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Full Length Article

Comparison of Soil Enzyme Activity and Microbial Community Structure between Rapeseed–Rice and Rice–Rice Plantings

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Abstract

Rice (*Oryza sativa* L.)–rapeseed (*Brassica campestris* L.) intercropping is a routine farming practice in Southern China, and its advantages include more efficient and productive land use as well as an improved soil environment. Field experiments were carried out on two cropping systems (rapeseed–rice rotation and rice–rice continuously) between July 2015 and October 2015. Laboratory tests were conducted to compare rapeseed–rice rotation with rice–rice continuous cropping as the control to investigate enzyme activity and the structure of bacterial communities in the soils. Two sets of soil samples were collected and tested to show that, compared to rice–rice, the catalase, urease, phosphatase and cellulase contents of the soil from rapeseed–rice rotation are increased by 11.92, 24.94, 18.80 and 20.34%, respectively, and the sucrase content decreased by 17.93%. DNA from soil microbial communities was extracted for analysis of microbial diversity using the high-throughput sequencing technique. Microbial communities in the rapeseed–rice soil were significantly different in composition to those in the rice–rice soil. Crop rotation increased the size of bacterial populations and altered their proportionality in the bacterial community and decreased the population size of Chlorobi and Chloroflexi. Canonical correspondence analysis showed that there are significant differences in bacterial population size between the two cropping systems. Firmicutes, Latescibacteria, Verrucomicrobia, Gemmatimonadetes and Acidobacteria were the principal driving force microorganisms. Rape–rice soil showed more Firmicutes and Latescibacteria in the July samples and Gemmatimonadetes and Acidobacteria in the October samples whereas the rice–rice soil showed more Verrucomicrobia and Gemmatimonadetes in the July samples and Latescibacteria and Verrucomicrobia in the October samples. These results suggest that in comparison with a rice–rice planting system, long-term rapeseed–rice rotation changes the composition and structure of microbial communities, affects soil enzyme activity, supports a more abundant and diverse microbial population, and consequently maintains higher soil quality and fertility.

Keywords: Crop rotation; Continuous cropping; Soil enzyme activity; Microbial community structure; Soil microorganism diversity; High throughput sequencing

Introduction

Paddy–upland rotation is a dominant cropping system in the southern regions of China and other Asian countries. It is estimated that in Southeast Asia the overall area used in paddy–upland rotation is approximately 27 million hectares (Timsina and Connor, 2001). Rapeseed–rice rotation is one pattern of paddy–upland rotation which is typically used in the Yangtze River basin in China. Rapeseed–rice rotation and an alternative pattern, rice–rice continuous cropping, are illustrated in Fig. 1.

Crop rotation can change soil composition and soil properties. Crop rotation directly influences substrate, particle, microorganisms, enzymes, organic matter and nutrients (Aziz *et al*., 2014; Zhang *et al*., 2014). For these changes of the crop rotation that roots plays a key role in adjustment of soil enzymatic activities and microorganism. Plants supply organic matter through their roots, which promotes the growth of microorganisms which in turn promotes plant growth by providing nutrients, siderophores and phytohormones or by suppressing phytopathogens (Haney *et al*., 2015). Crop rotation is an effective way to maintain soil habitat balance through regulating the soil micro-ecosystem (Lars *et al*., 2013). Rice–upland crop rotation can change the content and fractions of soil nutrients as well as the physicochemical properties of the soil, and thereby affect the community of soil microorganisms (Vanegas *et al*., 2013).

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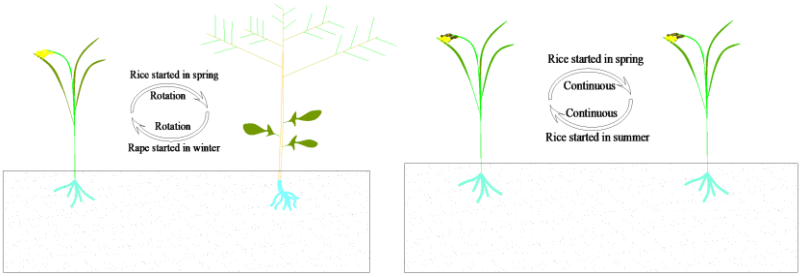

