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ORIGINAL PAPER

Microbial utilization of rice root exudates: ^{13}C labeling and PLFA composition

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Abstract The soluble components of rhizodeposition—root exudates—are the most important sources of readily available carbon (C) for rhizosphere microorganisms. The first steps of exudate utilization by microorganisms define all further flows of root C in the soil, including recycling and stabilization. Nevertheless, most studies have traced root exudates C much later after its initial uptake by microorganisms. To understand microbial uptake and utilization of rice root exudates, we traced ^{13}C incorporated into microbial groups by ^{13}C profiles of phospholipid fatty acids (PLFAs) within a short time (6 h) after $^{13}CO_2$ pulse labeling. Labeling was conducted five times during three growth stages: active root growth (within the 21 days after transplanting), rapid shoot growth (37 and 45 days), and rapid reproduction (53 and 63 days). ^{13}C was quickly assimilated throughout the rhizosphere microorganism, and the incorporation rate increased with rice maturity. Despite low redox conditions in paddy soil, fungi outcompeted bacteria in utilizing the root exudates. At all growth stages, fungal PLFAs (18:2 w6, 9c/18:0) showed the highest ^{13}C levels, whereas actinomycete PLFAs (16:0 10-methyl) showed the lowest ^{13}C incorporation. Principal component analysis revealed that the rhizosphere microbial community differed among rice growth stages, whereas the whole microbial community remained stable. In conclusion, the rapid incorporation of carbon from root exudates into microorganisms in paddy soils depends on the growth stage of the rice plant and is the first step of C utilization in rice rhizosphere, further defining C utilization and stabilization.

Keywords Rice (*Oryza sativa* L.) · Rhizodeposition · $^{13}CO_2$ labeling · Microbial utilization · Phospholipid fatty acids (PLFAs) · Paddy soils

Introduction

Rhizodeposits consist of a wide range of compounds, including root exudates, mucilage, sloughed-off cells and tissue, cell lysates and root debris (Kuzyakov and Larionova 2005; Gregory 2006). Exudates are photosynthetic products released continuously and passively from roots, and account for 5–30 % of the total photosynthetically fixed C in the soil (Kuzyakov and Domanski 2000; Kuzyakov et al. 2003; Nguyen 2003). The main components of root exudates are soluble sugars (Fischer et al. 2010; Palacios et al. 2014; Gunina and Kuzyakov 2015), which serve as a primary energy source and an important source of C for soil microorganisms in the vicinity of growing roots (Högberg et al. 2008; Werth and Kuzyakov 2010; Pii et al. 2015). Photosynthates assimilated in leaves are actively loaded through phloem to the roots and passively released into the rhizosphere. The time interval between photoassimilation in leaves and direct transport to roots can be predicted from phloem transport rates, which range from 0.5 to 1.0 m h^{-1}, and vary between species and growth stages (Thompson and Holbrook 2003; Nobel 2005;

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Barnard et al. 2007; Kuzyakov and Gavrichkova 2010), depending on environmental conditions. In contrast, the release of exudates from roots is very fast and occurs nearly without any time delay (Gavrichkova and Kuzyakov 2012). The amount and quality of exudates are strongly controlled by photosynthesis and depend on plant species, age, and external biotic and abiotic factors (Gavrichkova and Kuzyakov 2012; Ge et al. 2012; Meng et al. 2013). In general, the amount of root exudate is highest during fast vegetative growth and decreases with plant age (Yevdokimov et al. 2006; Garbeva et al. 2008).

Many researchers have analyzed belowground C partitioning through rhizodeposition and the incorporation of that C into microbial biomass (Paterson et al. 2007; Hannula et al. 2012; Benesch et al. 2015). Several molecular and biochemical methods have been developed over the past decade to study the diversity of soil microorganisms, most of which are unknown and unculturable (Ganzert et al. 2011; Shade et al. 2012). For example, the composition of microbial phospholipid fatty acids (PLFAs) mirrors the general composition of soil and aquatic microbial communities (Lu et al. 2007; Abraham 2014). Stable C isotope techniques coupled with PLFA analysis allows study of both the function and structure of microbial communities. ^{13}C-PLFA reveals information about the portion of the microbial community that uses ^{13}C-labeled substrates (Webster et al. 2006; Dungait et al. 2011; Yao et al. 2015).

Nearly all ^{13}C-PLFA studies have been conducted in dryland soils and with crops such as maize, wheat, ryegrass, or barley to determine of the amount of root-derived C entering the soil and its subsequent microbial utilization. ^{13}C pulse-chase labeling combined with PLFA analysis of ryegrass has shown that the responses of microbial communities to rhizodeposition depend on plant age (Butler et al. 2004; Philippot et al. 2013). The role of rhizodeposition in the relationship between the composition of microbial community and photosynthesis in paddy soils has been evaluated in only a few studies (Tian et al. 2013). Even fewer studies have focused on such fast process as deposition of root exudates and implications for associated microbial communities in rice paddies.

Paddy soils cultivated with rice (*Oryza sativa* L.) make up the largest anthropogenic wetlands on earth (Kögel-Knabner et al. 2010) and are a primary food source for nearly half of the world's population (FAO 2011). Paddy ecosystems have great potential for sequestering atmospheric CO_2 (Lal 2004) and are very sensitive to climate change (Ge et al. 2012, 2015). Paddy soils are different from upland soils because they are regularly flooded and intermittently irrigated. Rice is cultivated under continuous flooding and anaerobic conditions, but the rhizosphere of rice plants can have physicochemical conditions that differ from those found in the absence of roots. The roots of rice plants promote diffusion of atmospheric oxygen into the rhizosphere. This oxygenation capability of rice roots in the rhizosphere is largely affected by the age and phenological stage of the plant (Doran et al. 2006). Differences in the rhizosphere redox potentials also contribute to changes in the physicochemical soil properties (Nikolausz et al. 2008), thereby affecting the microbial composition and the succession of microbial communities. Paddy soils are characterized by relatively slow C turnover; however, only few studies have shown the biological processes determining soil organic matter stabilization and turnover in paddy soil, especially at the microbial level (Sollins et al. 1996; Gale et al. 2000). Better understanding of the release of root C by rice, its distribution and transformation belowground, and its microbial utilization are critical for understanding global C cycling and the ecological functions of paddy ecosystems.

