as nonspecific PLFAs that exist in a wide variety of microorganisms. PLFA 18:2ω6,9 was used as an indicator for fungi (Kaiser et al. 2010), whereas 18:1ω9 was used as an indicator for G^+ bacteria (Frostegård et al. 2011). The 10Me-branched PLFAs were used as specific actinobacterial biomarkers (Esperschütz et al. 2009).

Calculations

^{13}C in rice-soil system

The ^{13}C incorporation into the plant and soil samples was calculated as follows:

$$^{13}C = \left[\left(\text{Atomic}^{13}C\%\right)_{s,\,L} - \left(\text{Atomic}^{13}C\%\right)_{s,\,UL}\right] \times C_s/100,$$

where $(\text{Atomic } ^{13}C\%)_{s,\,L}$ and $(\text{Atomic } ^{13}C\%)_{s,\,UL}$ are the atomic $^{13}C\%$ in labeled and unlabeled samples, respectively, and C_s is the total C content of the samples.

The ^{13}C incorporation into the shoots, roots, and soil pools was expressed as the percentage of ^{13}C recovery on the sampling day. The total ^{13}C recovery after sampling was calculated as the sum of ^{13}C in the shoots, roots, and soil (Tian et al. 2013a, b).

Rice-derived C in microbial biomass

The ^{13}C incorporated into the microbial biomass (^{13}C-MBC) was calculated as the difference in ^{13}C between fumigated and unfumigated soil extracts divided by 0.45 (Lu et al. 2002b):

$$^{13}\text{C-MBC} = \left\{\left[\left(\text{Atomic}^{13}\text{C}\%\right)_{\text{f, L}} - \left(\text{Atomic}^{13}\text{C}\%\right)_{\text{f, UL}}\right] \times C_{\text{f}} - \left[\left(\text{Atomic}^{13}\text{C}\%\right)_{\text{uf, L}} - \left(\text{Atomic}^{13}\text{C}\%\right)_{\text{uf, UL}}\right] \times C_{\text{uf}}\right\}/100/0.45,$$

where *(Atomic $^{13}C\%$)* is the atomic $^{13}C\%$ in a soil extract, and C is the total C content of the soil extract. Subscript letters f, uf, L, and UL indicate fumigated, unfumigated, labeled, and unlabeled samples, respectively.

Rice-derived C in PLFA

The amount of ^{13}C incorporation into each PLFA (mg ^{13}C kg^{-1} soil) was determined using a mass balance approach:

$$^{13}C_{\text{PLFA}} = \left[\left(\text{Atomic}^{13}\text{C}\%\right)_{\text{PLFA, L}} - \left(\text{Atomic}^{13}\text{C}\%\right)_{\text{PLFA, UL}}\right]/100 \times C_{\text{PLFA}},$$

where C_{PLFA} is the ^{13}C content of an individual PLFA in the labeled samples. The relative ^{13}C distribution to specific microbial groups was calculated as follows (Tian et al. 2013a):

$$^{13}C\% = {}^{13}C_{\text{PLFA}}\text{–groups}/\sum {}^{13}C_{\text{PLFA}} \times 100.$$

Statistical analysis

Data are expressed as the means of three replicates ± standard error (SE). Analysis of variance in conjunction with Duncan's test was performed to identify differences at $P < 0.05$. Pearson's correlation test and linear regression were carried out to identify significant associations ($P < 0.05$) between ^{13}C-SOC (^{13}C content of soil organic C), ^{13}C-MBC, ^{13}C-PLFA, and the rice root biomass at various N fertilization rates. PLFA profiles were examined using canonical analysis after redundancy analysis (RDA) for data grouping. All analyses were performed using SPSS 10.5 (IBM, Chicago, IL, USA).

Results

Shoot and root biomass and rice biomass C and N content

Shoot and root biomass increased with the increasing N fertilization rates ($P < 0.05$; Fig. 1). Shoot biomass ranged from 1.58 (N0) to 4.35 (N60) g per pot and root biomass ranged from 1.05 (N0) to 2.44 (N60) g per pot. The root-to-shoot ratio of N0 plants was significantly higher than that of N-fertilized plants, but there were no differences in root-to-shoot ratio between the four N fertilization levels (range 0.52 to 0.59; Fig. 1).

