

# Microbial community dynamics and function associated with rhizosphere over periods of rice growth

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## ABSTRACT

A field experiment was conducted to illustrate the different degree and dynamics of microbial community structure and function in the rhizosphere across four growing stages (before plantation and three growth stages) using a combination of biochemical (enzyme assay and microbial biomass carbon) and molecular approaches of qPCR and PCR-DGGE (polymerase chain reaction-denaturing gradient gel electrophoresis). Rice plant cultivation promoted higher enzyme activities (invertase and urease), microbial biomass carbon ( $C_{mic}$ ), bacterial (16S rRNA) and fungal (ITS rRNA) genes abundances in the rhizosphere compared to unplanted soil. Principal component analyses of PCR-DGGE profile also revealed that structures of bacterial and fungal communities of rice planted soil were well distinct from unplanted soil. Moreover, enzyme activities showed a significant positive correlation with the total microbial biomass in the rhizosphere throughout growth stages of rice plant. Relative fungal: bacterial ratios were significantly higher in rice planted soil compared to unplanted soil, suggesting rice plantation enhanced the fungal community in the rice rhizosphere environment. These results further suggest a significant linkage between the microbial community dynamics and function in the rhizosphere associated with rice plant over time.

**Keywords:** *Oryza sativa*; temporal effect; spatial effect; symbiosis of plants and soil microflora; enzyme activities; field ecosystems

Rice ecosystems remain flooded through a major part of the cropping period and are distinctive from upland soils in several physicochemical and biological properties. Therefore, flooded rice fields became a model system to study soil microbial ecology (Leisack et al. 2000). In planted soil the rice aerenchymatous tissue is also responsible for the leakage of oxygen creating an oxic rhizosphere within the anoxic bulk soil (Revsbech et al. 1999). Moreover, plant rhizodeposition in the rhizosphere results in increased microbial population size and community structures distinct from that in bulk soil (Bais et al. 2006). Hence, oxygen and carbon-releasing aerenchymatous rice plants may affect the abundance, structures and function of the microbial community in the rhizosphere of waterlogged paddy soil. The changes in overall microbial community structures and composition can be correlated with changes in certain functions both under controlled laboratory conditions (Avrahami et al. 2003) and in the field

(Marschner et al. 2001). But our understanding about the linkage between microbial community structure and function in the rhizosphere of rice crop is poorly understood. A significant research was done about the influence of plant on the dynamics of bacterial communities based on 16S rRNA analysis (Marschner et al. 2001, Smalla et al. 2001). Few studies investigated the structures and ecosystem functioning of fungal communities in the paddy soil; to our knowledge, none have investigated the relative fungal and bacterial abundances in rhizosphere across rice growth stages under field conditions. The objectives of this study were therefore (i) to evaluate the linkage between total microbial biomass and function across different growing stages; (ii) to compare the structures of bacterial and fungal communities in rice planted soil and unplanted soil; (iii) to assess the relative abundances of fungal and bacterial populations in planted soil and unplanted soil at various growth stages.

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## MATERIAL AND METHODS

**Site description and experiment layout.** The experiment was carried out at a rice farm located in Yixing City, Yifeng Village (31°24.26'N, 119°41.36'E), Jiangsu Province, China. Derived from lucstrine deposit, the soil was a typical high-yielding paddy soil classified as a hydroagric Stagnic Anthrosols and an entic Halpudept according to Soil Survey Staff, USA. The basic properties of the studied top soil (0–10 cm) are mentioned in Table 1. One month old seedlings of rice (*Oryza sativa*), Wugeng13 cultivar, were planted in randomly selected plots at a plant density of 20 plants/m<sup>2</sup>. The experiment used a randomized block design with three replicate plots. The size of each plot was 4 m × 5 m. The field was kept most of the time flooded with irrigation water. No chemicals were applied for plant protection and the plots were weeded by hand. Rhizosphere and bulk soil samples were collected on 45 days after planting (tillering stage-S1), 81 days after planting (grain filling stage-S2) and 107 days after planting (ripening stage-S3). Unplanted bulk soil (S0) was also collected before rice plantation from each plot to investigate the effect of the rice plant cultivation on the microbial communities.

