Impact of carbon and nitrogen application in paddy-soil ecosystem: $^{13,14}C$ labeling, zymography, pH mapping and PLFA

Dissertation

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Contents

Summary

Rice (*Oryza sativa* L.), a major cereal crop, is cultivated on more than 140 million hectares worldwide. Consequently, rice growing paddy fields are major consumers of nitrogen (N) fertilizer and the biggest users of agricultural water. Compared to other non-flooded agricultural lands, paddy soils contain 12%–58% higher soil organic matter (SOM) content, which makes them an important carbon (C) sink. Rice straw retention, nitrogen fertilizer application and over-flooded water are the three most important factors responsible for the higher C sink in paddy fields. Together, they have great impact on rice-soil ecosystems. This thesis therefore presents three studies within the confines of these key factors.

The two studies (Chapter 2 and 3) were designed to reveal: 1) the effects of N fertilization, and 2) the long term effects of rice straw retention on the distribution of photosynthates in varies soil C pools. Soil C and N availed by N fertilizer application and rice straw retention affect microbial composition and activities, resulting in altered SOM decomposition and plant assimilates allocation. Experiments conducted focused on the interactions between C and N availabilities and the consequent effects on rhizodeposition and microbial community in paddy soil. Using carboxymethyl cellulose (CMC) as long term rice straw decomposition mimic, treatments: CMC (+C), $(NH_4)_2SO_4$ (+N), their combination (+CN), and unfertilized soil (control) were designed. Rice were continuously labeled with $^{13}CO_2$ and the tracer (^{13}C)incorporated into both above- and belowground plant biomass, SOM, dissolved organic matter (DOC), microbial biomass (MBC), and phospholipid fatty acids (PLFAs) was quantified.

The long term degradation of rice straw as mimicked by single CMC application (+C) led to mobilization of a 3% of total N from SOM and a positive N priming effect. This finding supported the microbial N mining hypothesis. The highest rice yield increase occurred in +CN treatment despite smallest root biomass and lowest

assimilation of ^{13}C into roots, DOC, SOM, and MBC. Additionally, +CN altered microbial community composition. Specifically, +CN application decreased 1) Gram-positive (G+)/ gram-negative (G-) ratios, and 2) G+ bacteria and fungi abundance. Contrary, G- and actinomycetes were stimulated by N fertilization. Fertilization and plant growth stage are the two factors that explained 81% of the variance in the microbial communities. Fertilization was responsible for 36.5% of the variance in the composition of microorganisms.

Flooding, as another key factor in paddy field, creates anaerobic conditions, which changed root morphology and soil physiochemical properties such as root iron plaque, rhzodepositions and pH. Since the impact of flooding on paddy fields remain unknown, we introduced triple combination of ^{14}C imaging, pH mapping and zymography for the first time in paddy soils (Chapter 4). This combination enabled the water effects from root iron plaque, rhzodepositions and pH on five enzyme activities involved in carbon (C) (β-glucosidase, cellobiohydrolase， xylanase), nitrogen (N) (leucine aminopeptidase), and phosphorus (P) (phosphatase) cycling to be evaluated. Varying the H_2O content from <25% to oversaturation, we confirmed the hypotheses: 1) flooding increases root biomass but decreases water use efficiency; 2) flooding increases rhzodeposition (^{14}C) but decreases pH in both rhizosphere and bulk soil; 3) flooding is the dominant factor determining the spatial distribution patterns of enzyme activities. Through diffusion effects, flooding evenly distributed enzyme substrates. Through 3D mesh and contour map, we simultaneously evaluated the correlations of enzymes involved in C, N and P cycling successfully. The cancelling effect of flooding resulted in loss of several optimal combination peaks of C, N and P related enzymes through diffusion. This flooding effect ultimately narrowed the optimal combination area. Concluding, water effects improved formation of root iron plaque, increased rhzodepositions and decreased pH.

This PhD thesis therefore introduced new concepts such as cancelling effects and developed new triple combination of enzyme zymography, ^{14}C imaging and pH mapping approach. The study improved the understanding on how the three key factors: 1) rice straw retention, 2) N fertilization, and 3) flooding impact the rice-soil

ecosystem, and enabled further guidance on countering the challenges brought about global climate change.

Zusammenfassung

Wasserreis (Oryza sativa l.) ist eine wichtige Getreidepflanze, die weltweit auf mehr als 140 Millionen Hektar wächst. Das Reisfeld ist ein wichtiger Verbraucher von Stickstoffdünger und der größte Wasserverbraucher in der Landwirtschaft. Sein Gehalt an organischer Substanz im Boden ist 12-58% höher als der anderer landwirtschaftlicher Nutzflächen, und er ist eine wichtige Kohlenstoffsenke. Strohmulchen, Stickstoffausbringung und Überflutung sind drei Schlüsselfaktoren, die den Reisertrag beeinflussen. Sie haben wichtige Auswirkungen auf das Reis-Boden-Ökosystem. In diesem Papier werden drei Schlüsselfaktoren untersucht.

Die erste und zweite Studie (Kapitel 2 und 3) zielten darauf ab, die Auswirkungen von Stickstoffdünger und langfristigem Strohmulchen aufzudecken. Die Verfügbarkeit von Bodenkohlenstoff und Stickstoff (Stickstoffdünger und Strohmulchen) beeinflusst die Zusammensetzung und Aktivität der Mikroorganismen, was zur Zersetzung der organischen Bodensubstanz (SOM) und zur Verteilung der pflanzlichen Photosyntheseprodukte führt. In den Kapiteln 2 und 3 wurde die Wechselwirkung zwischen Kohlenstoff- und Stickstoffverfügbarkeit, Wurzeldeposition und mikrobieller Gemeinschaft in Rohböden untersucht. Carboxymethylcellulose (CMC) (+C) wurde verwendet, um die langfristige Zersetzung von Stroh zu simulieren. Es wurden vier Behandlungen durchgeführt: Carboxymethylcellulose (CMC) (+C), (NH4)2SO4 (+N), die gleichzeitige Zugabe von beiden (+CN) und die Kontrolle ohne Düngung. Wasserreis wurde kontinuierlich $^{13}CO_2$-markiert. Gleichzeitig wurden die ^{13}C-Gehalte in oberirdischer und unterirdischer Pflanzenbiomasse, organischer Substanz, gelöster organischer Substanz (DOC), mikrobieller Biomasse (MBC) und Phospholipid-Fettsäuren (PLFAs) verfolgt.

Die Ergebnisse in Kapitel 2 und Kapitel 3 zeigen, dass als Indikator für den langfristigen Strohabbau die einmalige Ausbringung von Kohlenstoffdünger (CMC) den Anregungseffekt von Stickstoff erzeugt, d.h. 3% Stickstoff im Boden werden

durch Mikroorganismen aufgrund des Strohmulchens aus SOM freigesetzt. Dieser Befund unterstützt die Hypothese von microbial N mining. Die gleichzeitige Anwendung von +CN maximierte den Reisertrag, führte aber auch zu einer minimalen Wurzelbiomasse und reduzierte den ^{13}C-Gehalt im Wurzelsystem, DOC, SOM und MBC. In Bezug auf die Struktur der mikrobiellen Gemeinschaft reduzierte die +CN-Düngung das Verhältnis von Gram-positive (G+)/Gram-negative (G-), was zu einer Abnahme der Häufigkeit von G+-Bakterien und -Pilzen führte, während die Häufigkeit von G- und Aktinomyzeten durch die Anwendung von Stickstoffdünger stimuliert wurde. Düngung und Pflanzenwachstum erklärten 81% der Variation in der mikrobiellen Gemeinschaft. Unter ihnen hingen 36.5% der mikrobiellen Variation mit der Düngung zusammen.

Überschwemmungen, ein weiterer Schlüsselfaktor in Reisfeldern, erzeugen anaerobe Bedingungen, die die Wurzelmorphologie und die physikalisch-chemischen Eigenschaften des Bodens verändern, wie z.B. die Eisenmembran der Wurzeln, die photosynthetische Kohlenstoffsekretion und den pH-Wert. In der dritten Studie stellten wir zum ersten Mal die Kombination von 14C-Bildgebung, In-situ-pH-Bildgebung und Enzymspektrum im Reiserde vor. Damit ist es möglich, die Auswirkungen von Staunässe auf den Eisenfilm von Pflanzenwurzeln, die photosynthetische Kohlenstoffabscheidung und den pH-Wert der Rhizosphäre zu bewerten. Fünf Enzyme sind an der Studie beteiligt: β-Glucosidase, Fibrinosaccharid-Hydrolase, Xylanase, Leucin-Aminopeptidase und Phosphatase. Durch den Vergleich des Wassergehalts von 25% mit dem der Staunässebehandlung bestätigten wir die folgende Hypothese :1) Staunässe erhöht die Wurzelbiomasse, verringerte aber die Effizienz der Wassernutzung. 2) Staunässe erhöht die photosynthetische Kohlenstoffablagerung in der Rhizosphäre und Nicht-Rhizosphäre und senkt den pH-Wert. 3) Staunässe ist der dominierende Faktor bei der Bestimmung des räumlichen Verteilungsmusters der Enzymaktivität. Durch den Diffusionseffekt des Wassers wird das Substrat des Enzyms gleichmäßiger verteilt. Wir evaluierten erfolgreich die Korrelation zwischen den Enzymen, die an den C-, N- und P-Zyklen beteiligt sind, unter Verwendung eines dreidimensionalen Gitters und einer

Konturkarte. Das Fluten durch Diffusion eliminiert mehrere optimale Kombinationspeaks von C-, N- und P-verwandten Enzymen (Offsetting-Effekt). Die Überflutung reduzierte schließlich die Fläche des optimalen Kombinationspeaks. Die Ergebnisse zeigten, dass das Wasser die Bildung der Eisenmembran der Wurzeln und die Akkumulation der photosynthetischen Kohlenstoffablagerung förderte und auch den pH-Wert des Bodens senkte.

Daher werden in diesem Papier neue Konzepte (wie die ausgleichende Wirkung von Wasser) vorgeschlagen und neue Methoden entwickelt (die dreifache Kombination von Enzymspektrum, ^{14}C-Bildgebung und In-situ-pH-Bildgebung). Gleichzeitig zeigt diese Studie die Auswirkungen von drei Schlüsselfaktoren der Reisstroh-Erhaltung, der Stickstoffdüngung und die Staunässe auf das Reisboden-Ökosystem auf und liefert damit eine Anleitung zur Bewältigung der Herausforderungen, die der globale Klimawandel mit sich bringt.

List of figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Abbreviations

C	Carbon
N	Nitrogen
DOC	Dissolved organic matter
SOC	Soil organic carbon
SOM	Soil organic matter
MBC	Microbial biomass carbon
MBN	Microbial biomass nitrogen
PLFA	Phosphor lipid fatty acid
ANOVA	Analysis of variance
CMC	Carboxymethyl cellulose

Chapter 1

General introduction

1.1 Importance of paddy soils

Mitigating global climate change and achieving sustainable use of natural resources are the most serious challenges facing the world nowadays (Zhang and Wen, 2008). The carbon cycle of soil-plant ecosystem has arisen great research interests. It plays an important role in regulating the change of atmospheric greenhouse gas emission, in affecting the system stability and productivity, and in maintaining soil fertility and sustainable agricultural development (Smith et al., 2007).

Carbon (C) in soil organic matter (SOM) is three times higher than that in the atmosphere (Fischlin et al., 2007). The composition and transformation of SOM affect the maintenance of soil fertility, the stability of ecosystem, the sustainable development of agriculture, and the regulation of greenhouse gas emission (Swift et al., 2004).

As a major cereal crop (Smith and Dilday, 2002), rice (Oryza sativa L.) production exceeded 506 million metric tons annually. Paddy soil, as hydragric anthrosols, is cultivated on more than 140 million hectares worldwide (Rice Statistics database; IRRI, 2018). Compared with other agricultural soils, paddy soil has a 12%–58% higher SOM content (Liping and Erda, 2001). Thus, with large cultivation areas and higher SOM content, paddy soil is of great importance when researching mitigation of global climate change and the stabilization of sustainable natural resources(Liu et al., 2006).

With higher SOM content in paddy fields, researches regarding to SOM cycle in paddy soil mainly focused on three aspects: (1) the dynamics and carbon sequestration of soil organic carbon (SOC, Zhang et al., 2007); (2) the mechanisms of carbon sequestration affected by biophysical factors, such as the increasing C input derived from straws and different fertilization management (Zhang et al., 2007); (3) SOC dynamics related to composition and biodiversity of microorganisms (Bambaradeniya and Amerasinghe, 2003). For example, it was preliminarily identified that the microorganisms in the rhizosphere, was a key factor in regulating the accumulation of new carbon (photosynthate) (Schimel and Schaeffer, 2012).

Related to those three aspects mentioned above, rice straw retention, nitrogen fertilizer applications and over-flooded water are the most important features in paddy fields. Hence, quantifying the impact on paddy fields (higher amount of SOM, microbial community etc.) under those three factors is of great importance for gaining a better understanding of future researches.

1.2 Key factors in paddy fields

1.2.1 Rice straw retention

More than 506 million metric tons (FAO, 2018) of rice were generated annually. For each ton of rice grain harvested, 1.35 tons of straw remained in the field (Kadam et al., 2000). The disposal of such large amount of straws (740 to 1111 million tons per year, Abraham et al., 2016) can be problematic.

With low digestibility, low protein and high lignin contents (Kausar et al., 2011), rice straw is not an optimal source for livestock fodder. Thus, straws were typically burnt in the field, which can cause air and water pollution, contamination, and greenhouse gas emissions (Gadde et al., 2009; Qu et al., 2012). The retention of rice straw in paddy fields has become an increasingly prevalent practice that can facilitate improvements in soil fertility, physical and chemical properties (Mahmoud et al., 2009), and enhance crop yield (Fusi et al., 2014; Kanchikerimath and Singh, 2001; Wang et al., 2015).

Rice straw consists of 32% cellulose, 24% hemicelluloses, and 18% lignin (Howard et al., 2003), and these constituents are strongly intermeshed and chemically bonded by non-covalent forces and covalent cross-linkages (Pérez et al., 2002) to form a lignocellulosic matrix structure (Fig. 1).

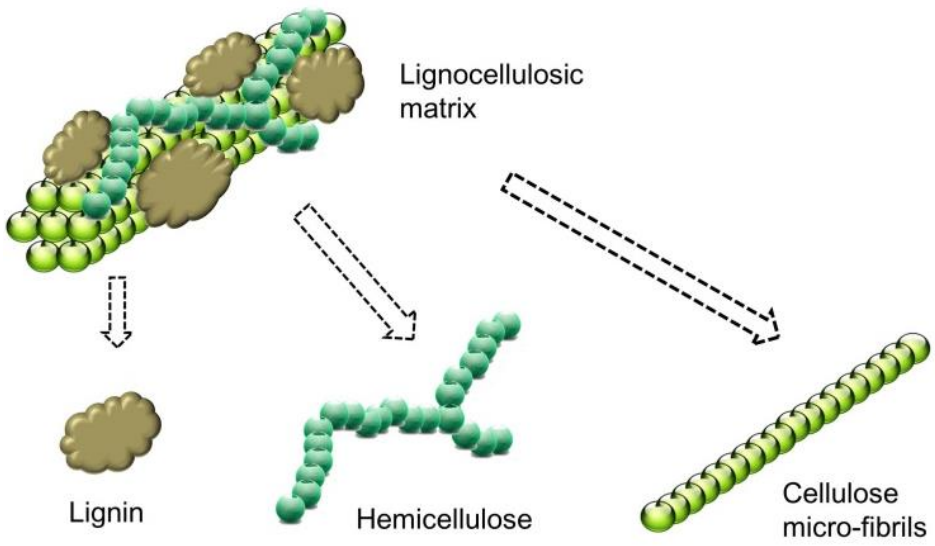

Fig.1 Structure of rice straw framework, lignocellulosic matrix of cellulose, hemicellulose and lignin

Cellulose is the main structure in lignocellulosic matrix, and combined by D-glucose through β-1,4 glucosidic bond (Sánchez, 2009). The molecular formula of the straight cellulose polymer chain is $(C_6H_{10}0_5)_n$, where n is the number of glucose. The n ranges between 8000 ~ 10000 or even greater. This structure makes the cellulose with macromolecules. Hemicellulose are also macromolecules with a lower molecular weight than cellulose. It is formed from D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, 4-O-methyl-glucuronic, D-galacturo- nic and D-glucuronic acids, through β-1,4- and sometimes by β-1,3-glycosidic bonds(Pérez et al., 2002). Lignin, which linked to both hemicellulose and cellulose (Bugg et al., 2011), formed a physical seal as an impenetrable barrier, to give resistance against microbial attack and oxidative stress(Martínez et al., 2005). With those large macromolecules of cellulose, hemicellulose, and the protection from lignin, it is hard for microorganisms to penetrate and degrade (Fig. 2).

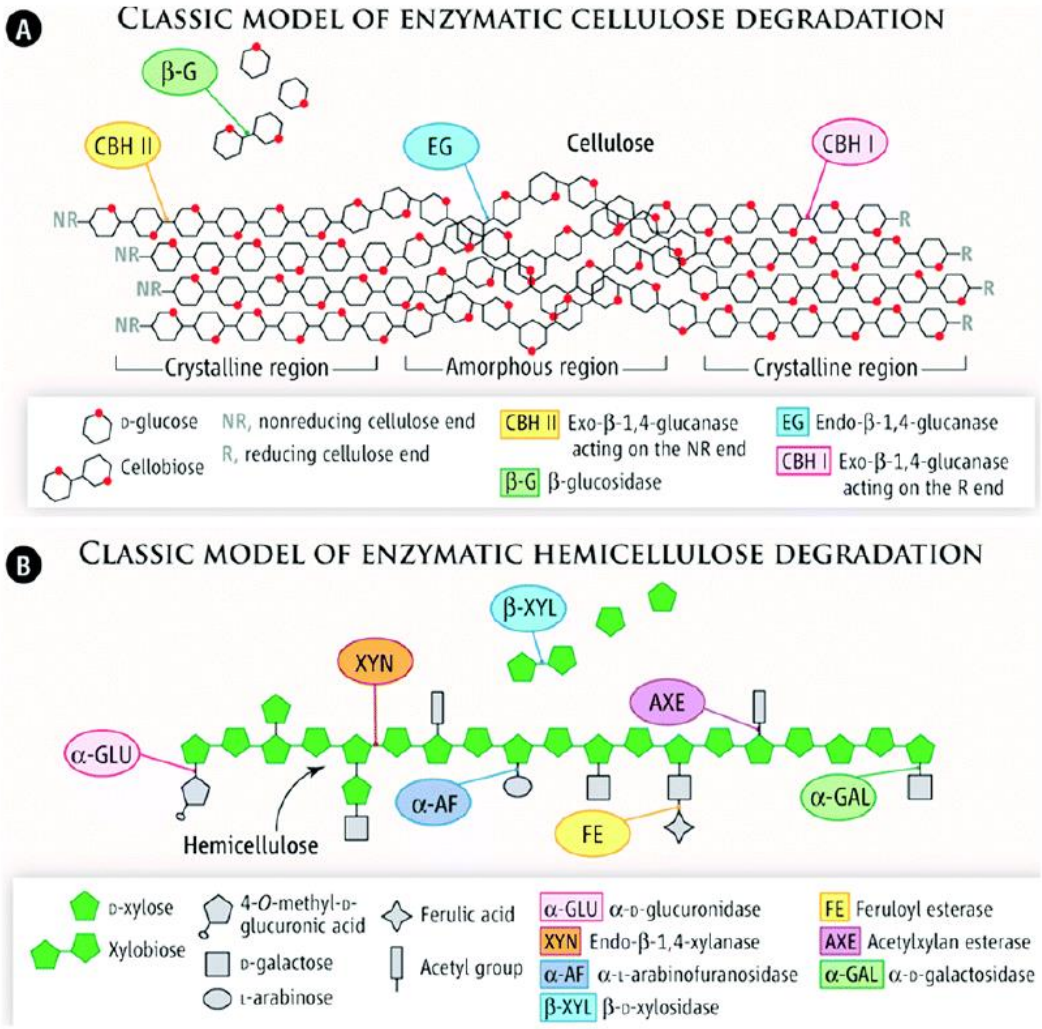

Fig.2 Classic models of composition and enzymatic degradation of cellulose and hemicellulose (Alves et al., 2017)

Thus, rice straw is typically slow to degrade (Kausar et al., 2011) with slow decomposition rate, especially under anaerobic environment created by over-flooding in paddy fields (Dev êvre and Horw áth, 2000). Therefore, evaluating the effects of rice straw retention on soil C pools (rhizodeposition, SOM etc.) requires long-term studies with continuous or pulse ^{13}C or ^{14}C labelling (Kuzyakov, 2001). However, those labeling are generally conducted over short periods of weeks or months.

By adding carboxymethyl cellulose (CMC) to mimic the cellulose (the major component of rice straw, Howard et al., 2003) with continuous ^{13}C labeling in present studies (chapter 2 and 3), it facilitates to research the effects of rice straw retention on

rice-soil ecosystem in a relatively short term (39days).

1.2.2 Nitrogen fertilizer application in paddy field

Rice planted area in China accounts for 19% of the world's total paddy fields (Peng et al., 2009), yet the grain yields reaches for more than 29% of the world's total rice yield (Liu et al., 2011). However, this success achieved at the expense of large amounts of fertilizer application, especially N addition. China's nitrogen fertilizer consumption accounts for 30% of the world's total N addition, which makes China the world's largest N fertilizer consumer (Liu et al., 2011). Since the 1980s, China has been consuming a staggering 35% of the world's N on 7% of the world's arable land (Peng et al., 2006). China's annual grain production increased by 76% from 1981 (325 million tons, Walker, 1984) to 2011 (571 million tons, Li et al., 2014), while the consumption of N fertilizer nearly tripled. At present, the average application rate of N fertilizer in paddy field in China is 180 kg per hm^2, 75% higher than that in the world (Peng et al., 2002). N application in some high-yielding rice fields in China ranges from 270 to 300 kg per hm^2, and some even reach 450 kg per hm^2, and such trend is still increasing (Xuejun and Fusuo, 2011).

At present, the absorption rate of N fertilizer in China is only 30 - 45% of the total N applied in the soil, in some regions, it is even less than 30% (Zhang et al., 2008). Whereas, the N absorption rate in paddy fields in other countries is as high as 50% ~ 60% (Liu et al., 2013). Thus, large loss of N fertilizer in China occurs and may cause a decrease in economic benefit in that aspect.

After N application, rice plant absorb 25% ~ 50%, 10% ~ 35% of N remain in the soil as residual, the rest of the fertilizer is lost through ammonia volatilization, nitrification, de-nitrification and nitrate leaching (Ju et al., 2009; Zhang et al., 2008).

The root is the main absorbing organ. N from the soil and N applied into the rice field can only be absorbed by the root system into the rice plant (Yoneyama, 1950). The vertical distribution characteristics of rice roots are closely related to N

absorption. The development of finely branched roots is an important way to improve N use efficiency.

N in rice leaves is closely related to photosynthesis, and N in chloroplasts accounts for 80% of total leave N (Imai et al., 2008). Photosynthates are the only source of organic matter in crops, about 90% of the crop yield is derived from photosynthetic product. With the decrease of N metabolism, photosynthesis also decreases, as N and C metabolisms are tightly linked (Shand, 2007). Former researches mainly focused on the rice genotypes with high N utilization efficiency, high photosynthetic rate per unit of nitrogen or chlorophyll. The effects of such large amount of N application on the distributions of photosynthates in varies soil C pools, and on the microbial communities are scarcely studied. By combining N addition, continuous ^{13}C labeling and ^{13}C-PLFA in this thesis (chapter 2 and 3), those questions are well answered.

1.2.3 Over-flooded water in paddy field

Rice is a typical semi-aquatic plant with strong demand of water (Parent et al., 2010). Paddy field has the largest grain crop planting area in China, it consumes about 54% of the total water consumption, and accounts for over 65% of the total agricultural water consumption (Sun et al., 2017).

Once the paddy field is flooded, the tillage layer saturated with water, air is removed with gas exchange blocked, and the oxygen content drops sharply (Nishiuchi et al., 2012). Rice roots exhaled carbon dioxide and other gases, which were accumulated, and making the soil in a reductive state. Consequently, nitrogen and nitrate were reduced (Hasebe et al., 1987), followed with the reduction of manganese and iron nitrides (Mandal, 1961). And finally, the reduction of sulfate and the formation of methane (Dalsgaard and Bak, 1994; Wang et al., 1997). The whole reduction process can be divided into two stages: the first stage is the decomposition of organic matter, mainly conducted by both the aerobic and anaerobic microorganisms (Peters and Conrad, 1996). In the second stage, with the decrease of

redox potential, the decomposition of organic matter was dominated by obligate anaerobic microorganisms (Trolldenier, 1977).

The length of the first reduction stage depends on the ratio of readily decomposable organic matter content to iron oxide content in the soil. In soils with high iron oxide content, the first stage was prolonged. However, the soil with high content of organic matter that is easy to decompose has a short duration in the first stage and will soon enter into the reduction process in the second stage (Takai and Kamura, 1966).