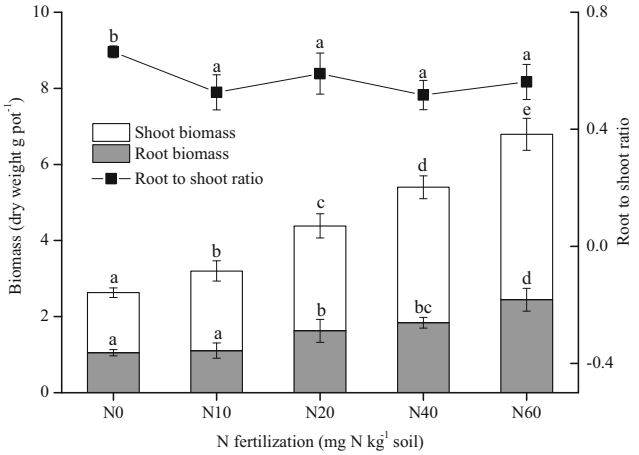

Fig. 1 Effects of N fertilization rates on rice root and shoot biomass (dry weight) and root-to-shoot ratios. *Letters* indicate significant differences among treatments at $P < 0.05$. *Error bars* represent one standard errors of the means ($n = 3$). N0, N10, N20, N40, and N60 correspond to 0, 10, 20, 40, and 60 mg N kg^{-1} soil, respectively

The percentage of total biomass C consisting of ^{13}C-SOC (^{13}C-SOC/rice biomass C × 100) in the N0 treatment was significantly lower than those observed in all of the N fertilization treatments. The percentage of total C that was ^{13}C-SOC varied with fertilization as follows: N20 > N40 > N60 > N10 > N0. However, there were no significant differences between the N20 and N40 (Table 1).

^{13}C distribution in plant biomass and soil

Total ^{13}C incorporated into the rice-soil system (the sum of ^{13}C in shoots, roots, and soil) ranged from 63.4 (N0) to 241 (N60) mg ^{13}C per pot (Fig. 2) and increased with N fertilization rates ($P < 0.05$; Fig. 2). The percentage of ^{13}C recovery from the shoots of N0, N10, and N20 was higher than that from the shoots of N40 and N60. The ^{13}C incorporation into roots at N0, N10, and N20 was lower than that at N40 and N60 ($P < 0.05$; Fig. 2). Overall, the ^{13}C incorporation into the soil

Table 1 Effects of N fertilization rates on rhizodeposition as a percentage of total plant C (^{13}C soil organic C/rice biomass C)

Nitrogen fertilization* (mg N kg^{-1} soil)	^{13}C-SOC/rice biomass C (%)
N0	1.39 ± 0.01 a
N10	1.45 ± 0 b
N20	1.66 ± 0.01 c
N40	1.61 ± 0.02 c
N60	1.51 ± 0 b

Letters indicate significant differences among the treatments at $P < 0.05$.

* N0, N10, N20, N40, and N60 correspond to 0, 10, 20, 40, and 60 mg N kg^{-1} soil, respectively