**Soil sampling.** For sampling of rhizosphere soil, ten rice plants with root-soil systems were randomly sliced down 10 cm deep in the middle from each plot and shaken up in plastic bags until approximately four-fifths of the initial weight (100 g) was in the bag; this portion was considered as bulk soil. The remaining fifth that was still attached to the root system and was considered as rhizosphere soil (Butler et al. 2003). The rhizosphere and corresponding bulk soil samples of the same plot at the same plant growth stage were subsequently pooled to make one composite sample, respectively. Soil samples were sieved (< 2 mm) and stored at 4°C for analysis of microbial biomass C, N and enzymes activity and at –20°C for DNA extraction, and part of sieved soil samples was air dried for physical and chemical analyses.

**Microbial biomass C and N determination.** Microbial biomass carbon ( $C_{mic}$ ) was determined by the fumigation-extraction method as described by Vance et al. (1987).  $C_{mic}$  was calculated from (extractable carbon/0.45, where extractable carbon = (C extracted from fumigated soil) – (C extracted

from non-fumigated soil). Microbial biomass N ( $N_{mic}$ ) was estimated as (extractable nitrogen)/0.54, where extractable nitrogen = (total N extracted from fumigated soils) – (total N extracted from non-fumigated soils).

**Enzyme activity assay.** Enzyme activities were determined on fresh moist sieved (< 2 mm) soils within 15–20 days from the collection of the samples. Two enzymatic activities (invertase and urease) were analyzed according to the protocols described by Gu et al. (2009). In case of invertase, 5 g of soil (< 2 mm) was mixed with 0.2 mL of toluene and 5 mL of phosphate buffer (pH 5.5) in 50-mL Erlenmeyer flasks, then 15 mL of the 8% sucrose solution was added, and the flasks were swirled for a few seconds. The flasks were covered with stopper and placed in an incubator at 37°C for 24 h. After incubation, solution contents were passed through filter paper. The 1 mL of filtrate was pipetted into 50 mL test tube and 3 mL 3, 5-dinitrosalicylic acid monohydrate solution was added. Then all tubes were placed into a boiling water bath for 5 min and allowed to cool at room temperature with tap water. Further, the final volume of the each solution was made up to 50 mL with ddH<sub>2</sub>O. The glucose produced was determined colorimetrically at 508 nm and expressed as mg glucose/g soil/24 h.

Urease activity was determined using 5 g of soil (< 2 mm) that was mixed with 1 mL of toluene in a 50-mL Erlenmeyer flask, and allowed to stand for 15 min. Then 10 mL 10% urea solution and 20 mL citrate buffer (pH 6.7) were added and mixed well. The flasks were covered and placed in an incubator at 37°C for 24 h. After incubation, soil solution contents were passed through filter paper. Moreover, 3 mL of aliquot from filtrate was added into a 50-mL test tube along with 4 mL sodium phenol solution and 3 mL sodium hypochlorite solution and tubes were swirled well for mixing. After 20 min, the final volume of each solution was made to 50 mL with ddH<sub>2</sub>O. The released ammonium was measured colorimetrically at 578 nm and expressed as mg NH<sub>4</sub>-N produced/g soil/24 h. Control tests with autoclaved soils were carried out to evaluate the spontaneous or abiotic transformation of substrates. All chemical determinations and enzymatic activities were determined in triplicate.

Table 1. Basic physico-chemical properties of soil (0–10 cm)

pH (H <sub>2</sub> O)	CEC (cmol <sub>+</sub> /kg)	Clay (< 0.002mm) (g/kg)	Bulk density (g/cm <sup>3</sup> )	Total carbon (g/kg)	Total nitrogen (g/kg)	Alkali-releasable nitrogen (mg/kg)
6.7	18.05	399.9	1.1	20.2	2.99	276.1

Table 2. Primer sets and thermal profiles used for the DGGE and qPCR analysis

Target gene	Primer set	Size (bp)	Thermal cycling profile	Reference
16S rRNA (Bacteria)	338-F-GC <sup>a</sup> 518-R	250	94°C (5 min); 35 cycles of 94°C (60 s), 55°C (60 s), and 72°C (60 s)	Muyzer et al. (1993)
18S rRNA (Fungi)	NS1 <sup>a</sup> Fung-GC	470	94°C (5 min); 35 cycles of 94°C (60 s), 58°C (60 s), and 72°C (60 s)	May et al. (2004)
16S rRNA (Bacteria)	338-F <sup>b</sup> 518-R	250	95°C (5 min); 40 cycles of 94°C (30 s), 55°C (60 s), and 72°C (60 s)	Fierer et al. (2005)
ITS rRNA (Fungi)	ITS-F <sup>b</sup> 5.8s-R	350	95°C (5 min); 40 cycles of 94°C (30 s), 53°C (60 s), and 72°C (60 s)	Fierer et al. (2005)