Reductive state in paddy soil is helpful for rice nutrients absorption. For example, in the reduction state, inorganic nitrogen almost exists in the form of ammonium nitrogen, which is beneficial to the absorption and utilization of rice (Kirk, 2003). In addition, under the reduction condition, the solubility of phosphorus, iron, manganese and other elements can be improved for rice absorption and utilization (Patrick and Mahapatra, 1968). Iron phosphate is difficult to dissolve in water, in the reduction condition, iron phosphate is reduced to a more soluble ferrous phosphate. Therefore, the viscous alluvial soil with developed reduction layer has more available phosphorus, while the sandy soil, with strong seepage, strong acid and more active iron and aluminum, has less available phosphorus (Manzoor Alam, 1999).

However, when the reduction state is too strong, the content of ferrous iron, organic acid and hydrogen sulfide are excessive produced, which will have a toxic effect on rice roots and inhibit the absorption of water, phosphorus, potassium, calcium and other substances in rice roots (Becker and Asch, 2005). Redox potential in paddy soil is influenced by many factors (Flessa and Fischer, 1992; Gao et al., 2002; Jiao et al., 2006).

The traditional rice cultivation is to maintain the flooded water layer. The water lost through natural evaporation accounts for about 80% of the irrigation water in the rice field (Li, 2001). This kind of management not only wastes a large amount of water, but also hinders the economical water utilization and affects the development of rice production potentially. Thus, Since the 1990s, measures of rice water-saving irrigation have been popularized and applied in various places (Zhang et al., 2004). However, those un-flooded and flooded cultivation methods may have strong impact on soil pH,

rice roots, photosynthates released from roots, and enzyme activities from both roots and microorganisms. Those impacts can be revealed by combining C pulse labeling, pH mapping and zymography in this thesis (chapter 4).

Rice straw retention, N fertilization, and flooding are key factors responsible for higher C sink in paddy fields. The first and second studies (chapter 2&3) focused on the impact of N fertilization and long term rice straw degradation on paddy fields. Considering the low degradation rate of rice straw and the large amount of N fertilizer applied to these soils, this study aimed to assess: 1) how the mineral N and organic C fertilization affects the distribution and dynamics of photosynthesis-derived C in paddy soils during five stages of rice growth, 2) the effect of mineral N and organic C addition on the composition of soil microbial communities, and 3) if N priming effects can be triggered by organic C application. The hypotheses tested were: 1) long-term rice straw degradation increases the photosynthate distribution and recovery in the rice–soil system, 2) different C and N fertilization management affects the recovery and partitioning of photosynthate in plants and various soil C pools (highest in +CN, and lower in +C and +N addition), and 3) different C and N fertilization management affects the structures of soil microbial communities utilizing photosynthates.

When compared with non-flooded soils, frequent flooding in paddy fields results in relatively lower pH in rhizosphere and bulk soils. Being main factor in paddy fields, pH affects structures of microbial communities and photosynthates distribution. Gradients around roots are expected to be extremely different in flooded soils. The third study (chapter 4) was designed to 1) reveal the *in situ* pH by contrasting flooded and non-flooded paddy soils in presence of rice root, 2) evaluate rhizodepositions in flooded and non-flooded soils and the effects of hotspots on enzyme activities through ^{14}C labeling, and 3) describe the quantity and spatial distribution patterns of enzyme activities in paddy soils under the influence of pH and rhizodepositions (^{14}C) through zymography. We hypothesized that 1) flooding lowers pH in rhizosphere and bulk soil, 2) flooding leads to higher root biomass with resultant increase in rhizodeposition (^{14}C) and stimulation of enzyme exudation from both roots and microorganisms, and

3) flooding results in high degree of enzyme diffusion, leading to moderate spatial distribution patterns of enzyme activities.

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Chapter 2

Carbon and nitrogen availability in paddy soil affects rice photosynthate allocation, microbial community composition, and priming: Combining continuous ^{13}C labeling with PLFA analysis

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2.1 Abstract

Background and Aims Carbon (C) and nitrogen (N) availability in soil change microbial community composition and activity and so, might affect soil organic matter (SOM) decomposition as well as allocation of plant assimilates. The study was focused on interactions between C and N availability and consequences for rhizodeposition and microbial community structure in paddy soil.

Methods Rice continuously labeled in a $^{13}CO_2$ atmosphere was fertilized with either carboxymethyl cellulose (CMC) (+C), ammonium sulfate (+N), or their combination (+CN), and unfertilized soil was used as a control. ^{13}C was traced in aboveground and belowground plant biomass, soil organic matter, and microbial biomass. Microbial community composition was analyzed by phospholipid fatty acids (PLFAs).

Results +CN application led to a higher yield and lower root C and N content: ^{13}C assimilated in shoots increased for 1.39-fold and that in roots decreased for 0.75-fold. Correspondingly, after +CN addition, ^{13}C from rhizodeposits incorporated into SOM and microorganisms decreased by 0.68-fold and 0.53-fold, respectively, as compared with that in the unfertilized soil. The application of +C or +N alone resulted in smaller changes. CMC led to a 3% of total N mobilized from SOM and resulted in a positive priming effect. Both fertilizations (+C, +N, or +CN) and plant growth stages affected soil microbial community composition. With decreasing microbial biomass C and N, and PLFA content under +CN amendment, +CN fertilization decreased Gram-positive (G+)/ gram-negative (G-) ratios, and resulted in lower G+ bacteria and fungi abundance, whereas G- and actinomycetes were stimulated by N fertilization.

Conclusions Organic C fertilization led to N positive priming effect. Organic C and mineral N application decreased C input by rhizodeposition followed with lower ^{13}C recovery in SOM and microbial incorporation. C and N addition also altered microbial community composition, as +CN decreased content of microbial groups, such as G+ bacteria and fungi, yet, +N stimulated G- bacteria and actinomycetes.

Keywords: GC-IRMS; Continuous $^{13}CO_2$ labeling; Belowground photosynthate

allocation; Rice rhizodeposition; N priming effect; phospholipid fatty acid analysis

2.2 Introduction

Photosynthesized carbon (C) released from plant roots (a process known as rhizodeposition) is an important C source in the soil, which serves as a C and energy source for microorganisms (Curl and Truelove 1986; Lynch and Whipps 1990). Studies quantifying rhizodeposits in cropland soils have shown that 10% of net photosynthesized C is allocated to the roots, and in this 10% of photosynthesized C allocated to roots, 70% of them enters the soil (Pausch and Kuzyakov 2018). Compared with upland soils, flooded paddy soil creates specific conditions conducive to the accumulation of photosynthates as well as for soil organic matter (SOM) stabilization. This conditions include considerably reducing oxygen content and suppressing the activity of oxidizing enzymes, resulting in slower mineralization of new organic C inputs and old organic matter (Freeman et al. 2001; Kemmitt et al. 2008; Wei et. al., 2017). Additionally, active iron oxides in paddy soils increase the stabilization of root-derived organic matter via complexation and co-precipitation (Pan et al. 2003). Therefore, SOM content in paddies is 12%–58% higher than that in corresponding upland soils (Liping and Erda 2001), and thus it can serve as an important C sink for mitigating the effects of global climate change (West and Marland 2002; Xie et al. 2007). The amount of photosynthates entering the soil is affected by a number of factors, including light intensity (Kuzyakov and Cheng 2001), temperature (Bhattacharyya et al. 2013), CO_2 concentration (Van Ginkel et al. 2000), soil and air moisture (Tian et al. 2013), and nutritional status (Carvalhais et al. 2011), as well as plant variety and growth stage. For paddy soils, Ge et al. (2015) found that the amount of photosynthesis-derived C converted into SOM proportionately increased with N fertilization and rice root biomass.

Organic amendments (e.g., rice straw, plant residue, and manure) are frequently applied to paddy soils (Mikha and Rice 2004; Pan et al. 2009; Li et al. 2010), and this also changes root growth, rhizodeposition (Liu et al. 2013b, a), and SOM accumulation (Dong et al. 2012). Through physical and chemical associations with

hemicellulose and lignin, cellulose in manure forms a lignocellulosic matrix structure. This lignocellulosic matrix, prevents the decomposition and constitutes 22%–34% of cow and swine manure (Sarko 1986; Himmelstein 1991; O'Sullivan 1997; Sun and Cheng 2002; Liao et al. 2008). Cellulose is the most abundant compound (30%–50% of plant dry weight) in plant residues. Thus, cellulose serves as an important C source for soil microorganisms. Owing to its polymeric molecular structure, three enzymes (endo-β-1,4-glucanase, cellobiohydrolase, and β-D-glucosidase) (Pérez et al. 2002; Sun and Cheng 2002) are required for degrading cellulose to soluble glucose that is accessible to microorganisms. A soluble analog of cellulose – carboxymethyl cellulose (CMC), with similar molecular structure, is more readily utilized by microbes, as only one enzyme (endoglucanase) is needed for its degradation (Robson and Chambliss 1989). Thus, CMC can be used to mimic the microbial utilization of macromolecular organic C compounds, where the effects of organic amendments (straw, plant residues, and manure) on plant growth stages and microorganisms are of interest. Plant growth stages has strong effects on the distribution of photosynthates (Kuzyakov 2002). Keith et al. (1986) showed that approximately 50% of the photosynthates was transferred to the soil from young wheat, but only 3% was transferred from mature wheat. However, how the simultaneous application of mineral N and organic fertilizers affects the dynamics of photosynthesis-derived C and the fate of these nutrients in paddy soils during plant growth stages is unknown until date.

The rhizosphere is the primary region in soil, wherein plants interact with microorganisms via rhizodeposits, and the rhizosphere has a complex composition, creating a high degree of spatial heterogeneity (Hinsinger et al. 2009; Kuzyakov and Blagodatskaya 2015). Rhizodeposits affect the composition and functioning of microbial communities (Paterson et al. 2007), leading to the development of fast-growing species, mainly G- bacteria (Dippold et al. 2014), which are capable of utilizing large amount of low-molecular-weight organic compounds as well as complex substances (Watt 2009). The abundance and activity of microorganisms in

the rhizosphere are strongly correlated with the amount of rhizodeposits and their composition as well as with environmental factors (e.g., temperature, moisture, O_2 concentration, nutrient content). Another factor that considerably affects the composition of microorganisms in the rhizosphere is the presence of mineral and organic fertilizers. Mineral N application increases the proportion of phospholipid fatty acids (PLFAs) of actinomycetes in soil (Zhang et al. 2007). In contrast, higher abundance of actinomycetes has also been reported in unfertilized soils (Clegg 2003). A previous study also found that organic fertilizers increase specifically fungal and actinomycete abundance (Dong et al. 2014b). However, the effects of organic C and mineral N on microbial community structure in paddy soils, especially in the presence of living plants, remain unknown.

The aim of the study was to answer the following questions: (i) How does mineral N and organic C fertilization affect the distribution and dynamics of photosynthesis-derived C in paddy soils during five stages of rice growth? (ii) What's the effect of mineral N and organic C addition on the composition of soil microbial communities? (iii) Can N priming effects be triggered by organic C application?

2.3 Materials and methods

2.3.1Experimental design

A typical paddy soil (plowing Anthrosol) developed from granitic red soil was collected from the plow layer (0–20 cm) of a rice field located in the Changsha Research Station for Agricultural and Environmental Monitoring, Hunan Province, China (28°33′04″N, 113°19′52″E, 80 m a.s.l.). The paddy field had been annually rice-fallow-rice cropped for over 30 years. The climate is typically subtropical and the area has an annual mean temperature of 17.5 ℃ and annual rainfall of 1300 mm. Soil chemical and physical properties are a pH of 5.56 (1:2.5, soil: water ratio), 20.6 g C kg^{-1} soil, 2.6 g N kg^{-1} soil, 0.45 g phosphorus kg^{-1} soil, 6.7% clay, 69.4% silt, and 24.0% sand.

Soil samples were sieved (4 mm) under moist conditions and visible plant residues, algae, and stones were removed. Moist soil (water content of 41%, equivalent to 1.45 kg of dry soil) was filled in PVC pots (10 cm inner diameter and 20 cm height). Each pot was planted with four rice plants (*Oryza sativa* L., two-line hybrid rice Zhongzao 39) at a similar height and weight at the seeding stage. All pots were irrigated with de-ionized water with a 2–3 cm water layer maintained above the soil surface during the entirety of the labeling period.

Four treatments, each with three replicates, were established: (1) control, unfertilized; (2) C addition (+C), where 2000 mg C in the form of CMC kg^{-1} dry soil was added; (3) N addition (+N), a total amount of 100 mg N kg^{-1} dry soil was added; and (4) CMC+N addition (+CN), where 2000 mg C kg^{-1} dry soil of CMC and sufficient $(NH_4)_2SO_4$, such that 100 mg N kg^{-1} dry soil was simultaneously added. All the four treatments were destructively sampled at the 1st, 5th, 10th, 25th and 39th labeling days, respectively.

2.3.2 ^{13}C continuous labeling

An automatically controlled gas-tight growth labeling chamber (110 × 250, 180 cm height) has been previously described by Ge et al. (2012) and was used in the present study. All pots were transferred into a single labeling chamber. Temperature was adjusted to the outdoor temperature (28 ℃) via air conditioning with temperature sensors (SNT-96S, Hangzhou T-Domain Electronics Co. LTD), which were placed both inside and outside the chamber. To obtain a reference for the natural abundance of ^{13}C and ^{15}N, control pots that were not subjected to ^{13}C labeling were maintained at a distance of 10–15 m from the labeling chambers and cultivated under the same temperature and moisture conditions.

Continuous labeling was performed over 39 days, and $^{13}CO_2$ was produced in the chamber via the reaction of 80 mL 1 M 10 atom% ^{13}C $NaHCO_3$ and 90 mL 1 M HCl in a plastic beaker placed inside the chamber every day from 9 am to 12 am. During

the labeling, CO_2 was released into the chamber only when the CO_2 concentration in the chamber was lower than 380 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), and excess CO_2 was absorbed. All pots in the labeling chamber were exposed to natural sunlight, and artificial light was used on cloudy days.

2.3.3 Harvesting and soil sampling

Plant and soil samples were destructively taken on the 1st, 5th, 10th, 25th, and 39th labeling days respectively. 39 days of labeling including rice pre tillering stage, post tillering stage, and elongation and panicle formation stage. All the above ground rice tissues (shoots) were cut at the soil surface to separate from the roots. Roots were separated from the soil and carefully washed with de-ionized water to remove adhered mineral particles. Shoots and roots were dried at 60 ℃, weighed, and milled (Yevdokimov et al. 2006). All soils in each pot were thoroughly mixed and divided into four subsamples, and 50 g soil samples were oven dried at 105 ℃ to determine soil water content. Fresh soil samples (100 g) were used for microbial biomass C (MBC) analysis, and 200-g soil samples were carefully wrapped with aluminum foil, instantly frozen in liquid nitrogen, and vacuum freeze-dried for PLFA analysis. The remaining soils were air-dried and used for C and N analysis.

2.3.4 Analytical methods

Soil pH was analyzed using a pH meter (Delta 320; Mettler-Toledo Instruments Co. Ltd., China). MBC was determined by the chloroform fumigation-extraction method (Wu et al. 1990), and the C and N content of soil, shoots, and roots were analyzed using an automated C/N analyzer (vario MAX, Elementar Analysensysteme GmbH, Germany). The N priming effect was calculated from soil total N in control

minus that in +C addition. The $\delta^{13}C$ values of shoots, roots, and soil were determined using an isotope ratio mass spectrometer (Finnigan^{TM} MAT253, Thermo Electron Corporation) coupled with an elemental analyzer FLASH 2000 (Thermo Fisher Scientific, US).

PLFAs were assayed according to Tian et al. (2013) based on previous studies (Bossio and Scow 1995; Zelles 1997, 1999). Briefly, total lipids were extracted from 3 g soil samples, with 25 μL of internal standard one added (1 μg μL^{-1}, 19:0 phospholipid) (before extraction), using one phase buffer, which was prepared by mixing chloroform, methanol, and citric acid (0.15 M, pH 4.0) in the ratio 1:2:0.8 (v/v/v). Extracted lipids were then separated into neutral-, glyco-, and phospholipids on a silica column. Collected phospholipids were saponified (0.3 M solution of BF_3 in methanol) and methylated (1 M solution of NaOH in methanol) and the PLFAs thus obtained were extracted using hexane. Cleaned PLFAs were dried under an N_2 stream and re-dissolved in toluene (185 μL), with internal standard two added (15 μL of 13:0 fatty acid methyl ester, 1 μg μL^{-1}). PLFA content was measured using gas chromatography-mass spectrometry (GC-MS).

2.3.5 Calculations and statistical analysis

2.3.5.1 ^{13}C in a rice system

The $\delta^{13}C$ and ^{13}C atom% values were used for ^{13}C assimilation calculations in the plant and soil samples. The following equations were used (Lu et al. 2002a, b; Wu et al. 2009):

$$^{13}C_x = \left[\left(^{13}C_{atom}\%\right)_{x,L} - \left(^{13}C_{atom}\%\right)_{x,UL}\right]/100 \times C_x$$

where, $_L$ and $_{UL}$ indicate labeled and unlabeled samples, respectively; $^{13}C_x$ is the total ^{13}C content in the plant and soil samples (mg ^{13}C pot^{-1} and mg ^{13}C kg^{-1} dry soil,

respectively). C_x is the total C content (mg C pot^{-1}; mg C kg^{-1} dry soil). $^{13}C_{atom}$ is the atom percents of C samples (%).

2.3.5.2 *^{13}C in microbial biomass and community classification*

The amount of ^{13}C in MBC, MBC and microbial biomass nitrogen (MBN) was calculated as the difference between fumigated and unfumigated soil extractions and was divided by a factor of 0.45 (for C) or 0.38 (for N) (Lu et al. 2002a, b).

PLFAs were divided into six groups (Leckie 2005; Gunina et al. 2017): Gram-positive bacteria (G+) (i14:0, i15:0, a15:0, i16:0, i17:0, and a17:0) (Yao et al. 2000, 2012, Zhang et al. 2007, 2012); Gram-negative bacteria (G-) (17:1ω8c) (Moss and Daneshvar 1992; Li and Pang 2010); arbuscular mycorrhizal fungi (AMF) (16:1ω5c) (van Diepen et al. 2010), fungi (18:2ω6,9c, 18:1w9c) (Vestal and White 1989; Yao et al. 2000; Zhang et al. 2007, 2012), anaerobes (cy17:0, cy19:0ω8c) (Vestal and White 1989); and actinomycetes (10Me17:0, 10Me18:0, and 10Me16:0) (Zhang et al. 2007, 2012). Ratios of total G+/G- bacteria were calculated by dividing the sum of the bacterial groups (G+ and actinomycetes) that belong to G+ bacteria (i14:0, i15:0, i16:0, i17:0, a15:0, a17:0, 10Me16:0, 10Me17:0, and 10Me18:0) by the sum of the bacterial groups (G- and anaerobes) that belong to G- bacteria (17:1ω8c, cy17:0, and cy 19:0) (Yao et al. 2000; Zhang et al. 2007, 2012). PLFA content was calculated based on the calibration curves obtained for the external standards and presented as nmol g^{-1} soil (Apostel et al. 2015). To determine microbial community structure in soils at various stages of labeling and amendment, principal component analysis (PCA) was performed using SPSS19 (SPSS inc., Chigaco, IL, USA) and plotted with Canoco for Windows 4.5 (Biometris, Wageningen, The Netherlands), with response variables of all the 20 PLFAs (including 14:0, 15:0, 16:0, 17:0, and 18:0) (mol%) after $\log_e$ transformation.

2.3.5.3 Statistical analysis

All data in the resulting figures is expressed as the mean of three replicates ± standard error (SE). One-way ANOVA with subsequent Tukey tests was used to identify means and differences between the treatments at a particular sampling date at a significance level of 0.05. The residuals were checked for normal distribution with a Shapiro-Wilk test and homogeneity was checked with a Levene test. All the analyses were performed using SPSS for Windows.

2.4 Results

2.4.1 Effects of C and N addition on plant properties

Both C and N stock in shoots and roots increased substantially during the plant growth in all treatments. Shoot C and N stocks increased faster after +CN addition than they did in the separate +C or +N fertilization. Combined +CN addition decreased root biomass (C and N stocks) compared to individual additions or the control (Fig. 1). The +CN treatment led to a greater incorporation of shoot C (70%–79% of total plant C) and N (69%–79% of total plant N) stock as compared with those of +C, +N, or the control. The highest rate of biomass growth (59–72 mg C pot^{-1} day^{-1} in shoots and 14–20 mg C pot^{-1} day^{-1} in roots) and N (1.7–3.0 mg N pot^{-1} day^{-1} in shoots and 0.72–0.97 mg N pot^{-1} day^{-1} in root) occurred between the 10^{th} and 25^{th} labeling days.

2.4.2 Effects of C and N addition on photosynthate distribution in the rice–soil system

The amount of photoassimilated ^{13}C in rice shoots and roots increased substantially during the labeling period in all additions compared to unfertilized Control (Fig. 2A;

2B). +CN application led to the greatest incorporation of ^{13}C in shoots and the least ^{13}C incorporation in roots as compared with the single-fertilizer additions. The +CN addition resulted in a relatively higher shoot ^{13}C/root ^{13}C ratio (5–10 times) than that in the +C, +N, and control treatments. The highest plant photosynthate ^{13}C incorporation rates (1–1.5 mg pot^{-1} day^{-1} in shoots and 0.3–0.4 mg pot^{-1} day^{-1} in roots) occurred between day 10 to day 25.

During the entire labeling period, the ^{13}C amount incorporated into the SOM gradually increased in all additions, with the most rapid rate being recorded between day 10 to day 25 in all the amendments (Control, +C, +N and +CN)(Fig. 2C). As compared with the unfertilized soil, all amendments resulted in a lower ^{13}C incorporation into SOM, with the lowest ^{13}C incorporation observed after the +CN addition.

Similarly, +CN application also led to a lower ^{13}C accumulation in the microbial biomass carbon (MBC) (Fig. 2D) than in any other amendment, and the highest amount of ^{13}C incorporated into MBC was detected in unfertilized soil. The most rapid incorporation rate of four amendments (Control, +C, +N and +CN) occurred between the 5th and 10th labeling day, which was earlier than that for ^{13}C incorporation into SOM. In summary, simultaneous C and N application (+CN) resulted in a relatively higher shoot ^{13}C allocation and lower ^{13}C recovery in roots, SOM, and microorganisms as compared with that in single fertilizer addition (+C, +N) and non-fertilized soils.

2.4.3 Positive N priming effect following CMC application

A positive N priming effect was induced by CMC addition, as 3% of soil total N mineralized from SOM, when compared with that in the unfertilized soil (Fig. 3). Of this N mineralized from SOM in +C amendment, 16% were taken up by rice plants. Compared with the unfertilized soil, less soil total N was found in the +N and +CN amendments at the end of labeling.

2.4.4 Effects of C and N addition on soil microbial community

The effects of C and N addition on PLFA biomarker composition were evaluated by PCA (Fig. 4), which revealed a clear separation of microbial community structure according to each amendment and sampling stage. Microbial composition in +CN-amended and unfertilized soils (control) was highly different from that in +C- and +N-amended soils, which had comparable compositions. Fertilization had a stronger effect on microbial composition than rice development.

The amount of MBC and MBN (Fig. 5 A, C) gradually increased during the entire labeling period, following the same order as that of increase in ^{13}C content of SOM (Fig. 2C): control > +N > +C > +CN. Addition of +CN led to a relatively lower MBC and MBN content during microbial proliferation as compared with that of single applications or non-fertilized soils.

The amounts of PLFA biomarkers (Fig. 5 B) increased substantially during the 39 days of growth. +CN application resulted in minimum PLFA content as compared with that in the +C, +N, and control soil (control > +N > +C > +CN).

The G+/G- ratio (Fig. 5D) in the control soil and +C and +N amendments steady increased until the end of labeling. The G+/G- ratio in +CN soil slightly decreased throughout the entire labeling period. After 39 days of rice growth, soils with +C and +N applications had similar G+/G- ratios. Addition of +CN led to the lowest G+/G- ratio recorded during the entire labeling period.

Application of C or N or their combination decreased the PLFA biomarker contents for most microbial groups (Fig. 6). The strongest decrease after the additions of C or N or their combination were found in PLFA groups of G+ and fungi. +N application led to a specific increase of PLFAs responsible for G- bacteria and actinomycetes.

2.5 Discussion

2.5.1 Effects of C and N addition on the rice–soil system

The stimulating effect of C and N addition on crop yield (Fig. 1) has been shown in many previous studies (Pan et al. 2009; Zhang et al. 2012; Wang et al. 2014a, b). In contrast, lower root C (F value:7.55, *p* value 0.01 in 39th labeling day), root N (F value:7.27, *p* value 0.12), and ^{13}C photosynthate incorporation in root (F value:8.92, *p* value 0.006) (Fig. 1 B, D; Fig. 2 B) was observed following the addition of +C, +N and +CN. According to the Optimal Partitioning Theory (OPT) (Bloom et al. 2012), when nutrients in soil limited plant growth, plants invest preferentially in root growth at the expense of shoot growth (Janeček et al. 2014; Wang et al. 2014c). Addition of +C, +N and +CN mitigated nutrients limitation for plant growth in soil, and so resulted in less root C, root N, and ^{13}C photosynthate allocation. Root development depends on external factors such as availability of mineral N and other nutrients, pH, oxygen availability, and redox potential (Bloom et al. 2002). Many studies have reported that high mineral N levels inhibit root development and elongation (Britto and Kronzucker 2002; Linkohr et al. 2002; Li et al. 2010). Symptoms of root inhibition caused by NH_4^+ generally appear with external NH_4^+ concentrations greater than 0.1 to 0.5 mM (Katwijk et al.; Peckol and Rivers 1995). In the +N and +CN addition, high NH_4^+ content in soil solution (up to 3 mM) was detected. NH_4^+ can inhibit root activity through physiological mechanisms: rhizosphere acidification, nutrient imbalance, photosynthesis system damage, and carbohydrate limitation (Gerendás et al. 1997; Britto and Kronzucker 2002), as well as at the genetic level: inhibition of GDP-mannose pyrophosphorylase activity (Barth et al. 2010; Li et al. 2010). Thus, +N and +CN decreased ^{13}C photosynthate incorporation into the roots and consequently decreased ^{13}C rhizodeposition, which ultimately led to a reduction in the ^{13}C level in MBC and SOM (Fig. 2 C).