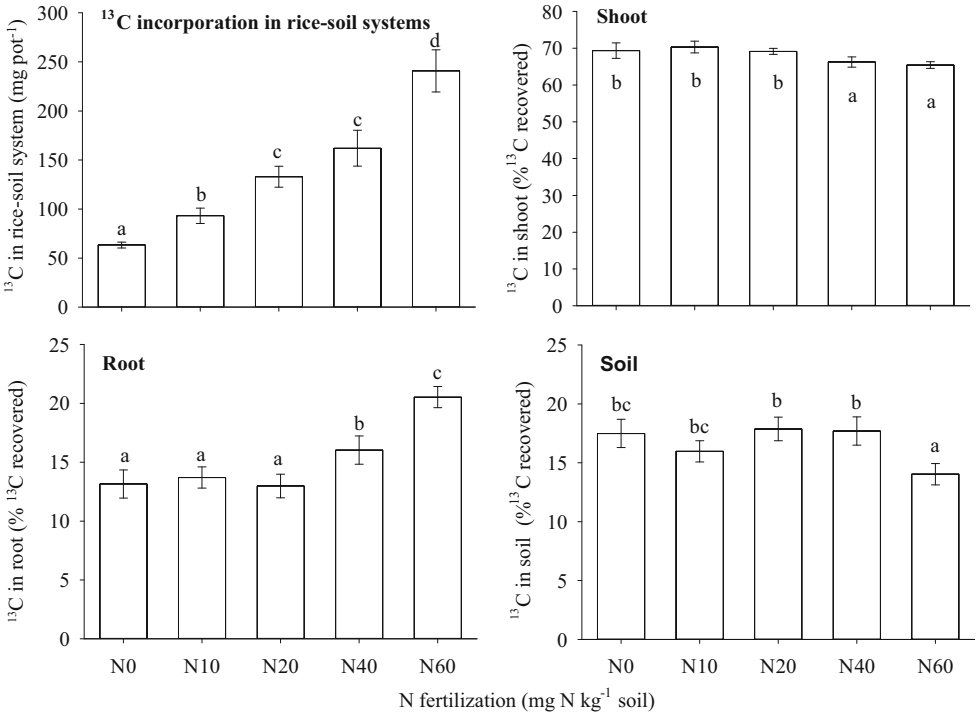

Fig. 2 Effects of N fertilization rates on ^{13}C incorporation into the C pools of rice-soil systems and ^{13}C recovery in rice shoots, and rice roots, and soil. *Letters* indicate significant differences among treatments at $P < 0.05$. *Error bars* represent one standard error of the mean ($n = 3$). N0, N10, N20, N40, and N60 correspond to 0, 10, 20, 40, and 60 mg N kg^{-1} soil, respectively

at N60 (14.0 % of ^{13}C incorporation into rice-soil mesocosms) was lower than that at N0 (17.5 %), N10 (16.1 %), N20 (17.9 %), and N40 (17.7 %; $P < 0.05$; Fig. 2).

Soil MBC, total PLFAs, and microbial community structure

Soil MBC and total PLFAs were greater for all N fertilization treatments than in N0 control (Table 2). The PLFA content of N10, N20, N40, and N60 was 6.3, 25.1, 20.8, and 31.8 % higher than that of N0, respectively (Table 2). The abundance of G$^-$ bacteria and fungi increased with increasing N fertilization rates ($P < 0.05$; Table 2). However, the abundance of G$^+$ bacteria, actinomycetes, and arbuscular mycorrhizal (AM) fungi was not affected by N. The fungi/bacteria ratio (F/B) was strongly affected by N fertilization (Table 2). The F/B ratios of N10, N20, N40, and N60 were 1.05, 1.62, 2.01, and 1.96 times higher than that of N0, respectively ($P < 0.05$; Table 2).

Incorporation of rhizodeposits into soil MBC and total PLFAs

The total ^{13}C recovered from MBC and total PLFAs decreased with the increasing N fertilization rates ($P < 0.05$), although

Table 2 Effects of N fertilization rates on the abundance of microbial groups in soil

	N0	N10	N20	N40	N60
Microbial biomass C (mg kg^{-1})	584.7 ± 23.4 a	627.3 ± 30.1 b	635.8 ± 32.0 b	657.4 ± 21.1 c	607.0 ± 24.1 b
Total PLFA (mg C kg^{-1})	44.7 ± 2.3 a	47.5 ± 4.0 a	55.9 ± 1.2 b	54.0 ± 1.2 b	58.9 ± 3.6 c
PLFA groups (mg C kg^{-1})					
Gram-positive bacteria (G$^+$)	11.3 ± 0.8 a	12.2 ± 0.6 a	11.5 ± 0.5 a	11.4 ± 1.20 a	11.7 ± 0.4 a
Gram-negative bacteria (G$^-$)	4.3 ± 0.3 a	5.5 ± 0.5 b	5.7 ± 0.4 b	5.9 ± 0.2 b	7.0 ± 0.6 c
Fungi	5.3 ± 0.2 a	6.4 ± 0.3 b	9.5 ± 1.1 c	11.8 ± 0.1 d	12.5 ± 0.3 d
AM fungi	0.47 ± 0.05 a	0.46 ± 0.06 a	0.43 ± 0.05 a	0.38 ± 0.03 a	0.50 ± 0.02 a
Actinomycetes	4.9 ± 0.2 a	4.1 ± 0.2 a	4.6 ± 0.3 a	5.1 ± 0.4 a	5.4 ± 0.7 a
Fungi/bacteria	0.34 ± 0.02 a	0.36 ± 0.03 a	0.55 ± 0.04 b	0.68 ± 0.04 c	0.67 ± 0.05 c