<sup>a</sup>primer set used for DGGE (denaturing gel gradient electrophoresis); <sup>b</sup>primer set used for qPCR (quantitative polymerase chain reaction)

**Molecular and statistical analysis.** Three DNA extractions of each soil sample (0.5 g) from the same replicate field plot were made using PowerSoil™ DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, USA) following the manufacturer's instructions. Relative bacterial and fungal abundances were estimated using real time PCR (qPCR), with primers and the thermal cycling conditions described in Table 2. The DNA concentration was measured at 260 nm using UV spectrophotometer (Bio Photometer, Eppendorf, Germany). Each qPCR reaction was performed in a 25 µL volume containing 10 ng of DNA, 0.2 mg/mL BSA, 0.2 µmol of each primer and 12.5 µL of SYBR premix EX Taq™ (Takara Shuzo, Shinga, Japan). The size of PCR product was confirmed by melting curve analysis and electrophoresis in a 1.5% agarose. A plasmid containing target region of bacterial (16S rRNA) and fungal (ITS rRNA) gene was used to construct a standard curve. The standard curves were generated using triplicate 1:5 serial dilutions of plasmid DNA ranged from  $3.41 \times 10^4$  to  $2.13 \times 10^7$  copies for bacterial 16S rRNA gene and  $2.21 \times 10^3$  to  $1.38 \times 10^6$  copies of template for fungal ITS rRNA gene per assay, respectively. Denaturing gradient gel electrophoresis (DGGE) was performed to assess the bacterial and fungal communities structure using 338F-GC and 518R primers set (Muyzer et al. 1993), and the NS1 and Fung-GC (May et al. 2001), respectively. For DGGE analysis, PCR products were separated on 8% (w/v) polyacrylamide gels (acrylamide-bisacrylamide [37.5:1]) containing denaturing gradients of 35% to 70% for bacteria and 10% to 40% for fungi using the Bio-Rad D-Code universal mutation detection system. A 100% denaturant was defined as 8% acrylamide containing 7 mol/L urea and 40% deionised formamide. DGGE was performed using 20 µL of the PCR product in 1 × TAE buffer at 60°C, 200 V for 5 min, then 100 V

for 12 h (bacteria) and 140 V for 5.5 h (fungi). Gels were stained with silver staining and were scanned with gel document system (Bio-Rad, Hercules, USA). All statistical analyses were performed using Minitab v.15.1. The significance level was fixed at  $P < 0.05$ .

**Variation in enzyme activities and total microbial biomass in rhizosphere across rice growth stages.** Soil enzyme activities are often used as indices of microbial function and soil fertility and are greatly affected by quality and quantity of root exudates depending on the plant species, cultivar, plant growth stage and physiological status of the plant (Saxena et al. 1999). Enzyme activities (invertase and urease) increased with rice plant at the grain filling stage (S2) and then declined at the ripening stage (S3) (Figure 1). This phenomenon was also observed by Gu et al. (2009) in paddy soil under field conditions. The reduced enzyme activities at later growth stages of rice plant may appear due to the reason that roots release less organic compounds into soil to support enzyme activities in paddy soil (Lu et al. 2002). A significant positive correlation was observed between enzyme activities (urease and invertase) and total microbial biomass ( $C_{mic}$ ) in rhizosphere across all sampling time points (Table 3). Several studies also reported that changes in overall microbial community structures and composition can be correlated with the changes in certain functions both under controlled conditions (Avrahami et al. 2003) and in the field (Marschner et al. 2001). Moreover, the temporal variability of microbial biomass dynamics is complex and poorly understood in rice soils. Previous studies revealed that with development of rice plant,  $C_{mic}$  was increased (Hoque et al. 2001) or decreased (Reichardt et al. 1997), or no difference between planted and unplanted soils (Witt et al. 2000) was observed. In accordance with the findings by Lu