Fig. 1: Rapeseed–rice rotation and rice–rice continuous cropping

Crop rotation changes soil habitat because the rotated crops, since they are different, extract different nutrients, have different root depths, and produce different amounts of residue that remains in the soil. Crop rotation can stimulate soil biodiversity and increase biological activity in comparison with monoculture (Balota *et al*., 2004). Previous studies (Chen *et al*., 2012; Wu *et al*., 2015) have shown that the structure of microbial communities in paddy soils with rapeseed–rice rotation differs from the structure of microbial communities in soils that are monocultured (e.g., rice–rice continuous planting). Chen *et al*. (2012), using terminal restriction fragment length polymorphism (T-RFLP), showed that the structure of microbial community in paddy soils with rapeseed–rice rotation differed from that of microbial community in monoculture soil. Wu *et al.* (2015) showed that microorganisms responsible for CO_2-fixationin paddy soils with rapeseed–rice rotation were different from those in a monoculture planting. Chen *et al.* (2012), Wu *et al.* (2015) explored the differences between microbial communities in paddy soils with rapeseed–rice rotation and those in monoculture planted soils, but the composition of microbial communities in the rice rhizosphere with rapeseed–rice rotation has not been fully elucidated. The relationship between crop rotation and the composition of microbial communities in soil has been little studied, particularly with respect to determining the effect of paddy–upland crop rotation on microbial community structure and soil enzyme activity. Microorganisms and enzymes are important indicators of soil health and quality (Abbas *et al.*, 2015). Microorganisms are important in the biogeochemical cycling of plant nutrients and exudates in the soil (Chaudhary *et al*., 2015). Soil enzymes catalyse the many reactions necessary to soil microorganism life processes, such as the decomposition of organic residues (Dick *et al*., 1994).

To reduce this research gap, comparative studies were carried out in South China to investigate soils that have supported 30 years of rapeseed–rice rotational cropping and soils that have supported 30 years of rice–rice continuous cropping. The objective of this study was to examine in depth soil enzyme activities and the structure of microbial communities under different crop planting practices and tillage systems. We show that they can provide an improved microecology suitable for better crop growth and yield. We hypothesized that long-term rapeseed–rice rotation would increase enzyme activity and change the composition of microbiota in the soil. The study attempted to identify specific soil improvement due to crop rotation so that future agricultural practices can be modified to make them more efficient and to increase soil productivity. The results offer insight into the effect of long-term crop rotation on plant growth and soil properties.

Materials and Methods

Experimental Details and Measurements

Experiment location: The experiment, which began in March 1985, was carried out in a field in Dukou village, Hunan province, China (26°37'N, 113°20'E). Experimental planting followed local farming practices in the study area. There are two major cropping systems: rapeseed‑rice rotation (DY) and rice–rice continuous planting (D). The field, 20 m × 8 m, was divided into 6 plots: 3 plots for planting DY and 3 plots for planting D. The annual planting–harvesting sequence for DY was: rice was planted in April and harvested in July; consequently, rapeseed was planted in October and harvested in the following April. The annual planting–harvesting sequence for D was: rice was planted in April and harvested in July; a second rice crop was planted in July and harvested in October. Application of fertilizer, irrigation and field management accorded with the general cultivation methods used in the locality.

Experiment soil: Three rhizosphere soil samples were taken from the field for each cropping system in July 2015 and in October 2015, giving twelve samples in total. Sampling was carried out according to the methodology of Restovich *et al.* (2012). The soil samples for the analysis of enzyme activity were sieved with a 2 mm sieve and stored at 4°C. Aquantity of 50 g soil was taken from each soil sample and stored at −80°C for subsequent high-throughput sequencing analysis. The soil samples collected from the DY plots were labelled DY_J (July 2015 samples) and DY_O (October 2015 samples). The soil samples collected from the D plots were labelled D_J (July 2015 samples) and D_O (October 2015 samples).

Measurement of soil enzyme activity: Catalase and sucrase activity were measured using the methods described by Jin *et al.* (2009), Zhang *et al.* (2011). Catalase activity was determined after performing the following steps. First,

40 mL distilled water and 5 mL 0.3% H_2O_2 were added to 2 g soil that had been stored at 4°C to produce a mixture which was then shaken for 20 min (at 120 rpm) and immediately filtered (Whatman 2V filter paper). Subsequently, the reaction in the filtrate was quenched with 0.1 mol L^{-1} sulfuric acid and titrated with $KMnO_4$. The results were expressed as 0.1 mol $KMnO_4$ g^{-1} soil 20 min^{-1}.

