Effect of long-term fertilisation on enzyme activities and microbial community composition in the rice rhizosphere

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ABSTRACT
Mineral fertilisers differ in changing soil properties, and revealing how the rhizosphere and non-rhizosphere respond could provide a robust assessment of fertiliser regimes. Rhizosphere and non-rhizosphere soils were sampled from five fertilisation treatments in a long-term (24 year) experiment. Enzyme activities and total phospholipid fatty acids (PLFA) content in the rhizosphere soil were 85.8% and 51.3% higher than in the non-rhizosphere soil, respectively. Fertilisation increased enzyme activities, especially the N-cycling enzyme β-1,4-N-acetylglucosaminidase in NP fertilised soil (1.5 and 2.5 times for rhizosphere and non-rhizosphere soil, respectively). The PLFA composition indicated that fungi dominated in the rhizosphere fertilised with P, whereas bacteria were more common in the non-rhizosphere soil. The PLFA contents and enzyme activities in the rhizosphere of P-fertilised plants were lower than those in the non-rhizosphere soil because P availability was lower in the rhizosphere. The redundancy analysis showed that the microbial community in the rhizosphere soil was different from that of the non-rhizosphere soil, mainly because there were differences in the 15:1ω6c and 16:0. Long-term (24 year) fertilisation strongly increased nutrient contents, and microbial biomass and activity in paddy soil. It is advisable to apply P fertiliser in the root zone to increase fertiliser use efficiency.

Introduction

Microbial community composition and activities are sensitive to nutrient availability and both are usually used to evaluate the effects of fertilisation. A meta-analysis of 55 studies about fertilisation in paddy rice systems indicated that the application of mineral fertilisers could increase microbial biomass C and change microbial community structure by altering soil properties (Geisseler et al. 2017). The degree of change in microbial communities depended on the fertilisation regimes. For example, the long-term application of mineral fertiliser increased the iC15:0 fatty acids content, while mineral fertiliser combined with manure or rice straw decreased its content but increased aC15:0 fatty acids (Tang et al. 2018). Some of these studies mainly focused on the effects of fertilisation on non-rhizosphere soil. However, the rhizosphere is the interface between root and soil and has important effects on plant growth, but there is only a limited understanding about the impacts of fertilisation on the rhizosphere.

The altered nutrient fluxes to the roots and the organic C released by the roots mean that the microbial community composition in rhizosphere soil differs from that in non-rhizosphere soil (Zhao et al. 2019), and their response to fertilisation is different (Ai et al. 2012; Liu et al. 2019). The application of nitrogen fertiliser had a greater effect on the diversity of the bacterial community in the non-rhizosphere soils than in the rhizosphere (Zhai et al. 2018). However, whether the degree of change in rhizospheric microorganisms that are stimulated by fertilisation differs from non-rhizosphere soils remains unclear (Liu et al. 2019; Wang et al. 2020).

Microbial biomass C represents the amount of active microorganisms, and these organisms play important roles in the nutrient cycle because they secrete a series of enzymes. Soil enzymes are important contributors
to nutrient cycling and their activities mirror the intensity and direction of soil biochemistry. Most of the enzymes are secreted by microorganisms and plant roots, and are closely related to microbial biomass and soil nutrients availability. Fertilisation influences enzyme activities by enhancing microorganisms and plant growth. Francioli et al. (2016) reported that chitinase and phosphatase activities, and their biomass-specific activities, increased after applying mineral fertiliser for more than a century. However, the impact of fertilisation on enzyme activities in rhizosphere soil is mediated by root exudates (Ai et al. 2012). Fertilisation with P decreased acid phosphatase activity in the non-rhizosphere soil, but this effect was lower in the rhizosphere (Spohn et al. 2015). Generally, enzyme accumulation and persistence in soil depend on fertilisation. Plants can utilise this legacy of fertilisation and form specific rhizosphere enzymes conditions. However, understanding the effects of long-term fertilisation on enzyme activities in rhizosphere is limited.