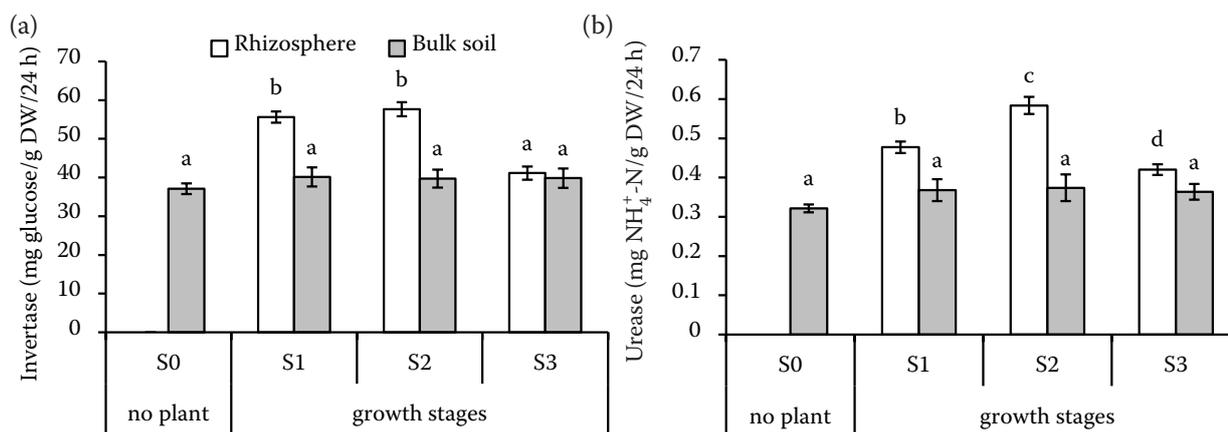


Figure 1. Invertase (a) and urease (b) activities in the rhizosphere and bulk soil at the S1 (tillering), S2 (grain filling) and S3 (ripening) growth stages. Different letters indicate significant differences by ANOVA at  $P < 0.05$  ( $n = 3$ ; error bars are  $\pm$  SD)

et al. (2002) and Zeng et al. (2005), we observed that  $C_{mic}$  in rhizosphere increased during earlier growing periods (S1-tillering and S2-grain filling) and declined at the later stage (S3-ripening stage, Table 4).

#### Relative bacterial and fungal abundance in rhizosphere during growth periods of rice plant.

As  $C_{mic}$  measures the total microbial biomass in soil, including bacterial, archaeal, and fungal components, A real-time PCR approach was used to further estimate the total bacterial population (16S rRNA gene), total fungal population (ITS rRNA gene) and relative fungal: bacterial ratio. The cultivation of rice crops had a strong stimulating effect on the numbers of total bacteria and fungi in the paddy soil (Figure 2). The number of 16S rRNA and ITS rRNA gene copies in the rhizosphere were significantly higher than in bulk soil across each growth stage. This may be due to the rhizodeposition of carbohydrates from plant roots increasing microbial growth in comparison with that in the bulk, a phenomenon well known as rhizosphere effect (Smalla et al. 2001, Mougel et al. 2006). It is also well known that rice roots release oxygen through aerenchymatous tissue at rates sufficient to support non-specific aerobic microbial processes (Arth et al. 1998). On the contrary, in the bulk soil

there could only be microbial respiration but lower oxygen concentrations. The relative fungi: bacteria ratio was also used as potential tool to study the state of soil microbial community in response to different environmental stresses in various ecosystems (Fierer et al. 2005). Lauber et al. (2008) argued that relative fungal: bacterial ratios (rRNA-qPCR based) were not significantly different across the land-use types (hardwood and pine forests, cultivated and livestock pasture lands). Our study revealed that the ratio of fungi to bacteria (rRNA-qPCR based) ranged from 0.03 to 0.06 across all growth stages, which indicated that bacterial population dominated the rice ecosystem (Figure 2c). We observed non significant difference in fungi to bacteria ratio across the rice plant growth stages, suggested that the observed patterns were not driven by any particular microbial group. Although real-time PCR is flexible, simple and rapid promising molecular tool for the quantification of soil microbial communities, it has some important limitations. These limitations include DNA extraction bias, the availability of sequence data, and adequate preparation of inhibitor-free target DNA. However, qPCR provides reproducible method to monitor differences and changes in microbial population size within growth stages.