We tested two hypotheses: (1) root exudates and their utilization by microbes change during plant growth, and (2) rhizosphere fungi outcompete bacteria for initial uptake of root exudates because of their larger biomass and because the diffusion of atmospheric oxygen by rice roots favors fungal growth. We performed ^{13}C pulse-chase labeling of wetland rice at three growth stages under field conditions. To test the first hypothesis, the pattern of ^{13}C photosynthate uptake into shoot, roots, total soil organic matter, and microbial biomass were measured and compared during the growth stages. To test the second hypothesis, the incorporation of exudates into different microbial groups was traced by analyzing PLFAs shortly after ^{13}C assimilation aboveground.

Materials and methods

Soils and sampling

Typical Stagnic Anthrosol soil, developed from highly weathered granite, was collected from a rice field (113° 19′ 52″ E, 28° 33′ 04″ N, 80 m a.s.l.) located at the Changsha Research Station for Agricultural and Environmental Monitoring. The site has a subtropical climate with an annual mean temperature of 17.5 °C, 1300 mm rainfall, 16.63 h day^{-1} sunshine, and a frost-free period of 274 days. The soil at the research station was derived from quaternary red clay with a bulk density (BD) of 1.31 g cm^{-3}. Moist soil samples were collected from the plow layer (0–20 cm) and sieved (<4 mm) to remove coarse plant residues. The soil consisted of 7.51 % clay, 68.4 % silt, and 24.1 % sand. The soil contained 18.1 g kg^{-1} organic C, 1.80 g kg^{-1} total N, 0.43 g kg^{-1} total P, and had a pH of 5.56 (1:2.5, soil/water ratio). The extractable nutrients and other basic properties of this soil are listed in Table 1.

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

Rice growth conditions and $^{13}CO_2$ labeling were as described by Ge et al. (2012, 2015) with some modifications. Briefly,

Table 1 Soil physicochemical properties at the start of the experiment and during the rice growth

Days after transplantation	Eh (mV)	Available N (mg kg^{-1})	Olsen P (mg kg^{-1})	Available K (mg kg^{-1})	Fe (II) (mg kg^{-1})	Mn (II) (mg kg^{-1})	CEC (c mol kg^{-1})
0	–	57.39 ± 3.59b	13.02 ± 0.75a	100.29 ± 2.95d	315.2 ± 18.1a	5.07 ± 1.03a	11.72 ± 0.86a
21	−275 ± 3a	45.26 ± 2.43a	20.18 ± 0.25c	56.93 ± 1.22c	338.5 ± 18.1a	5.55 ± 0.60a	12.37 ± 0.64a
37	−247 ± 6b	44.42 ± 1.14a	20.42 ± 0.44c	50.90 ± 3.48b	494.3 ± 16.9b	5.30 ± 0.10a	12.43 ± 0.49a
45	−235 ± 5c	42.35 ± 2.54a	17.35 ± 0.48b	45.03 ± 2.37ab	712.7 ± 29.1c	5.19 ± 0.59a	12.17 ± 0.19a
52	−251 ± 5b	40.69 ± 1.03a	17.28 ± 0.59b	39.05 ± 3.34a	735.8 ± 26.6c	5.15 ± 0.39a	12.43 ± 0.37a
63	−255 ± 4b	41.14 ± 2.98a	17.67 ± 0.38b	35.31 ± 1.37a	724.7 ± 20.2c	5.53 ± 0.94a	11.60 ± 0.26a

Means followed by the same letter are not significantly different ($P > 0.05$) between the soils

three 30-day-old rice seedlings (*Oryza sativa* L.; two-line hybrid rice Zhongzao 39; average dry weight 0.10 g per plant) were transplanted into each of 30 pots (filled with 1 kg soil; dry weight) on April 29, 2013. An initial basal fertilizer, consisting of $(NH_4)_2SO_4$, calcium superphosphate, and KCl, was applied at a rate of 60 mg N, 20 mg P, and 80 mg K kg^{-1} of soil and mixed thoroughly. Deionized water was used for irrigation, and a 1–2-cm water layer was maintained above the soil surface throughout the growing season. Any weeds in the soil were carefully removed by hand. Control soil was held outside of the labeling chambers.

Fifteen pots were used for labeling: $^{13}CO_2$ pulse labeling was performed five times at 21, 37, 45, 52, and 63 days after transplantation, covering the complete growth period of rice plants (Lu et al. 2002a, 2007). These times correspond to the three typical rice growth stages (rapid root growth stage (up to 21 days after transplantation), rapid shoot growth (between 37 and 45 days after transplantation), and reproduction (between 52 and 63 days after transplantation)). The plants in three independent pots were labeled on each date. Plants were labeled with $^{13}CO_2$ for 6 h (09:00–15:00) in an automatically controlled gas-tight growth chamber system (area 110 cm × 250 cm, height 180 cm). No specific precautions, such as leak checks, were taken to ensure that shoots and roots were isolated because downward diffusion of $^{13}CO_2$ into acid floodwater (pH < 6.0) was negligible (Lu et al. 2002a). The $^{13}CO_2$ used in the experiment was generated through reaction of $NaH^{13}CO_3$ (99 atom% ^{13}C, Cambridge Isotope Laboratories, Inc.) and HCl (1 M) in a plastic beaker placed inside the growth chamber (Shsen-QZD, Qingdao, China). During labeling, CO_2 was released into the chamber only when the CO_2 concentration in the chamber was lower than 360 μL L^{-1}. At CO_2 concentrations greater than 380 μL L^{-1}, a switch diverted gas flow so that it passed through CO_2 traps (NaOH solution), where excess CO_2 was absorbed. The other 15 pots and a control soil without plant were prepared as references for natural ^{13}C abundance to calculate the ^{13}C atom % excess and were placed outdoors, 10–15 m away from labeled plants. The soil properties and the $\delta^{13}C$ signature did not change in the control soil during the test period.