In subtropical China, paddy soils derived from Quaternary red clays generally lack nutrients, especially N and P (Li et al. 2000). The effects of fertilisation on soil properties were studied in a long-term experiment that was performed in a paddy field. Differences in the ecological habitat of rhizosphere and non-rhizosphere soils shape diverse microbial communities, and the sensitivity of these different microbial communities to fertiliser application can vary greatly. We hypothesised that (i) the effects of long-term fertilisation and rice cultivation lead to specific rhizosphere microbial communities and activities, and these are related to rhizosphere C accumulation; and (ii) P fertiliser has the greatest effect on rhizosphere microbial composition and C and N cycling enzyme activities in P-limited paddy soil. To test these hypotheses, rhizosphere and non-rhizosphere soils were sampled from the long-term fertilisation experiment. The microbial community composition was evaluated by measuring the phospholipid fatty acids (PLFA) and the activities of four enzymes: β-glucosidase and β-xylanase, associated with C cycling, β-1,4-N-acetyl-glucosaminidase, which is involved in N cycling, and acid phosphatase, which is involved in P cycling.

Materials and methods

Site description and soil sampling

A long-term field experiment was established in 1990 at the Yingtan National Agroecosystem Field Experiment Station (28°15′30″ N, 116°55′30″ E) in Yujiang County, Jiangxi Province, China. The site has a subtropical monsoon climate with a mean annual temperature of 17.6°C and a mean annual rainfall of 1800 mm. Double-cropped rice (Oryza sativa L.) (i.e. early and late season rice) is planted from early April to the end of October and the field remains fallow for the rest of the period. The soil, which is a Dystric Clayic Plaggic Anthrosol (World Reference Base for Soil Resources, 2014), is derived from Quaternary red clay and is low in nutrients, especially P (Table 1).

Five fertilisation treatments were selected: (1) no fertiliser application (Control); (2) mineral N fertilisation (N) with 115 kg N (urea) ha⁻¹ year⁻¹; (3) mineral N and P fertilisation (NP) with 115 kg N (urea) ha⁻¹ year⁻¹ and 30 kg P ha⁻¹ year⁻¹; (4) NK mineral fertiliser, with 115 kg N (urea) ha⁻¹ year⁻¹ and 35 kg K ha⁻¹ year⁻¹; and (5) NPK mineral fertiliser, with 115 kg N (urea) ha⁻¹ year⁻¹, 30 kg P ha⁻¹ year⁻¹, and 35 kg K ha⁻¹ year⁻¹. The P and K fertilisers were applied as a basal dressing, whereas the N fertiliser was applied at 61 kg N ha⁻¹ as a basal dressing and at 54 kg N ha⁻¹ as a top dressing. The experiment was a randomised complete block design with three replications and each plot was 30 m² in size. The rhizosphere and non-rhizosphere soils were separately sampled after the late rice harvest in November, 2015. The rhizosphere soil was collected by the shaking roots according to Barililot et al. (2013). Briefly, 10 bundles of rice stubble were pulled from the soil in each plot, gently shaken to remove any excess surface soil, and then brushed to gently remove the 1–2 mm thick soil adhering to the root surface. This was the rhizosphere soil sample. Five soil cores (3.5 cm diameter × 20 cm deep) were sampled from the plow horizon (0–20 cm depth) in each plot following a ‘W’ pattern and then merged into one non-rhizosphere soil sample. Each soil sample was divided into three sub-samples: the first was freeze dried at –80°C for the PLFA analyses, the second was stored at field moisture content and 4°C for the enzyme activity analysis, and the third was air dried at room temperature for chemical analysis.

Chemical properties analyses

The chemical properties of the soil were measured according to the routine methods described by Lu (1999). The soil pH was determined using a 1:2.5 soil: water suspension; the K₂Cr₂O₇ + H₂SO₄ oxidation

| Table 1. Physico-chemical properties of the Ap horizon (0–20 cm depth) of the initial soil (after 24 years). |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| SOC | TN | TP | TK | AN | AP | AK | Silt % |
| pH | g kg⁻¹ | g kg⁻¹ | g kg⁻¹ | mg kg⁻¹ | mg kg⁻¹ | mg kg⁻¹ | % |
| 4.5 | 3.3 | 0.43 | 0.65 | 13.4 | 90.2 | 5.6 | 105.9 | 38 |

Note: SOC: soil organic carbon; TN: total nitrogen; TP: total phosphorus; TK: total potassium; AN: available nitrogen; AP: available phosphorus; AK: available potassium.
method was used to determine the soil organic C (SOC); total and available N were determined using the Kjeldahl N method; and total K and available K were measured by HF–HClO₄ digestion and ammonium acetate extraction (flame photometer), respectively. The total P and available P were determined by HF–HClO₄ digestion and sodium bicarbonate extraction (molybdenum blue method), respectively.