Table 3. Pearson correlation between enzyme activities and microbial abundances in rhizosphere across all sampling time points ( $n = 4$ )

	$C_{mic}$ (m/kg)	Bacterial 16S rRNA gene copies/g dry soil	Fungal ITS rRNA gene copies/g dry soil
Urease	<b>0.956*</b> (0.044)	0.932 (0.068)	<b>0.973*</b> (0.027)
Invertase	<b>0.966*</b> (0.034)	0.825 (0.175)	0.887 (0.113)

\*significant coefficients ( $P < 0.05$ ) are highlighted in bold font. Values in bracket show the level of significance

Table 4.  $C_{mic}$  (mg/kg),  $N_{mic}$  (mg/kg) and  $C_{mic}/N_{mic}$  ( $n = 3$ ; mean  $\pm$  SD) in rhizosphere and bulk soil at different sampling points

	S0	S1		S2		S3	
		R	B	R	B	R	B
$C_{mic}$	596.9 $\pm$ 4.0 <sup>d</sup>	694.8 $\pm$ 4.0 <sup>b</sup>	615.26 $\pm$ 13.7 <sup>d</sup>	711.2 $\pm$ 2.2 <sup>a</sup>	620.15 $\pm$ 8.5 <sup>d</sup>	677.6 $\pm$ 4.1 <sup>c</sup>	618.1 $\pm$ 13.2 <sup>d</sup>
$N_{mic}$	62.1 $\pm$ 4.5 <sup>b</sup>	70.0 $\pm$ 1.3 <sup>a</sup>	65.6 $\pm$ 4.2 <sup>b</sup>	71.5 $\pm$ 2.3 <sup>a</sup>	65.7 $\pm$ 3.9 <sup>b</sup>	68.7 $\pm$ 2.5 <sup>ab</sup>	65.93 $\pm$ 2.2 <sup>b</sup>
$C_{mic}/N_{mic}$	9.61 $\pm$ 0.27 <sup>a</sup>	9.92 $\pm$ 0.17 <sup>a</sup>	9.40 $\pm$ 0.71 <sup>a</sup>	9.94 $\pm$ 0.33 <sup>a</sup>	9.42 $\pm$ 0.44 <sup>a</sup>	9.87 $\pm$ 0.36 <sup>a</sup>	9.41 $\pm$ 0.4 <sup>a</sup>

S0 – before plantation bulk soil; S1 – tillering stage; S2 – grain filling stage; S3 – ripening stage; R – rhizosphere; B – bulk soil. Different letters in the same row indicate significant differences ( $P < 0.05$ )

**Dynamics of bacterial and fungal community structure in response to rice developmental stages.** The use of molecular approaches provides valuable information about the characterisation of naturally occurring microbial communities without cultivation (Štursa et al. 2009). Microbial communities in the rhizosphere are dynamic and susceptible to changes in plant conditions. In response to changing root exudation patterns with the development of plant, the microbial community structure and composition in the rhizosphere also change with time and varies during the life cycle and with the seasonal response of plants (Germida and Siciliano 2001). In the current study, principal component analysis of DGGE profile of the 16S rRNA gene (bacterial) and the 18S rRNA gene (fungal) revealed differences in both the bacterial

and fungal community structures across different growth stages (Figure 3). We found that bacterial and fungal communities of unplanted soil (S0) and planted soil were well separated, showing the well profound plant rhizosphere effect. In fact, the introduction of plants to soil affects the physical-chemical properties and the biological parameters of the soil environment close to the growing roots by continuously producing and excreting organic compounds (Hinsinger et al. 2006). Houlden et al. (2008) performed a field study to explore microbial community structure and activity in the rhizosphere of three field crops (pea, wheat and sugar beet) over a period of growth. By using DGGE and BIOLOG techniques, they observed remarkable temporal shifts in diversity and relative activity in rhizosphere bacterial and fungal communities with