Root growth under mechanical impedance displaces soil particles, and as a result, root elongation decreases with increasing soil strength and density (Hodge et al. 2009).

As a soil conditioner, water retainer, and remediating agent (Mishra et al. 2018), CMC increases soil glutinousness and water-retention capacity (Ang 1991). Through the binding of mineral particles via organic polymers, CMC application increases the cohesion of aggregates and their average diameter (Chenu et al. 2000; Subbian et al. 2000), which increased soil strength and density. Thus, +C addition may impede root elongation and development and consequently lead to reduced incorporation of ^{13}C photosynthates into roots, SOM, and microbial biomass. Moreover, CMC, due to its macromolecular structure, serves as a slow-release energy source for microorganisms. In +C-amended soil, microbes use labile C to decompose recalcitrant SOM and thereby acquire N, a process called "microbial N mining" (Fontaine et al. 2003; Moorhead and Sinsabaugh 2006). As high mineral N levels inhibit root development and elongation (Britto and Kronzucker 2002; Linkohr et al. 2002; Li et al. 2010), long-term mining of mineral N by soil microbes from SOM may trigger relatively lower root biomass, which can also lead to less ^{13}C incorporated into roots, SOM, and microbes in +C-amended soil.

The maximum ^{13}C accumulation rate in roots and SOM occurred at the same time (between the 10^{th} to 25^{th} labeling day) (Fig. 2 B, C), as plant photosynthates released by roots (rhizodeposited C) are one of the primary sources of SOM formation (Finzi et al. 2015). Rhizodeposited C strongly affects ^{13}C incorporation into microbial biomass, as microorganisms preferentially use labile rhizodeposits over other substrates (Lambers et al. 2009; Jones et al. 2009). Rather than use photoassimilates for plant development, plants primarily release labile photosynthate C into the rhizosphere for microorganisms, which stimulates nutrient cycling for further plant nutrient uptake and growth (Paterson et al. 2007). Thus, the maximum ^{13}C incorporation into microbial biomass occurred earlier (5^{th} and 10^{th} labeling days) (Fig. 2 D) than that of ^{13}C in roots and SOM.

Experiments have been performed to examine the effects of long-term fertilization on the fertility of paddy soils (Whitbread et al. 2003). Studies have, however, yielded inconsistent results. Some studies have shown enhanced soil

fertility under long-term fertilizer application (Whitbread et al. 2003), whereas other have reported severe degradation of red soils with high acidity and low nutrient content (Dawe et al. 2000; Kumar and Yadav 2001). By taking living roots into account and using CMC as a mimic to evaluate long-term organic fertilizer addition, our study has demonstrated lower photosynthate input into paddy soil.

2.5.2 N mining by positive priming effect induced by CMC application

Priming effects (PEs) have been defined by Kuzyakov et al. (Kuzyakov et al. 2000) as "strong short-term changes in the turnover of SOM caused by comparatively moderate treatments of the soil," and are now well documented (Fontaine et al. 2003; Kuzyakov 2010). Most of these studies primarily focus on the PE of SOM caused by exogenous addition of glucose or other substances (Hamer and Marschner 2005; Fontaine et al. 2007; Dilly and Zyakun 2008). Addition of organic substances to soil not only promotes the PEs of SOM, but also affect soil N (Liu et al. 2017). Organic C (CMC) application resulted in 3% of soil total N being released from SOM over 39 days (Fig. 3). Organic fertilizers increase N mineralization because microbes shift from SOM to added organic fertilizer for energy and N (Ekschmitt et al. 2005; Chen et al. 2014). However, SOM can substitute N source when pure N free organic substances (CMC) are added. +C amendment promotes microbial decomposition of SOM to acquire N (Fontaine et al. 2003; Moorhead and Sinsabaugh 2006). Hence, positive N PEs occurred owing to N mining by microorganisms (Chen et al. 2014). In +C amendment, 16.2% of the newly released mineral N was taken up by the plants (Fig. 4), which is in line with previous reports of enhanced plant N uptake as a result of organic fertilizer application (Dijkstra et al. 2009).

2.5.3 Effects of C and N addition on soil microbial community

Nutrient availability is a strong determinant of microbial community composition (Drenovsky et al. 2004; Dong et al. 2014a) The +C, +N, and +CN amendments also

strongly affected soil microbial community structure over time (Fig. 4), which is consistent with the findings of previous studies (Marschner et al. 2003; Houlden et al. 2008).

The addition of low molecular weight organic substances, such as glucose, can be taken up by microorganisms from solution within a few hours (Jones and Edwards 1998; Jones and Hodge 1999; Fischer and Kuzyakov 2010; Gunina et al. 2014), which rapidly stimulates microbial growth. In contrast, the addition of CMC introduced slow but steady release of energy to microorganisms, leading to microbial proliferation similar to that as by plant C inputs. With lower root C inputs, +C and +CN led to lower MBC, MBN, and PLFA content than that in unfertilized soils (Fig. 5 A, B, C).

In situ stable isotope probing revealed that G- and eukaryotic microorganisms are the most active assimilators of root-derived photosynthetic C in paddy soil, whereas G+ microorganisms are more important in bulk soil (Lu et al. 2007). Compared to +CN addition, higher G+/G- ratio in +C and +N (Fig. 5 D) indicate that during microbial proliferation, single C or N application promoted the growth of G+ bacteria in bulk soil over that of G- bacteria in the rhizosphere. In contrast, +CN addition led to a reverse effect.

Changes in microbial community structure are linked to microbial metabolism with respect to soil C and N availabilities. G+ bacteria are reported with specialized activities in plant promotion (Kloepper 1980). As either C or N fertilization resulted in relatively slower root development (Fig. 1 B, D; Fig. 2 B), G+ bacteria PLFAs decreased most at the 39^{th} growth day (Fig. 6). G+ bacteria decreased with increasing soil organic C availability from crops, straw and manure were reported (Bossio and Scow 1998; Peacock et al. 2001; Buyer et al. 2010). When compared to +N amendment, C addition (+C and +CN) resulted in relatively smaller G+ bacteria PLFAs, as cellulose (CMC addition) is the major organic component of rice straw (Howard et al. 2003). . Either C or N fertilization can led to decreasing Fungi PLFAs (Fig. 6), as Fungi is more capable of colonizing in nutrient poor soils with their

wide-ranging enzymatic capabilities(Frey et al. 2003). Compared to +N amendment, C addition (+C and +CN) resulted in relatively smaller Fungi PLFAs (Fig. 6). As in anoxic environments (e.g., paddy soil), bacteria are almost exclusively responsible for the degradation of cellulose (Leschine 1995; Lynd et al. 2002). In this regard, bacteria outcompete fungi by producing inhibitors such as hydrocyanic acid, antibiotics, lytic enzymes, and volatiles, as well as nutrient-sequestering molecules, such as iron-chelating siderophores (Whipps 2001; Wheatley 2002).

+N fertilization specifically stimulated G- bacteria (Fig. 6) which is in consistence with result reported by Zhang et al. (2007), who also found increasing G- bacteria PLFAs in paddy soil under N application with the absence of C fertilization. As fewer root derived C (compared to control) and less C source (compared to +C and +CN amendments), proliferation of G- bacteria in +N application indicated that G- bacteria can be active C assimilators in low C availability soils. With less root derived C and fewer available C, in order to gain access to energy source, +N fertilization stimulated proliferation of actinomycetes (Fig. 6), as hyphal growth of actinomycetes can enable efficient penetration through soils via pores and thereby facilitate access to energy sources (Lynd et al. 2002; De Boer et al. 2005).

2.6 Conclusions

By using CMC as a mimic of straw degradation for evaluating slow organic C mineralization, either a single application of +C or +N, or their combined addition (+CN) decreased root biomass (Fig. 1B). The decrease in root biomass led to lower incorporation of photosynthates transported to the roots, which resulted in less ^{13}C incorporation into rhizodeposits (Fig. 7). Lower ^{13}C in microbial biomass with a small delay lead to lower ^{13}C incorporation into SOM confirming that microbial necromass is a significant source of C sequestered in soil (Fig. 7). Single CMC addition also released 3% of N stored in SOM through positive N priming effects. C and N availability in soil decreased the G+/G- ratio and PLFA content. By impeding root

development and decreasing the input of rhizodeposits, increased C and N availability decreased content of microbial groups, such as G+ and fungi, N fertilization stimulated G- bacteria and actinomycetes, thereby altering the composition of the soil microbial community.

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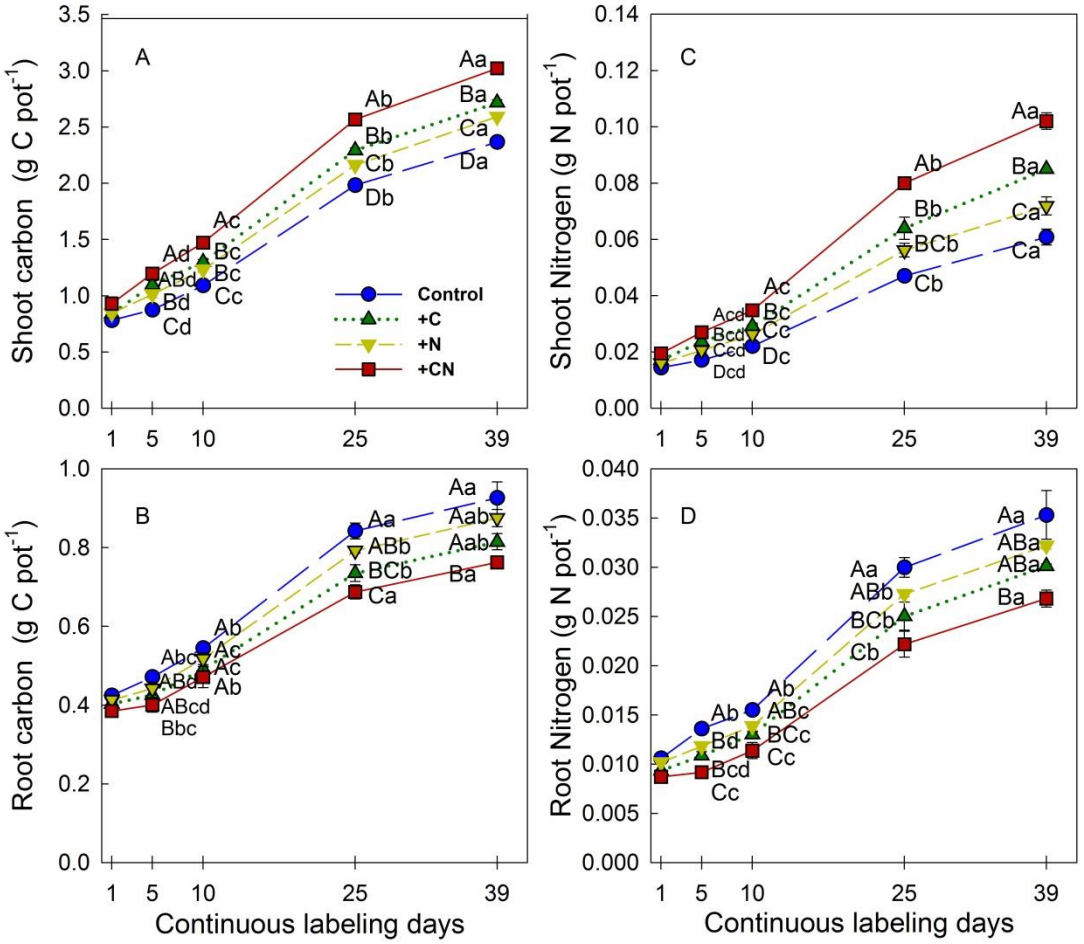

Fig. 1 Total C and N in shoots and roots (g pot^{-1}) of rice in Control (no addition), CMC addition only (+C), N fertilizer only (+N), and combined C and N application (+CN) on days 1, 5, 10, 25, and 39 of continuous $^{13}CO_2$ labeling. A) shoot C; B) root C; C) shoot N; D) root N. Data points represent means (n = 3), and error bars represent standard errors. Capital letters adjacent to the points represent significant differences ($p < 0.05$) in the same labeling day between treatments, and lowercase letters represent significant differences ($p < 0.05$) in the same treatment between different labeling days.

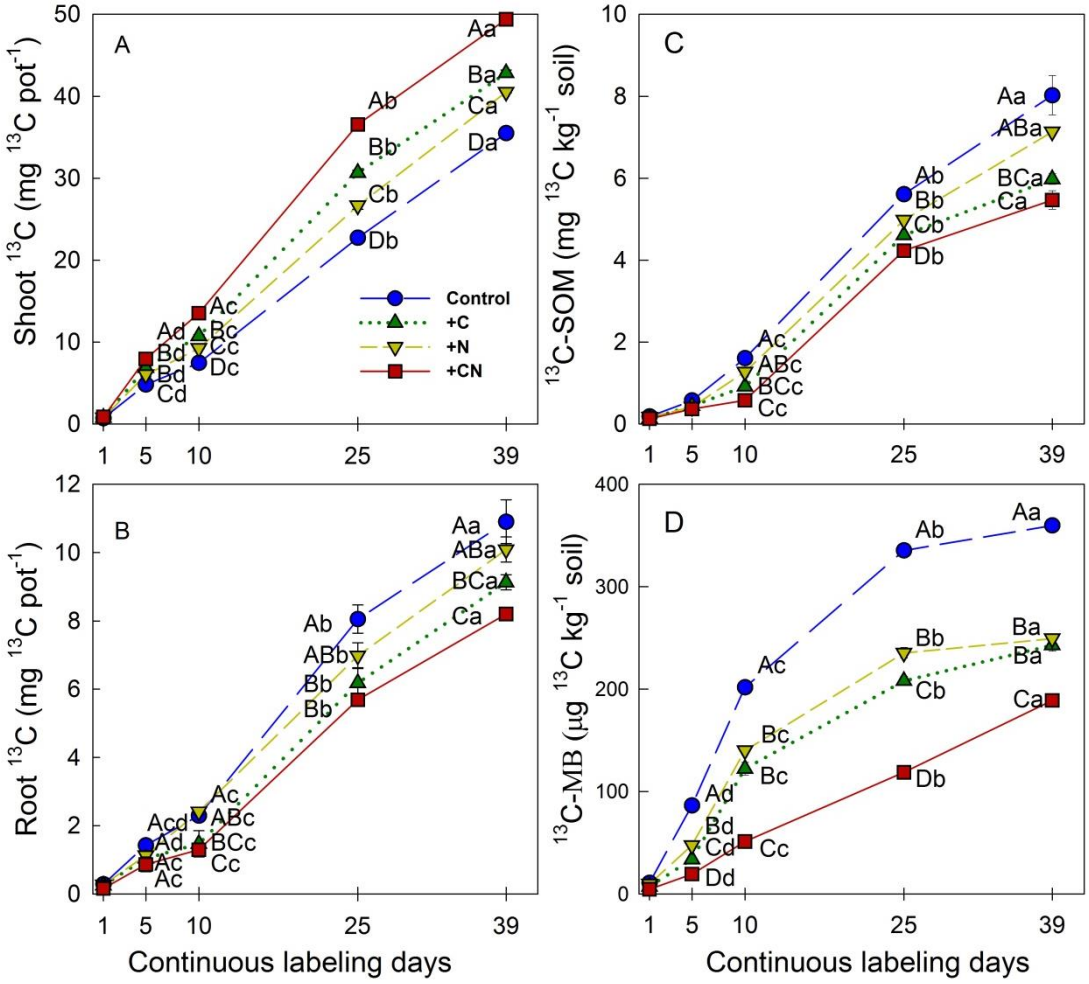

Fig. 2 ^{13}C in rice shoots (mg pot^{-1}), roots (mg pot^{-1}), SOM (mg kg^{-1}), and MB (μg kg^{-1}) in four treatments: Control (no addition), CMC addition only (+C), N fertilizer only (+N), and combined C and N application (+CN) on days 1, 5, 10, 25, and 39 of continuous $^{13}CO_2$ labeling. A) shoot ^{13}C; B) root ^{13}C; C) ^{13}C in SOM D) MB^{13}C. Data points represent means (n = 3), and error bars represent standard errors. Capital letters adjacent to the points represent significant differences ($p < 0.05$) in the same labeling day between treatments, and lowercase letters represent significant differences ($p < 0.05$) in the same treatment between different labeling days.

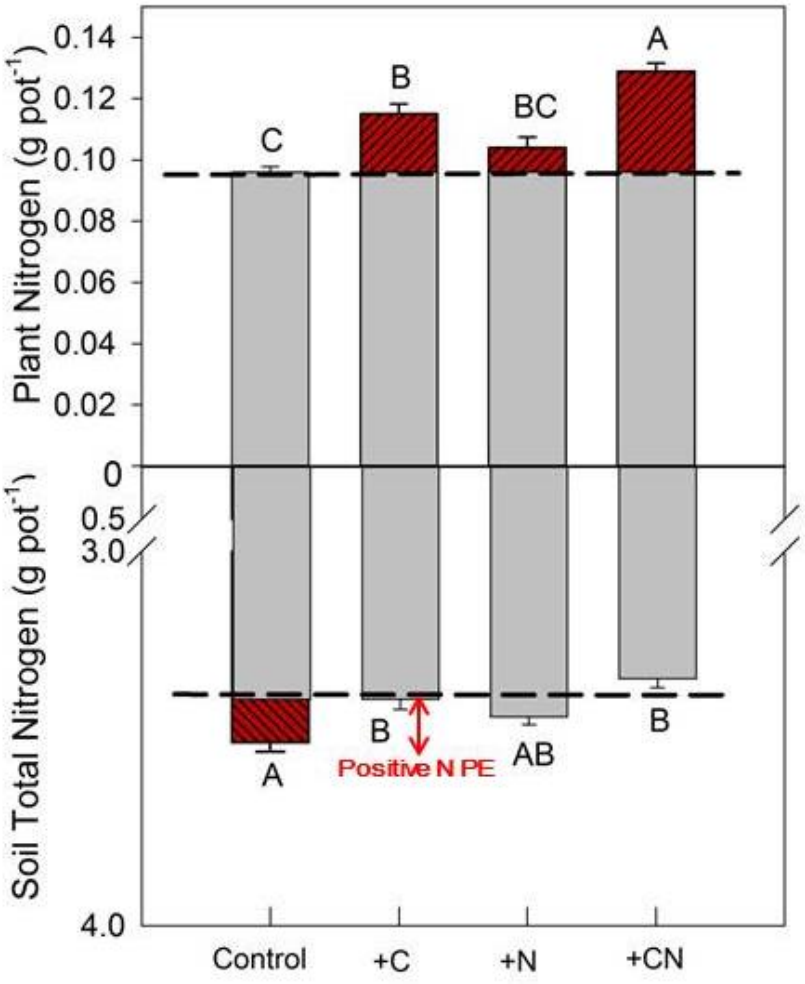

Fig. 3 Positive N priming effect in the CMC-amended (+C) soil on the 39th day of continuous $^{13}CO_2$ labeling. Four treatments: Control (no addition), CMC addition only (+C), N fertilizer only (+N), and combined C and N application (+CN). Red regions represent differences compared with the control. Data points represent means (n = 3), and error bars represent standard errors. Capital letters adjacent to the error bars represent significant differences ($p < 0.05$) between treatments.

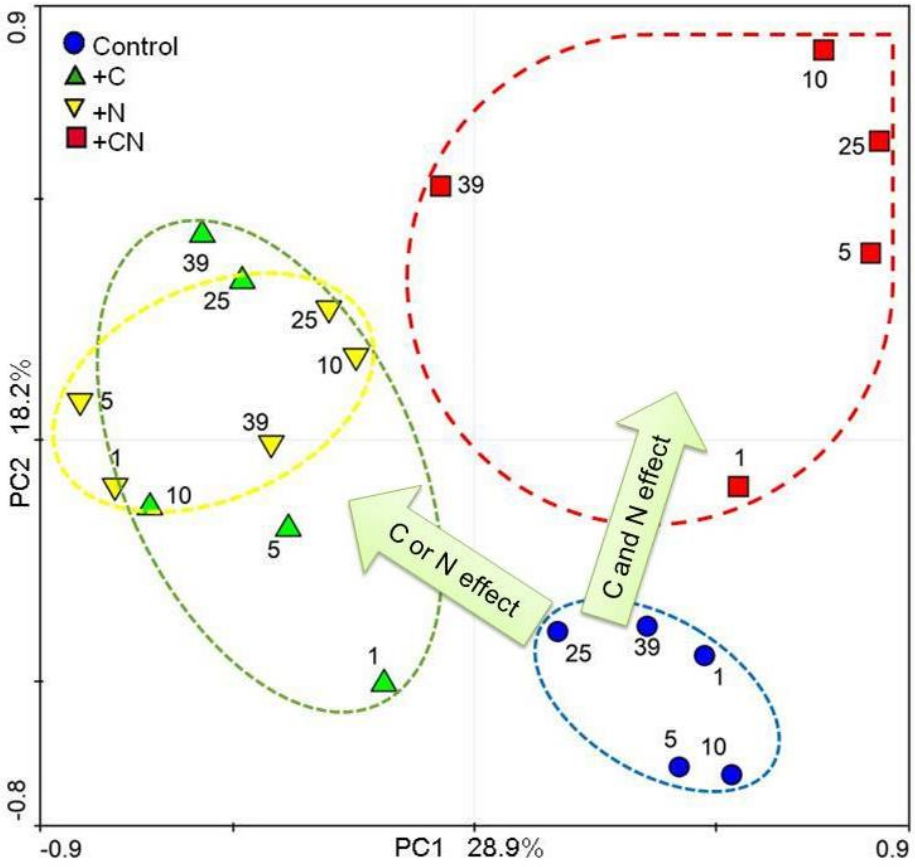

Fig. 4 Principal component analysis (PCA) of PLFAs compositions in soil without addition (Control), CMC addition only (+C), N fertilizer only (+N), and combined C and N application (+CN) on days 1, 5, 10, 25, and 39along principal component axes PC1 and PC2.

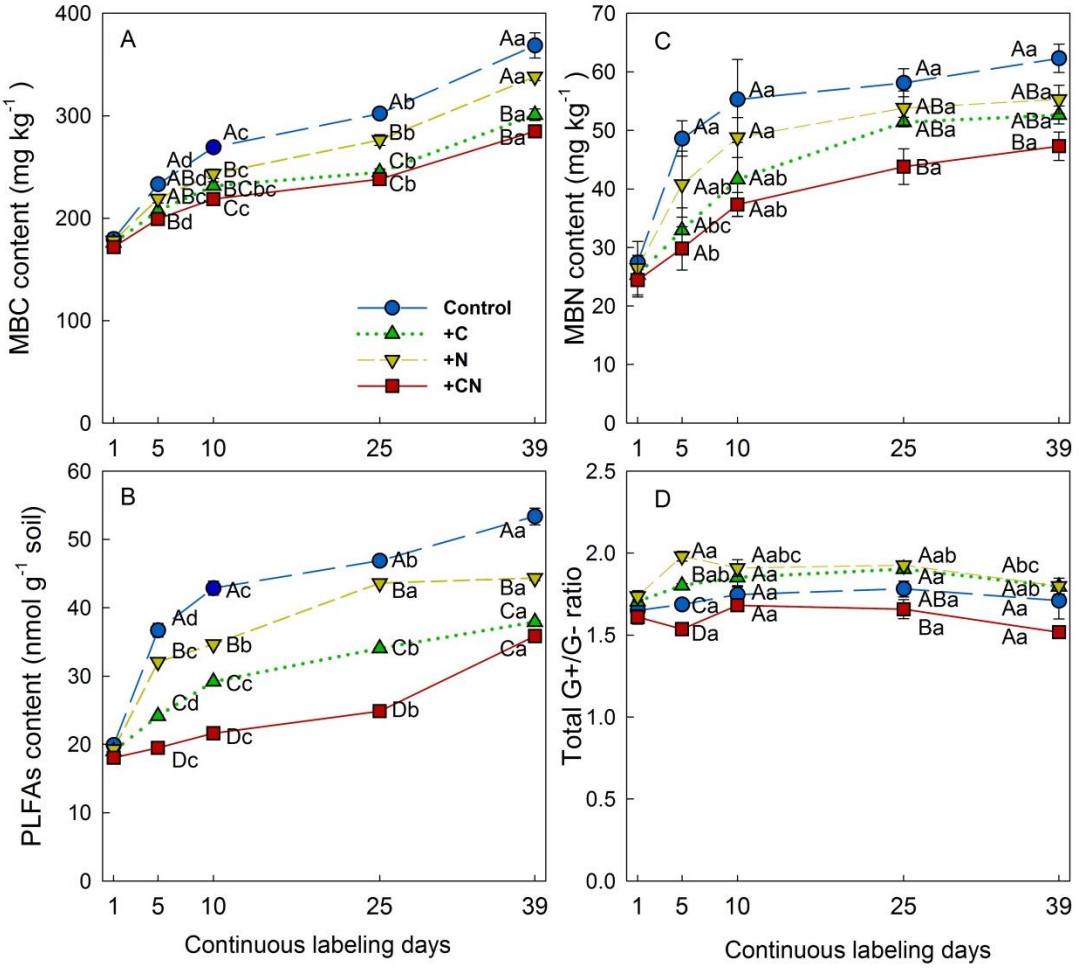

Fig. 5 Microbial biomass carbon content (mg C kg^{-1}), microbial nitrogen content (mg N kg^{-1}), PLFA content (nmol g^{-1}) and total G+/G- ratio in the four treatments: Control (no addition), CMC addition only (+C), N fertilizer only (+N), and combined C and N application (+CN) on days 1, 5, 10, 25, and 39 of continuous $^{13}CO_2$ labeling. A) MBC; B) PLFA content; C) MBN; D) Total G+/G- ratio. Data points represent means (n = 3), and error bars represent standard errors. Capital letters adjacent to the points represent significant differences ($p < 0.05$) in the same labeling day between treatments, and lowercase letters represent significant differences ($p < 0.05$) in the same treatment between different labeling days.