Letters indicate significant differences among treatments at $P < 0.05$. N0, N10, N20, N40, and N60 correspond to 0, 10, 20, 40, and 60 mg N kg^{-1} soil, respectively

the ^{13}C recovery from MBC and PLFAs was similar for N0 and N10 (Fig. 3). The maximum ^{13}C recovery from MBC was 1.7 % (N0) and that from total PLFAs was 0.26 % (N10). The ^{13}C incorporation into SOM, microbial biomass, and PLFA increased with root biomass ($r^2 = 0.95$, 0.93, and 0.93, respectively; $P < 0.01$; Fig. 4).

Incorporation of rhizodeposit C into microbial groups

^{13}C distributions differed between microbial groups depending on N fertilization (Table 3). The ^{13}C incorporation into all fungal groups ranged from 14.1 % (N10) to 39.6 % (N40) of total ^{13}C-PLFAs. Overall, the highest ^{13}C percentage was recovered in fungal PLFA (26 %; 18:2ω6,9c and 18:1ω9c), followed by G^+ (11 %; i16:0, i17:0, a17:0, i14:0, i15:0, and a15:0) and G^- bacteria (9 %; 16:1ω7, 18:1ω7c, cy17:0, and cy19:0) (Table 3).

As N fertilization increased, relative ^{13}C incorporation into G^+ and G^- bacteria decreased, whereas that into actinomycetes increased ($P < 0.05$; Table 3). The relative ^{13}C incorporation into all AM fungi of fertilized treatments was 67 % (N10), 55 % (N20), 62 % (N40), and 22 % (N60) higher than that of the unfertilized control (Table 3).

The first and second axes in the RDA explained 98.1 % of the variance (Fig. 5). The ^{13}C incorporation into microbial communities was clearly grouped by N fertilization rate ($P = 0.002$ by Monte Carlo permutation test within RDA; Fig. 5).

Discussion

Effects of N fertilization on plant biomass and ^{13}C allocation and distribution in rice-soil systems

Rice shoot and root biomass increased with N fertilization rate (Fig. 1), which led to an ^{13}C increase in SOC (Fig. S2). N availability regulates root activity, architecture (Xu et al. 2012), morphology, and consequently, rhizodeposition (Kuzyakov and Domanski 2000; Jones et al. 2009). Liu et al. (2014) reported that rice plants grown under low to intermediate N fertilization rates develop more fine roots than those grown under high N (Liu et al. 2014). Large roots may divert more C from the shoots than small roots, limiting aboveground plant biomass C (Coque and Gallais 2006). However, in the present study, rice allocated proportionally more C into the roots under high N fertilization than under lower rates (Fig. 2). The percentage of total ^{13}C recovered in the soil did not differ among the N0, N10, N20, and N40 rates; however, the ^{13}C percentage recovered in the soil was significantly lower for the N60 treatment (Fig. 2). Thus, N fertilization increased the belowground portion of plant C but did not increase the C input into the soil.

Relatively large ^{13}C amounts in SOC derived from rice occur at high N fertilization rates because rhizodeposition increases with root biomass, and less C is lost due to respiration from large roots than from small roots. Indeed, the incorporation of recent photosynthates into the roots under high N fertilization rates was greater than that under low N fertilization rates (Fig. 2, Fig. S2).