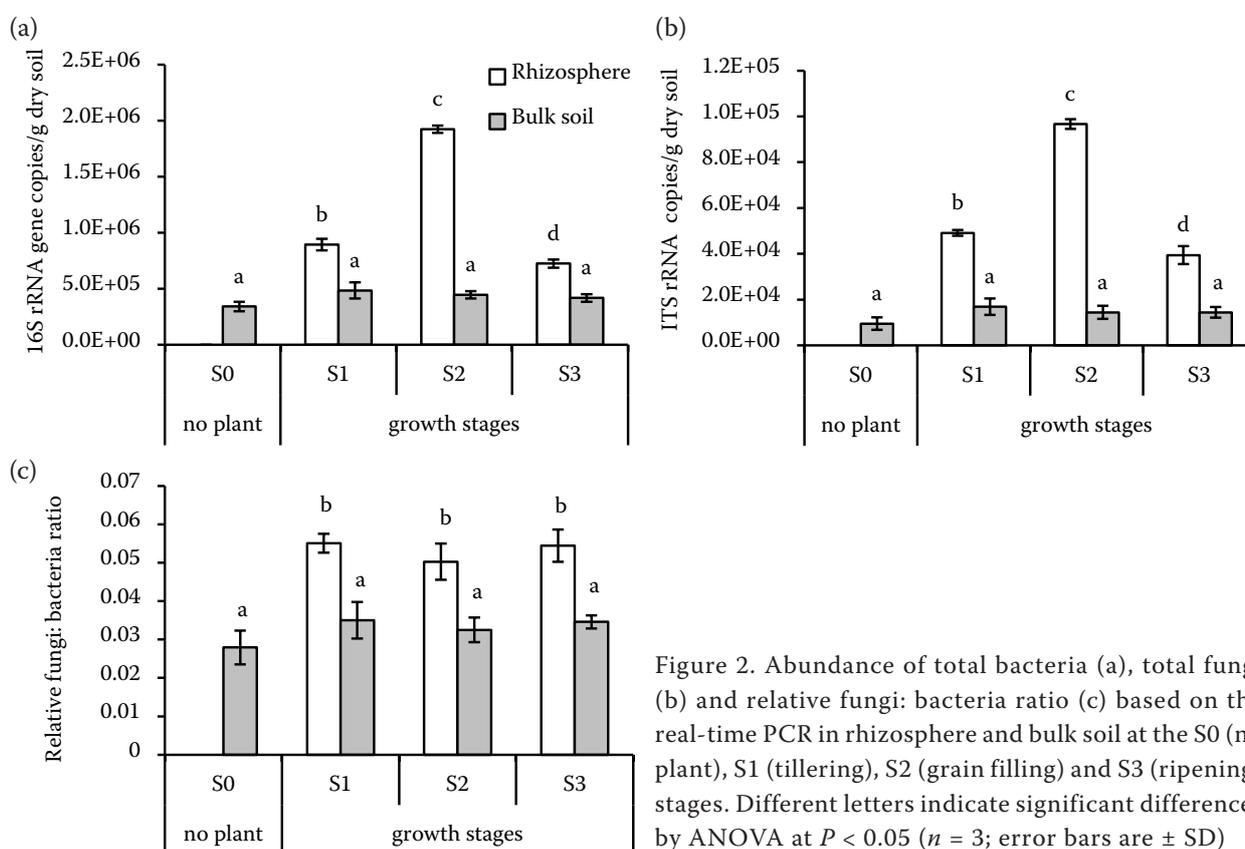


Figure 2. Abundance of total bacteria (a), total fungi (b) and relative fungi: bacteria ratio (c) based on the real-time PCR in rhizosphere and bulk soil at the S0 (no plant), S1 (tillering), S2 (grain filling) and S3 (ripening) stages. Different letters indicate significant differences by ANOVA at  $P < 0.05$  ( $n = 3$ ; error bars are  $\pm$  SD)