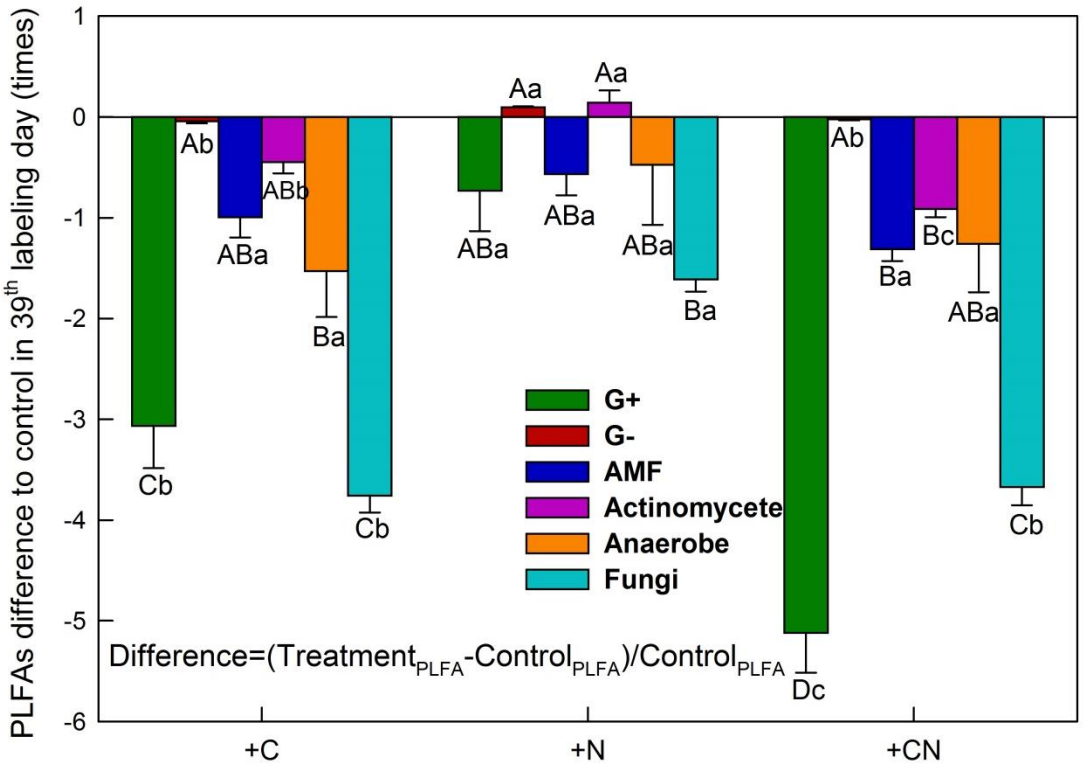

Fig. 6 Changes in PLFA content (number of times) relative to the control in three fertilization compared to control (no addition): C fertilizer only (+C), N fertilizer only (+N), and combined C and N fertilizer (+CN) at the end of 39 days of continuous $^{13}CO_2$ labeling. Difference = (Treatment$_{PLFA}$-Control$_{PLFA}$)/Control$_{PLFA}$. Data points represent means (n = 3), error bars represent standard errors. Capital letters adjacent to the points represent significant differences ($p < 0.05$) in the same treatment between different microbial PLFAs groups, and lowercase letters represent significant differences ($p < 0.05$) in the same microbial PLFAs groups between different treatments.

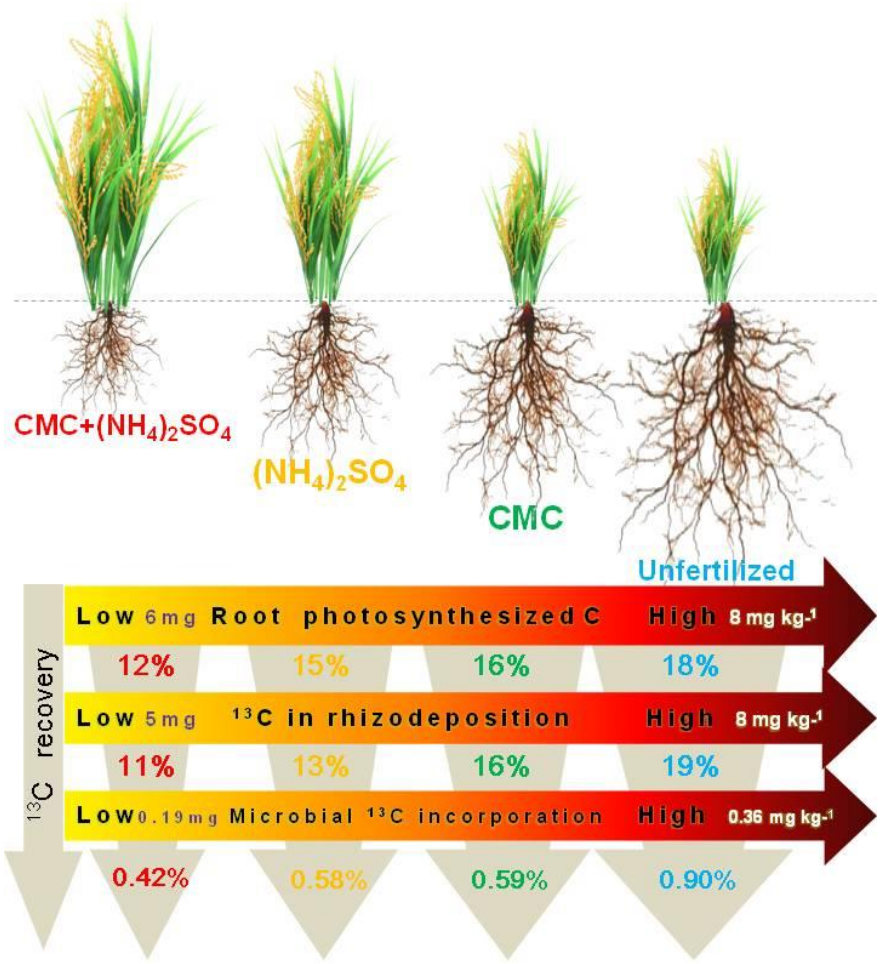

Fig. 7 Morphology, recovery of root photosynthesized C, ^{13}C in rhizodeposition, and microbial ^{13}C incorporation in soil under rice on the 39th labeling day in four treatments—Control, CMC (+C), $(NH_4)_2SO_4$ (+N), CMC+$(NH_4)_2SO_4$ (+CN). Numbers under each arrow represent ^{13}C recovery in roots, soil, and microorganisms (%) in the four treatments.

Supplymentary

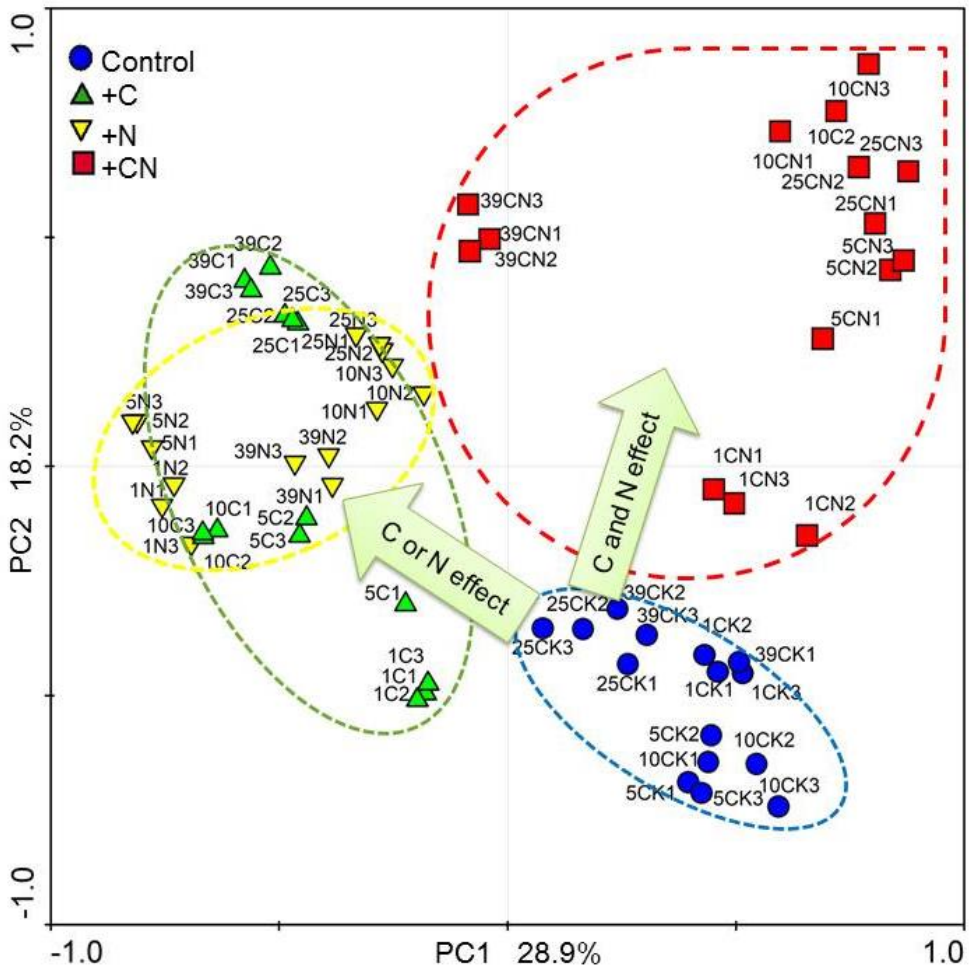

Fig. S1 Principal component analysis (PCA) of PLFAs compositions in soil without addition (Control: CK), CMC addition only (+C), N fertilizer only (+N), and combined C and N application (+CN) on labeling days 1, 5, 10, 25, and 39 along principal component axes PC1 and PC2. Numbers (1, 2, 3) after CK, +C, +N and +CN indicate the replicates in each treatment.

Chapter 3

Long-term effects of rice straw degradation in paddy fields: Above- and belowground rice ^{13}C budget and microbial utilization of rhizodeposits

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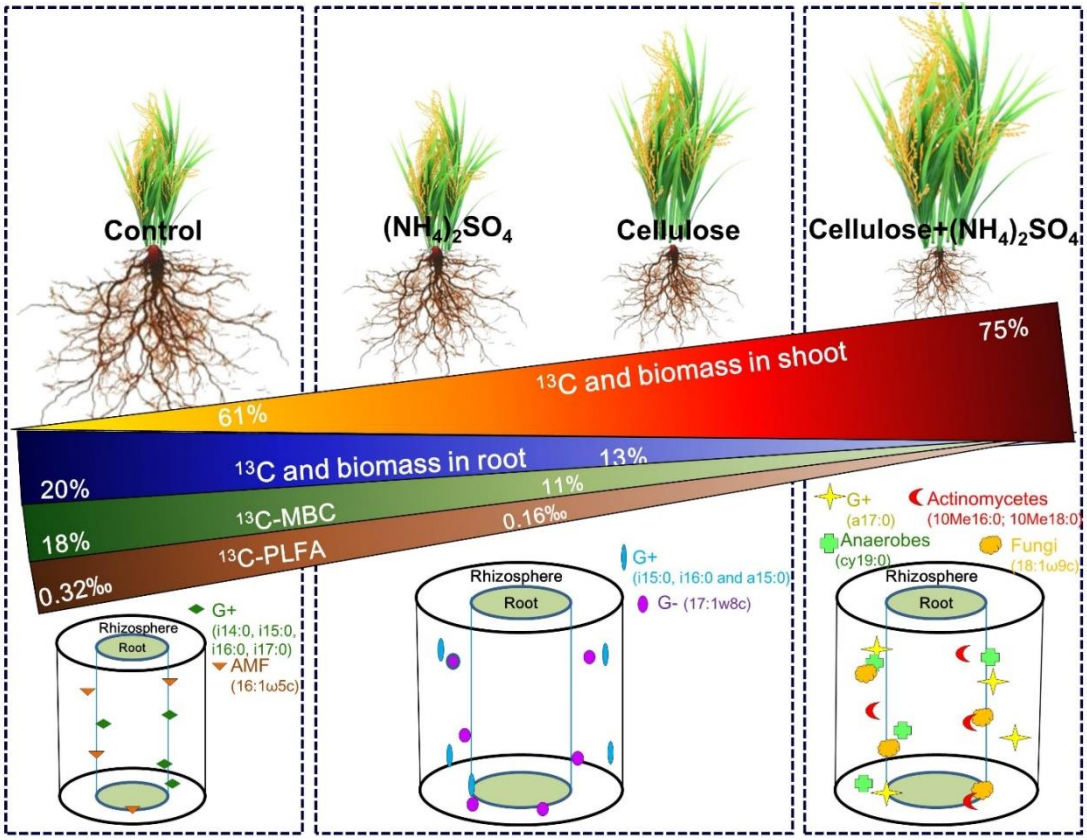
3.1 Graphical abstract



3.2 Abstract

Although rice straw is widely applied in paddy fields, the long-term effects of straw decomposition on photosynthate (^{13}C) recovery in the rice–soil ecosystem and microbial community responses are unknown. We determined the effects of application of cellulose (+C), $(NH_4)_2SO_4$ (+N), and their combination (+CN) on photosynthate (^{13}C) recovery in soil for various rice growth stages and photosynthate utilization by microbial communities under continuous $^{13}CO_2$ labelling. Combined +CN application maximally increased shoot biomass and shoot ^{13}C incorporation but minimally increased photosynthate recovery in roots and soil C pools [soil organic matter (SOM), dissolved organic C (DOC), microbial biomass C (MBC), and phospholipid fatty acids (PLFA)]. Consequently, despite enhancing plant yield, cellulose with N fertilization decreased rice C allocation into available and stable SOM pools. Redundancy, hierarchical clustering, and heat map analyses of ^{13}C-PLFA revealed that different fertilization regimes dominated specific microbial groups utilizing root exudates. Single +C or +N addition increased the relative percentages of G- (17:1w8c) and G+ (i15:0, i16:0, and a15:0) groups. The +CN application stimulated groups of G+ (a17:0), anaerobes (cy19:0), fungi (18:1ω9c), and actinomycetes (10Me16:0 and 10Me18:0). Two factors, fertilization and plant growth stage, explained 81% of the variance in the microorganisms utilizing root exudates. Fertilization decreased root biomass; affected MBC, DOC, SOM, and soil organic and inorganic N; and was responsible for 36.5% of the variance in microbial community structure. Comparisons with ^{13}C-PLFA in unfertilized soils indicated that fertilization also decreased microbial utilization of photosynthates by limiting root ^{13}C input. Thus, by decreasing the recovery and distribution of photoassimilates in the rice–soil system and by regulating plant and soil properties, fertilization shifted microbial group dominance and decreased the microbial biomass associated with rhizodeposit utilization.

Keywords: Continuous $^{13}CO_2$ labelling; Photosynthate recovery; Phospholipid fatty

acid analysis; Straw decomposition; Carbon and nitrogen fertilization

3.3 Introduction

Rice (*Oryza sativa* L.), as a major cereal crop, is cultivated on more than 140 million hectares worldwide (Rice Statistics database; IRRI, 2018), with an annual grain production exceeding 506 million metric tonnes (FAO, 2018). Annually, substantial amounts of rice straw are generated, as for each tonne of grain harvested, 1.35 tonnes of rice straw remain in the field (Kadam et al., 2000). With a worldwide production of some 740 to 1111 million tonnes of rice straw per year (Abraham et al., 2016), its disposal can be problematic. Given its low digestibility, low protein and high lignin contents (Kausar et al., 2011), rice straw is not an optimal source for livestock fodder. Typically, it is burnt in the field after harvest, which can lead to air and water pollution, contamination, and greenhouse gas emissions (Gadde et al., 2009; Qu et al., 2012). As an alternative to *in situ* burning, the retention of rice straw in paddy fields has become an increasingly prevalent practice that can facilitate improvements in soil fertility and physicochemical properties (a decrease in soil salinity and increases in Ca^{2+}, K^{+}, and organic matter; Mahmoud et al., 2009) and enhance crop yield (Fusi et al., 2014; Jia et al., 2015; Kanchikerimath and Singh, 2001).

Rice straw consists of 32% cellulose, 24% hemicelluloses, and 18% lignin (Howard et al., 2003), and these constituents are strongly intermeshed and chemically bonded by non-covalent forces and covalent cross-linkages (Pérez et al., 2002) to form a lignocellulosic matrix structure. Owing to its polymeric molecular structure, rice straw is typically slow to degrade (Kausar et al., 2011), especially considering the slow decomposition processes in paddy soils under irrigation. It has, for example, been demonstrated that only half of the straw is mineralized after 160 days of incubation in paddy soils (Devêvre and Horwáth, 2000), despite being subjected to high temperatures commonly encountered in the subtropics. Although numerous microorganisms are associated with the degradation and utilization of cellulose and hemicellulose (Sánchez, 2009), considerably fewer have the ability to break down

lignin, which is the most recalcitrant component of plant cell walls (Sánchez, 2009). Therefore, the degradation of rice straw, particularly under the oxygen-limiting conditions that typically prevail in paddy fields (Supplementary S2), is especially slow. Only a relatively small group of microorganisms, such as white-rot fungi, have sufficiently strong oxidative activity and the requisite ligninolytic enzymes to break down lignin (Ten Have and Teunissen, 2001). Thus, evaluating the effects of rice straw application typically necessitates long-term studies. Although rhizodeposition is an important C and energy source for microorganisms, there are no studies on the long-term effects of straw decomposition on rhizodeposition, as the quantification of rhizodeposition requires isotopic techniques, i.e. continuous or pulse ^{13}C or ^{14}C labelling (Kuzyakov, 2001), which are generally conducted over short periods of weeks or months. Nevertheless, cellulose, the major component of rice straw (Howard et al., 2003), can be utilized by microorganisms in a relatively short time (months) (Blagodatskaya et al., 2014) compared with the slow degradation of the lignocellulosic matrix (years). Hence, cellulose can be used to mimic the effects of long-term rice straw degradation in paddy soils, thereby facilitating study of the above- and belowground budgeting of rice-derived C and microbial utilization of rhizodeposits.

Maize, ryegrass, and barley are often used for the quantitative analysis of rhizodeposits in croplands to determine photosynthate inputs below ground and their subsequent transformations (Pausch and Kuzyakov, 2018). Compared with upland soils, flooded paddy soils generally have a 12%–58% higher soil organic matter (SOM) content (Liping and Erda, 2001), as the low oxygen content suppresses the activity of oxidizing enzymes, thereby retarding the mineralization of new organic inputs and old organic matter (Freeman et al., 2001; Kemmitt et al., 2008). Hence, quantifying the input and subsequent transformations of rice photosynthates into and in the soil is of considerable importance for gaining an understanding of the effects of straw addition in paddy soil. Review of $^{13/14}C$ partitioning under continuous labelling have indicated that rice plants allocate 72% of net photosynthesized C to shoots, 17%

to the roots, 10% to the soil, and 1.3% into microbial biomass (Liu et al., 2019). The amount of assimilated C input belowground can be affected by soil C and nitrogen (N) nutrient content (Bhattacharyya et al., 2013; Carvalhais et al., 2011; Kuzyakov and Cheng, 2001; Tian et al., 2013), including nutrient input from the decomposition of plant residues. However, it is still unclear how the application of organic and/or mineral fertilizers affects patterns of rhizodeposit' utilization in paddies.

Based on single and combined applications of cellulose and $(NH_4)_2SO_4$, this study aimed to answer the following questions: (i) How does long-term rice straw degradation affect the dynamics of photosynthate distribution and recovery in the rice–soil system? (ii) How does C and N fertilization affect photosynthate recovery and partitioning between various soil C pools? (iii) How does C and N fertilization affect the composition of soil microbial communities that utilize photosynthates?

3.4 Materials and Methods

3.4.1 Sampling and experimental design

The experimental setup was similar to that previously reported by our group (Zhao et al., 2018). Briefly, soil samples (ploughing Anthrosol) were collected from a paddy field (0–20 cm soil layer) located at the Changsha Research Station for Agricultural and Environmental Monitoring, Hunan Province, China (28°33′04″N, 113°19′52″E, 80 m a.s.l.). The paddy fields at this station have been cropped on a rice-fallow-rice rotation for over 30 years. The chemical and physical properties of the paddy soil are as follows: pH 5.56 (1:2.5, soil:water ratio), 20.6 g C kg^{-1} soil, 2.6 g N kg^{-1} soil, 0.45 g phosphorus kg^{-1} soil, 6.7% clay, 69.4% silt, and 24.0% sand. Prior to using the soil samples for experiments, we removed visible plant residues, algae, and stones, and sieved the soil (4-mm mesh) under moist conditions.

Each treatment consisted of three PVC pots (17 cm inner diameter and 40 cm height) as replicates. The four treatments applied were as follows: (i) Control

(unfertilized soil), (ii) +C: addition of cellulose (2000 mg C kg^{-1} dry soil), (iii) +N: addition of ammonium sulphate (100 mg N kg^{-1} dry soil), and (iv) +CN: combined addition of cellulose and ammonium sulphate (2000 mg C kg^{-1} dry soil + 100 mg N kg^{-1} dry soil). Each PVC pot was filled with 1.45 kg of dry soil, moistened to 41% of the water-holding capacity, into which were planted four rice seedlings (*Oryza sativa* L., two-line hybrid rice Zhongzao 39) of similar height and weight. During the experiment, a 5–7 cm depth of de-ionized water was maintained above the soil surface.

3.4.2 Continuous $^{13}CO_2$ labelling

The type of continuous labelling chamber used in the present study has been described previously by Ge et al. (2012). All experimental plants (with the exception of unlabelled plants as controls) were continuously labelled with $^{13}CO_2$ for 39 days. Labelling was performed each day from 09:00 to 12:00 at a temperature of 28 ℃, via generation of $^{13}CO_2$ through the reaction between $NaHCO_3$ (80 mL, 1 M 10 atom% ^{13}C) and H_2SO_4 (90 mL, 1 M). When the CO_2 concentration was lower than 380 μL L^{-1}, a fresh consignment of $^{13}CO_2$ was released into the chamber, whereas when CO_2 concentrations exceeded 380 μL L^{-1}, a switch diverted gas flow to pass CO_2 through NaOH solution to trap the excess CO_2. Air in the growth chamber was continuously circulated by two fans. To obtain the natural ^{13}C abundance of CO_2, the pots containing control plants were placed at a distance of 10–15 m from the labelling chambers under the same irrigation and temperature conditions.

At the end of the 1st, 5th (pre-tillering stage), 10th (post-tillering stage), 25th (elongation), and 39th (early panicle formation) days of labelling, the soil in each pot was sampled, separated from rice roots, and thoroughly mixed. The plants were separated into shoots and roots by cutting at the interface between the soil and the irrigating water layer. Roots were washed with de-ionized water and carefully separated from the adhering soil and mineral particles. Both shoots and roots were

dried at 60 ℃ for 24 h, weighed (dry weight of shoot and root biomass), and milled (Yevdokimov et al., 2006). The soil samples were divided into four subsamples: 50 g was dried at 105 ℃ to determine soil water content; 100 g was used to determine dissolved organic C (DOC, ^{13}C DOC) and microbial biomass C (MBC, ^{13}C MBC); 200 g was wrapped, instantly frozen in liquid nitrogen, and freeze-dried for subsequent phospholipid fatty acid (PLFA) and ^{13}C PLFA extraction; and the remainder was air-dried and used for soil C (SOM. ^{13}C SOM) and N analyses.