N fertilization affects the partitioning and allocation of plant photosynthates (Kuzyakov et al. 2002; Ge et al. 2015). N60 treatment resulted in lower ^{13}C recovery from the soil than N0, N10, N20, or N40 (Fig. 2). In agreement with previous studies (Ge et al. 2015), very high rates of N fertilization led to relatively low allocation of photosynthates belowground, thus regulating microbial response and distribution within the organic matter pools. The maximum ^{13}C recovery in soil (16.6 %; Fig. 2) was obtained for the N40 fertilization and was ~2-fold higher than that in a previous study with rice (7.3 %) (Ge et al. 2015) and 3-fold higher than that in pulse labeling studies with 100 mg N kg^{-1} fertilization (Tian et al. 2013a, b). These differences between studies reflect the effects of two labeling approaches (continuous vs. pulse labeling) and labeling durations. In addition, N fertilization rate, plant growth stage, and soil properties also affect C allocation and distribution in rice-soil systems (Meharg and Killham 1988; Swinnen et al. 1994; Ge et al. 2012) and explain the differences between these studies. Because the duration of continuous labeling in the present study was much longer than that

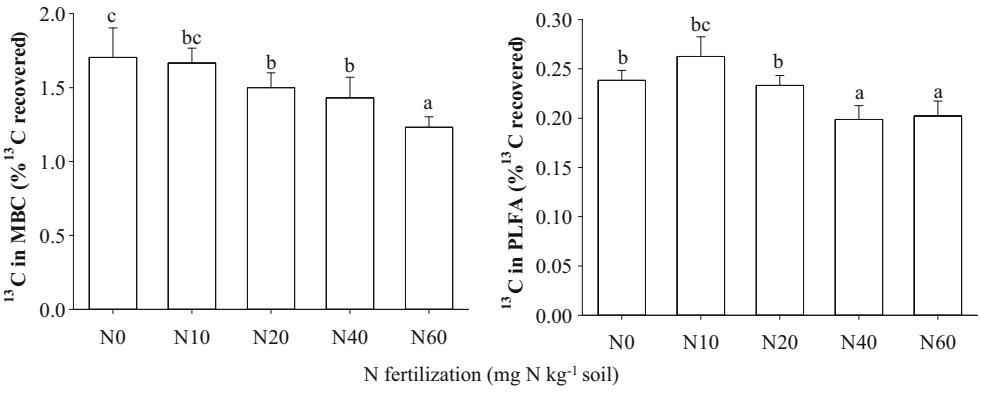

Fig. 3 Effects of N fertilization rates on ^{13}C incorporation into microbial biomass (^{13}C-MBC) and total phospholipid fatty acids (^{13}C-PLFAs) in rice-soil systems. *Letters* indicate significant differences among treatments at $P < 0.05$. *Error bars* represent one standard error of the mean ($n = 3$). N0, N10, N20, N40, and N60 correspond to 0, 10, 20, 40, and 60 mg N kg^{-1} soil, respectively

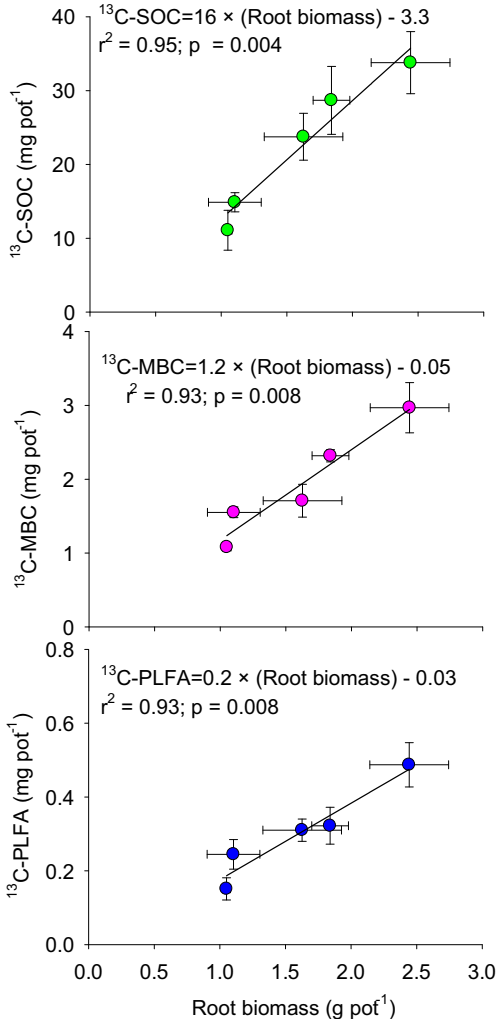

Fig. 4 Relationship between rice root biomass and ^{13}C in soil organic C (*SOC*), microbial biomass C (*MBC*), and phospholipid fatty acids (*PLFAs*) at five N fertilization rates. *Lines* indicate linear regression. *Error bars* represent standard errors of the means ($n = 3$). N0, N10, N20, N40, and N60 correspond to 0, 10, 20, 40, and 60 mg N kg^{-1} soil, respectively

in most previous studies, and the most intensive root growth and rhizodeposition occurs during tillering, we concluded that the quantification of rhizodeposition in the present study was more reliable than those reported previously.