Sucrase activity was determined using 8% glucose solution as a substrate. First, a mixture of 5 g soil that had been stored at 4°C, 15 mL substrate, 5 mL 0.2 *M* phosphate buffer (pH 5.5) and 5 drops of toluene were incubated for 24 h at 37°C. Then the mixture was filtered (Whatman 2V filter paper) and 1 mL filtrate was reacted with 3 mL 3, 5-dinitrolsalicylate in a volumetric flask and heated for 5 min. When the solution in the flask reached room temperature, it was quantified by ultraviolet spectroscopy at 508 nm (Ren *et al*., 2016). The results were expressed as mg glucose g^{-1} 24 h^{-1}.

Urease activity was determined using the method described by Wang *et al*. (2014). A total of 10 mL 10% urea and 20 mL citrate buffer (pH 6.7) were added to 5.0 g soil that had been stored at 4°C, incubated in distilled water at 37°C, shaken thoroughly and immediately filtered. Then 3.0 mL filtrate was transferred into a 50 mL volumetric flask and 10 mL distilled water, 4.0 mL sodium phenolate (1.35 mol L^{-1}), and 3.0 mL sodium hypochlorite (activated chlorine 0.9%) were added to the flask (Liu *et al*., 2014). The flask stood for 20 min and then the mixture was diluted to 50 mL. The NH_4^+ ions produced by the enzymatic hydrolysis of urea develop a blue color during the ammonia-phenol-hypochlorite reaction; when the solution turned blue (<1 h), its optical absorbance (OA) was measured at 578 nm using a spectrophotometer. A standard curve was constructed from a standard NH_4^+OA and the spectrophotometer reading obtained was translated into urease activity. For the soil quality correction, the mass of the dry soil was measured after drying the soil in an oven at 105°C.

Cellulase activity was determined using the method described by Xu and Zheng (1986) with the following modification: 10 g soil that had been stored at 4°C was mixed with 20 mL 1% carboxymethyl cellulose solution at 37°C and stored for 72 h before the addition of 5 mL phosphate buffer (pH5.5) and 1.5 mL methylbenzene. The mixture was immediately filtered and 3 mL 3, 5-dinitrosalicylic acid solution and 5 mL distilled water were then added to the filtrate, resulting in 2 mL supernatant in the tube. All tubes were incubated in boiling water for 5 min and then cooled to room temperature. The absorbance of the reaction solution was measured using a UV-visible spectrophotometer at 508 nm. The results are expressed as μg glucose g^{-1} h^{-1} (Karasawa *et al*., 2015).

Phosphatase activity was determined using the method described by Tabatabai and Bremner (1969). One gram 2 mm sieved soil was placed in a 50 mL Erlenmeyer flask and the following were added: 4 mL modified universal buffer (pH 6.5), 0.25 mL toluene, and 1 mL P-nitro phenol (PNP) solution as substrate for the enzyme. The contents of the flask were mixed for several seconds, and the flask was covered and placed in an incubator for 1 h at 37°C. Then the mixture was removed and 1 mL 0.5 *M* $CaCl_2$ and 4 mL 0.5 *M* NaOH were added, swirled thoroughly for a few seconds, and filtered. The yellow color intensity of the filtrate was measured using a spectrophotometer at 420 nm (Baddam *et al.*, 2016).

The rate of increase (R) of the enzyme activities for catalase, urease, cellulose and phosphatase in crop rotation soils (DY) compared to monoculture soils (D) are calculated by:

$$R = (E_{DY} - E_D)/E_D$$

Where E_{DY} and E_D are the enzyme activity in DY and D, respectively.

Soil Microbial Diversity Analysis

DNA extraction and high-throughput sequencing: Total DNA of the soil microorganisms was isolated according to the instructions in the Power Soil DNA Isolation Kit (Mo Bio, USA). The primer pair 515F (5'-3' GTGCCAGCMGCCGCGG) and 806R (5'-3' GGACTACHVGGGTWTCTAAT), specific to the V4 regions of the 16S rRNA gene, was used to produce16S rRNA gene for PCR amplification. Barcode oligonucleotide sequences were fused to the 5' end of the primers. PCR amplification was performed in a 20 μL reaction system containing 0.5 μL DNA template, 2.5 μL dNTPs (2.5 mmol/L), 0.5 μL of each primer (0.4 mmol/L), 0.5 μL Taq polymerase (2.5 U) and 5 μL 10× PCR buffer that was mixed in deionized ultrapure water (Yang *et al.*, 2012). The reaction was as follows: pre-denaturation for 3 min at 95°C, denaturation for 30 s at 94°C, annealing for 30 s at 56°C, and extension for 30 s at 72°C. There were 40 cycles, followed by an overlap extension for 5 min at 72°C (Langefors *et al.*, 2000).