Sampling and harvesting

At the end of the ^{13}C pulse labeling, the plants and soils from three independent pots were destructively sampled after the measurements of soil redox potentials (Eh) by using a platinum electrode against a standard Ag/AgCl electrode (Mettler Toledo. Switzerland), at soil depths of 5–10 cm. This corresponded to 6 h after the start of assimilation. At harvest, the shoots were cut off at the stem base, allowing for separation of the roots, shoots, and soil components. Because roots and fungi share several of common PLFA biomarkers, it was necessary to make sure of the PLFA profiles obtained and their $\delta^{13}C$ values were representative of the soil microbial community rather than plant material. To do this, any soil adhering to the roots was removed as described by Butler et al. (2002), suspended in 40 ml of 50 mM phosphate buffer (pH 6.8), centrifuged at 8400×*g* for 5 min, and the supernatant was decanted to remove the root cell contamination (the floatable, plant-derived debris). To assess whether this method was effective enough to remove most of the fine living roots from soils, 15 g of soil from each sampling pot was examined under a dissecting microscope (SM-1BSZZ-64S, AmScope, USA). If the fine roots were not found in samples, the sieving-centrifugation procedure was considered effective enough to remove most of the fine living roots in comparison with visual separation under a microscope. If not, the fine living roots were removed manually after the resuspension and centrifugation. All obtained fresh soil samples were mixed thoroughly and divided into three portions. One portion was immersed immediately in liquid nitrogen and stored at −70 °C for future PLFA analysis. Another was used immediately for determination of soil Mn (II), Fe (II), and microbial biomass C. The remaining portion was air-dried, ground, sieved through a 100 mesh, and used for determining the ^{13}C-soil organic carbon (SOC) and other soil

physiochemical properties. Visible dead roots were included in the total root mass because it was impossible to separate fragments of dead roots from living roots. Therefore, all analyses were carried out with mixed (living and dead) root material (Yevdokimov et al. 2006). Shoot and root materials were dried in an oven for 72 h at 70 °C, weighed, and pulverized.

The soil physicochemical properties and ^{13}C in plant–soil–microbial system

The C content of independent soil, shoot, and root samples were analyzed using an automated C/N analyzer (Vario MAX, Elementar Analysensysteme GmbH, Germany). The soil cation exchange capacity (CEC) was measured by titration (Rhoades 1982), total P by NaOH fusion and colorimetric determination (Olsen and Somers 1982), total K by NaOH fusion flame photometry (Allen 1989), and available N by alkali hydrolytic diffusion (Keeney and Nelson 1982). Olsen P was extracted using sodium bicarbonate and determined using the Mo-Sb colorimetry method (Colwell 1963). Available K was extracted by ammonium acetate and determined by flame photometry (Knudsen et al. 1982). Soil exchangeable Mn (II) was extracted by NH_4OAc (1 M at pH 6.0) and determined by atomic absorption spectroscopy (Ragnarsdottir and Hawkins 2006), and available Fe (II) was extracted by HCl (0.5 M) and determined by 1,10-phenanthroline colorimetric assay (Fadrus and Malý 1975).

Prior to analysis for $\delta^{13}C$, dry samples of the soil, shoots, and roots were ground to a fine powder. The stable C isotope ratios of plant and soil materials were measured using a MAT253 isotope ratio mass spectrometer coupled with an elemental analyzer FLASH HT (ThermoFisher Scientific, USA). Soil microbial biomass C (MBC) together with dissolved organic carbon (DOC) extracted by 0.5 M K_2SO_4 were measured by the fumigation extraction method (Wu et al. 1990) with subsequent $\delta^{13}C$ analysis.

PLFA extraction and analysis

Twelve soil samples, representing the three typical rice growth stages, were selected for soil microbial PLFA analysis. The soil microbial PLFAs were extracted, fractionated, and purified according to the method described by Wu et al. (2009), with minor modifications. Briefly, approximately 2 g of freeze-dried soil was extracted twice using 22.8 ml single-phase chloroform/methanol/citrate buffer system (1:2:0.8 *v*/*v*/*v*; pH 4.0). The phospholipids were then separated from neutral lipids and glycolipids using silica acid columns (Supelco, Bellefonte, PA, USA). Phospholipids were methylated using mild alkaline methanolysis (White et al. 1979). Following methylation of the phospholipids, the PLFA methyl esters (FAME) were separated and identified using a gas chromatograph (N6890; Agilent, Santa Clara, CA, USA) fitted with a MIDI Sherlock microbial identification system (Version 4.5; MIDI, Newark, DE, USA). For quantification of the phospholipids, methyl nonadecanoate fatty acid (19:0) was added prior to derivatization as an internal concentration standard. Microbial phospholipid fatty acid markers (Tunlid and White 1992; Zelles et al. 1992; Frostegård et al. 1993; Olsson et al. 1999; Hill et al. 2000; Grayston et al. 2001; Priha et al. 2001) are shown in Table 2. The remaining fatty acids were not assigned to any microbial group, including monounsaturated fatty acids (16:1 w7c/16:1 w6c, 16:1 w5c, 18:1 w9c, 18:1 w7c, 20:1 w9c), cyclopropane fatty acids (17:0 cyclo, 19:0 cyclo w8c), anteiso (15:0 anteiso,17:0 anteiso), and iso (14:0 iso,15:0 iso,16:0 iso.17:0 iso). A Trace GC Ultra gas chromatograph (GC) with a combustion column attached via GC Combustion III to a Delta V Advantage isotope ratio mass spectrometer (IRMS; Thermo Finnigan, Germany) was used to analyze the $\delta^{13}C$ of individual PLFAs. The equipment and running conditions were as described in detail by Thornton et al. (2011).