**Enzyme activities assay**

The β-glucosidase, β-xylosidase, β-1,4-N-acetyl-glucosaminidase, and acid phosphatase activities were quantified fluorometrically using the MUF-linked substrates: 4-MUF-β-D-glucoside, 4-MUF-β-D-xylopyranoside, 4-MUF-N-acetyl-β-D-glucosaminide, and 4-MUF-phosphate, respectively (German et al. 2011; Razavi et al. 2016). This method was described in detail by DeForest (2009). Briefly, 1 g of fresh soil was added to 125 mL 50 mM acetate buffer (pH 5.0) and homogenised for 2 min with an OMNI mixer (Omni, Los Angeles, CA, USA). Then the soil slurry was continuously stirred using magnetic stirrer, and 50 μL of 200 μM MUF-linked substrate was dispensed into black polystyrene 96-well filters which served as the quench standard and the reference standard. Then, 50 μL acetate buffer was dispensed in wells containing 200 μL of soil slurry and acetate buffer, which served as the sample assay and substrate control. A total of 50 μL 10-μM MUF (4-methylumbelliferone) solution was added to the wells containing 200 μL of soil slurry and acetate buffer, which served as the quench standard and the reference standard. Then, 50 μL acetate buffer was dispensed in wells containing 200 μL of soil slurry or acetate buffer, which served as the soil control and the blank control, respectively. The solutions were thoroughly mixed and then incubated in the dark at 20°C for 2 h. Finally, their fluorescence was measured with 365-nm excitation and 450-nm emission filters using a microplate fluorometer (SpectraMax i3x, Molecular Devices, San Jose, CA, USA) (Saiya-Cork et al. 2002). Enzymatic activities were expressed in nmol g⁻¹ h⁻¹.

**Phospholipid fatty acid analysis**

A PLFA analysis was performed to assess the microbial community composition based on the Bligh-Dyer method with modifications (Bossio and Scow 1998). Briefly, phospholipids were extracted from 3 g of freeze-dried soil using 15.2 mL of buffer (chloroform: methanol:phosphate; ratio: 1:2:0.8, v/v/v). Then, the polar lipids were separated from the extracted phospholipids in chloroform using a silica-bonded phase column (SPE-Si, Supelco, Poole, UK) and transformed into fatty acid methyl esters (FAMEs) by an alkaline methanolysis trans-esterification reaction. Finally, the FAMEs were analysed by a gas chromatograph mass spectrometer (Agilent 7890, Santa Clara, CA, USA) and a MIDI Sherlock Microbial Identification System (MIDI, Newark, DE, USA) following the manufacturers’ instructions. The PLFAs were separated into various taxonomic groups following previously published PLFA biomarker data (Table S1).

**Data analysis**

One-way ANOVA with Tukey’s test was used to investigate significant differences among the response variables for the fertiliser treatments. A t-test was used to evaluate the significant differences between the rhizosphere and non-rhizosphere soils for each response variable. A two-way ANOVA was performed to determine significant differences between the soil locations (rhizosphere and non-rhizosphere) and among fertiliser treatments. The ANOVAs were conducted using SPSS ver. 16.0 for Windows (SPSS Inc., Chicago, IL, USA). A redundancy analysis (RDA) with forward selection was used to test the influence of soil chemical parameters on the microbial community composition and a SIMPER analysis was performed to test the contribution made by the PLFAs to the variations in microbial community between the rhizosphere and non-rhizosphere soils. The RDA and SIMPER analyses were conducted using the ‘vegan’ package in R (version 3.2.5; R Development Core Team) software.

**Results**

**Effects of fertilisation on organic C and nutrients in rhizosphere and non-rhizosphere soil**

Applying NP or NPK significantly increased the organic C and nutrient contents in the rhizosphere and non-rhizosphere soils compared to the unfertilised control (Figures 1 and 2). The SOC, available N content, and the C/P ratio in the rhizosphere soil were higher than in the non-rhizosphere soil, but the available P content showed an inverse tendency (p < 0.001). The total P and available P increases (1.1 and 4.0 times, respectively) in response to P fertilisation were higher in the rhizosphere soil than the corresponding increases (0.8 and 2.3 times, respectively) in the non-rhizosphere soil (p < 0.05).