3.4.3 Analytical methods

MBC was determined using the chloroform fumigation-extraction method (Wu et al., 1990). The C and N in shoots, roots, and soil were determined using an automated C/N analyser (vario MAX; Elementar Analysensysteme GmbH, Germany). A Finnigan^{TM} MAT253 isotope ratio mass spectrometer (Thermo Electron Corporation) coupled with a FLASH 2000 elemental analyser (Thermo Fisher Scientific, US) was used to determine the $\delta^{13}C$ values of shoots, roots, soils, DOC, MBC, and PLFAs. PLFAs were extracted and measured according to Zhao et al. (2018), based on the procedures described in previous studies (Bossio and Scow, 1995; Zelles, 1999, 1997). Gas chromatography–mass spectrometry (GC–MS) was used to measure PLFA content.

3.4.4 Calculations and statistical analyses

3.4.4.1 ^{13}C in the rice system

The $\delta^{13}C$ and ^{13}C atom% values of shoots, roots, soil, DOC, MBC, and PLFA were calculated using the following equation (Lu et al. 2002a, b; Wu et al. 2009):

$$^{13}C_x = \frac{(^{13}C_{atom}\%)_{x,L} - (^{13}C_{atom}\%)_{x,UL}}{100} \times C_x,$$

where, $_L$ and $_{UL}$ are labelled and unlabelled samples, respectively; $^{13}C_x$ is the total ^{13}C content in the plant and soil samples; and C_x is the total C content in plant and soil samples.

$^{13}C_{atom}$% was calculated as follows:

$$^{13}C_{atom}\% = \frac{(\delta^{13}C + 100)R_{PDB}}{(\delta^{13}C + 100)R_{PDB} + 1} \times 100\%,$$

where R_{PDB} is the $^{13}C/^{12}C$ ratio of the standard international Pee Dee Belemnite (R_{PDB} = 0.0112372; Zhu et al. 2016).

$\delta^{13}C$ was calculated as follows:

$$\delta^{13}C = \frac{R_s - R_{PDB}}{R_{PDB}} \times 1000,$$

where Rs is the $^{13}C/^{12}C$ ratio of the plant or soil samples.

3.4.4.2 ^{13}C-PLFA community classification

Twenty PLFA biomarkers were used to determine the microbial community structure. The classification of ^{13}C-PLFAs used in the present study has been described previously by Zhao et al. (2018). Briefly, ^{13}C-PLFAs were divided into the following six groups: gram-positive bacteria (G+) (i14:0, i15:0, a15:0, i16:0, i17:0, and a17:0); gram-negative bacteria (G-) (17:1ω8c); arbuscular mycorrhizal fungi (AMF) (16:1ω5c), fungi (18:2ω6,9c, 18:1w9c), anaerobes (cy17:0, cy19:0); and actinomycetes (10Me17:0, 10Me18:0, and 10Me16:0). The data for all 20 PLFA biomarkers (including non-specific saturated PLFAs 14:0, 15:0, 16:0, 17:0, and 18:0) were used in redundancy analysis (RDA), heat map and hierarchical clustering analyses. For the purposes of these analyses, we used the relative percentage of each ^{13}C-PLFA biomarker in the total ^{13}C-PLFA.

3.4.4.3 Statistical analyses

One-way ANOVA followed by Tukey tests were used to determine significant differences ($p < 0.05$) between treatments for each time point separately. The Shapiro–Wilk test was used to assess the residuals for normal distribution. Homogeneity was assessed using the Levene test. Correlation analysis was conducted using SPSS 19 (SPSS Inc., Chicago, IL, USA). Figures were generated using Sigmaplot 11 (Systat Software, Inc.). RDA maps were plotted using Canoco 5 (Biometris, Wageningen, The Netherlands). R 3.4.0 was used to produce heat maps and hierarchical clustering figures. The means of three replicates ± standard error (SE) were calculated and are shown in the figures.

3.5 Results

3.5.1 Shoot and root biomass and root/shoot ratio

In fertilized and unfertilized soils, shoot and root biomass increased by 2.93- to 3.11-fold and 1.89- to 2-fold, respectively, which resulted in a 0.6- to 0.68-fold decrease in root/shoot ratio (Fig. 1). Compared with the single applications of +C or +N, simultaneous +CN fertilization increased shoot biomass by up to 3.11-fold at the most, but resulted in the smallest increase in root biomass (1.89-fold). Compared with single +C fertilization, +N amendment led to greater root development and resulted in a comparatively smaller root/shoot ratio.

3.5.2 Dynamic recovery of ^{13}C in plants, soil pools, and microbial biomass

Recovery of ^{13}C in shoots followed the order: +CN > +C > +N > Control,

whereas ^{13}C recovery in roots followed the opposite trend, which indicates that fertilization generally decreases plant investment in rhizodeposits (Fig. 2A, B).

+CN led to minimal ^{13}C recovery in SOM. In both fertilized and unfertilized soils, the ^{13}C recovery in SOM decreased during the first 5 days of labelling but had increased two-fold by the end of the labelling period (Fig. 2 C). This clearly indicates that there is a switch from rhizodeposit input from root exudates (1^{st} to 5^{th} days of labelling) to the input of photosynthates derived from root exudates, mucilage, and dead tissues (5^{th} to 39^{th} days of labelling).

In both fertilized and unfertilized soils, the ^{13}C recovery in PLFA and in MBC was strongly reduced at the tillering stage (1^{st} to 10^{th} days of labelling), and resulted in a similar ^{13}C recovery (4%) in PLFA at the end of the labelling (Fig. 2F). This pattern reveals that microorganisms switch from proliferation to rapid nutrient turnover during plant development.

3.5.3 Correlations among C or ^{13}C in roots, SOM, and MBC

Total soil C was closely related to root C (Fig. 3A). Application of cellulose (+C and +CN) led to higher amounts of SOM, which was derived from cellulose decomposition. ^{13}C incorporation into the soil was shown to be directly related to ^{13}C recovered in root biomass (Fig. 3C), and fertilization had negligible effects on this relationship. With the exception of +CN application, ^{13}C-MBC was exponentially related to total soil ^{13}C (Fig. 3D). The addition of +CN decreased the correlation between ^{13}C-MBC and ^{13}C in soil, which clearly indicates that, in the absence of fertilization, microorganisms are mainly stimulated by rhizodeposits. Thus, fertilization stimulates ^{13}C incorporation into microorganisms through both rhizodeposition and C and N addition.

3.5.4 Microbial communities associated with rhizodeposit utilization

Redundancy analysis of ^{13}C-PLFAs revealed that fertilization and plant growth stage had strong impacts on the soil microbial communities associated with photosynthate utilization (Fig. 4). Both factors together explained 81% of the variance in microbial community composition. Compared with plant growth stage, fertilization had a greater effect on microbial community composition. The plant and soil properties listed in Fig. 4 collectively accounted for 36.5% of the variance in microbial community composition.

Hierarchical clustering of ^{13}C-PLFA biomarkers (right panel in Fig. 5) confirmed that single +C or +N application led to the formation of similar microbial community compositions, and that these were distinct from those characterizing both +CN-amended and unfertilized soils. A heat map of ^{13}C-PLFA biomarkers (left panel in Fig. 5) indicates that fertilization activated specific members of the microbial community, with the addition of +C or +N stimulating G+ (a15:0, i15:0 and i16:00) and G- (17:1w8c) bacteria, and the addition of +CN stimulating G+ bacteria (a17:0), fungi (18:1ω9c), anaerobes (cy19:00), and actinomycetes (10Me16:0 and 10Me18:0). Addition of combined +CN and single additions of +C or +N resulted in the dominance of different ^{13}C-PLFA biomarkers, which were responsible for their variance in clustering classification.

For most microbial groups, application of fertilizers reduced the incorporation of rhizodeposit-derived ^{13}C into PLFA (Fig. 6). The most pronounced reduction was observed for G+ bacteria, and followed the order +C < +N < +CN. In contrast, N application specifically increased the incorporation of ^{13}C into G- bacteria.

3.6 Discussion

^{13}C or ^{14}C pulse labelling can be well used to estimate the distribution of recently photoassimilated C only at a fixed plant growth stage (Kuzyakov and Domanski,

2000). Given that photosynthate distribution patterns change during the course of plant growth (Weiner 2004), continuous labelling is particularly appropriate for estimating the distribution of net photosynthates among belowground soil C pools over a prolonged growth period (Liu et al., 2019; Meharg, 1994). Here, we used continuous $^{13}CO_2$ labelling over four rice growth stages (pre-tillering, post-tillering, elongation, and panicle formation stage), when photosynthate was being actively transferred into root and belowground C pools and utilized by microorganisms.

3.6.1 C and N fertilization regulates photosynthate distribution in the rice–soil system

Fertilizations led to an average of 27% of net photosynthesized C allocated to belowground roots and soil C pools (SOM, DOC, and MBC) (Fig. 2). This is broadly consistent with the reported 20% to 30% C allocation reported for annual agricultural crops such as wheat and barley (Kuzyakov, 2001; Liu et al., 2019; Pausch and Kuzyakov, 2018). However, fertilization resulted in only 12.7% of the net photosynthesized C being allocated to soil C pools (Fig. 2. CDE), which is half as much as the corresponding percentages (23%–28%) for rice growing seasons estimated by Wang et al. (2017). This difference can be attributed to the decreased root growth in response to the fertilizations (+C, +N, and +CN), which accordingly led to a lower input of rhizodeposits into soil C pools. Low root biomass typically occurs if NH_4^+ concentrations in the soil solution exceed 0.5 mM (Katwijk et al., n.d.; Peckol and Rivers, 1995). The concentration of mineral N in soil solution under +N and +CN fertilization was 3 mM (Fig. S1), which is sufficient to suppress root growth. In contrast, in the soil with only +C fertilization, plants depend on microbial activity to depolymerize and mineralize N, as N is bound in organic compounds that have minimal bioavailability for plants (Jacoby et al., 2017). Thus, +C fertilization leads to limited availability of N (Fig. S1C) and consequently reduced root development and a decrease in the allocation of ^{13}C to roots and belowground C pools. The +C application therefore promotes a strong N-mining response mediated by the close

interactions between microorganisms and roots. When compared with the single +C or +N additions, combined +CN application led to the largest increase in shoot biomass (Fig. 1A), which is consistent with a simultaneous maximal yield increase by organic C and mineral N fertilization (Pan et al., 2009; Zhang et al., 2012). Thus, +CN amendment reduces the input of photoassimilates into the soil as a consequence of decreased root growth.

Microorganisms in soil are generally in a dormant state (Blagodatskaya and Kuzyakov, 2013), as they are limited by the availability of labile C and energy sources (Hodge et al., 2000; Schimel and Weintraub, 2003). Here, photosynthates were ^{13}C labelled and released as low-molecular weight organic substrates in the form of root exudates, mucilage, and dead tissues (Dennis et al., 2010), which provide easily available sources of C to stimulate microbial growth in the rhizosphere. Simultaneous reductions in ^{13}C-DOC (Fig. 2D) and ^{13}C-SOM (Fig. 2C) during the first 5 days of labelling indicate that ^{13}C-DOC derived from root exudation is the main source of C used by microorganisms during this period. In contrast, the increase in ^{13}C-SOM after the 5^{th} day of labelling indicated the use of ^{13}C released from mucilage and dead tissues to support microbial growth, in addition to C derived from root exudates.

Newly synthesized ^{13}C-PLFA is an indicator of microbial proliferation (Zelles, 1997). The decreasing percentage of ^{13}C-PLFA in ^{13}C-MBC from the 1^{st} to 10^{th} days of labelling (Fig. 2F) also indicates that microorganisms switched from growth and proliferation to a rapid microbial turnover. Simultaneous application of +CN led to higher ^{13}C-PLFA incorporation (Fig. 2F) than did single application of either +C or +N, thereby indicating that the combined addition of C and N meets the microbial C:N stoichiometric requirements(Cleveland and Liptzin, 2007; Qiu et al., 2016; Zhu et al., 2018). However, even though +CN application accelerated the microbial nutrient turnover, the ^{13}C incorporated into PLFA accounted for only 0.16‰–0.32‰ of total net photosynthesized C in the rice–soil ecosystem at the end of labelling. The smallest ^{13}C-MBC (Fig. 2E) and maximum shoot ^{13}C biomass (Fig. 1A) in response to +CN addition revealed that the rate of nutrient turnover by MB, rather than the MB

content , is the key driver of aboveground plant growth and the rate-limiting step in productivity (Schimel and Bennett, 2004).

3.6.2 C and N fertilization regulates the quantity and quality of SOM

With a low oxygen content and large input of rice straw, the decomposition rates of added organic fertilizers and SOM are slow in paddy fields, which favours SOM accumulation (Kögel-Knabner et al., 2010). Numerous studies have reported that straw addition increases C sequestration in soils (Follett, 2001; Janzen et al., 1998), which is consistent with our findings (Fig. 3A). Fertilization did not affect the correlations between ^{13}C-SOM and root-^{13}C (Fig. 3C); however, the addition of cellulose (+C and +CN) leads to higher correlations between SOM and root-C (Fig. 3A). This clearly indicates that the increase in SOM, in response to the addition of cellulose, is derived from cellulose decomposition rather than from rhizodeposition or dead root tissues.

The mean residence time of root-derived C (rhizodeposition, mucilage, and dead tissues) in SOM has been found to be 2.4 times longer than that of straw-derived C (cellulose addition) (Rasse et al., 2005). Whether SOM undergoes stabilization or mineralization is dependent on the relative proportions of root-derived and straw-derived C in the SOM (Rasse et al., 2005). Here, fertilization decreased the amounts of root-derived C in SOM (Fig. 2C) via decreases in root biomass (Fig. 1B) and ^{13}C in roots (Fig. 2B). Moreover, fertilization also increased the percentage of cellulose-derived C in SOM (Fig. 2A), thereby indicating that in addition to enhancing soil C sequestration, fertilization also increases the SOM instability. Accordingly, fertilization of paddy fields challenge the attempt to establish a balance between pursuing agricultural economic productivity and aiming to promote environmental protection, as fertilization increases both the yield of rice (Fig. 1A) and SOM instability (Fig. 3). The simultaneous addition of +CN substantially decreased the correlation in ^{13}C between MBC and soil (Fig. 3D), which indicates that

fertilization strongly regulates the microbial community that utilizes rhizodeposits (Fischer and Kuzyakov, 2010). Thus, fertilization decreases SOM stability by increasing cellulose-derived C in SOM and decreasing the incorporation of rhizodeposit-derived ^{13}C into microorganisms (Hinsinger et al., 2009; Kuzyakov and Blagodatskaya, 2015).

3.6.3 C and N fertilization regulates the composition and abundance of the microbial community

RDA of ^{13}C-PLFAs revealed that fertilization and plant growth stage had the strongest effects on the microbial communities associated with photosynthate utilization (Fig. 4). Collectively, the factors relating to fertilization and plant growth stage explained 81% of the variance in the composition of the microbial community associated with rhizodeposit utilization. Furthermore, fertilization affected shoot and root biomass (Fig. 1), MBC, DOC, SOM (Fig. 2), and soil inorganic and organic N (Fig. S1), which are responsible for 36.5% of the variance in the microbial community. RDA revealed closer ^{13}C-PLFA clustering in response to single additions of +C and +N compared with that in +CN amended or unfertilized soil. These findings were corroborated by hierarchical clustering of the ^{13}C-PLFAs (Fig. 5). It showed that soils fertilized with either +C or +N have similar microbial community compositions. And these microbial community compositions following +C or +N addition were clearly distinct from those detected following +CN application or in unfertilized soils. The heat map analysis (Fig. 5) revealed that simultaneous +CN or single +C/+N fertilizations stimulated and led to the predominance of discrete microbial groups (based on the relative percentage of ^{13}C-PLFAs). Namely, single addition of +C/+N increased the relative percentage of G- (17:1w8c) and G+ (i15:0, i16:0 and a15:0) bacteria, whereas simultaneous application of +CN specifically increased the relative percentage of G+ bacteria (a17:0), anaerobes (cy19:0), fungi (18:1ω9c), and actinomycetes (10Me16:0 and 10Me18:0). In addition to promoting the dominance of

specific microbial groups through increasing the relative percentage of specific ^{13}C-PLFAs, each fertilization type substantially decreased the ^{13}C incorporation in PLFAs in most microbial groups (Fig. 6) compared with that in unfertilized soil. The contrast between the increase in the relative percentage of ^{13}C-PLFAs (Fig. 5) and the decrease in ^{13}C-PLFA content (Fig. 6) indicates that fertilization affected both the composition and abundance of the microbial communities.

3.7 Conclusions

Continuous $^{13}CO_2$ labelling of rice plants was used to measure the combined effects of cellulose degradation (to mimic the effects of long-term rice straw decomposition) and $(NH_4)_2SO_4$ application on the distribution of photosynthates (^{13}C) in a rice–soil system (Fig. 7). Cellulose (+C and +CN) addition increased soil C content through decomposition of cellulose, and simultaneously decreased the root-derived ^{13}C (rhizodeposition) in SOM. Thus, despite its positive effects on shoot biomass, the application of cellulose can destabilize SOM and reduce its quality. Compared with single +C or +N amendment, +CN maximally promoted shoot biomass and ^{13}C incorporation into shoots, which led to the smallest root biomass and root to shoot ratio. In turn, simultaneous +CN led to the minimal recovery of rhizodeposits (^{13}C) in root, soil (DOC and SOM), and microbial C (DOC, MBC, and PLFA) pools. Fertilization stimulated microbial proliferation during the first 5 days of labelling through rhizodeposition, and markedly reduced ^{13}C recovery in DOC and SOM. RDA, hierarchical clustering and heat map analyses of microbial communities (^{13}C-PLFA) revealed that, single (+C, +N) or combined (+CN) fertilization promoted the dominance of specific microbial groups associated with the utilization of rhizodeposits. Single +C or +N addition specifically increased the relative percentages of G- (17:1w8c) and G+ (i15:0, i16:0, and a15:0) bacteria, whereas combined +CN application specifically increased the percentages of G+ bacteria (a17:0), anaerobes (cy19:0), fungi (18:1ω9c), and actinomycetes (10Me16:0 and 10Me18:0).

Fertilization largely decreased the ^{13}C incorporation into PLFAs, thereby indicating that fertilization affects both the composition and abundance of microbial communities. Factors related to fertilization and plant growth stage collectively explained 81% of the variance in microbial community composition. Plant (shoot and root biomass) and soil properties (MBC, DOC, SOM, and N properties) were affected by fertilization and were responsible for 36.5% of the variance in the shift in microbial communities. Thus, by modifying the recovery and distribution of photoassimilates (^{13}C) in the rice–soil system and by regulating plant and soil properties, fertilization ultimately promotes a shift in the dominance of microbial groups, and reduces the abundance of microbial biomass associated with rhizodeposit utilization.

3.8 Acknowledgments

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Figure legends

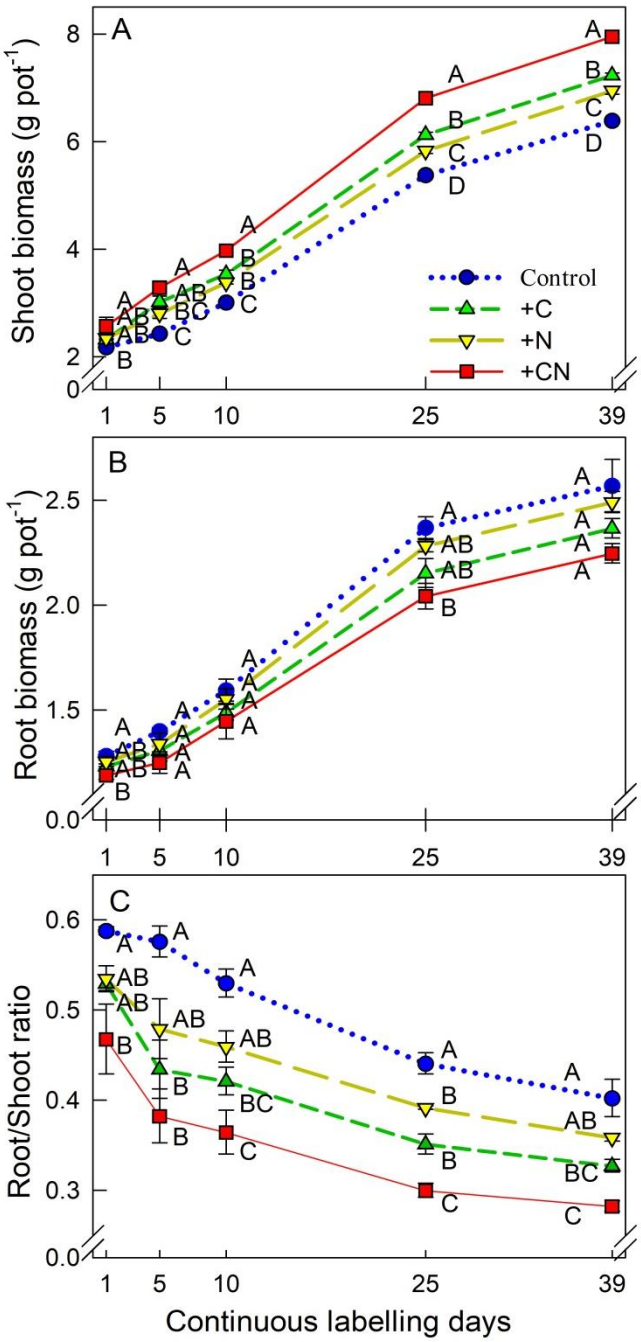

Fig. 1. Dynamics of plant properties during 39 days of continuous $^{13}CO_2$ labelling. A: Shoot biomass (g pot^{-1}). B: Root biomass (g pot^{-1}). C: Root/Shoot ratio. Lines and symbols in the legend represent treatments: Control (no addition), +C (cellulose addition), +N (ammonium sulphate addition), and +CN (combined cellulose and ammonium sulphate addition). Data points represent means (n = 3), error bars represent standard errors. Capital letters adjacent to the points represent significant

differences ($p < 0.05$) between treatments for the same labelling day.

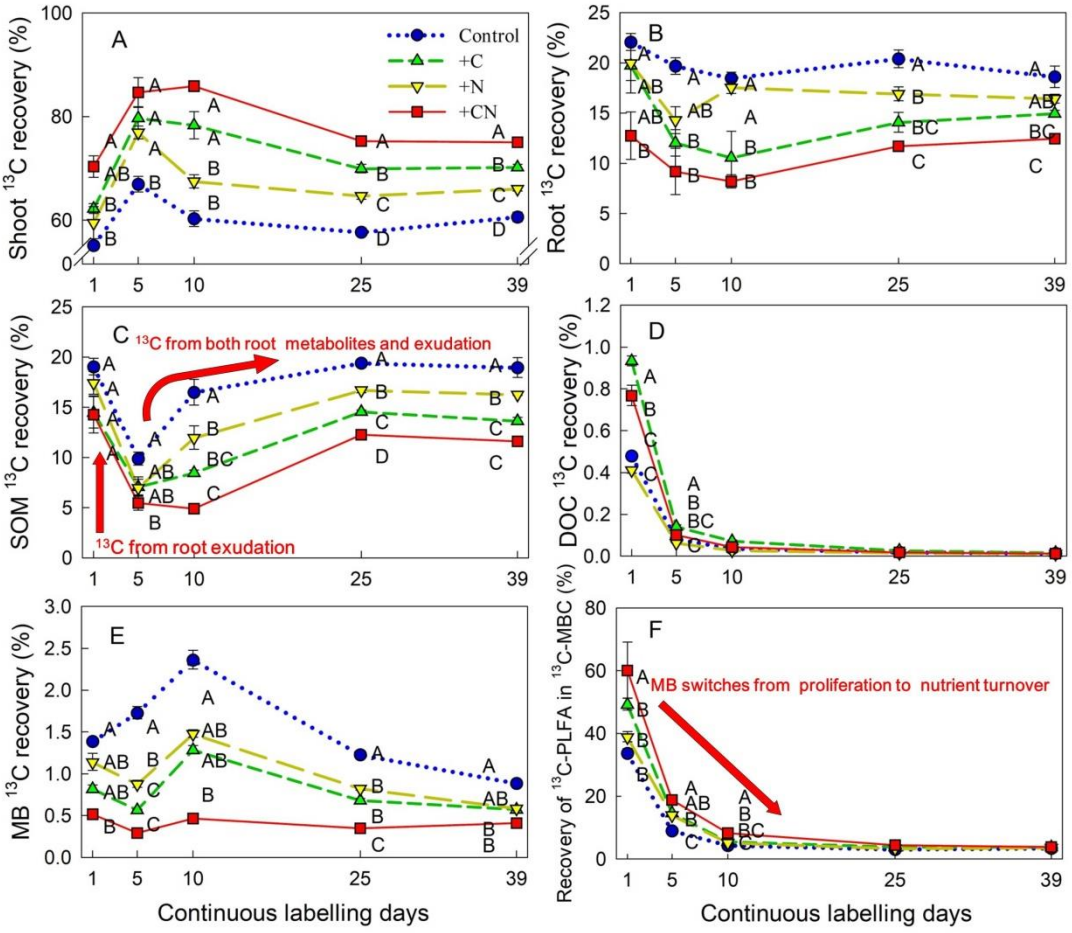

Fig. 2. Dynamics of above- and belowground rice ^{13}C budget and microbial utilization of rhizodeposits during 39 days of continuous $^{13}CO_2$ labelling. From figure A to E are the percentage of ^{13}C recovery (% of total ^{13}C assimilated) in shoot, root, SOM (total ^{13}C-SOM in soil without ^{13}C in MBC and DOC), DOC, and MBC, respectively. Figure F is the percentage of ^{13}C-PLFA in ^{13}C-MBC. Lines and symbols in the legend represent treatments: Control (no addition), +C (cellulose addition), +N (ammonium sulphate addition), and +CN (combined cellulose and ammonium sulphate addition). Data points represent means (n = 3), error bars represent standard errors. Capital letters adjacent to the points represent significant differences ($p < 0.05$) between treatments for the same labelling day.

