Impact of Bt-transgenic rice (SHK601) on soil ecosystems in the rhizosphere during crop development

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ABSTRACT

In contrast to other transgenic *Bacillus thuringiensis* (Bt) crops (e.g. Bt maize and cotton), risk assessments of Bt rice on soil ecosystem are few. To assess the influence of Bt rice on rhizosphere soil ecosystems, soil samples from Bt, non-Bt and controls were taken at seedling, tillering, booting, heading and maturing stages. The activities of dehydrogenases, invertase, phenol oxidases, acid phosphatases, ureases and proteases showed no significant differences between Bt and non-Bt rice. A Biolog system was used to evaluate the effect of Bt rice on functional diversity of microbial communities. Although there were differences in carbon substrate utilization between Bt and non-Bt rice at seedling, tillering and heading stages, these differences were transient and not persistent. Denaturing gradient gel electrophoresis (DGGE) fingerprint patterns showed that Bt rice had little effect on the dominant rhizosphere bacterial, fungal and actinobacterial communities. The richness and consistency of microbial communities according to carbon substrate utilizations and DGGE band patterns did not differ significantly between Bt and non-Bt rice, and were close to that of control soil. There was no evidence to indicate apparent effects of Bt rice on soil enzyme activities, microbial community composition and functional diversity in this study.

Keywords: risk assessment; enzyme activities; microbial community; Biolog EcoPlate; denaturing gradient gel electrophoresis

The potential risks of genetically modified (GM) plants to environmental and human health have become a public concern in recent years, due to the release of transgenic crop plants worldwide and their replacement of traditional crops (Nap et al. 2003). The introduction of GM plants into agricultural ecosystems raised a number of questions, including the ecological impact on soil ecosystems. Soil-borne communities are dominated by microorganisms, which account for > 80% of the total biomass in soil (Kowalchuk et al. 2003). They are involved in numerous important processes, including decomposition of organic matter, nutrient mineralization, regulation of plant pathogens and improvement of soil structure (Bruinsma et al. 2003). Changes in the structure or function of microbial communities have a major impact on soil ecosystems and biogeochemical processes. Microbial community structure and function in rhizosphere soil, which is directly influenced by root exudates of GM plants, are often proposed as an early and dynamic indicator of GM risk assessment on soil ecology, and used increasingly for sensitive responses (Nannipieri et al. 2003).

Rice is the staple diet for nearly two billion people worldwide and the major food for over half of those living in Asia. The introduction of Bt rice will not only greatly increase rice production, but also reduce the use of insecticides. Many rice varieties have been transformed with genes encoding various Bt crystal proteins and have been shown to be resistant to one or more lepidopteran pests of rice. However, no Bt rice or other transgenic rice varieties have been released for commer-

Supported by the Key Technologies R & D Program of the Shanghai Agricultural Commission, Project No. 2009-6-4; by the Science and Technology Commission of Shanghai Municipality, Project No. 10DZ2294103, and by the Youth Fund of the Shanghai Academy of Agricultural Sciences, Project No. 2011-16.

cialization due to controversy over biosafety, yet. Although there were several reports on impacts of transgenic Bt rice on soil enzyme activities and microbial composition in the rhizosphere (Wu et al. 2004a,b, Liu et al. 2008), a comprehensive study on risk assessment of transgenic Bt rice on soil ecosystem is lacking.

The objective of this study was to investigate the effects of Bt rice on soil biochemical processes and microbial communities in a flooded paddy soil under laboratory conditions. Denaturing gradient gel electrophoresis (DGGE) was used to analyze the effects of Bt rice on soil microbial community composition. Soil enzyme activities and carbon (C) substrate utilization by soil microorganisms were measured to determine the effects of Bt rice on metabolic capabilities and functional diversity of the microbial community in rhizosphere soil.

MATERIAL AND METHODS

Soil and plants materials. Soil was collected from the top layer (0–20 cm) of an experimental rice field at the Baihe Experiment Station of the Shanghai Academy of Agriculture Sciences, Shanghai, China, where no transgenic rice had ever been planted. The soil was air-dried at room temperature, passed through a 2-mm sieve and then homogenized by mixing three times. The soil contained 21.2 g/kg of total organic C content, 1.3 g/kg of total nitrogen (N), 170 mg/kg of available N, 1.4 g/kg of total phosphorus (P), 17 mg/kg of available P, 13.6 g/kg of total potassium (K), 150 mg/kg of available K, and had pH of 6.89.

The Bt rice used in the tests was line SHK601, which contains the synthetic version of the insecticidal *cry1Ac* gene. It was derived from a Chinese rice variety Shuhui 527, and transformed by the *Agrobacterium* method under the control of 35S cauliflower mosaic virus promoter. The seeds of the two lines were provided by the Rice Institute of Sichuan Agricultural University, Sichuan Province, China.