**Effects of fertilisation on enzyme activities in rhizosphere and non-rhizosphere soil**

The β-glucosidase, β-xylosidase, β-1,4-N-acetylglucosaminidase, and acid phosphatase activities in the rhizosphere were higher than those in the non-rhizosphere soil (p < 0.001). The combined N and K fertiliser application...
increased the β-xilosidase and acid phosphatase activities in the non-rhizosphere soil compared to the unfertilised control, but there were no increases in the rhizosphere soil (Figure 3). The treatments with phosphorus fertilisation increased the activity of β-glucosidase and β-1,4-N-acetylglucosaminidase in the rhizosphere and non-rhizosphere soils compared to the treatments without phosphorus (Figure 3). Furthermore, the largest increase (1.5 and 2.5 times for the rhizosphere and non-rhizosphere soils, respectively) in enzyme activity was for β-1,4-N-acetylglucosaminidase in the NP fertilised soil.

**Effects of fertilisation on the microbial communities in the rhizosphere and non-rhizosphere soils**

The actinomycetes, fungi, and bacteria PLFA contents and total PLFAs were higher in the P fertilisation (NP and NPK) samples than in the unfertilised control samples (Figure 4). The actinomycetes, fungi, and bacteria PLFA contents and the total PLFAs in the rhizosphere soil were higher than those in the non-rhizosphere soil (p < 0.001). Both the fertilisation regimes and soil sampling parts (rhizosphere and non-rhizosphere), and their interactions influenced total PLFAs, bacteria, the Gram-positive/Gram-negative bacteria ratio (G+/G-), and fungi/bacteria levels in the soil (Table S2). The greatest increase in PLFA biomarkers in the rhizosphere soil was fungi in NPK soil, which accounted for 73.8% of the variance. The largest increase in PLFA biomarkers in the non-rhizosphere soil was general bacteria in NP soil, which increased by 1.1 times compared to the unfertilised control.

The RDA showed that the microbial community in the rhizosphere soil was separated from that in the non-rhizosphere soil along the RDA1 axis, which explained 52.6% of the variation (Figure 5). The 15:1ω6c (Gram-negative bacteria) and 16:0 (general bacteria) were the
major fatty acids leading to this separation (Figure 6). The soil organic carbon (SOC) and available N contents were the major contributors to these differences in microbial community structure.

**Discussion**

The results showed that the changes in soil nutrients contents and biological properties strongly depended on fertilisation regime after applying mineral fertilisers to P-limited paddy soil for 24 years. Fertilisation with P (NP and NPK) had positive effects on soil nutrients, as shown in Figures 1 and 2. A similar result was also reported by Zhong and Cai (2007) who reported that soil nutrients and microbial properties were improved after applying P fertiliser over 13 years. Phosphorus fertilisation directly increased the available P content in P limited soil (available P: 5.6 mg P kg$^{-1}$) and increased crop yields (unpublished). An increase in aboveground and root biomass and remaining stubble led to higher organic C levels in the soil. The SOC content in the rhizosphere was higher than that in the non-rhizosphere soil because of inputs from rhizodeposits and microbial biomass accumulation in the rhizosphere (Rovira 1969; Gregorich et al. 1996; Pausch and Kuzyakov 2018). Such a localised C input into the rhizosphere (Pausch and Kuzyakov 2012) will be redistributed throughout the whole soil volume when the soil is next plowed. The available N content in the rhizosphere soil was higher than that in the non-rhizosphere soil (Figure 2). This pattern occurred because water flux to the roots leads to nutrient accumulation around the roots (Lynch and Whipp 1990). In contrast, the available P concentrations were depleted in the rhizosphere soil because phosphate diffusion is very slow, especially when the soil iron content is high. Consequently, P uptake by roots cannot be compensated for by diffusion.