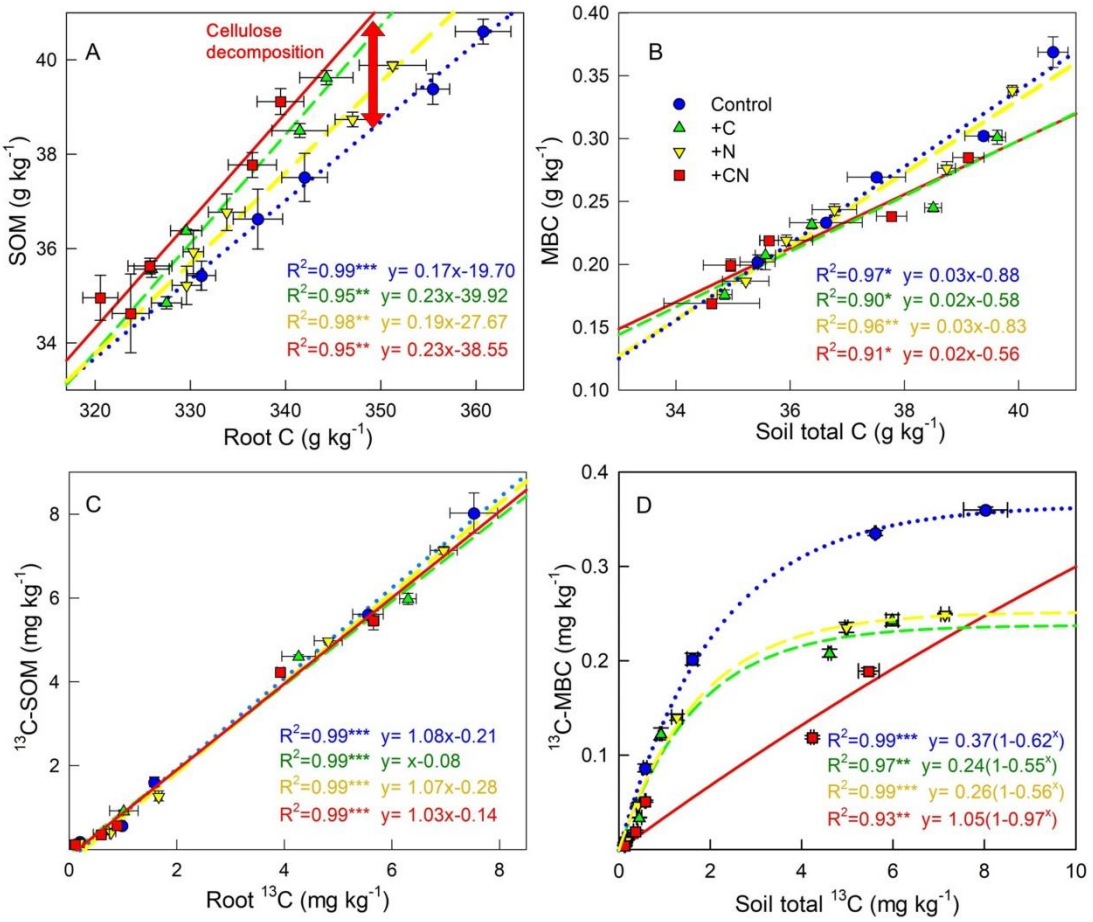

Fig. 3. Relationships between root-C and SOM, SOM and MBC, root ^{13}C and ^{13}C-SOM, ^{13}C-SOM and ^{13}C-MBC. Lines and symbols in the legend represent treatments: Control (no addition), +C (cellulose addition), +N (ammonium sulphate addition), and +CN (combined cellulose and ammonium sulphate addition). Data points represent means (n = 3), the crossed standard errors represent the standard error from x and y axis, respectively. Formulae in the legends are the equations of each regression line. R^2 values are coefficients of determination of the regression lines. The *p*-value is the asymptotic significance of each regression line. *, **, and *** represent the statistical significance at the probability levels of 0.05, 0.01, and 0.001, respectively.

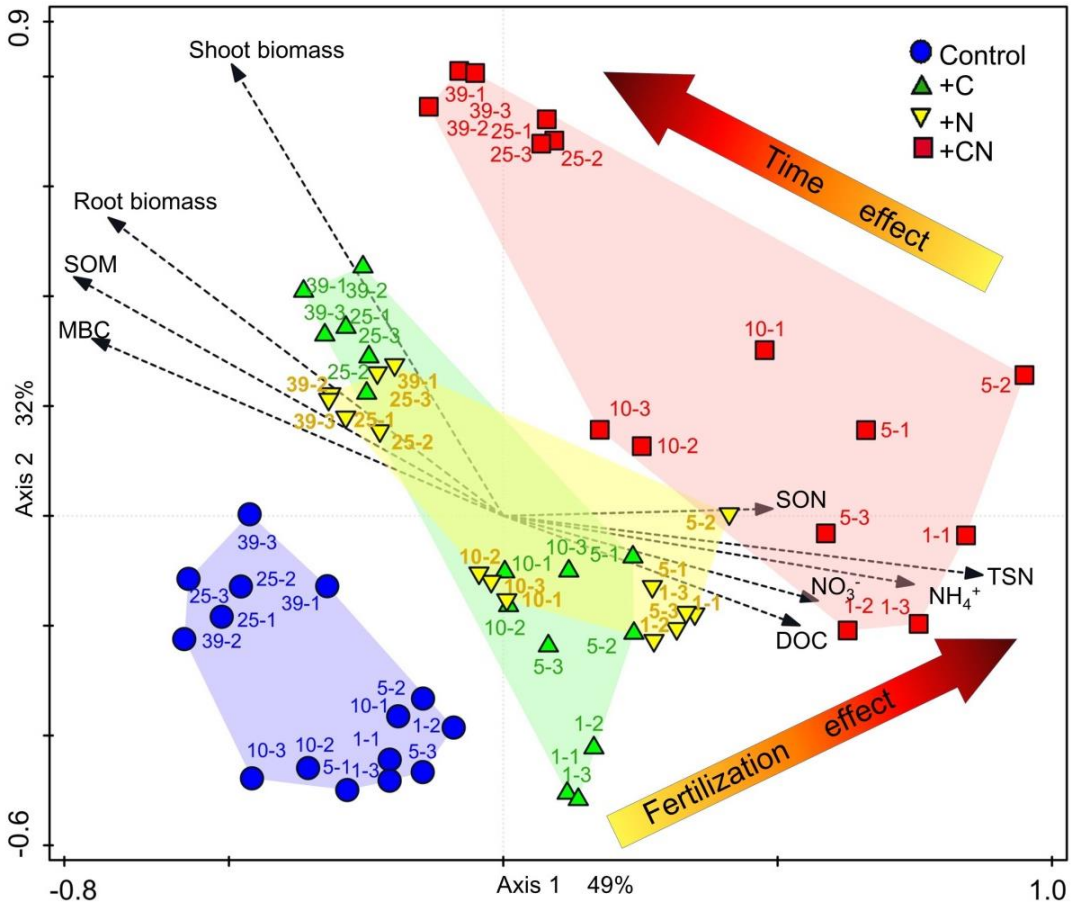

Fig. 4. Redundancy analysis of ^{13}C-PLFA biomarkers on the 39th day of labelling. Symbols in the legend represent treatments: Control (no addition), +C (cellulose addition), +N (ammonium sulphate addition), and +CN (combined cellulose and ammonium sulphate addition). Numbers before the dash are the labelling days (1, 5, 10, 25, and 39), whereas numbers after the dash are the replicates of each labelling day (1, 2, and 3). 49% and 32% are the explained fitted variation along axis 1 and axis 2, respectively.

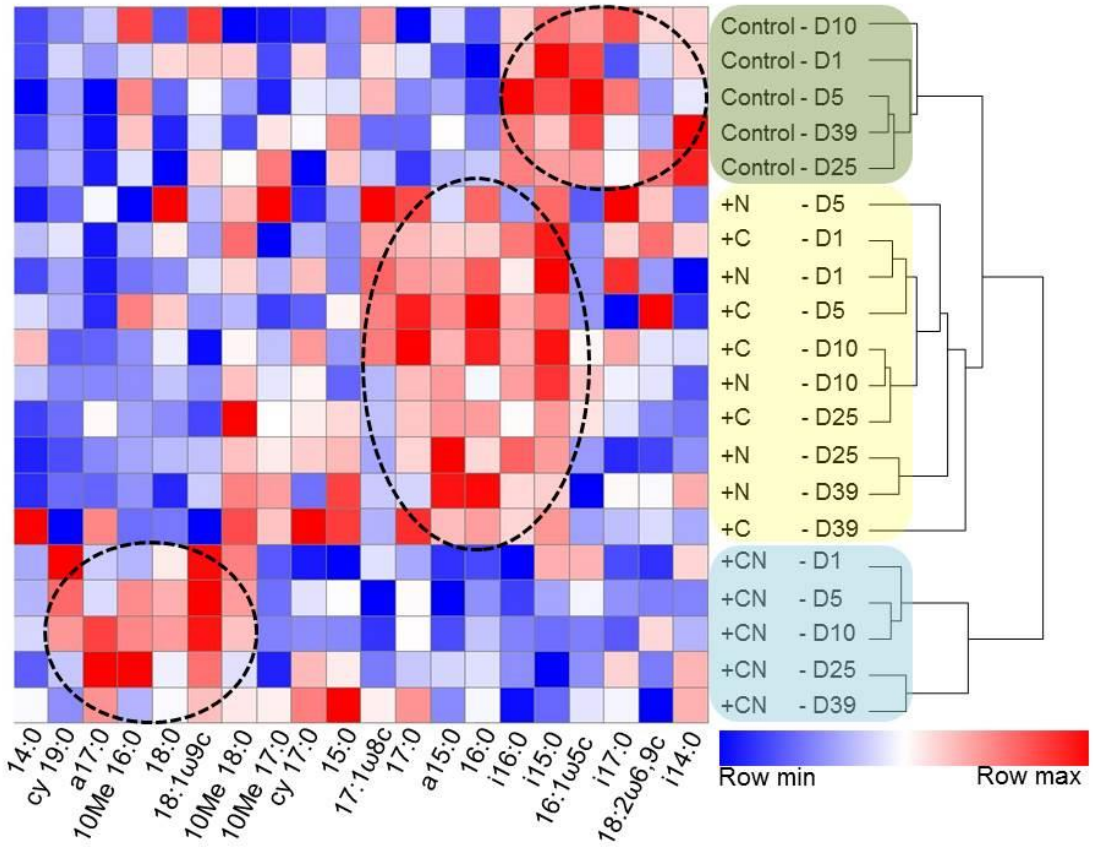

Fig. 5. Heat map and hierarchical clustering of ^{13}C-PLFA biomarkers. The heat map shows all the relative amounts of ^{13}C PLFA biomarkers in each treatment. Each coloured square of the ^{13}C-PLFA biomarkers in the heat map is the relative percentage of a particular ^{13}C-PLFA biomarker in each treatment (amount of each ^{13}C-PLFA biomarker/total amount of ^{13}C-PLFA biomarkers in each treatment $\times 100$). Colour bar: row min and row max represent the highest and lowest relative percentage of ^{13}C-PLFA biomarkers in each treatment. Hierarchical clustering on the right-hand side shows the treatment groups that have a similar composition of ^{13}C-PLFA biomarkers (control, +C and +N addition and +CN addition). Control: no addition, +C: cellulose addition, +N: ammonium sulphate addition, and +CN: combined cellulose and ammonium sulphate addition. Black circles show the dominant biomarkers in each clustering branch. Numbers after the letter D (day) represent the labelling days (1, 5, 10, 25, and 39).

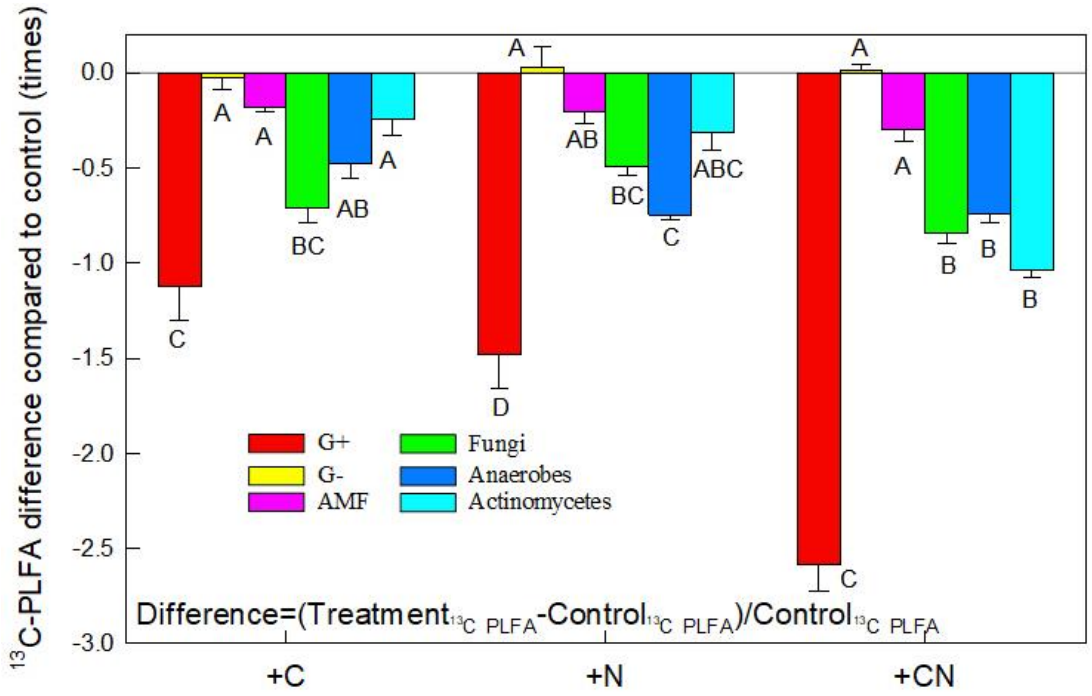

Fig. 6. Changes in ^{13}C-PLFA content relative to the control soil at the end of continuous $^{13}CO_2$ labelling. Relative changes were calculated as (treatment^{13}C-PLFA-Control^{13}C-PLFA)/Control^{13}C-PLFA. Microbial groups are as follows: G+ (gram positive bacteria); G- (gram negative bacteria); AMF (arbuscular mycorrhizal fungi), Fungi, Anaerobes, and Actinomycetes. Letters on the x-axis represent fertilization treatments: +C (cellulose addition); +N (ammonium sulphate addition), and +CN (combined cellulose and ammonium sulphate addition). Each column represents the mean of the difference to the control (n = 3). Capital letters adjacent to the columns represent significant differences ($p < 0.05$) between microbial groups based on the ^{13}C-PLFAs for the same treatment.

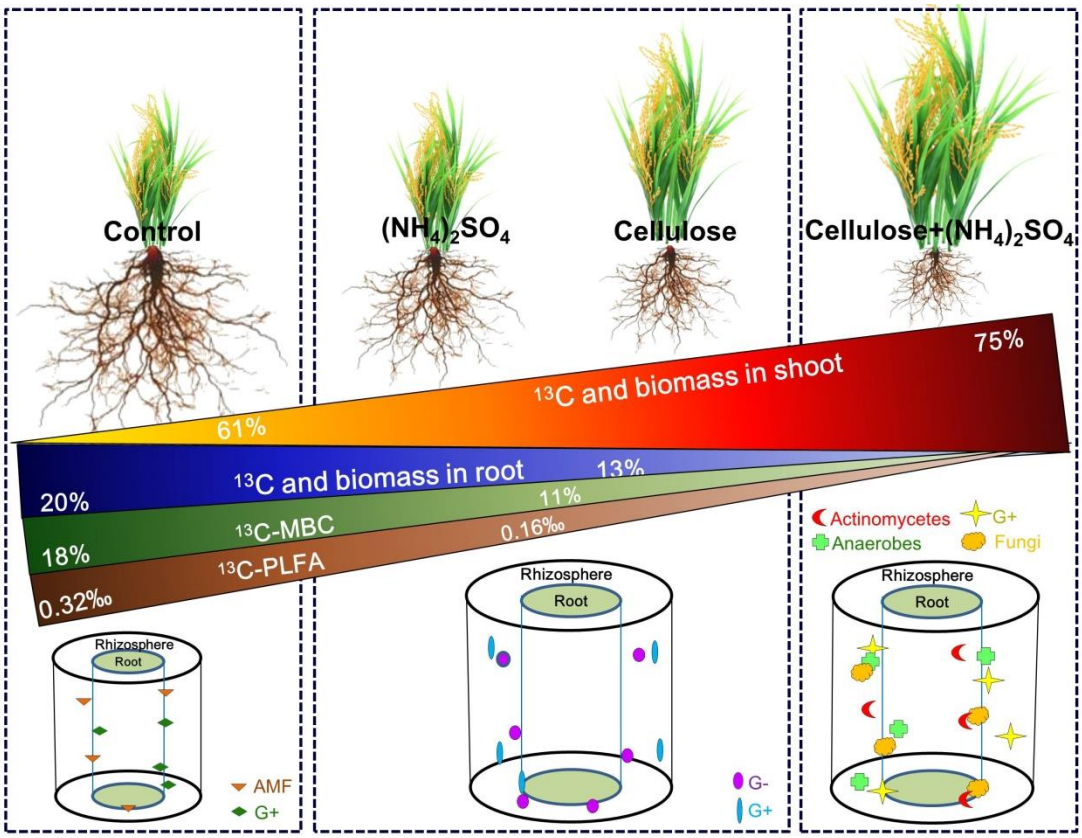

Fig. 7. Conceptual schematic of morphology of rice plants, recovery of ^{13}C in rice–soil system on the 39th labelling day, and descriptions of microbial community compositions related to photosynthate utilization depending on C and N fertilization. Four treatments: Control, Cellulose, $(NH_4)_2SO_4$, and Cellulose + $(NH_4)_2SO_4$ application. Coloured geometrical shapes indicate ^{13}C-PLFA biomarkers representing microbial groups.

Chapter 4

Water effects on enzyme activities in paddy soil: triple combination of ^{14}C imaging, pH mapping and zymography

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4.1 Abstract

Flooding in paddy field creates anaerobic conditions, which changed root morphology and soil physiochemical properties, such as root iron plaque, rhzodepositions and pH. The impact of those key factors on enzyme activities in paddy field remain unknown. For the first time, we introduced triple combination of ^{14}C imaging, pH mapping and zymography in paddy soils. It enabled to evaluate the water effects from root iron plaque, rhzodepositions and pH on five enzyme activities involved in carbon (C) (β- glucosidase, cellobiohydrolase), nitrogen (N) (leucine aminopeptidase), and phosphorus (P) (phosphatase) cycling. By contrasting under 25% and oversaturated H_2O content, we confirmed the hypotheses: 1) flooding increased root biomass with decreased water use efficiency; 2) flooding improved rhzodeposition (^{14}C) with decreased pH in both rhizosphere and bulk soil; 3) flooding is the dominant factor to determine the spatial distribution patterns of enzyme activities. Through diffusion effects, flooding evenly distributed enzyme activities vertically. The water effects increased enzyme activities through forming of iron plaque, increased rhzodepositions and decreased pH, both of which also enhanced by flooding. Through 3D mesh and contour map, we successfully evaluated the correlations of enzymes involved in C, N and P cycling simultaneously. Flooding resulted in water canceling effects, which canceled several optimal combination peaks of C, N and P related enzymes through diffusion. And flooding ultimately narrowed the optimal combination area. Conclusions can be made that, water effects improved formation of root iron plaque, increased rhzodepositions and decreased pH, both of which, in turn, dominated both the quantity and distribution of enzyme activities in paddy field.

Keywords: ^{14}C labeling, pH mapping, zymography, hotspots, enzymes, water effects

4.2 Introduction

Rice as the staple food for half of the world's population (Huang et al., 2011; Kögel-Knabner et al., 2010), is grown on more than 155 million ha of the world's surface (IRRI 2015). Paddy soils differentiate from dry land soils for their continuous or intermittent flooding. This flooding creates anaerobic conditions by oversaturated water, and induces limited gas exchange in both soil and root zone. These long-term flooding process led to physical and chemical changes in soils, such as increased extractable Fe, pH and redox potential (Eh) fluctuation (Hemati Matin and Jalali, 2017), higher organic matter content (SOM) (Liping and Erda, 2001), and higher microbial biomass (Kabra et al., 2007; Kashem and Singh, 2004; Zheng and Zhang, 2011). Flooding conditions also led to the evolution of rice root to adapt typify semiaquatic structures with specialized tissues (e.g. sclerenchyma, aerenchyma) to permit root growth during water oversaturated conditions. Enzymes, which excreted by both roots and soil microorganisms (Nannipieri et al., 2007; Sinsabaugh et al., 2008), can be mediated by abiotic factors (water content, pH) and biotic factors (enzyme synthesis and secretion by microbes and root) (Burns et al., 2013). Given the implications of microenvironment changes in rice root and soil properties by flooding, it is relevant to expect distinctive enzyme patterns in both quantity and spatial distributions in paddy soils. By using the *in situ* zymography in present study, it enables determining both the spatial distribution and the general quantity of enzyme activities in paddy soils (Ge et al., 2015; Razavi et al., 2016).

Rather than the former nature of hydragric anthrosols (IUSS Working Group WRB, 2006), paddy soil is more sensitive to anthropogenic managements, such as continuous or intermittent flooding, rice straw retention and fertilization (Barnes, 1990; Kawaguchi and Kyuma, 1977; Kögel-Knabner et al., 2010). Anoxic conditions prevail either in continuous or intermittent flooding during most time of rice growth (Kögel-Knabner et al., 2010). Conclusion drawn by Quantin et al. (2008) indicate that the overall effect of continuous and intermittent flooding either increased or decreased

pH, and it is affected by numerous factors (organic matter, elemental composition, element speciation etc.) (Ethan, 2015). This pH fluctuation also severely affected soil enzymes, as each enzyme has a certain optimal range of pH for activity (Caldwell, 2005). However, limited *in situ* results were shown the impact of changed pH by flooding on enzymes in paddy soils. We combined *in situ* pH mapping with zymography in present study to reveal the influence on enzymes from pH by contrast of un-flooded and flooded paddy soils with rice root existed.

Flooding also plays an important role in the release and consequent impacts of root exudates (Song et al., 2012). Root exudates (rhizodeposition) prepared easily available energy source for microbial activity, and create a biological niche, where high abundance, activity, and diversity of microorganisms exist (Pausch and Kuzyakov, 2011a). With averaged 10% of net photosynthesized C (labile C) allocated into roots (Holz et al., 2018), the rhizosphere is defined as an important hotspot (Kuzyakov and Blagodatskaya, 2015a), where higher microbial activity with higher enzyme exudation can be expected. By triple combined pulse ^{14}C labeling with *in situ* pH mapping and zymography, it is able to track the rhizodeposition and its impact on enzyme hotspots.

Flooded soil have a 12%–58% higher SOM content when compared to upland soils (Liping and Erda, 2001). And results showed that the higher SOM is mainly derived from straw decomposition rather than from rhizodeposition (Zhao et al., 2018). Cellulose and hemicellulose together, accounts for more than 56% constructional component in rice straw (Howard et al., 2003). For present study, when selecting representative enzymes related to C cycling in paddy field, β-glucosidase, cellobiohydrolase and xylanase should be taken into account. As xylanase, β-glucosidase and cellobiohydrolase are responsible for hemicellulose and cellulose degradation, respectively. Leucine aminopeptidase, which is involved in protein degradation in the N-cycle, was selected. Phosphatase, which catalyzes the hydrolysis of organic P into phosphate esters, was selected. With five enzymes involved in C, N

and P cycling, and by triple combination of ^{14}C labeling, pH mapping and zymography, this study was designed to 1) reveal the *in situ* pH by contrasting flooded and un-flooded paddy soils with the present of rice root; 2) evaluate rhizodepositions in flooded and un-flooded soils and the effects of hotspots on enzyme activities through ^{14}C labeling; 3) to describe the quantity and spatial distribution patterns of enzyme activities in paddy soils under the influence of pH and rhizodepositions (^{14}C) through zymography. We hypothesized that 1) flooding may led to lower pH around both rhizosphere and bulk soil; 2) flooding may led to higher root biomass with more rhizodeposition (^{14}C), and stimulate enzyme exudation from both roots and microorganisms; 3) flooding may result in high degree of enzyme diffusion, which may led to moderate spatial distribution patterns of enzyme activities horizontally and vertically.