The use of plant pots and an enclosed growth chamber may slightly decrease plant growth (Norby et al. 2001). We used a relatively small soil volume (approximately one third of the soil volume explored in the field), which might have restricted nutrient uptake. The slightly decreased light intensity in the growth chamber, which may have been accompanied by increased relative humidity and decreased gaseous exchange relative to unlabeled control plants, might have restricted photosynthesis. However, if any, these effects were of very minor importance as the plants in the growth chamber did not show any stress symptoms or early senescence.

Effect of increasing N fertilization rates on soil MBC and microbial groups

Similar to previous rice-paddy studies, microbial biomass increased with the increasing N fertilization rates (Table 2) (Ge et al. 2015). However, in upland soils, microbial biomass and activity decreased with N fertilization (Bowden et al. 2004; Knorr et al. 2005). Therefore, the observed positive effect of N on microbial biomass depended on the specific conditions of rice cultivation. Unlike upland soils, paddy soils are anaerobic and rich in SOM and have relatively low biological activity (Smolander et al. 2005). Therefore, the quantity and quality of SOM, root biomass, rhizosphere oxygen availability, and rhizodeposition are key factors that affect microbial activity. N fertilization promotes rice growth and increases rhizodeposition (Fig. 2), thereby increasing C availability for microorganisms. Moreover, N fertilization can directly stimulate the growth of fungi and G$^-$ bacteria (Aira et al. 2010; Tian et al. 2012; Yuan et al. 2013; Paungfoo-Lonhienne et al. 2015). Low N availability leads to fine roots, whereas N fertilization increases root diameter (Liu et al. 2014) and the size of aerenchyma cells (Yamauchia et al. 2013).

N fertilization decreases soil pH (Rousk et al. 2011; Fig. S1). Soil pH is one of the most powerful determinants of microbial community composition (Rousk et al. 2010a, b). Fungal growth is favored at low pH, whereas bacterial growth is favored at neutral pH (Rousk et al. 2009). The fungi/bacteria ratio increased with increasing N fertilization (Table 2). The RDA analysis of the microbial community (Fig. 5) suggests that the growth of some microbial groups increased after N fertilization and that these groups were distributed in hot spots, where the root C and soil NH_4^+ were localized. ^{13}C-SOC derived from rice rhizodeposition increased with N fertilization, resulting in increased C availability and improved growth conditions for soil microorganisms, especially fungi (Yuan et al. 2016). Some mycorrhizae of the genus *Glomus* survive in waterlogged conditions. Our previous study showed that fungi outcompete bacteria or actinomycetes for ^{13}C derived from rhizodeposition, which increases with rice plant age (Yuan et al. 2016).

Effect of increasing N fertilization rates on the incorporation of rhizodeposited C into microbial groups

N fertilization affected microbial properties (biomass and community composition) (Figs. 3 and 5). We recovered less ^{13}C from MBC and total PLFAs in the N20 and N60 groups than in the N0 and N10 groups (Fig. 3). C sequestration is a function of C input by plants and utilization by microorganisms. In our previous study, we used $^{14}CO_2$ continuous labeling to show that incorporation of ^{13}C into microbial biomass is maximized at a low N fertilization rate (10 mg N kg^{-1} soil) (Ge et al. 2015). In

Table 3 Effects of N fertilization rates on the relative ^{13}C incorporation into microbial groups after continuous labeling