Consistent with the 2nd hypothesis, the response of enzyme activities to fertilisation differed among fertilisation regimes. As shown in Figure 3, our results present new findings related to $\beta$-1,4-N-acetylglucosaminidase was the most stimulated by P fertilisation. The possible reason for this is that applying P to a P-limited paddy soil increases the levels of microbial functional genes encoding C, N, and P cycling enzymes (Su et al. 2015). Similarly, the N-acetylglucosaminidase activity increased more in NPK fertilised soil than in soils subjected to other fertilisation regimes (farm yard manure fertilisation, and combined farm yard manure and mineral fertilisation) (Francioli et al. 2016). This enzyme is important in N cycling because it hydrolyses chitin (e.g. from fungal hyphae) to amino sugars, a major source of mineralisable N in soil (Ekenler and Tabatabai 2002). Plants can alter rhizosphere microbial activities by competing with microorganisms for nutrients such as nitrogen (N), which leads to nutrient limitation in the rhizosphere and stimulates microorganisms to mine the stored nutrients (Kuzyakov and Xu 2013).
Consistent with the 1st hypothesis, the potential enzyme activities in the rhizosphere soil were 0.3–1.7 times higher than those in the non-rhizosphere soil. The reason for this increase was because most enzymes are secreted by roots and microorganisms, which are most abundant and active in the rhizosphere (Burns 1982; Mendes et al. 2011; Kuzyakov and Razavi 2019). The results showed that NP or NPK fertilisation increased enzyme activities in both the rhizosphere and non-rhizosphere soils, but the increase was lower in the rhizosphere. This could be due to P being more limited in the rhizosphere relative to the non-rhizosphere, which would restrain microorganism proliferation and related enzyme release. Another possible reason is that root exudates could mediate the degree influence of fertilisation regimes on the soil microbial community and extracellular enzyme activities (Ai et al. 2012).

The total PLFA content in the rhizosphere soil was higher than that in the non-rhizosphere soil, as shown in Figure 4. Previous studies have also reported that the total PLFA content in wheat rhizosphere soil was higher than that in non-rhizosphere soil (Ai et al. 2012; Zhao et al. 2019). This could be due to the fact that the input of organic C from the roots led to the rapid proliferation of microorganisms, resulting in accumulation of microbial biomass (De Nobili et al. 2001; Blagodatskaya and Kuzyakov 2013). Microbial communities differed significantly between fertiliser treatments and between soil parts, with soil organic carbon being the dominant factor in this difference (Figure 5). The increase in SOC over time in the fertilised soil is considered to be the dominant explaining factor for the increases in microbial biomass C (Yuan et al. 2013; Li et al. 2019). Yuan et al. (2016) reported that the effect of fertilisation on microbial biomass C was species specific because each species has distinct eco-physiological characteristics. Consistent with the 1st hypothesis, Gram-negative bacteria monounsaturated 15:1ω6c and saturated fatty acids 16:0 were the major fatty acids leading to the differences in microbial community composition between the rhizosphere and non-rhizosphere soils (Figure 6). One possible reason for this is the different requirements of microbes for environmental conditions for growth (e.g. oxygen and nutrients).

The interaction between fertilisation and location (rhizosphere and non-rhizosphere soils) clearly influenced the bacteria, the G+/G- ratio, and fungi/bacteria (Table S2). Phosphorus fertilisation increased the PLFA contents in both the rhizosphere and non-
rhizosphere soils, but the increase was lower in the rhizosphere soil. This difference was probably due to the lower available P content in the rhizosphere soil compared to that in the non-rhizosphere (Figure 2), which would limit the proliferation of rhizosphere microorganisms. Fungi in the rhizosphere and general bacteria in the non-rhizosphere soil were most stimulated by P fertilisation, limited by microbial function and

**Figure 4.** Effects of fertilisation on microbial community composition in the rhizosphere and non-rhizosphere soil. CK, N, NK, NP, and NPK treatments represent the unfertilised control and fertilisation with N; N and K; N and P; and N, P, and K, respectively. Each bar represents the mean ± standard error (n = 3). Lowercase letters show significant differences among bars with the same colour (p < 0.05).

**Figure 5.** Redundancy analysis (RDA) of the relationship between the nutrients and PLFA contents. CK, N, NK, NP, and NPK treatments represent the unfertilised control and fertilisation with N; N and K; N and P; and N, P, and K, respectively. SOC represents soil organic C. AP, TP, TN, and AN denote available phosphorus, total phosphorus, total nitrogen, and available nitrogen, respectively.

**Figure 6.** SIMPER analysis of the contribution made by the PLFA biomarkers to the differences in microbial community composition between the rhizosphere and non-rhizosphere soils. G+ and G− represent Gram-positive and Gram-negative bacteria, respectively.
physiological-ecological properties. Consistent with our study, phosphorus fertilisation strongly increased the abundance of the rhizosphere fungi *Scutellospora* and *Racocetra*, and these taxa are known to play important roles in soil P solubilization/acquisition (Silva et al. 2017). The higher fungi biomass content and enzyme activities in rhizosphere improved the plant nutrient uptake in the nutrient limited soil.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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