Calculations

^{13}C incorporation into plant and soil samples was calculated according to Eq. (1).

$$^{13}C = \left[\left(Atomic^{13}C\%_{L} - Atomic^{13}C\%_{UL}\right) \times C\right]/100 \quad (1)$$

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

The ^{13}C-MBC was calculated as the difference in ^{13}C between fumigated and non-fumigated soil extracts, divided by a factor of 0.45, as shown in Eq. 2 (Lu et al. 2002b).

$$\begin{aligned} ^{13}C\text{–}MBC = \big[&\left(Atomic^{13}C\%_{f,L} - Atomic^{13}C\%_{f,UL}\right) \\ &\times C_f - \left(Atomic^{13}C\%_{uf,L} - Atomic^{13}C\%_{uf,UL}\right) \\ &\times C_{uf}\big] \Big/ (100 \times 0.45) \end{aligned} \quad (2)$$

where $Atomic^{13}C\%$ f, *uf*, *L*, and *UL* indicate fumigated soil extracts, non-fumigated soil extracts, extracts from labeled samples, and extracts from unlabeled samples, respectively. C_f and C_{uf} are the total C contents of the fumigated and non-fumigated soil extracts.

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

$$^{13}C_{PLFA} = Atomic^{13}C\%_{PLFA,L} - Atomic^{13}C\%_{PLFA,UL}\big)/100 \times C_{PLFA} \quad (3)$$

where $Atomic^{13}C\%$ *PLFA*, *L*, and *UL* indicate PLFA, extracts from labeled samples, and extracts from

Table 2 Microbial phospholipid fatty acids and corresponding microbial groups in tested soils

Microbial groups	Unique phospholipid fatty acids	References
Gram-negative bacteria	16:1w5c, 16:1w7c/16:1w6c, 18:1w7c, 17:0 cyclo, 19:0 cyclo w8c	Zelles et al. (1992) Frostegård et al. (1993)
Gram-positive bacteria	14:0 iso,15:0 iso, 15:0 anteiso,16:0 iso, 17:0 iso, 17:0 anteiso	Zelles et al. (1992) Frostegård et al. (1993)
Fungi	18:1w9c, 18:2w6, 9c/18:0 ante	Frostegård et al. (1993)
Actinomycetes	16:0 10-methyl, 17:0 10-methyl, 18:0 10-methyl	Tunlid & White (1992)
Arbuscular mycorrhizal fungi	16:1w5c	Olsson et al. (1999)

unlabeled samples, respectively. We calculated the relative ^{13}C distribution in each specific microbial group according to the following equation (Tian et al. 2013):

$$^{13}C\% = {}^{13}C_{PLFA}\text{-}group / \sum {}^{13}C_{PLFA} \times 100 \quad (4)$$

where $^{13}C_{PLFA}$-group is the amount of ^{13}C-PLFA incorporated into each specific microbial group and $\sum {}^{13}C_{PLFA}$ is the total amount of ^{13}C-PLFA incorporated into soil microbes.

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

Statistical analysis

Data were processed using Excel 2010 for the means and standard errors. After normality testing on the data with the W-test, multiple comparison tests were made using one-way ANOVA followed by Duncan's multiple range test ($P<0.05$). Correlation analyses were done using the Pearson correlation method with significance defined at the 0.05 level unless otherwise stated. All analyses were performed using SPSS 13.0 software for Windows XP.

Principal components analysis (PCA) and redundancy analysis (RDA) were performed with CANOCO 5.0 for Windows (Microcomputer Power, Ithaca, NY, USA) based on the PLFA profiles during each rice growth stage to identify the effects of soil physiochemical parameters and root exudates, including ^{13}C-SOC, ^{13}C-root, and soil extractable nutrients on the soil rhizosphere microbial communities.

Results

The soil physiochemical properties at rice stages

Rice growth had an effect on the soil chemical properties (Table 1). Eh increased during active root and shoot growth and then slightly decreased during the reproductive stage; a similar trend was found for Fe (II) content, which significantly increased at the root and shoot growth stages and then remained constant through the reproductive stage. The available N and K contents decreased during the experiment, while the Olsen P content increased during the first 21 days (root growth) and decreased until day 45 (shoot growth). Other soil physiochemical parameters such as CEC and Mn (II) contents did not greatly vary ($P>0.05$) during the rice growth period.

Shoot and root biomass and allocation of ^{13}C photosynthates in the plant–soil system

Shoot and root biomass increased with rice growth (from 1.6 to 11.8 and 1.2 to 5.5 g C per pot, respectively; Fig. 1): overall, biomass of both shoots and roots increased throughout the life of the plant until final harvest (63 days after transplantation). Allocation of assimilated ^{13}C in shoots and roots was depended on the rice growth stage. The amount of ^{13}C in shoots and roots ranged from 18.6 to 163 and from 2.9 to 13.7 mg C per pot, respectively. The ^{13}C in roots and shoots increased with rice growth (Fig. 1). The ^{13}C-SOC content ranged from 2.7 to 6.9 mg C per pot and increased as plants grew, with the greatest increase occurring as shoot growth transitioned to reproductive growth (Fig. 2). The ^{13}C incorporated into DOC (^{13}C-DOC) was 9.2–12.6 μg C per pot and ^{13}C-MBC was 6395–1310 μg C per pot. The increases in ^{13}C-MBC occurred in concert with plant growth and, as for DOC, there was no significant difference during the plant growth period (Fig. 2). Correlation parameters showed significant correlation among ^{13}C-SOC, ^{13}C-MBC, and ^{13}C in the roots ($P<0.01$; Fig. 3). Some soil extractable nutrient variables and plant ^{13}C-photosynthetic products were also significantly correlated (Table 3). Available K was significantly correlated with ^{13}C in the shoots, root, SOC, and MBC, and a significant correlation was also observed between Available N and ^{13}C-SOC (see Table 3).