Relative ^{13}C distribution (%)	N0	N10	N20	N40	N60
Fungi	14.8 ± 3.0 a	14.1 ± 3.0 a	31.8 ± 4.1 c	39.6 ± 3.1 d	28.4 ± 2.6 b
Gram-positive bacteria (G^+)	12.6 ± 0.1 b	11.4 ± 0.8 b	10.1 ± 0.7 b	9.8 ± 0.8 a	9.5 ± 1.2 a
Gram-negative bacteria (G^-)	10.1 ± 1.2 c	8.2 ± 1.2 a	8.3 ± 0.6 a	9.4 ± 0.8 a	8.7 ± 0.7 a
AM fungi	0.49 ± 0.06 a	0.82 ± 0.09 d	0.76 ± 0.08 c	0.79 ± 0.07 d	0.59 ± 0.06 b
Actinomycetes	0.54 ± 0.02 a	0.59 ± 0.06 b	0.77 ± 0.03 c	0.87 ± 0.09 c	1.02 ± 0.05 d
Nonspecific PLFAs	61.5 ± 7.2 d	65.0 ± 5.7 d	48.4 ± 6.2 b	39.6 ± 4.3 a	51.8 ± 5.7 c

Letters indicate significant differences among treatments at $P < 0.05$. N0, N10, N20, N40, and N60 correspond to 0, 10, 20, 40, and 60 mg N kg^{-1} soil, respectively

the present study, ^{13}C recovery from MBC (1.5 %) was considerably higher than that from total PLFAs (0.23 %), regardless of the N fertilization rate. In addition, there is generally less C contained in PLFA than in microbial biomass.

Incorporation of root-derived ^{13}C into SOM, microbial biomass, and PLFAs increased with root biomass (Fig. 4). Our previous study showed that ^{14}C-SOC and ^{14}C-MBC contents were also positively correlated with the root biomass (Ge et al. 2012, 2015). Already Shamoot et al. (1968) reported a positive relationship between root biomass and rhizodeposition after ^{14}C labeling. Recovered ^{13}C was not evenly distributed among microbial groups. At medium and high N fertilization rates, fungi incorporated more ^{13}C than other microbial groups (Table 3). This suggests that, unlike in upland systems, fungi uptake newly rhizodeposited C more intensively than other microorganisms and are important initial C sinks also in paddy soils (Denef et al. 2009; Jin and Evans 2010). Fungi prefer to grow in moist, but not flooded, soils because they are obligate aerobes, susceptible to desiccation and ion imbalance (Yao et al. 2012). Rice roots in paddy soils can release oxygen that greatly influences the oxygen availability and redox potential in the soil surrounding the roots (Eickhorst and Tippkötter 2009). The radial O_2 diffusion from the rice roots into the soil can lead to the proliferation of AM fungi and the promotion of plant growth, even under waterlogged conditions (Solaiman and Hirata 1995; Li et al. 2013). In the present study, the fungal groups that incorporated relatively large amounts of ^{13}C were mainly saprophytic and/or parasitic. Although mycorrhizal fungi tend to use easily available C obtained from mutualistic associations with roots, saprophytic fungi typically derive energy from the degradation of old complex organic C sources (Brundrett 2002). Fungal growth, but not bacterial growth, is directly stimulated by N fertilization (De Boer et al. 2005; Amaya-Carpio et al. 2009). Furthermore, fungal turnover is slower than bacterial turnover (Yuan et al. 2016), leading to a relatively high remaining ^{13}C. The ^{13}C in bacteria is more rapidly released as CO_2, recycled, or transferred to SOM pools than that in fungi, resulting in less ^{13}C remaining in G^+ and G^- bacteria than with fungi (Yao et al. 2012; Yuan et al. 2016). Fungi dominate the soil microbial biomass under most field conditions (Joergensen and Wichern 2008). The formation of SOM seems to be strongly influenced by fungi (Six et al. 2006) but with some exceptions (Strickland and Rousk 2010). Our previous study showed that fungi outcompete bacteria and actinomycetes for C assimilation from rice rhizodeposition (Yuan et al. 2016). In the present study, rice roots occupied the entire soil core, so it can be considered as rhizosphere soil. Rice plants deliver O_2 to roots through the aerenchyma and this O_2 can diffuse into soil. Therefore, fungi were probably not suppressed. In addition, most of the soil ^{13}C in the present study was recovered from fungal PLFAs (16:0, 18:2 w6, or 18:1 w9c. PLFAs 18:2w6, 9c, and 18:1w9c) at all levels of N fertilization. This is consistent with the results of Wang et al. (2016) and Yuan et al. (2016). In the rhizosphere, fungal hyphae have very large surface that allow them to mine for nutrients and oxygen to a greater extent than bacteria and Archaea. Further, mycorrhizal associations allow direct uptake of organics from roots, before release into the soil.