High-throughput sequencing of DNA obtained from PCR amplification was undertaken by Shanghai Majorbio Bio-pharm Technology Co., Ltd.

Statistical Analysis

Analysis of soil enzyme activity: SPSS19.0 software was used to identify any statistically significant differences in soil enzyme activity (for the enzymes catalase, sucrase, urease, cellulase, and phosphatase) between the two cropping systems (DY and D).

Analysis of bioinformatics: The program *mother* (Schloss *et al.*, 2009) was used to preprocess the16S rRNA sequences. The standard primers and barcodes of the raw sequence were trimmed off. Sequences shorter than 150 bp and low quality bases (quality score < 27) were removed. Chimeric sequences were also excluded using the default chimera parameters. The remaining valid sequences were 291 bp, aligned using the Needleman–Wunsch algorithm, and clustered using the SILVA database.

The sequences were clustered to OTUs (operational taxonomic units) at 97% sequence identity using mothur (furthest neighbour method). Principal composition analysis (PCA) diagrams were created using R software for cluster analysis. The relationship between microorganisms and samples from different treatments was analysed using Canoco 4.5 software for canonical correction analysis (CCA).

Results

Soil Enzyme Activity

Comparison of enzyme activity between different cultivation systems: Catalase activity, urease activity, cellulase activity and phosphatase activity were all significantly higher ($P<0.05$) in DY soils than in D soils whether the soil sample had been collected in July 2015 or October 2015 (Fig. 2A, B, C and E). Sucrase activity was significantly lower ($P<0.05$) in DY soils than in D soils whether the soil sample had been collected in July 2015 or October 2015 (Fig. 2D). There was no significant difference in eitherurease activity orcellulase activity between soil samples collected in July 2015 and soil samples collected in October 2015, but there are significant differences in activity between other enzymes. Fig. 2 shows that catalase and phosphatase activity in the soil were related depending on air temperature and humidity. Temperature and precipitation are both higher in July than in October in the area of the experiment.

Rate of increase in soil enzyme activities: The results show that the activity of catalase, urease, cellulase and phosphatase in DY soil were higher than in D soil. Table 1 shows data for enzyme activity in July 2015 and October 2015 and the average rates of increase in enzyme activities for catalase, urease, phosphatase, sucrase and cellulose were 11.92, 24.94, 18.80, −17.93 and 20.34%, respectively. Soil DY shows a larger increase in the rate of increase of urease activity than it does for catalase, phosphatase and cellulase. The results show that soil enzyme activities were highly correlated with the cropping system being used.

Soil Microorganism Analyses by OTUs from DNA Sequencing

Soil microbe phylum: The DNA sequences were clustered with UCLUST software. The sequence similarity was 0.97, which defines the cluster as a class. The operation taxonomic unit (OTU) is considered to be close to a species. Based on species classification and previous results, the graph showing the relative abundance of the 10 most common phyla (phyla) was generated. Cluster analysis of microbial similarities in soil samples collected in July 2015 and October 2015 was performed to produce the clustering tree (Fig. 3, left) which shows similarities between soil samples together with the numbers of microorganisms at the phylum level and the relative abundance of each species (Fig. 3, right). Each color represents a classification (phylum). The length of the color strip represents the relative proportion of microorganisms in each phylum. The following categories of soil microorganisms were found in DY and D soils: Proteobacteria, Acidobacteria, Choroflexi, Nitrospirae, Bacteria (unclassified), Verrucomicrobia, Gemmatimonadetes, Bacteroidetes, Actinobacteria, and Chlorobi. Soil samples collected in July 2015 showed that DY soils contained more Proteobacteria and Nitrospirae but less Choroflexi than D soils. Levels of Acidobacteria were not significantly different between DY and D soils. Soil samples collected in October 2015 showed that DY soils contained more Actinobacteria but less Choroflexi and Nitrospirae than D soils. There was no significant difference in levels of Proteobacteria between DY and D soils.