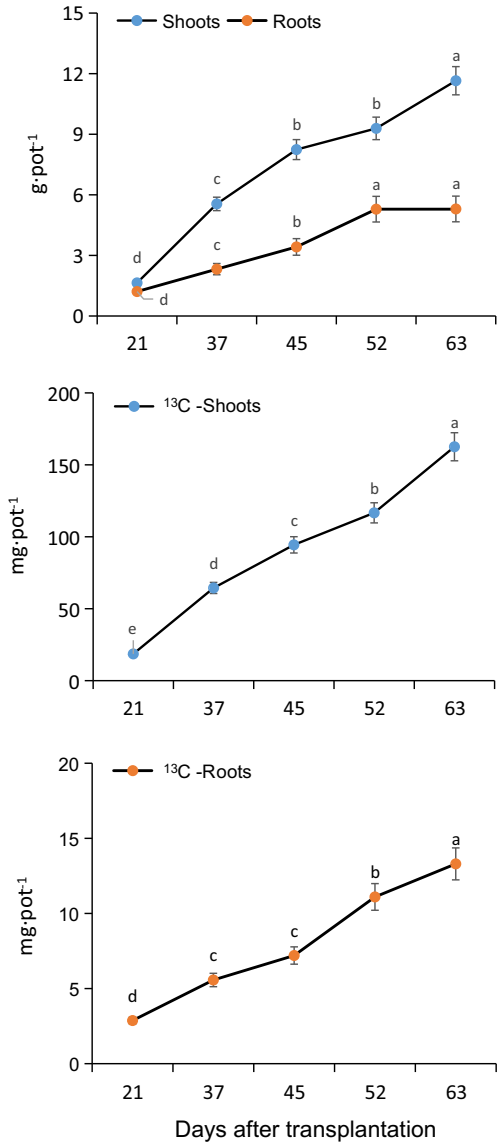

Fig. 1 Shoot and root biomass and allocation of photosynthesized ^{13}C into the shoot and root biomass at three rice growth stages

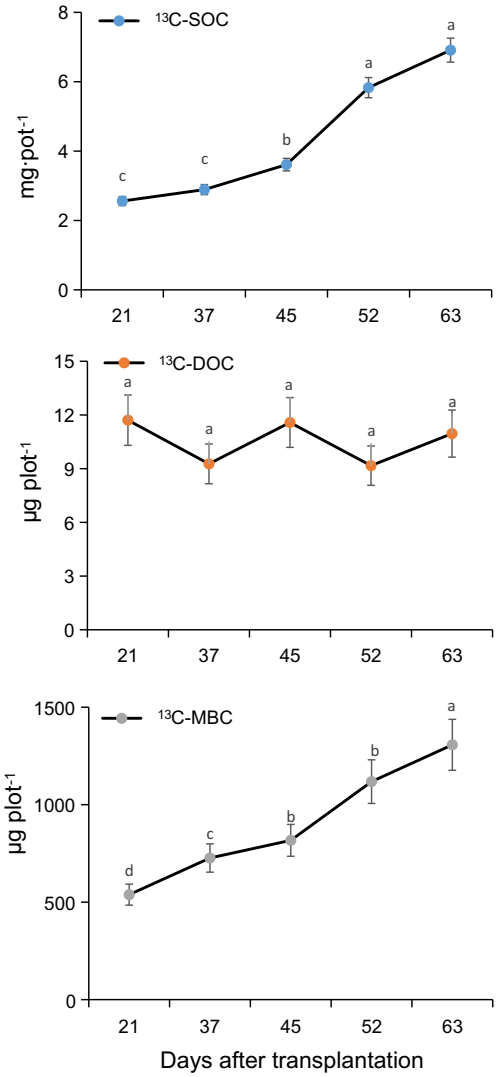

Fig. 2 Incorporation of belowground ^{13}C into soil organic C (*SOC*), dissolved organic C (*DOC*), and microbial biomass C (*MBC*) at five time points posttransplantation

Incorporation of rhizo-C in total PLFAs and microbial groups

Total PLFAs and ^{13}C incorporation into PLFAs ranged from 46.4 to 53.1 mg C kg^{-1} and from 107 to 165 μg C kg^{-1}, respectively (Table 4) and the recovery of ^{13}C-PLFAs for three of the sampling days differed significantly ($P<0.05$; Table 4).

The ^{13}C from root exudates was not evenly distributed among microbial groups, indicating that they differed in C uptake (Fig. 4a). For all three sampling days selected for soil microbial PLFA analysis, more than 40 % of ^{13}C recovered from the PLFAs was incorporated into fungi (18:2w6, 9c, 18:1w9c). Large amounts of ^{13}C were also incorporated into Gram-negative bacteria (16:1w7, 18:1w7c, cy17:0, cy18:0; Fig. 3b), which contained about 15 % of the total ^{13}C-PLFA. The actinomycetes (16:0 10-methyl) and mycorrhizal (AM) fungi (16:1w5c) only accounted for about 1 % of total ^{13}C-PLFAs, and the lowest percentage of ^{13}C-PLFA was incorporated into actinomycetes at day 63 (less than 0.5 %).

To determine the effects of rhizodeposition on microbial composition, PLFA and ^{13}C in PLFA data were subjected to PCA. The first two axes explained 91.3 % of the variance. The composition of rhizodeposit-dependent microbe communities (^{13}C-PLFA) was clearly grouped by sampling date, whereas the overall soil microbial diversity (PLFA) formed a single group (Fig. 5a). Furthermore, RDA results revealed that the soil Fe (II) and Olsen P values were the most significantly correlated with the rhizodeposit-dependent microbial composition ($P=0.002$ by the

