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Thirty-one years of rice-rice-green manure rotations shape the rhizosphere microbial community and enrich beneficial bacteria

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ABSTRACT

Green manure rotation is commonly used to increase soil fertility and improve crop yield. However, the effects of this management practice on the underground microbial ecosystem and the indirect impact on the aboveground crop growth have not been systematically analysed. In this study, we investigated the rice rhizosphere and bulk soil microbial community in a 31-year-old field experimental site treated with different green manures and rice rotations using both 16S rDNA high-throughput sequencing and quantitative PCR approaches. Four treatments have been setup in this experimental site since 1982, including a rice-rice-winter fallow treatment as a control and three green manure rotation treatments: rice-rice-Chinese milk vetch, rice-rice-rape and rice-rice-ryegrass. The qPCR results showed that the bacterial abundances in the rice rhizosphere of the green manure rotation treatments were all significantly higher than in the winter fallow ($p < 0.05$), but no significant differences were found among those three green manure rotation treatments. Moreover, α-diversity analysis revealed that green manure rotations decreased the microbial diversity (Shannon and Simpson indexes) and richness (Chao value) in the rice rhizosphere. Permutational Multivariate Analysis of Variance based on β-diversity revealed the microbial community was significantly switched in rice rhizosphere after long-term green manure rotation ($p < 0.01$). Additionally, the soil and plant characteristics contributed almost equally to the rhizosphere bacterial community based on a partial CCA-based variation partitioning analysis. At the genus level, the well-known plant-growth-promoting rhizobacteria *Acinetobacter* (31%–41%) and *Pseudomonas* (14%–28%) were the preponderant groups in green manure rotation treatments but accounted for only 4.4% and 2.5% in the winter fallow treatment. Overall, long-term rice-rice-green manure rotation shaped the microbial community in the rice rhizosphere; in particular, some beneficial bacteria, *Acinetobacter* and *Pseudomonas*, accumulated in the rhizosphere of green manure treatments.

1. Introduction

The application of green manure to paddy soil is considered a good management practice in agricultural production systems because it can increase soil sustainability by reducing soil erosion, ameliorating soil physical properties (MacRae and Mehuys, 1985), and increasing the soil organic matter, fertility (Biederbeck et al., 1998) and nutrient retention (Dennis et al., 2010; Drinkwater et al., 1998). It can also reduce the occurrence of plant diseases and insect pests (Larkin and Griffin, 2007; Naz et al., 2015). In addition, a cover crop such as milk vetch with a low C/N ratio may be a more favourable green manure since it minimizes the impact of CH_4 emissions and increases the rice productivity (Kim et al., 2012). For example, it was reported that a 28-year rice-green manure rotation increased rice yields by 18%–27% (Gao et al., 2013). Biological processes such as plant litter decomposition, gas

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emissions, soil fertility transformation and crops nutrition absorption require soil microbial activity; however, the knowledge about the effect of green manure rotations on microbial communities is still limited.

It is well known that soil microbial diversity is critical for maintaining the sustainability of an agricultural production system (Bending et al., 2004), but only a few studies have examined the effect of green manure on soil microbes by classical soil biological methods. Tejada et al. (2008) found that the soil microbial biomass, soil dehydrogenase, urease, β-glucosidase, phosphatase and arylsulfatase activities were significantly increased by amendment with green manure. Piotrowska and Wilczewski (2012) came to the similar conclusion that green manure rotation was a useful management practice for enhancing soil biological activity as evaluated by enzymatic activity. Stark et al. (2007) found that the addition of green manure improved the microbial biomass and activity and changed the soil microbial community significantly during the early days of the experiment. The soil microbial population was improved by a legume green fallow and cereal cropping system after 6 years based on a cultivable colony-count method (Biederbeck et al., 2005). A 26-year long-term planting of winter green manure significantly improved the soil microbial biomass carbon, microbial biomass nitrogen and enzyme activities in reddish paddy soil (Yang et al., 2011). Zhang et al. (2013) revealed significant effects of a long-term rotation of rice with milk vetch as a green manure on the endophytic microbial community using both culture-dependent and clone library methods. All of these studies suggested that soil amendment with green manure increases the soil microbial activity. However, these existing studies mainly used indirect methods such as biomass and enzyme activities or low throughput methods such as DGGE and clone library. Overall, the impact of green manure treatment on bacterial population dynamics and crop yields remains unclear.

The rhizosphere is the critical zone of soil surrounding the plant roots (Dennis et al., 2010). Microbes are more active in this “hot spot” in terms of higher abundance, more interactions and greater genetic exchange (Bulgarelli et al., 2013). The rhizosphere also attracts a variety of plant-associated bacteria from the soil environment, such as plant growth-promoting Rhizobacteria (PGPR) (Compant et al., 2010). Rhizosphere microbial communities are more closely associated with plant growth than those in bulk soil. Members of the genera *Bacillus*, *Pseudomonas*, *Enterobacter*, *Acinetobacter*, *Burkholderia, Arthrobacter* and *Paenibacillus* are the common PGPRs in the rhizosphere (Jha et al., 2009; Karagöz and Ates, 2012; Santoyo et al., 2016; Trotel-aziz et al., 2008). The application of green manure has been shown to improve soil quality and crop yields; however, the effects on the microbial communities in the rhizosphere are still unknown.