^{13}C incorporation into G^- and G^+ bacteria decreased slightly with increasing N fertilization rates. The decrease of ^{13}C incorporation into bacterial biomass likely resulted either from simple dilution of label within the growing microbial cells (G^-

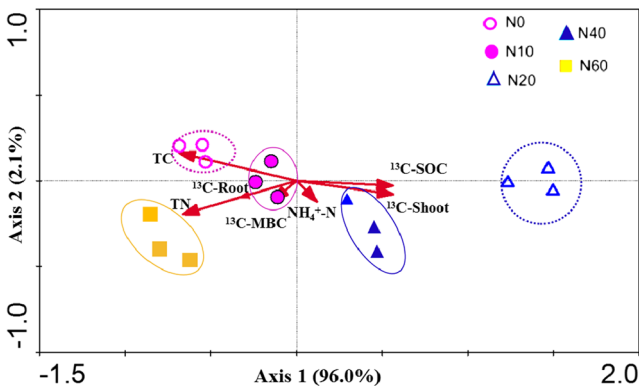

Fig. 5 Redundancy analysis (RDA) of ^{13}C-phospholipid fatty acid (PLFA) composition at five N fertilization rates. *Arrows* indicate variables. The *arrow directions* indicate the steepest increase of the variable, and the *length* indicates the strength relative to other variables. N0, N10, N20, N40, and N60 correspond to 0, 10, 20, 40, and 60 mg N kg^{-1} soil, respectively

bacteria (Table 2) or bacteria switching from rhizodeposits to other (nonlabeled) C sources. Microorganisms can be primed by rhizodeposition and then begin to utilize other SOM sources. G^+ bacteria may have changed C sources, as their biomass did not differ between N treatments (Table 2). In contrast to G^- and G^+ bacteria, the little ^{13}C incorporation into AM fungi and actinomycetes (Table 3) and the similar results reported by Tian et al. (2013a) suggest that these microorganisms efficiently utilize chitin from fungal cells and do not depend on plant-derived and other microbial-derived C sources (nonroot C sources). Therefore, Actinomycetes likely consume the dead hyphae of those fungal species that consume plant-derived C (Shirokikh et al. 2014). The ^{13}C to total C ratio in fungi, G^+ bacteria, and G^- bacteria increased significantly with the N fertilization, possibly reflecting changes in root exudate composition and microbial utilization (Table S1). ^{13}C incorporation into the microbial community was clearly grouped by the N fertilization rate (Fig. 5). However, the specific stabilization of C derived from individual microbial groups requires further investigations.

Conclusions

Rice shoot and root biomass strongly increased with N fertilization, as did rhizodeposition. In contrast, total ^{13}C incorporation into microorganisms decreased with N fertilization. The ^{13}C incorporation into microbial biomass, PLFAs, and SOM was positively correlated with the rice root biomass. The highest ^{13}C percentage was recovered in fungal PLFA, regardless of N application rate, suggesting that fungi were important primary rhizo C sinks in the paddy soils. In contrast, G^+ and G^- bacteria utilized less root-derived C at high N fertilization (40 and 60 mg N kg^{-1} soil) than at no N fertilization. Overall, we conclude that N fertilization in rice production systems affects the C input into the soil, the fate of rhizodeposited C, and its microbial utilization. We conclude that excessive N fertilization has not only consequences for environmental pollution but also decrease belowground C allocation by rice and rhizo C incorporation into microorganisms and SOM. Molecular techniques combined with DNA/RNA-SIP should be used in future studies to detect and express functional genes in the rhizosphere soil, including those that encode enzymes (Pathan et al. 2015), and to assess the effects of rhizodeposit composition on microbial functions as it relates to N fertilization.

Acknowledgments This study was supported by the National Natural Science Foundation of China (41671292; 41522107), Key Projects of International Cooperation in Science and Technology Innovation (S2016G0053), the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB15020401), Royal Society Newton Advanced Fellowship (NA150182), and the Recruitment Program of High-end Foreign Experts of the State Administration of Foreign Experts Affairs awarded to Y. K. (GDW20144300204).

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