Soil microbial community composition: The DNA sequences were classified using principal component analysis (PCA) in order to characterize the structures of the microbial communities by cropping system, as shown in Fig. 4. In July 2015 (Fig. 4, left) the contribution ratios of the first and second principal components were 42.09 and 21.48%, respectively. In October 2015 the contribution ratios of the first and second principal components were 51.5 and 20.85%, respectively. The first and second principal components are major constituents of the soil microbial communities. The PCA graphic representation was obtained by biplot on the two dimensions of the main principal components. Fig. 4 shows that for all soil samples collected in July 2015 there was no obvious clustering. For the soil samples collected in October 2015, DY has clustered, while D has not. This means that DY possibly influences the composition of soil microbial communities.

CCA analysis of soil microorganisms: Canonical correspondence analysis (CCA) provides a correspondence between microbial community composition and soil categories. OTU classification was determined according to the sequencing results. In the soil samples collected in July 2015 and October 2015, 13 and 14 species of microbes were selected respectively for CCA analysis (Fig. 5). Fig. 5A shows that Parcubacteria, Actinobacteria and Verrucomicrobia are positively correlated with the first axis of CCA (r=0.965, 0.920 and 0.766), and Latescibacteria, Cyanobacteria and Nitrospiraeare negatively correlated with the first axis of CCA (r=0.984, 0.945, 0.783). Acidobacteria, Chlorobi, Proteobacteria and Gemmatimonadetes are positively correlated to the second axis of CCA (r=0.976, 0.971, 0.819, 0.766) and Planctomycetales and Chloroflexiare negatively correlated to the second axis of CCA (r=0.951, 0.788). On the positive side, the first and second axes are defined by D, which is correlated with Verrucomicrobia and Gemmatimonadetes. On the negative side, the first and second axes are defined by DY, which is correlated with Latescibacteria, Chloroflexi and Firmicutes. Fig. 5B shows that Firmicutes, Latescibacteria, Chlorobi,

Table 1: Rate of increase in enzyme activities for DY compared to D (numbers are percentages)

Enzymatic Categories	July 2015	October 2015	Average
Catalase	12.24	11.59	11.92
Urease	26.19	23.68	24.94
Phosphatase	15.38	22.22	18.80
Sucrase	−18.18	−17.68	−17.93
Cellulase	6.74	23.94	20.34

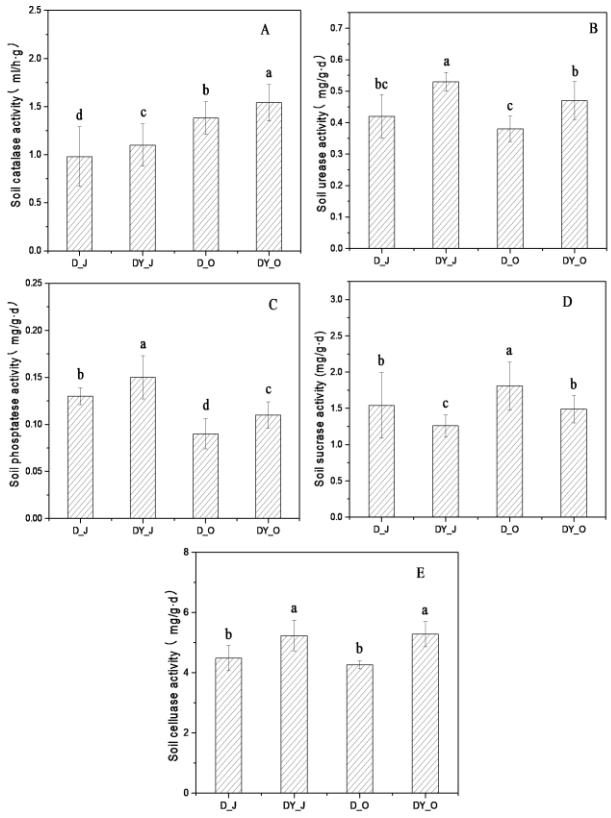

Fig. 2: Effects of different cultivation treatments on enzyme activity in soil: A soil catalase activity, B soil urease activity, C soil phosphatase activity, D soil sucrase activity, E soil cellulose activity; D_J and DY_J are soil samples collected in July 2015 and D_O and DY_O are soil samples collected in October 2015