The application of green manures to rice fields is a traditional agricultural practice in China. Long-term green manure and rice rotation experiments were set in the Key Field Monitoring Experimental Station for Red Soil Eco-environment of Ministry of Agriculture, Qiyang County of Hunan Province, in 1982. A series of recent studies showed that green manure rotations increased rice yields by 18.8–28.8% (Gao et al., 2013), improved soil nutrients and the structure of aggregates (Gao et al., 2011; Yang et al., 2012), enhanced enzyme activities and microbial biomass (Yang et al., 2011; Gao et al., 2015), and changed the structure of endophytic bacteria (Zhang et al., 2013). However, it remains unclear how the winter-growing green manure inputs affect the microbial communities in both the paddy and rice rhizosphere. In this study, we use high-throughput sequencing and quantitative PCR to investigate the effects of long-term green manure rotations on microbial communities in the paddy soils and rice rhizosphere. We hypothesized that 31 years of green manure field rotations would dramatically alter the quantity and the composition of the soil microbial community (especially the rhizosphere community) and significantly enrich bacteria in the rhizosphere, leading to an increase in rice yield.

2. Experimental procedures

2.1. Site description and sample collection

The site of the long-term green manure and rice rotation experiment is located at the Key Field Monitoring Experimental Station for the Red Soil Eco-environment of the Ministry of Agriculture, Qiyang County in Hunan Province (26°45′42″N, 111°52′32″ E). The soil is a typical red soil in the south of China, also known as a Ferralic Cambisol, that originally developed from Quaternary red clay, with the characteristics of low pH and poor fertility. Four treatments were established in 1982, specifically rice-rice-winter fallow (WF), rice-rice-Chinese milk vetch (MV), rice-rice-rape (RP) and rice-rice-ryegrass (RG), with these three green manure plants (Leguminosae, Cruciferae and Gramineae) commonly grown in the rice field. Each treatment was composed of three replicate plots of 37.5 m^2 (2.5 m × 15.0 m) and separated by a 60 cm cement bund. The initial soil pH was 6.5, the organic matter content was 20.1 g kg^{-1}, and the contents of total nitrogen, total phosphorus, total potassium, alkali-hydrolysable nitrogen, available phosphorus and available potassium were 0.94 g kg^{-1}, 0.66 g kg^{-1}, 11.5 g kg^{-1}, 156 mg kg^{-1}, 7.2 mg kg^{-1} and 176 mg kg^{-1}, respectively. From 1982 to 2013, the total fertilizer (basal plus topdressing fertilizer) applied to the early and late rice of each season included urea (153.0 kg/hm^2), P_2O_5 (84.0 kg/hm^2), and K_2O (129.0 kg/hm^2). A compound fertilizer [urea (84.0 kg/hm^2), P_2O_5 (84.0 kg/hm^2), and K_2O (84.0 kg/hm^2)] was used as a basal fertilizer, and urea (69.0 kg/hm^2) and K_2O (45.0 kg/hm^2) were used as topdressing. The basal fertilizer was applied before rice transplanting, and the topdressing was applied 6–10 days after rice transplanting. Green manure was sown in the winter, 10–15 days before the late rice was harvested. Rape, milk vetch and ryegrass seeds were spread onto the plot at 37.5 kg/hm^2, 7.5 kg/hm^2 and 15.0 kg/hm^2, respectively, which are the recommended local seeding quantities for each type of grass. The fresh green manure biomass was returned to the same plot 15 days before the early rice was transplanted. All straw (except the rice stubble) was removed from the plots after each seasonal rice harvest. No fertilizer was applied to the green manure planted in winter (Gao et al., 2011, 2013, 2015; Yang et al., 2012, 2014; Zhang et al., 2013). In October of 2013 (at the rice heading stage), three rice root samples were randomly collected in each plot and pooled, and the rice roots were shaken vigorously to remove loosely adhered soil (bulk soil). The soil that tightly adhered to the roots was regarded as rhizosphere soil, which was isolated by washing the roots with sterile water and then centrifuging at high speed (10,000×*g*, 10 min) to collect the sediment. The bulk soil was designated as SWF, SRP, SMV and SRG, and the rhizosphere soil was designated as RHWF, RHRP, RHMV and RHRG for the WF, RP, MV and RG treatments, respectively. All soil was stored at −80 °C. The bulk soil was used for soil characterization and nitrogenase activity analysis.

2.2. Soil characterization

Soil chemical properties were determined according to the methods described by Bao (2000). Briefly, the soil pH was determined with a glass electrode using a soil-to-water ratio of 1:2.5. The soil organic matter (SOM) was determined by the potassium dichromate method. The total N was determined by Kjeldahl digestion. Available N was determined by the alkaline hydrolysis