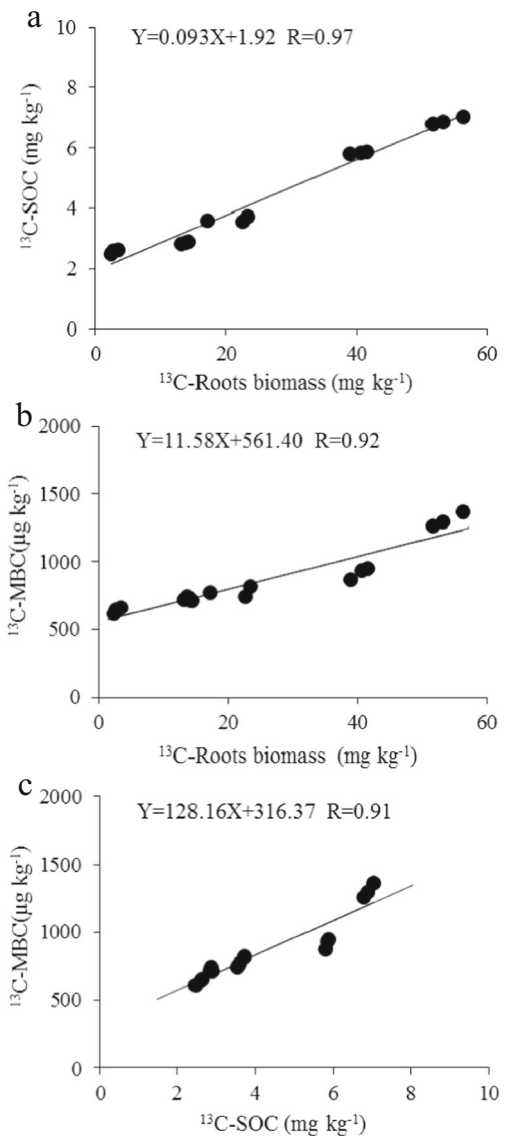

Fig. 3 Relationships between **a** root biomass and soil organic ^{13}C (^{13}C-SOC), **b** root biomass and microbial biomass ^{13}C (^{13}C-MBC), and **c** ^{13}C-SOC and ^{13}C-MBC in paddy soils sampled at five time points during growth of rice plants. Samples were collected within 1 h of ^{13}C pulse labeling of rice plants. *Points* represent individual tested soil means

Monte Carlo permutation test). However, Mn (II) levels were not significantly associated with the rhizodeposit-dependent microbial composition (Fig. 5b).

Discussion

^{13}C allocation in plant soil between growth stages of rice

For the high sensitivity of the PLFA-stable isotope probing (SIP) technique, soil sampling analysis of ^{13}C in microbial groups was performed only 6 h after the start of ^{13}C assimilation in order to investigate the early stages of root exudate utilization by rhizosphere microorganisms (Wiesenberg et al. 2010). Because of the short period, only a small part of the ^{13}C assimilated by the plants was dispersed belowground and incorporated into microbial PLFA. The distribution of photosynthates in plant–soil systems is affected by plant variety and growth stage (Kuzyakov and Cheng 2001; Lu et al. 2002a). In the present study, the total assimilated ^{13}C in this plant–soil system increased with rice age, but the proportion of ^{13}C incorporated into the roots decreased from 12 to 7 %. The highest rate of ^{13}C incorporation into SOC (10 %) occurred in the first growth stage and promoted microbial activity and growth (^{13}C-MBC, 3 %) by providing readily available substrates. The roots of young rice grew relatively quickly and, therefore, were a greater C sink than those of mature rice plants. Moreover, a shorter transport pathway in younger rice decreases the time interval between photoassimilation in leaves and release of root exudates compared to mature plants (Kuzyakov and Gavrichkova 2010; Gavrichkova et al. 2011). Therefore, young roots drew more photosynthates belowground than the roots of maturing rice. This is likely related to the greater C sink activity observed in the roots of younger plants than of older plants (Lu et al. 2002a).

Carbon (^{13}C or ^{14}C) incorporated into SOC and MBC during pulse or continuous labeling is positively correlated with rice biomass measured in greenhouse experiments (Ge et al. 2012). Pausch et al. (2013) successfully estimated the rhizodeposition-C in maize using ^{14}C labeling and suggested that rhizodeposition could be quantified at field scale by assuming a constant ratio of rhizodeposited C to root C. Thus, to estimate rhizodeposition under field conditions, it is necessary to use the linear relationship between rice biomass and ^{13}C-SOC with adjustment of its incorporation into MBC and DOC for the portion respired as CO_2. This will be verified in further studies on upland and paddy soils by examining more soil types, plant species, and depending on environmental conditions (Roth et al. 2013; He et al. 2015).

Incorporation of rhizodeposits into microbial groups at three growth stages of rice

Root exudates diffuse from root surfaces to microorganisms (Darrah 1991; Hafner et al. 2014). These easily available organic substances are utilized by rhizosphere microorganisms within a few minutes (Fischer et al. 2010; Moran-Zuloaga et al. 2015). Other relevant rhizodeposition processes, such as the sloughing off of

Table 3 Pearson correlations between the ^{13}C-photosynthetic products and physicochemical soil properties

	Eh	Available N	Olsen P	Available K	Fe(II)	Mn(II)	CEC	^{13}C-Shoots	^{13}C-Roots	^{13}C-SOC	^{13}C-DOC	^{13}C-MBC
Eh	1											
Available N	−0.456	1										
Olsen P	−0.511	0.883(*)	1									
Available K	−0.431	0.836	0.842	1								
Fe(II)	0.691	−0.768	−0.928[a]	−0.927[a]	1							
Mn(II)	−0.762	0.169	0.44	0.236	−0.524	1						
CEC	−0.06	0.453	0.44	0.636	−0.463	−0.48	1					
^{13}C-Shoots	0.444	−0.756	−0.781	−0.986[b]	0.897[a]	−0.146	−0.728	1				
^{13}C-Roots	0.324	−0.829	−0.774	−0.990[b]	0.868	−0.141	−0.655	0.980[b]	1			
^{13}C-SOC	0.129	−0.882(*)	−0.744	−0.950[a]	0.779	0.002	−0.675	0.911[a]	0.971[b]	1		
^{13}C-DOC	−0.007	0.02	−0.166	0.194	−0.072	0.291	−0.342	−0.152	−0.261	−0.221	1	
^{13}C-MBC	0.114	−0.793	−0.663	−0.929[a]	0.729	0.128	−0.784	.901[a]	0.924[b]	0.912[b]	−0.178	1

[a] Correlation is significant at the 0.01 level (two-tailed)

[b] Correlation is significant at the 0.05 level (two-tailed)

cells or the death of root hairs and fine roots, take much longer than exudation (days, rather than minutes; Kuzyakov and Domanski 2002; Högberg and Read 2006; Paterson et al. 2009) and could not have played a significant role within the 6-h labeling period used in this study. Most of the ^{13}C was recovered from fungal PLFAs (16:0, 18:2w6, or 18:1w9c; PLFAs 18:2w6, 9c, and 18:1w9c) on all three dates used for PLFA analysis (Fig. 4). This is consistent with the pulse labeling results of Wang et al. (2016).