Bacteria and Chloroflexiare positively correlated with the first axis of CCA (r=0.997, 0.933, 0.933, 0.743, 0.743) and Proteobacteria and Acidobacteria are negatively correlated to the first axis of CCA (r=0.829, 0.777). Verrucomicrobia, Parcubacteria, Cyanobacteria, Planctomycetales and Nitrospiraeare positively correlated with the second axis of CCA (r=0.956, 0.956, 0.857, 0.857, 0.766), and Gemmatimonadetes is negatively correlated with the second axis of CCA (r=0.961). On the positive side, the first and second axes are defined by D, which is correlated with Latescibacteria, Parcubacteria and Verrucomicrobia. On the negative side, the first and second axes are defined by DY, which is correlated with Acidobacteria, Gemmatimonadetes and Proteobacteria. The results of CCA analysis show that in soil samples from July 2015 Firmicutes and Latescibacteria were greatly influenced by DY and Verrucomicrobia and Gemmatimonadetes were greatly influenced by D. In the soil samples from October 2015 Gemmatimonadetes and Acidobacteria were greatly influenced by DY and Latescibacteria and Verrucomicrobia were greatly influenced by D.

Discussion

Soil microorganisms are an important component of a soil ecosystem. They directly or indirectly regulate the cycling of soil nutrients and the degradation of organic substances, thereby affecting the fertility of the soil (Shen *et al*., 2016). Soil microorganisms participate in many different biochemical processes in the soil in which

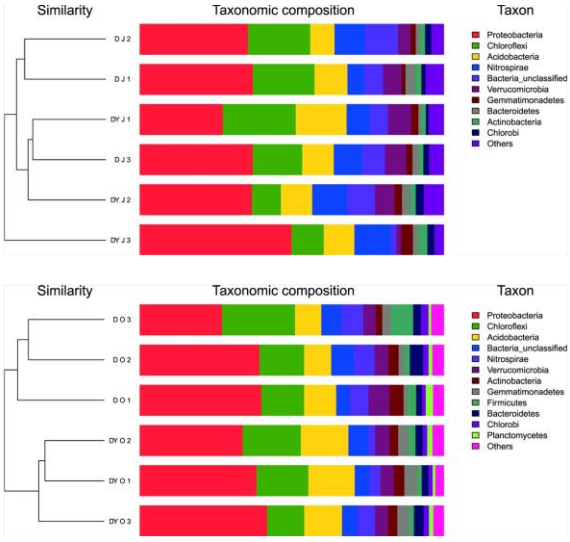

Fig. 3: The clustering tree (left) of DY and D soil samples collected in July (DYJ and D J, upper) and October (DYO and D O, lower; the numbers represent the soil sample) together with the taxonomic composition(and relative abundance) of soil microbes (right)

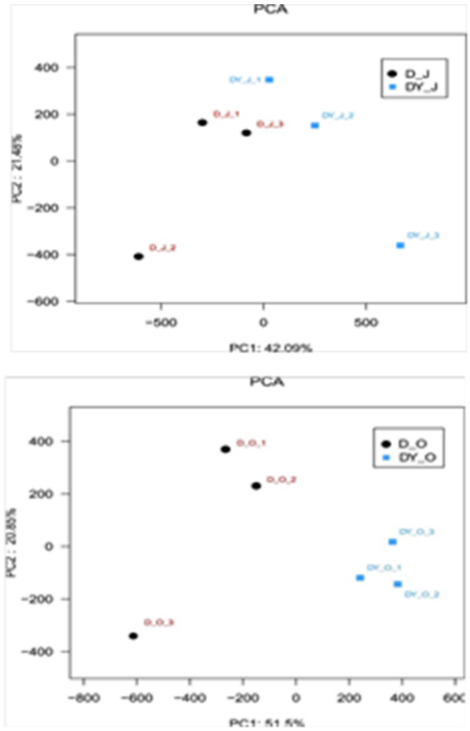

Fig. 4: PCA analysis using weighted UniFrac distances; D_J and DY_J are the soil samples collected in July 2015 (left), D_O and DY_O are the soil samples collected in October 2015 (right)