4.3 Materials and methods

4.3.1 Soil, plant preparation and treatments set up

The typical paddy soils (ploughing Anthrosol) were selected from a paddy field station located at Changsha Research Station for Agricultural and Environmental Monitoring, Hunan Province, China (28°33′04″N, 113°19′52″E, 80 m a.s.l.). The station climate is located in typical subtropical area with averaged 17.5 ℃ and annual rainfall of 1300 mm. The paddy fields followed a rice-fallow-rice rotation for over 30 years. Soils were collected from 0–20 cm soil layer, and had following properties: pH 6.95 (1:2.5, soil:water ratio), 16.8 g C kg^{-1} soil, 2.3 g N kg^{-1} soil, 0.49 g P kg^{-1} soil, 18.2 mg Olsen-P kg^{-1} soil, 6.7% clay, 72.2% silt, and 20.0% sand. Visible plant residues, algae, and stones were removed. Soils were thoroughly mixed, air-dried and sieved (4-mm mesh).

The four treatments were (1) no rice planted, 25% water content; (2) no rice planted, constantly flooding (water oversaturated, 4-6cm water layer); (3) rice planted, 25% water content; (4) rice planted, constantly flooding (water oversaturated, 4-6cm water

layer). Each treatment has 3 replicates. A total 12 rhizoboxes were prepared: 3 replicates ×4 treatments. To prevent water leaking, 1 kg sieved dry soils were filled in a transparent cuboid plastic bag (28×13×3.8 cm) and then put into the each rhizobox (25×13.5×4 cm). The water content was adjusted according to (1)-(4) described above during the entire experiment period. Common wild type rice seeds (*Oryza rufipogon* Griff.) were first germinated on wet filter paper in darkness for 72 h. Thereafter, the germinated seeds were transferred into 25ml centrifuge tubes with Hoagland's solution to cultivated for another 7 days in the climate chamber. The climate chamber had controlled temperature around 25 ± 1 ℃, 14 h daily light per day with 250 μ mol m^{-2} s^{-1} photosynthetically active radiation. After 7 days of cultivation, rice plants with similar heights and weights were selected and transferred into rhizoboxes in treatment (3) and (4), each rhizobox cultivated 1 rice plant. All the rhizoboxes in treatment (1)-(4) were 45° inclined during the whole experiment period to keep roots grow along the upper wall of rhizobox. Distilled water were irrigated from the top of rhizobox to maintain the required water content. All the rhizoboxes were kept in the climate chamber during the entire experiment period. After 20days of cultivation, ^{14}C pulse labeling was performed, followed with *in situ* pH mapping, zymography and ^{14}C imaging. After ^{14}C imaging, rice shoots and roots were cut at the interface between soil and the irrigating water layer. Shoots and roots were dried at 60 ℃ for 3 days and weighted.

4.3.2 $^{14}CO_2$ pulse labeling and ^{14}C imaging

All rhizoboxes were simultaneously pulse labeled in a labeling chamber, which previously described by Razavi et al., (2017). ^{14}C Pulse labelling was performed from 10:00 to 15:00 at a temperature of 25 ℃ with an air fan for intensive internal air circulation in the labeling chamber. During the whole labeling period, the transparent labeling chamber was kept under daylights equal to 250 μ mol m^{-2} s^{-1} photosynthetically active radiation. ^{14}C was generated in a vial with tubes connected to the labeling chamber. $^{14}CO_2$ released through reaction between Na_2 $^{14}CO_3$ (4.4

MBq of ^{14}C as $Na_2\ ^{14}CO_3$ in 10 ml 0.2 M Na_2CO_3solution) and H_2SO_4 (30 mL, 1 M). After the pulse labeling, the unassimilated $^{14}CO_2$ was trapped through pumping the air from the chamber to NaOH (100ml 1M) solutions.

The ^{14}C imaging process was previously described by Holz et al.(2019). Briefly, after labeling, each rhizobox was taken a ^{14}C image by putting an imaging plate (Storage phosphor screen, BAS-IP MS 2040 E, VWR) on for 15h in darkness. And then the imaging plates were scanned (FLA 5100 scanner, Fujifilm) with a spatial resolution of 50 μm to get a ^{14}C image.

4.3.3 pH mapping

After 14C imaging, the cuboid transparent plastic bag was carefully cut open along the upper part of the rhizobox. Then, the pH sensors (10×14 cm, SF-HP5R) were immediately attached on the root-soil surface. The pH mapping process was previously described by Ma et al. (2019). Briefly, by attaching to the soil surface, the pH sensors with embedded fluorescent indicator dye (Blossfeld and Gansert, 2007) can be activated under ultra-violet light with an excitation wavelength of 470 nm. A camera (VisiSens TD) connected to a PC then captured the activated fluorescence signals. Then the signals were analyzed by image capture software (VisiSens AnalytiCal, PreSens GmbH, Regensburg, Germany).

4.3.4 Soil zymography

After pH mapping, immediate *in situ* zymography was applied. Enzymes involved in C, N and P cycles were measured using previous protocol (Dong et al., 2007; Razavi et al., 2016b; Spohn et al., 2013). 4-methylumbelliferone (MUF) or 7-amino-4- methylcoumarin (AMC) combined substrates become fluorescent when it hydrolyzed by target enzymes (Dong et al., 2007): substrates 4-Methylumbelliferyl-β-D-glucoside (MUF-G) for enzyme β-glucosidase; 4-methylumbelliferyl-β-D-cellobioside (MUF-C) for cellobiohydrolase; 4-methylumbelliferyl-β-D- xylopyranoside (MUF-X) for xylanase;

L-leucine-7-amido-4-methylcoumarin hydrochloride (AMC-L) for leucine aminopeptidase; and 4-methylumbelliferyl-phosphate (MUF-P) for phosphatase. Briefly, each substrates separately dissolved in MES buffer (for MUF combined substrates, 12 mM, $C_6H_{13}NO_4SNa_{0.5}$) and TRIZMA buffer (for AMC combined substrates, $C_4H_{11}NO_3$ ·HCl, $C_4H_{11}NO_3$, 12 mM) (Sigma-Aldrich, Germany) (Koch et al., 2007). Then, fully saturate polyamide membrane (16×11.5 cm, pore size 0.45 mm, Tao Yuan, China) with the substrate in buffer. And attach the membrane to the root-soil surface for 1 h (Grierson and Comerford, 2000). Then, gently lift the membrane without any disturbance to the root-soil surface, and carefully remove any soil particles with tweezers. Then membrane can be placed under UV light (355 nm wavelength) in darkness, and image of zympgraphy taken by camera (EOS 5D, Canon) with 5 ms exposure time (Guber et al., 2018; Spohn et al., 2013). Enzymes detecting order followed as: β-glucosidase, cellobiohydrolase, xylanase, leucine aminopeptidase and phosphatase. During the entire zymography process, the camera kept in same height and settings, with same UV light wavelength.

4.3.5 Image and statistical analyses

The ^{14}C activities were transformed from grey value obtained in ^{14}C image according to standard calibration line using algorithm:

$$y = ax + b \qquad (1)$$

Where y is the ^{14}C activities (Bq), a is the slope of standard calibration line, x is the gray value of the ^{14}C image. Each treatment had their own calibration line by setting up the soils by adding increasing ^{14}C activities (6.5, 98, 1085, 5953, 10070 KBq). Then, the standard ^{14}C images were taken under the same situations as in ^{14}C imaging described above (Holz et al., 2019; Pausch and Kuzyakov, 2011b).

The pH values was calculated from *in situ* pH imaging using Boltzmann Equation(Blossfeld and Gansert, 2007; Faget et al., 2013). The calibration line was calculated by soaking pH sensors (2×2 cm) in increasing standard buffer solution of

pH (7.7, 7.6, 7.5, 7, 6.5, 6, 5.5) by mixing of $NaH_2PO4 \cdot H_2O$ and $Na_2HPO_4 \cdot 2H_2O$ (Ma et al., 2019). Then, the standard pH sensors were taken photographs under the same situation as in pH imaging.

Enzyme activities were transferred from the grey values of obtained images in zymography using standard calibration algorithm described by (Kuzyakov and Razavi, 2019). Under the same situation as in zymography, the calibration line was calculated by taking photograph of saturated membranes (2×2 cm) with standard concentrations of MUF (0, 0. 1, 0.2, 0.5, 1, 2, 4, 6, 10 mM) or AMC (0, 0.01, 0.2, 0.5, 1, 2, 3, 4, 5 mM). During calculation, the volume of MUF or AMC solutions taken up by each membrane had also been taken into account, and was calculated as pmol MUF or AMC /cm^2/h.

Both ^{14}C and zymography images were converted to 32-bit gray values with camera noises corrected. Each grey value of pixel points was transferred into ^{14}C or enzyme activities as described above. Top 20% enzyme activity was defined as a hotspot, and hotspot area was calculated based on the pixel size. pH images were split in to single R (red), G (green) and B(blue) channels and further transformed into 32-bit gray values, and then converted to pH values using Boltzmann Equation and calibration line described above. The images processed and analyzed using Fiji platform in image J (Schindelin et al., 2012).

Shapiro-Wilk's test and Levene's test were used to check the normality and homogeneity of data. One-way ANOVA followed with Duncan-test was used to determine significant differences ($p < 0.05$) between treatments. SPSS 19 (SPSS Inc., Chicago, IL, USA) were used during these tests. Figures were created using Sigmaplot 11 (Systat Software, Inc.). Heat map was plotted using R 3.4.0. Means of three replicates ± standard error (SE) was calculated.

4.4 Results

4.4.1 The response of roots, phosphatase activity (^{14}C) and pH to flooding

Oversaturated H_2O increased the H_2O content in both rhizosphere and bulk soils (Fig. 1E). Both 25% and oversaturated H_2O content resulted in lower percentage of H_2O in rhizosphere compared to bulk soil. And oversaturated H_2O also led to higher root and shoot biomass with higher root/shoot ratio. Flooding also formed an obvious iron plaque (yellowed roots, Fig. 1D) at the surface of the roots.

In ^{14}C imaging, root tips (caps and its' following meristem) are the primary and fundamental sources of rhizodeposition (^{14}C) in both flooded and un-flooded soils (Fig. 2 A, B). Flooding led to two times higher secretion of rhizodeposits (Fig. 2C) and relatively higher photosynthates distribution in bulk soil compared to un-flooded soils (Fig. 2 A, B).

In pH mapping, both flooded and un-flooded soils resulted in lower pH around rhizosphere than in bulk soils (Fig. 2D, E). And flooding also led to an averaged lower pH than that of un-flooded soil (Fig. 2F).

4.4.2 The response of enzyme distribution patterns to flooding

Fig.3 showed the overall distributions of five enzyme activities (β-glucosidase: Glu, cellobiohydrolase: Cello, xylanase: Xyl, leucine aminopeptidase: Leu, and phosphatase: Pho) that came from solely MB (un-planted) or root+MB (rice planted) released enzymes under two H_2O content: 25% and H_2O oversaturated. Flooding led to relatively more evenly distributed patterns regardless of soil depth, enzyme types and with or without rice roots (Fig. 4B, D). In un-flooded soils, without interference of rice roots (Fig. 4A), the dominant factor of distribution patterns by solely MB released enzymes is soil depth. Glu and Cello decreased along the soil depth (Fig. 4A), vise versa, microbes that released Pho increased along soil depth. In un-flooded soils, rice roots were the dominant factor that determined the spatial distribution patterns of

enzyme activities (Fig. 4C). Peaks of enzyme activities appeared between the depth of 3-12 cm, where existed higher amount of root branches and tips (Fig. 1). Flooding also increased the amount (Fig. 5A, C) and spreading area (Fig. 5B, D) of enzyme activities compared to un-flooded soil in both planted (MB released) and un-planted (root+MB released) soils. Flooding only slightly increased the percentage of hotspots area to the area of root surface (Fig. 6B), however, it substantially extended the enzyme spreading area by 4 – 6times (Fig. 6A).

4.4.3 Response of relationships between C, N and P related enzyme activities to flooding

Through 3D mesh and contour map, enzyme activities related to C (Glu+Cello+Xyl), N (Leu) and P (Pho) cycling were related (Fig. 7). Comparison of 3D mesh between 25% and H_2O oversaturated content (Fig. 7A, C) show the H_2O canceling effects, which canceled several optimal combination peaks of C, N and P related enzyme activities through diffusion. By comparing of contour map between 25% and H_2O oversaturated content (Fig. 7B, D), flooding narrowed the optimal combination area between C, N and P related enzyme activities. For un-flooded and flooded soils, the optimal range of enzyme activities related to C, N and P cycling are: C (4-16 pmol) – N(12-30 pmol) – P (6-15 pmol) and C (50-280 pmol) – N(20-90 pmol) – P (32-44 pmol) MUF or AMC cm^{-2} h^{-1}, respectively.

4.5 Discussion

4.5.1 H_2O effects on rice roots, ^{14}C rhizodeposition and pH

Flooding increased both root and shoot biomass and led to higher root/shoot ratio (Fig. 1) compared to un-flooded soil, which reveals that flooding developed more rice roots proportionally to the development of shoots yield. It indicates that oversaturated H_2O decreased the water use efficiency. This result also supports the contradictory

phenomenon that flooding improved rice yield and decreased water use efficiency simultaneously (Carrijo et al., 2017).

Root tip is the apical region with active meristem, which produces and secretes low molecular polysaccharide mucilage, and released 2–12% of the total rhizodeposition (Nguyen, 2009; Sievers et al., 2002). The lysis of sloughed off root tip cells may also account for 10% of the total rhizodeposition (Iijima et al., 2000). Thus, root tip secretion and sloughed off cells together provide substantial labile C for MB, and create soil hotspots (Kuzyakov and Blagodatskaya, 2015b). This is broadly consistent with the lit up root tips by ^{14}C and the hotspots created by root tips in present study (Fig. 2 A, B). However, with similar amount of root tips on the interface of ^{14}C imaging, flooding resulted in higher rhzodeposition (^{14}C) in bulk soils (Fig. 2B). This may attribute to higher total root biomass (Fig. 1) with larger root exudates, and the passive diffusion under flooding, which increased the motility of rhizodepositions. Bulk soil is usually contain less C compared to rhizosphere, therefore, the majority of MB in bulk soils are considered to be oligotrophic (Zelenev et al., 2005). This improved motility of rhizodepositions carried around by flooding stimulated microbial proliferation in bulk soil with higher MBC (Fig. S1). In need to meet C:N:P stoichiometric requirement, increased microbes may consequently start to 'mining' soil C pools in order to get access to N and P that bind in SOM (Chen et al., 2014). Therefore, the newly available N and P released from SOM may also feedback on the improvements of root biomass under flooding (Mooshammer et al., 2012).

In pH mapping, both flooded and un-flooded soils resulted in lower pH around rhizosphere than in bulk soils (Fig. 2D, E). There are mainly three mechanisms involved in acidification around rhizosphere: (i) Acid growth theory (Hinsinger et al., 2003b). In short, roots can release H^+ to compensate for an unbalanced cation uptake (e.g. K^+) at the root-soil interface (Hinsinger et al., 2003a; Tang and Rengel, 2003). (ii) Organic acids released from roots (Hoffland et al., n.d.). Citric, oxalic and malic acids are the most frequently referred organic acids that can accumulate around roots and led to rhizosphere acidification (Dinkelaker et al., 1989). (iii) Redox-coupled

acidification processes. Paddy soil is known for its' higher extractable Fe. Results in Fig. 1D strongly supported this mechanism, as flooding formed an obvious iron plaque (yellowed roots) around the root cell walls. The iron plaque came from precipitates through Fe^{II} oxidation (Chen et al., 1980; Green and Etherington, 1977). As rice developed typical semiaquatic structures (aerenchyma) under flooding, it enables roots to release O_2 around the root-soil interface (Ando et al., 1983; Armstrong, 1967). With O_2 provide by aerenchyma, Fe^{II} oxidation equation given by Ahmad and Nze (1990) clearly explained the redox-coupled acidification process in rhizosphere: $4Fe^{2+} + O_2 + 14H_2O \Leftrightarrow 4FeOOH + 8H_3O^+$. Begg et al. (1994) also detected strong acidification accompanied with rhizosphere oxidation, which is in line with results in present study (lower rhizosphere pH and iron plaque). The lower pH under flooding than that of un-flooded soil (Fig. 2F) may attribute to the co-impact from mechanisms (i) - (iii) as a result of higher root biomass.

4.5.2 H_2O effects on hotspots and spatial distribution patterns of enzyme activities

Enzyme activities can be influenced by multiple factors, such as soil physiochemical properties, microbial community (Kourtev et al., 2002), variety of plants (Sinsabaugh et al., 2002) and anthropogenic managements (Boerner et al., 2000). It is supported by the results of enzyme activities under normal H_2O content (25%) (Fig. 4A, C). The distribution patterns of enzyme activities vary with factors such as water content, enzyme types (C, N or P related enzymes), interference of roots (planted or un-planted), and soil depth. Soil depth is the dominant factor of enzyme distribution patterns in un-flooded and un-planted soils (Fig. 4A). β-glucosidase (Glu) and cellobiohydrolase (Cello) decreased along the soil depth (Fig. 4A), which indicates that microbes that released Glu and Cello may mainly come from aerobic favored microorganisms, as oxygen content decreased along soil depth. Conversely, microbes that released phosphatase (Pho) may mainly come from anaerobic favored microorganisms, as Pho increased along soil depth. Roots became the dominant determine factor of enzyme distribution patterns in un-flooded and planted soils (Fig.

4C). Peaks of enzyme activities appeared between the depth of 3-12 cm, which can be attributed to higher amount of root branches and tips existed (Fig. 1A, B).

However, when H_2O oversaturated, flooding should be considered as the most important factor in paddy field among other biotic (soil depth, pH) and abiotic (enzyme types, ^{14}C rhizodeposition and rice roots) environmental conditions. Flooding demonstrated evenly distributed vertical patterns (Fig. 4B, D) among the negligible influence from other factors (soil depth, enzyme types, pH, ^{14}C rhizodeposition and planted or un-planted roots). Flooding does not only determined the vertical patterns, it also increased the amount (Fig. 6A, C) and spreading area (Fig. 6B, D) of enzyme activities. Possible reasons could be the diffusion effects of flooding. Living microorganisms generally occupied less than 5% of the soil surface area, and account for only 0.001% of the potentially habitable soil volume (Ingham et al., 1985). Such concentrated microbial activity normally appeared around rhizodeposition and labile organic debris (Briar et al., 2011; Kim et al., 2008; Zoppini et al., 2005), and ultimately formed the so called 'hotspots'. This limited encounter of microorganisms with substrates is largely mediated by diffusion (Richard G Burns et al., 2013). Diffusion is a two-way dispersive process (Koch, 1990), which can both carry low molecular labile C towards microbial cells and move metabolic products away. Thus, flooding efficiently improved the possibilities between microorganisms and potential substrates. When standard by the area of root surface in the interface of imaging (Fig. 6), flooding only slightly extended the area of hotspots. This may explained by the lower water content in rhizosphere (Fig. 1E) with moderated diffusion.

4.6 Conclusions

In summary, triple combination of ^{14}C imaging, pH mapping and zymography enabled to evaluate the water effects in paddy soils. Flooding increased root biomass with decreased water use efficiency. Through higher root biomass, flooding increased rhzodeposition (^{14}C) with decreased pH in both rhizosphere and bulk soil. The

decreasing pH in rhizosphere is enhanced by aerenchyma of rice roots and iron plaque under flooding. Enzyme activities in normal water content (25%) is environmental specific (can be mediated by water content, enzyme types, root interference and soil depth). However, flooding is the most important determine factor under flooding. Through diffusion, flooding increased enzyme activities through forming of iron plaque, increased rhzodepositions and decreased pH. Furthermore, flooding also evenly distributed enzyme activities vertically and resulted to canceling effects. 3D mesh and contour map revealed that water canceling effects canceled several optimal combination peaks of C, N and P related enzymes through diffusion, and ultimately narrowed the optimal combination area. In conclusion, paddy soil is such a specific ecosystem, in future, when predicting and modeling the enzyme activities in paddy field, the water effects should be given specific considerations, as flooding improved forming of root iron plaque, increased rhzodepositions with decreased pH, both of them, in turn, dominated both the quantity and distribution of enzyme activities in paddy field.

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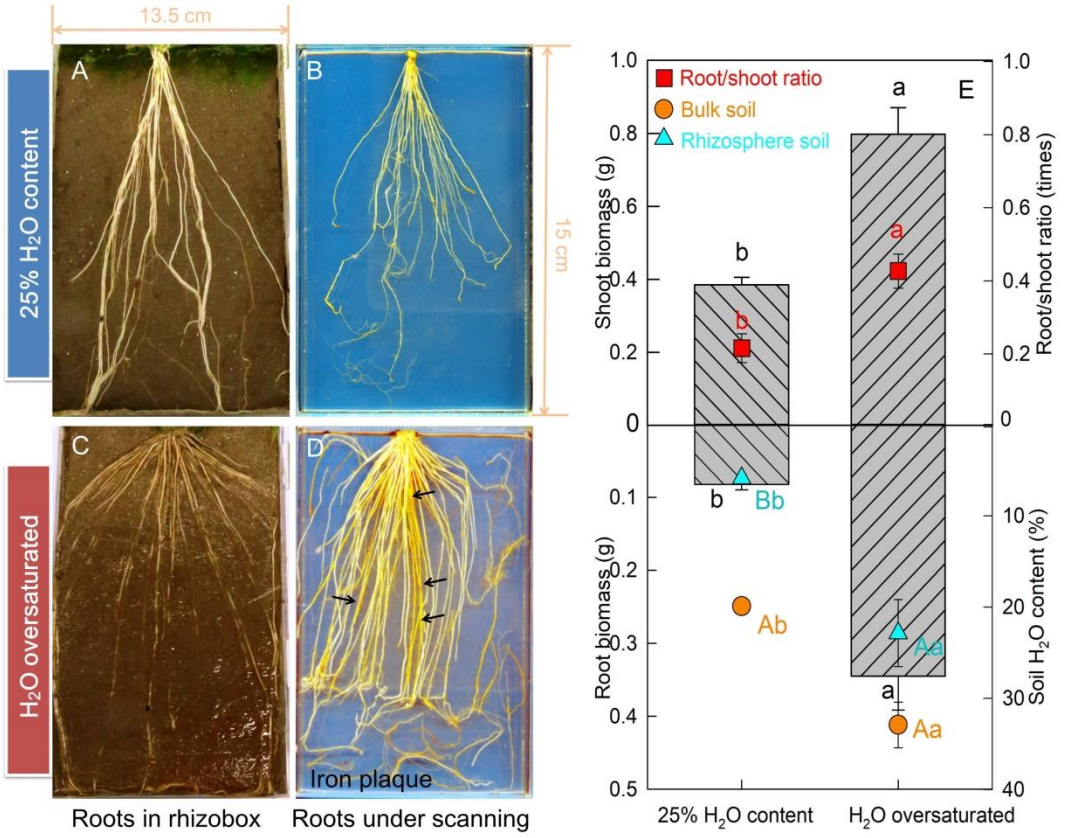

Fig. 1. Properties of rice roots in two H_2O contents: 25% and H_2O oversaturated. Number 10.5 and 15 cm is the width and length of the rhizobox. Left axis in right figures: histogram of shoot and root biomass (g) in two H_2O content: 25% and H_2O oversaturated. Right axis in right figures: point diagram of Root/Shoot ratio and H_2O content in rhizophere and bulk soil in two H_2O content: 25% and H_2O oversaturated. The capital and lower-case letters indicates significant differences within and between treatments ($p < 0.05$), respectively Error bars represent standard errors (±SE).

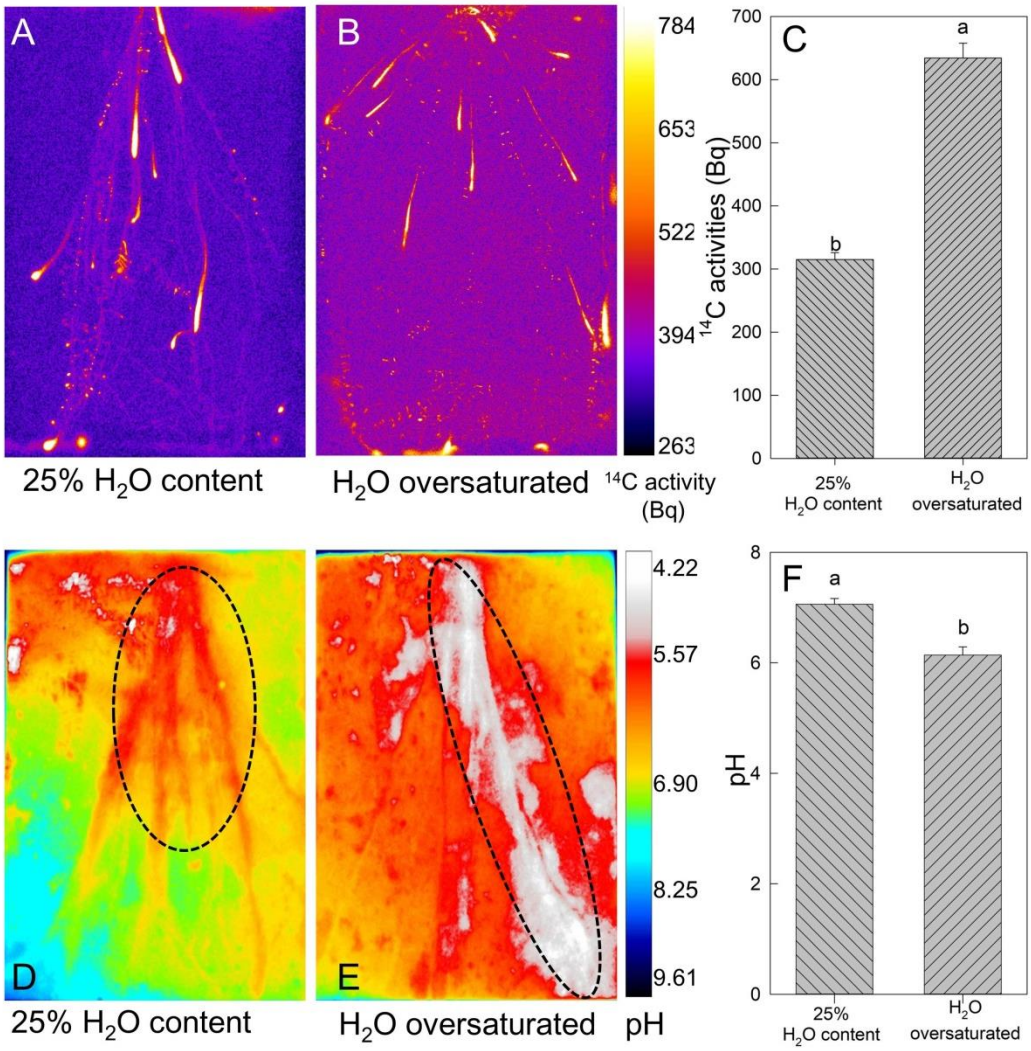

Fig. 2. ^{14}C imaging (A, B, C) and pH mapping (D, E, F) of rice roots in two H_2O content: 25% and H_2O oversaturated. Color bars are proportional to ^{14}C activities and pH values calibrated by standard ^{14}C activities and pH solutions, respectively. Figure C and F are the histogram of averaged ^{14}C activities and pH values generated from ^{14}C imaging (A, B) and pH mapping (D, E) in two H_2O content: 25%, H_2O oversaturated. The capital and lower-case lettersindicates significant differences within and between treatments ($p < 0.05$), respectively Error bars represent standard errors (±SE).