An enormous diversity of fungi can be found worldwide that have varied ecologies and life cycle strategies (Brundrett 2002, De Boer et al. 2005; Amaya-Carpio et al. 2009). Rice plants are continually involved in mycorrhizal associations under upland conditions. However, infection is rare under submerged conditions owing to the anoxic environment. In spite of this, some mycorrhizae belonging to the genus *Glomus* do survive in waterlogged conditions. Moreover, fungi were observed in the rhizosphere soil. Because the rhizosphere is well aerated by rice root parenchyma, fungi are not limited by anoxic conditions common in non-rhizosphere soil. With rice growth, dissolved nutrients in the soil decreased while reducing substances, such as Fe (II) increased. In the rhizosphere, fungal hyphae have very large surface areas, preferentially allowing fungi to mine for nutrients and oxygen (diffusion from rice plant roots) compared to bacteria and Archaea. Further, mycorrhizal associations allow organics to be absorbed directly from roots, before they can be released into the soil. A significant amount of ^{13}C was recovered from fungal PLFAs, which indicates that fungi might play a preeminent role in the utilization of root exudates. Recent studies have shown that fungi also play much more significant roles in C and N cycling under subanoxic soil conditions (Qin et al. 2014; Chen et al. 2015).

Effect of photosynthate inputs on soil microbial community composition

Rhizosphere microbial populations are significantly affected by photosynthate inputs and depend on plant communities and their growth stages (Göttlicher et al. 2006). However, this is not always true since soil properties can be more important than plant effects, particularly if the plant has been

Table 4 Total phospholipid fatty acids (PLFA) and ^{13}C-labeled PLFAs (^{13}C-PLFA)

Days after transplantation	PLFA (mg kg^{-1})	^{13}C-PLFA (μg kg^{-1})	^{13}C-PLFA/PLFA (%)	*Recovery of ^{13}C-PLFA (%)
21	53.1 ± 6.1a	107.1 ± 2.3a	0.10 ± 0.01a	0.45 ± 0.01c
45	46.4 ± 4.2a	149.9 ± 1.6b	0.16 ± 0.01b	0.13 ± 0.01b
63	51.1 ± 0.4a	165.3 ± 5.5b	0.16 ± 0.01b	0.07 ± 0.01a

Recovery of ^{13}C-PLFA is expressed as a percentage of total ^{13}C-PLFA (sum of the ^{13}C in the shoots, roots, and soil). Different letters within each column indicate significant differences. Values are means ± SE (n = 3)

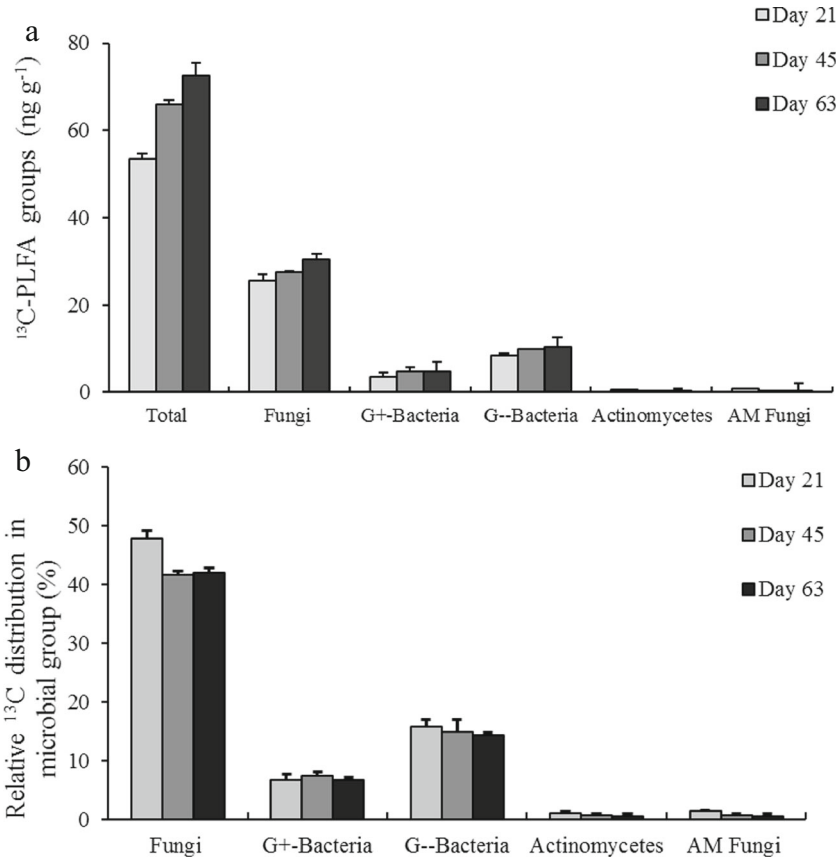

Fig. 4 ^{13}C distribution among microbial groups. **a** ^{13}C-PLFA recovered from soil at three rice growth stages. **b** Percentage of total ^{13}C-PLFA recovered from each microbial group. Days after transplantation are presented on X-axis. *Error bars* represent standard error of means ($n = 3$)