enzymes act as biocatalysts to facilitate a variety of biochemical reactions (Trasar *et al*., 2008). Thus enzyme activities and soil microorganisms are closely related in biochemical processes. Soil enzyme activity reflects the intensity of soil metabolism and changes in ecosystem functioning (Pandey *et al*., 2014). Approximately 60 enzymes have now been identified in soil, among which urease, phosphatase, invertase and cellulase have been shown to accelerate the decomposition of organic matter and increase the availability of carbon and nitrogen (Zhang *et al*., 2014). Catalase, which is actively associated with many soil microorganisms, catalyses the conversion of hydrogen peroxide radicals in soils to water and oxygen, thus reducing oxidative damage to organisms (Guo *et al*., 2015). This study analysed enzyme activity in soils that supported different cropping practices over a long period of rapeseed–rice rotation and rice–rice monoculture. The results show that the activities of catalase, urease, phosphatase and cellulase increased while the activity of sucrase decreased in the soils that supported crop rotation (Table 1). Changes in soil enzyme activities may lead to change in the composition and structure of soil microorganism communities. The structures of the microbial communities were analysed using high-throughput sequencing of the DNA extracted from soil samples. Our research shows that the composition of the soil microbial community differed between crop rotation soil and monoculture soil. The long-term rotation of rapeseed–rice increased the numbers of Proteobacteria and Acidobacteria and reduced the numbers of Choroflexi in the soil. Acidobacteria grow well in acid soil, and long-term rapeseed–rice rotation causes the soil pH to decrease (Yongsik *et al*., 2011), so the crop rotation is beneficial to Acidobacteria growth. The decrease in pH may be due to the release of acid radical ions in the root of rapeseed. In a crop rotation system, the roots of the different crops release different ions into the soil, creating a different soil environment that the microbial community is forced to adapt to (or die). Studies have shown that pH, soil type, soil enzyme activity and soil vegetation are the main factors that affect soil microorganisms (Koo *et al*., 2015). Microorganisms, through physiological and ecological processes, drive the decomposition of biomass and the transformation of plant nutrients, thereby altering the physical and chemical properties of the soil (Krome *et al*., 2010). Our results show that soil microorganisms respond to the rapeseed–rice rotation in ways that evidently benefit the microbial community (Fig. 3). The CCA plots (Fig. 5) explain the internal relationship between microbial community and soil types. They also reflect the ecological differentiation of soil microorganisms in rapeseed–rice cropping and rice–rice cropping (Fig. 5). The CCA results reflect how the different cropping systems can influence the relationship between soil categories and microbes.

Conclusion

This study has shown that soil enzyme activity is related to crop planting practice. Rapeseed–rice rotation can

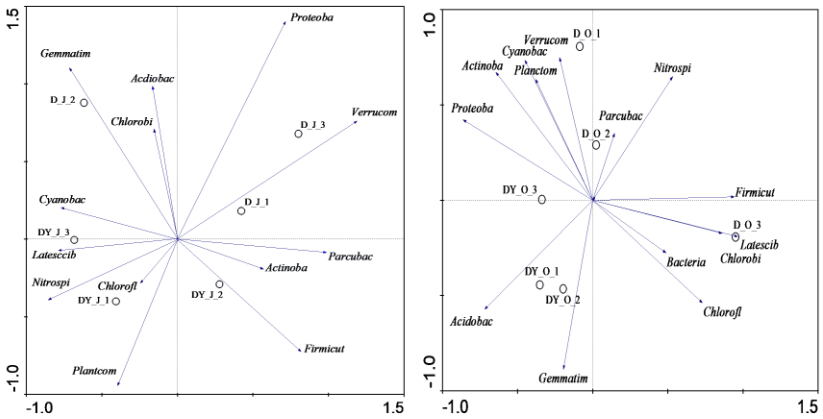

Fig. 5: CCAtriplots of soil samples considering microorganisms, OTU composition, and crop rotations: A soil samples collected in July 2015, B soil samples collected in October 2015;D_J and DY_J stand for soil sample collected in July 2015, D_O and DY_O stand for soil sample collected in October 2015

increase catalase, urease, phosphatase and cellulase activities in soil in comparison with a continuously planted rice crop. High-throughput sequencing results showed that there were significant differences in the composition of soil microbial communities between rapeseed–rice crop rotation and rice–rice monoculture. The results also show that several major types of microorganisms of different phyla in the soil can affect the structure of the microbial community. Further study is needed to find the mechanisms of change in soil microbiology that has been identified in this study.

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