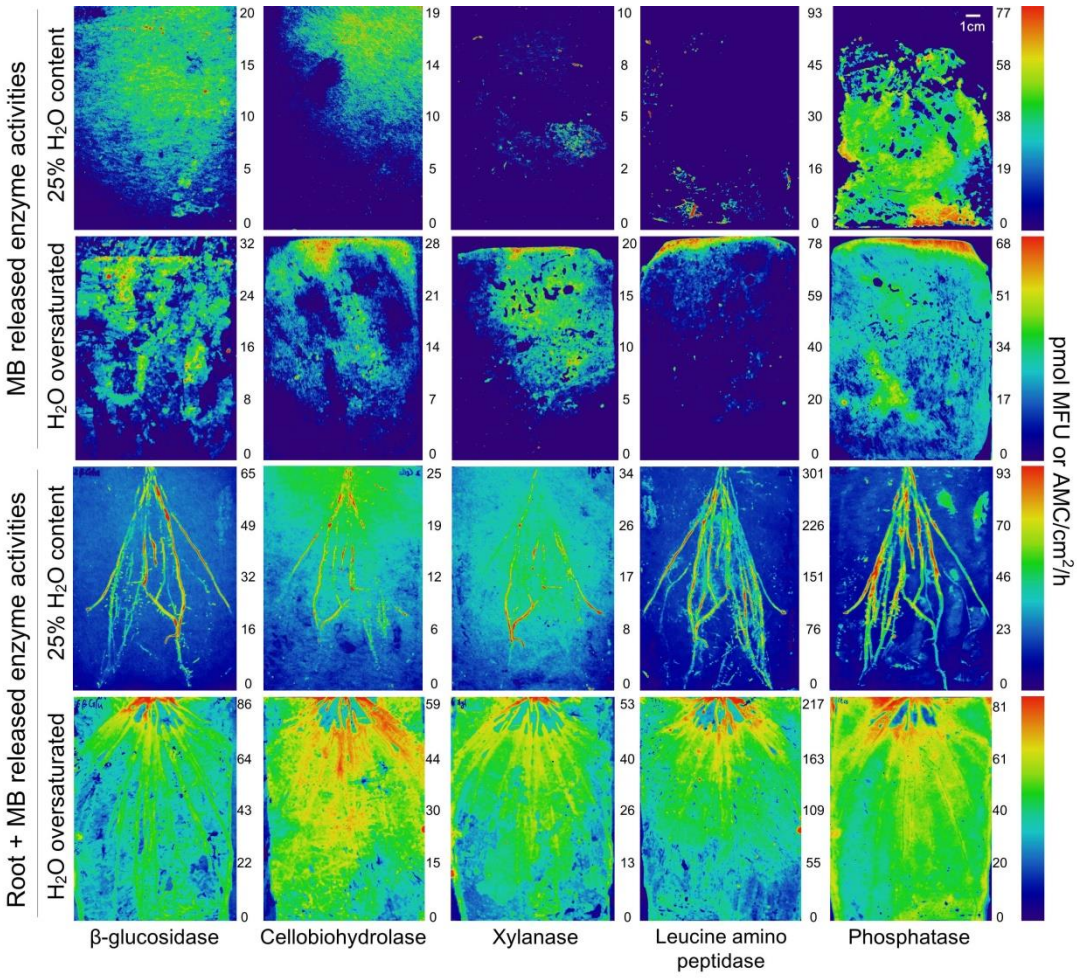

Fig. 3. Zymography of enyzme activities by microorganisms (MB) released only and by combined root+MB released in response to 25% H_2O content and H_2O oversaturated. Enzymes from left to right: β-glucosidase, cellobiohydrolase, xylanase, leucine aminopeptidase and phosphatase. Color bars are proportional to enzyme activities (pmol cm^{-2} h^{-1}). Numbers are the enzyme activities from low to high in each image according to the color bars.

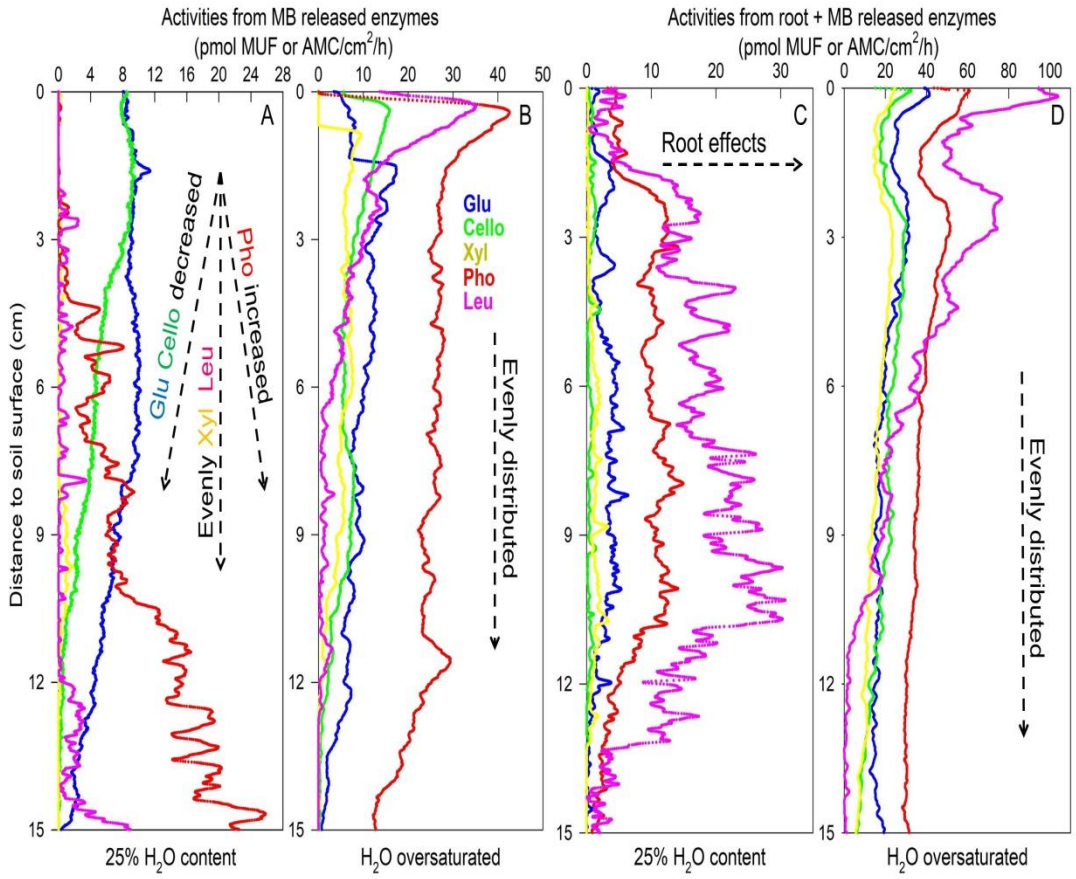

Fig. 4. Spatial distributions of enzyme activities in the distance to soil surface from single MB released or root+MB released enzymes under two H_2O content: 25% and H_2O oversaturated. Glu, Cello, Xyl, Pho, Leu is short for β-glucosidase, cellobiohydrolase, xylanase, phosphatase and leucine aminopeptidase, respectively. Black dashed lines indicates the vertical distribution patterns of different enzyme activities.

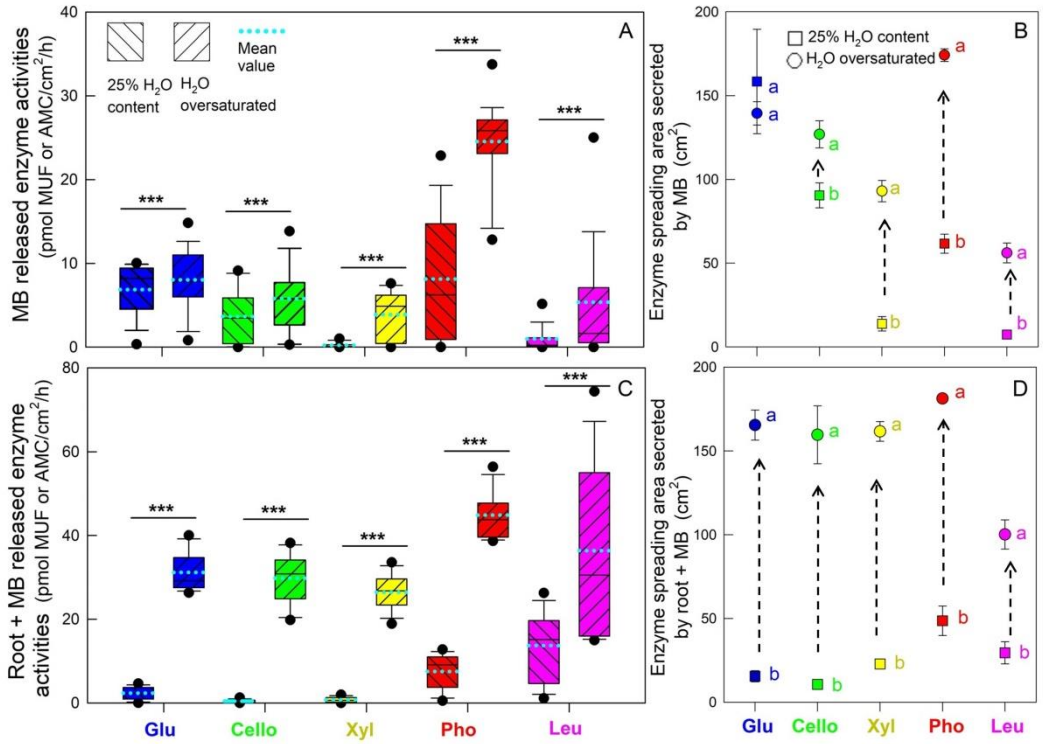

Fig. 5. Amounts (pmol MUF or AMC cm^{-2} h^{-1}) and spreading area (cm^2) of enzyme activities from single MB released or root+MB released enzymes in the rhizoboxes under two H_2O content: 25% and H_2O oversaturated. Glu, Cello, Xyl, Pho, Leu is short for β-glucosidase, cellobiohydrolase, xylanase, phosphatase and leucine aminopeptidase, respectively. *, **, and *** represent the statistical significance at the probability levels of 0.05, 0.01, and 0.001, respectively.

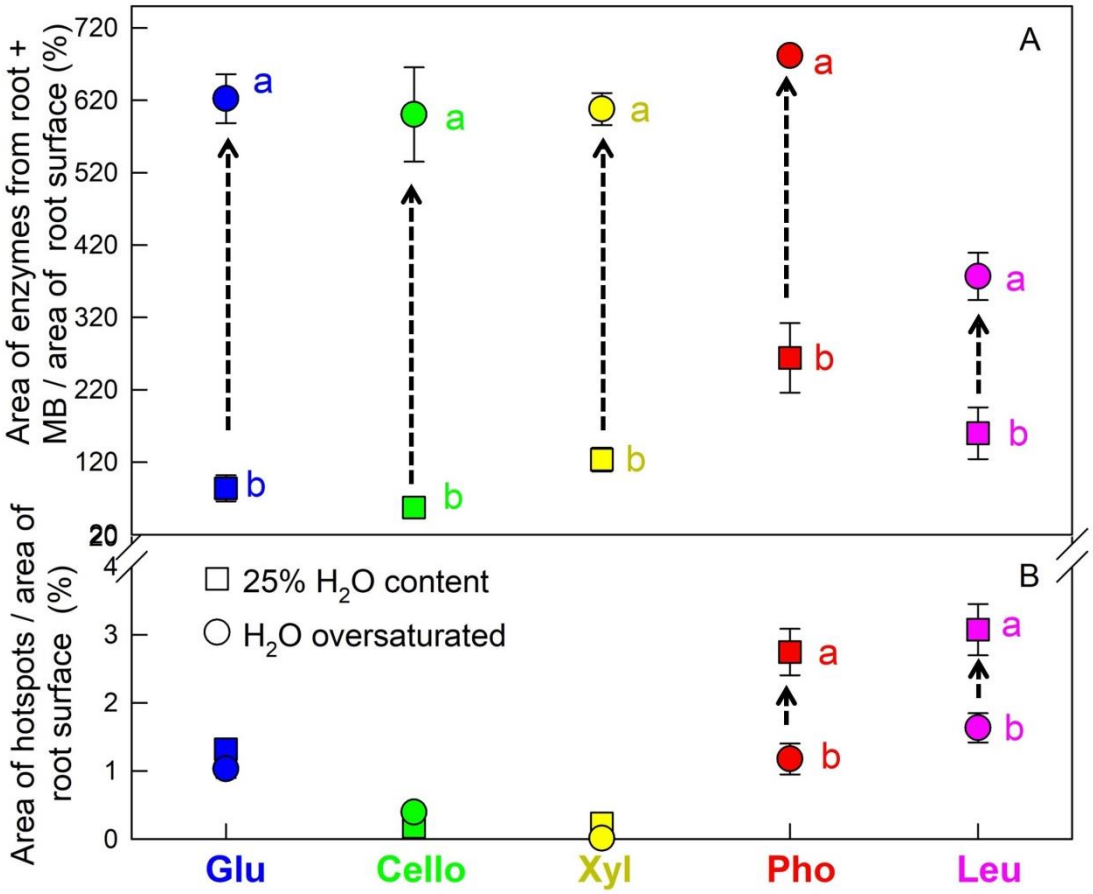

Fig. 6. Percentage (%) of area of root+MB released enzyme activities (cm^2) and area of hotspots (cm^2) to root surface (cm^2). Glu, Cello, Xyl, Pho, Leu is short for β-glucosidase, cellobiohydrolase, xylanase, phosphatase and leucine aminopeptidase, respectively. The letters indicate significant differences between two H_2O content: 25% and H_2O oversaturated ($p < 0.05$). Error bars represent standard errors (±SE).

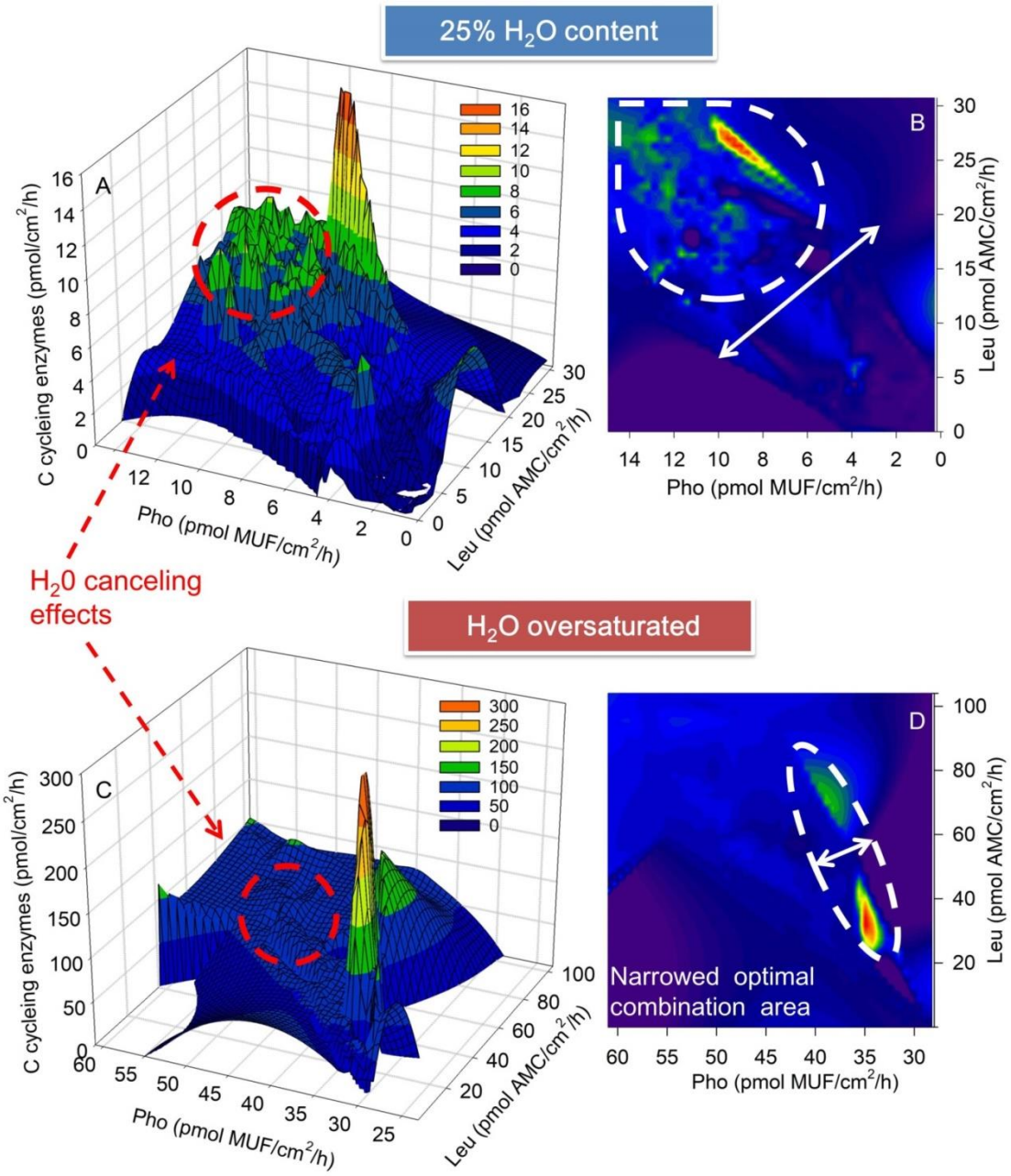

Fig. 7. 3D mesh (left) and contour map (right) of enzymes related to C (β-glucosidase, cellobiohydrolase, xylanase), N (leucine aminopeptidase) and P (phosphatase) cycling under 25% water content (up) and water oversaturated (down) treatments. X, Y, Z axes are enzyme activities of leucine aminopeptidase (Leu), phosphatase (Pho), and C cycling enzymes (sum up of β-glucosidase, cellobiohydrolase and xylanase), respectively

Chapter 5

Synthesis

5.1 Key findings

. Through these studies we conclude that the long-term effects of rice straw degradation (CMC) leads to mobilization of a 3% of total N from SOM and a positive N priming effect **(Chapter 2&3)**, a finding that support microbial N mining hypothesis. A combination of rice straw and nitrate fertilizer (+CN) produces highest rice yield. However, this combination lowers root biomass and C incorporation into roots, DOC, SOM, and MBC. Additionally, the combination alters microbial community composition. Specifically, +CN fertilization decreased Gram-positive (G+)/ gram-negative (G-) ratios as well as G+ bacteria and fungi abundance. Nonetheless, N fertilization stimulates G- and actinomycetes. Fertilization and plant growth stage are the two factors that explained 81% of the variance in the microbial communities. Fertilization accounted for 36.5% of the variance in the composition of microorganisms.

Compared to un-flooded soils, flooding, the dominant factor in paddy fields, increases root biomass but decreases water use efficiency (Chapter 4). Flooding also improves rhizodeposition (^{14}C) but decreases pH in both rhizosphere and bulk soil. Additionally, flooding is a dominant factor determining the spatial distribution patterns of enzyme activities through diffusion effects. The water effects increased enzyme activities through forming of iron plaque, increased rhizodepositions, and decreased pH. Through 3D mesh and contour map, we simultaneously evaluated the correlations of enzymes involved in C, N, and P cycling successfully. Finally, flooding induced cancelation of several optimal combination peaks of C, N, and P related enzymes, a concept we introduced for the first time as **water canceling effects**.

5.2 Implications

The Second State Soil Survey of China (SSSSC) conducted from 1980s to 2000

and data published in the last 5 years showed that (Xie et al. 2007) paddy soil has higher C density and C sequestration capacity compared with other farmland ecosystems in the same climatic zone. Paddy is the only type of soil in China, which has a mean SOM up to 25 g kg^{-1} in plough layer (Kögel-Knabner et al. 2010). The C sequestrated in paddy fields mainly comes from rhizodeposition (photosynthates) and rice straw retention. Our results (chapter 2 and3) showed that rice yield increase induced by fertilizer application reduces the photosynthates input into soil. This result clearly indicated that rice straw retention is the main reason responsible for the higher C density and C sequestration capacity in paddy field than rhizodeposition.

Nonetheless, the mean residence time of root-derived C (rhizodeposition) in SOM has been found to be 2.4 times longer than that of straw-derived C (rice straw retention) (Rasse et al. 2005). Although fertilization increases rice yield, it decreases the proportion of photosynthates (rhizodeposition) in SOM. This suggests that fertilization can potentially decrease the stability of SOM. The instability of SOM caused by large amounts of fertilizer application could also explain why paddy fields are important contributors of methane emission. Furthermore, long term rice straw degradation showed clear positive N priming effects. Thus, a single rice straw retention without external N fertilization can improve N availability for rice plants through microbial N mining from SOM.

China accounts for 7% of world agricultural land which consumes about 35% of the world's total N fertilizer (Zhang et al. 2013). This massive N fertilizer application leads to large amount of N loss through leaching and ammonia volatilization, which trigger a series of ecological environmental problems (Xing et al., 2002). Whereas China reduced rice cultivation area from 22% to 19% of the total world rice-planted lands between 1991 and 2010, the total rice yield increased by $11.5x10^6$ tons (A.A et al. 2015). The increase in rice yields is mainly attributed to excessive use of N fertilizer. Based on our results, single N addition does not increase rice yield when compared to single C addition or C and N combination. Worse still, single N application easily causes environmental

problems. Excessive chemical N fertilizer application severely acidifies the soil. This is illustrated by the typical rice fields soil pH in south China, which decreased sharply from 6.64 to 6.05 in recent years (Cai et al. 2015).

Originally, the hydroponic surface and redox layers produced by irrigation create an ideal salt-based buffer system for the paddy field. Oxides such as iron and manganese in the system reduce the concentration of hydrogen ions, effectively resisting acidification in paddy soils. However, excessive application of chemical N fertilizer as illustrated by a study using urea (Posch and Reinds 2009; Fujii et al. 2011), overcomes this buffering potential of the soil. One mole of urea hydrolyzes to 1mol of both $NH_4^+$and OH^-. A further conversion of NH_4^+ to NO_3^- through nitrification process produces 2 mol H^+. Root NO_3^- absorption releases 1 mole of OH^- to neutralize the net 1 mole of H^+ ions, maintaining the soil pH value. This implies that loss of NO_3^- from the excessively applied chemical N fertilizer $^-$ through leaching process and ammonia volatilization leaves H^+, which causes severe soil acidification.

This acidification process is further demonstrated by our results in chapter 4. Flooding in the soil collected from southern China under rice-fallow-rice rotation for over 30 years showed lower pH in both rhizosphere and bulk soil based on pH mapping. Long-term fertilization experiments on red soil in southern China have shown that soil acidification leads to serious suppression of crop growth (Wang et al., 2017). Our results indicate that soil acidification may be a long-term and prominent problem for future agricultural production in southern China.

In conclusion, flooding remains to be the dominant factor determining the spatial distribution patterns of enzyme activities through diffusion effects. Flooding effects increases both enzyme activities through forming of iron plaque and the rhizodepositions but decreases the soil pH.

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Declaration

I hereby declare, to the best of my knowledge and belief, that this thesis contains no material previously published or written by another person, except where due reference has been made in the text of the thesis. This thesis contains no material which has been accepted or definitely rejected for the award of any other doctoral degree at any university.

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