growing in the soil for a few years. Whether soil characteristics or specific plant properties are more important in shaping the bacterial community structure of the rhizosphere has been a topic of considerable debate (De Ridder-Duine et al. 2005; Delmont et al. 2014). Little research has been performed thus far to ascertain the effects of root exudates on the microbial community composition involved in plant-derived C cycling. Our study indicated that the allocation of photosynthesized ^{13}C into the soil affected the composition of the rhizo-coupled microbial community (Fig. 5). The changes in allocation of photosynthesized ^{13}C into the paddy soil ecosystem that occurred with growth stage resulted in obvious variations in the composition of the rhizodeposit-dependent microbial community. However, the whole soil microbial community composition did not change much during the study. Consequently, photosynthesized C inputs more easily affect the rhizo-coupled microbes than the overall microbial community. Among the physicochemical properties measured, multivariate statistical analysis showed that the major influences on the rhizodeposit-dependent microbial community were the soil Fe (II) and Olsen P values coupled with other soil extractable nutrients. However, the greatest influencing factors were changes in the soil Fe (II), Olsen P values, or Eh and ^{13}C-SOC that depended on the growth stage of the rice plant. In this study, the paddy soil pots were irrigated with deionized water, with a 2–3-cm water layer maintained above the soil surface throughout the growing season. Therefore, we suspect that the redox potentials and reduced forms of some elements (e.g., Fe (II)) were mainly affected by the rice roots. In the rhizosphere, organic compounds released from the rice plant root contribute to oxygen consumption mediated by heterotrophs, while atmospheric oxygen is diffused by the roots to lower the concentrations of these toxic-reduced substances. Both of the above processes are highly dependent on rice root age (Nawaz et al. 2012). Rice root uptake of nutrients from the soil solution differs during rice plant growth stages, from germination to harvest, and generally most of the nutrient uptake occurs before flowering (Ramanathan and Krishnamoorthy 1973). Correlation analysis also showed significant dependence between some of nutrients, microbial biomass, and ^{13}C-photosynthetic products (Table 3). Therefore, we expected that the physiological changes within the rice root through the stages of plant growth modify soil physiochemical conditions, in turn affecting the formation of the distinct rhizo-coupled microbial communities.

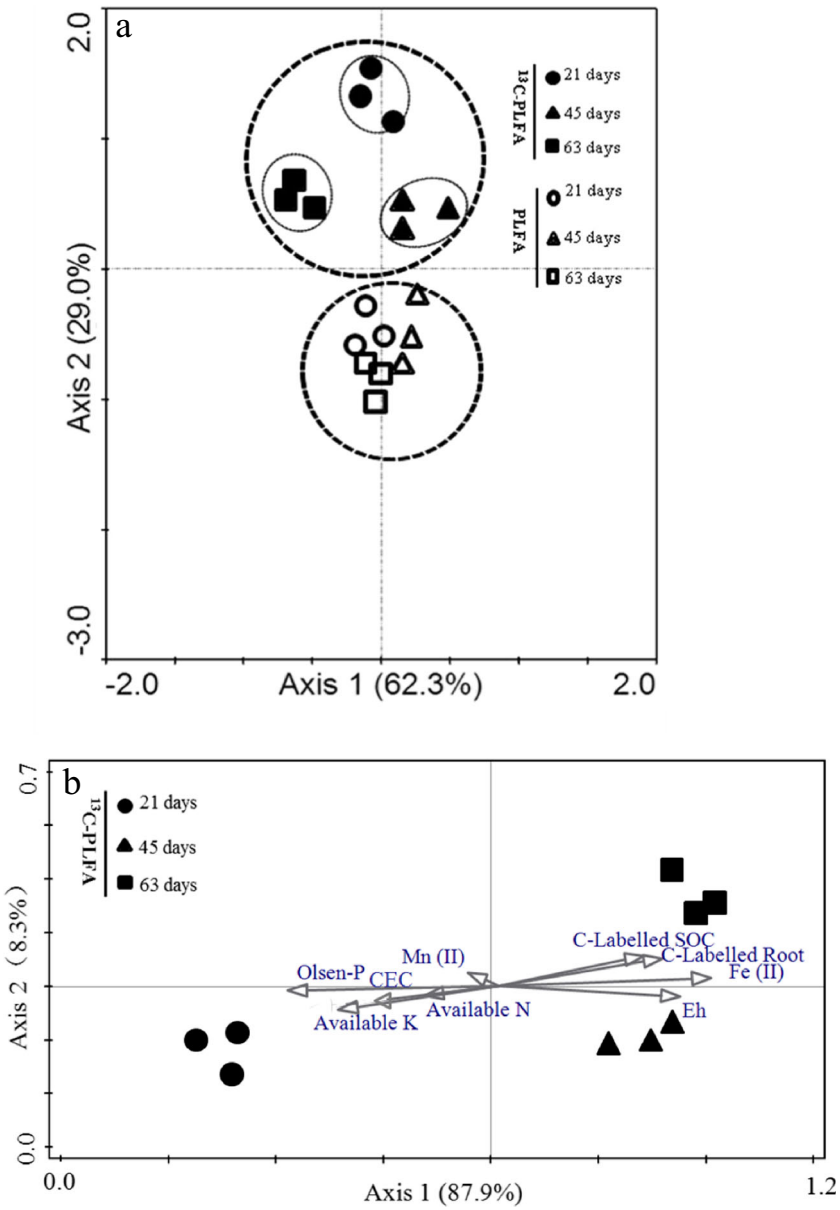

Fig. 5 Principal components (**a**) and redundancy analysis (**b**) of the ^{13}C in phospholipid fatty acids (^{13}C-PLFA) profiles of the rhizosphere microbial composition in the soil at three rice growth stages. *Arrows* indicate the variables. The *direction of an arrow* indicates the steepest increase of the variable, and the *length* indicates the strength relative to other variables

Conclusions

Rice rhizodeposits were incorporated into microbial community already 6 h after assimilation aboveground. Fungi appeared to outcompete bacteria for initial uptake of root exudates and are therefore responsible for the first step of microbial utilization of root-derived C in soil. Rhizodeposition can be well assessed under field conditions based on the root biomass, and therefore, the suggested approach can be used to estimate belowground C input and contributions to C sequestration. ^{13}C-PLFA analysis coupled with ^{13}C pulse labeling was a promising and effective approach for examining microbial dynamics associated with rhizosphere C cycling. Since the poor phylogenetic discrimination of the PLFA technique, the application of this methodology coupled with molecular techniques (RNA-SIP) in further studies will allow clarifying the taxonomy of fungi and other microbes involved in root-derived C cycle. These couplings of isotopic approaches with biomarkers have the potential to greatly enhance our knowledge of rhizosphere processes by focusing on the members actively involved in the cycling of C and nutrients within complex soil system.

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