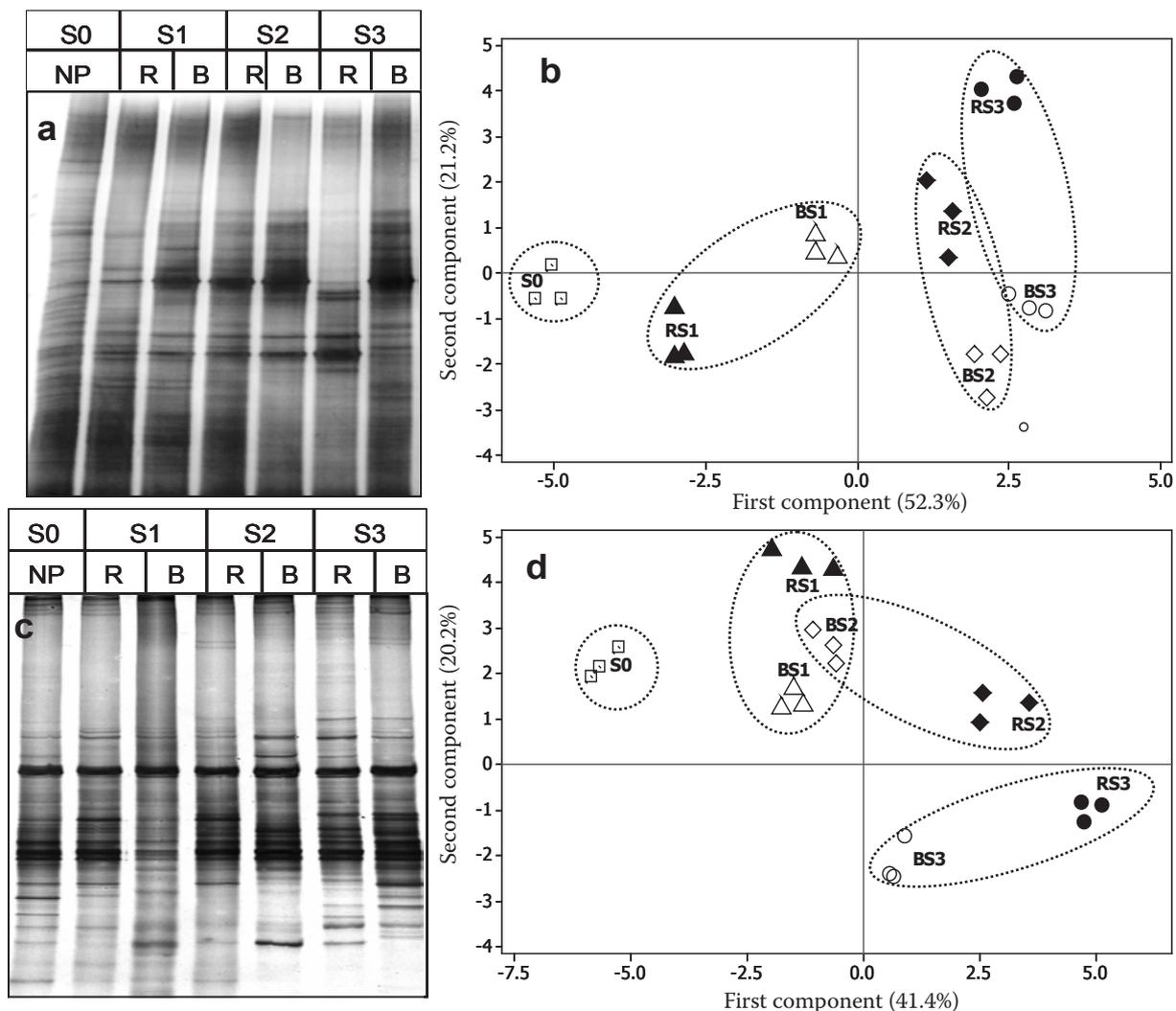


Figure 3. Principal component analysis (PCA) of denaturing gel gradient electrophoresis (DGGE) profile of bacterial (a, b) and fungal (c, d) communities in the rhizosphere (R) and bulk (B) soil at S0 (no plant, NP), S1 (tillering), S2 (grain filling) and S3 (ripening) stages of rice plant. Solid and hollow symbols indicate the rhizosphere (R); bulk (B) soil

developmental stage for all plant species. Further, pot and field experiments conducted by Xu et al. (2009) indicated that bacterial communities in the soybean rhizosphere changed with growth stages, and higher number of DGGE bands observed in early reproductive growth stages than in the other stages. Mougel et al. (2006) also characterized the bacterial and fungal community structure in rhizosphere of *Medicago truncatula* at five developmental stages in pot experiment by using the automated ribosomal intergenic spacer analysis (ARISA). Similarly, we found pronounced changes in the genetic diversity of bacterial and fungal communities during vegetative stage (tillering-S1) and reproductive stage (ripening-S3) of plant growth development (Figure 3). The difference in microbial communities structure between the growth stages may be due to a weaker rhizodepo-

sition during the reproductive stages than during the vegetative stage (Mougel et al. 2006).

A certain degree of succession occurs in the bacterial and fungal communities structures of the rhizosphere between vegetative (tillering) and reproductive (grain filling and ripening) stages. Interestingly, rice plant growing stages showed no significant effect on relative fungal: bacterial ratio, suggested that growth stage did not stimulate any particular microbial group. However rice plant stimulated higher relative fungal: bacterial ratio compared to unplanted treatment. This study illustrates the significance of rice ecosystem in context of rhizosphere microbial community dynamics and function over time. A future research is required to explore the ecological significance of different rice cultivars in terms of difference in microbial communities' dynamics and possible increased respiration rates.

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