Experimental design and soil sampling. A pot experiment under natural conditions was conducted to evaluate the impact of Bt rice on rhizosphere soil ecosystems on 30 June 2010. Each pot (20 cm × 20 cm) was packed with 5 kg of soil. After a week of flooding, Bt rice and non-Bt rice seedlings (three leaves) were transplanted into the pot soil. Pots without rice plants were used as the control treatment. Eight replicates were prepared for each treatment. The soil was kept flooded (2-cm deep) during the whole growing period. All pots were placed at random, and moved daily to ensure the same growing conditions.

Sampling (three replicates) of rhizosphere soils was performed at five stages in the rice growth period: seedling, tillering, booting, heading and maturing (22, 51, 70, 95 and 113 day, respectively). Plant was gently removed and rhizosphere soil was collected by gently shaking root to dislodge small soil clumps adhering to the root. Soil samples were stored immediately at −20°C before assay.

Enzymatic assay. Protease, urease, sucrase, dehydrogenase, catalase and polyphenol oxidase activities in rhizosphere soils were determined according to Tabatabai (1994). All determinations of enzymatic activity were performed in triplicate, and all values reported are averages of the three.

Determination of C substrate utilization. Biolog EcoPlates (Biolog Inc, Hayward, USA) were used to determine the C substrate utilization pattern by microorganisms in rhizosphere soil. Soil sample of 10 g was shaken in 90 mL of sterile water for 30 min and then adjusted to a final dilution of 10^{-3}. A 150-µL aliquot was inoculated in each microplate well. All plates were placed in polyethylene bags to reduce desiccation and incubated in darkness in growth chambers at 28°C. Each sample was performed in triplicate. The rate of utilization was indicated by reduction of tetrazolium, a redox indicator dye, which changes from colorless to purple. The absorbance at 590 nm was measured at 24 h intervals.

Genomic DNA extraction and PCR amplification. Total genomic DNA was extracted from 0.5 g of sample using the FastDNA spin kit for soil (MP Biomedicals, LLC, Solon, USA). For bacterial DGGE analysis, 16S rRNA fragment was amplified with the primers 341F-GC and 518R (Muyzer et al. 1996). The fungal internal transcribed spacer (ITS) region was amplified with the primers NS7-GC and NS8 (White et al. 1990). A group-specific primer, R513-GC and F243, was used to amplify 16S rRNA fragment of actinobacteria (Heuer et al. 1997). The PCR was performed with the following program: 5 min at 94°C, followed by 32 cycles at 94°C for 45 s, 55/50/63°C for bacteria/fungi/actinobacteria for 45 s, and 72°C for 45 s, with a final extension at 72°C for 7 min. PCR products were confirmed by electrophoresis on 1.5% agarose gels stained with ethidium bromide. The mixed PCR products from five replicate PCRs were used for DGGE to minimize deviation.

DGGE analysis. DGGE was carried out in a Dcode™ Universal Detection System (Bio-Rad, Hercules, USA) by a previously described method with slight modifications (Muyzer et al. 1996). PCR products

were resolved on polyacrylamide gels (7.5%, wt/vol) in 1 × TAE (20 mmol Tris-Cl, 10 mmol acetate and 0.5 mmol Na_2EDTA) using denaturing gradients of 30–70, 30–60 and 30–60% (for bacteria, fungi and actinobacteria, respectively) where 100% denaturant contained 7 mol/L urea and 40% formamide. Electrophoresis was carried out at a constant voltage of 160 V and a temperature of 60°C for 5 h. Gel was stained with SYBR Green in sterile water and photographed under UV light.

Statistical analysis. Microbial activity in each microplate was expressed as average well color development (AWCD) to eliminate variation in well color development caused by different cell densities:

$$AWCD = [\Sigma(C_i - R)]/31$$

Where: C_i – the mean value of the same three wells except for the control well; R – the value of the control well.

Principal component analysis (PCA) based on 120-h AWCD data were performed using the SPSS 13.0. Cluster analysis of DGGE banding patterns were performed with the unweighted-pair group method using NTSYS-pc software package, after band detection using Quantity One software (Bio-Rad, Hercules, USA). Shannon's (H'), Simpson's (D), McIntosh index (U) and Evenness (H' and U) were calculated according to Hacket and Grifiths (1997). Significant ($P < 0.05$) differences were analyzed by the Tukey's t-test with SPSS 13.0.

RESULTS AND DISCUSSION

Effect of Bt rice on enzyme activities in rhizosphere soil. The trends of the changes in enzyme activities of Bt rice, non-Bt rice and control were sim-

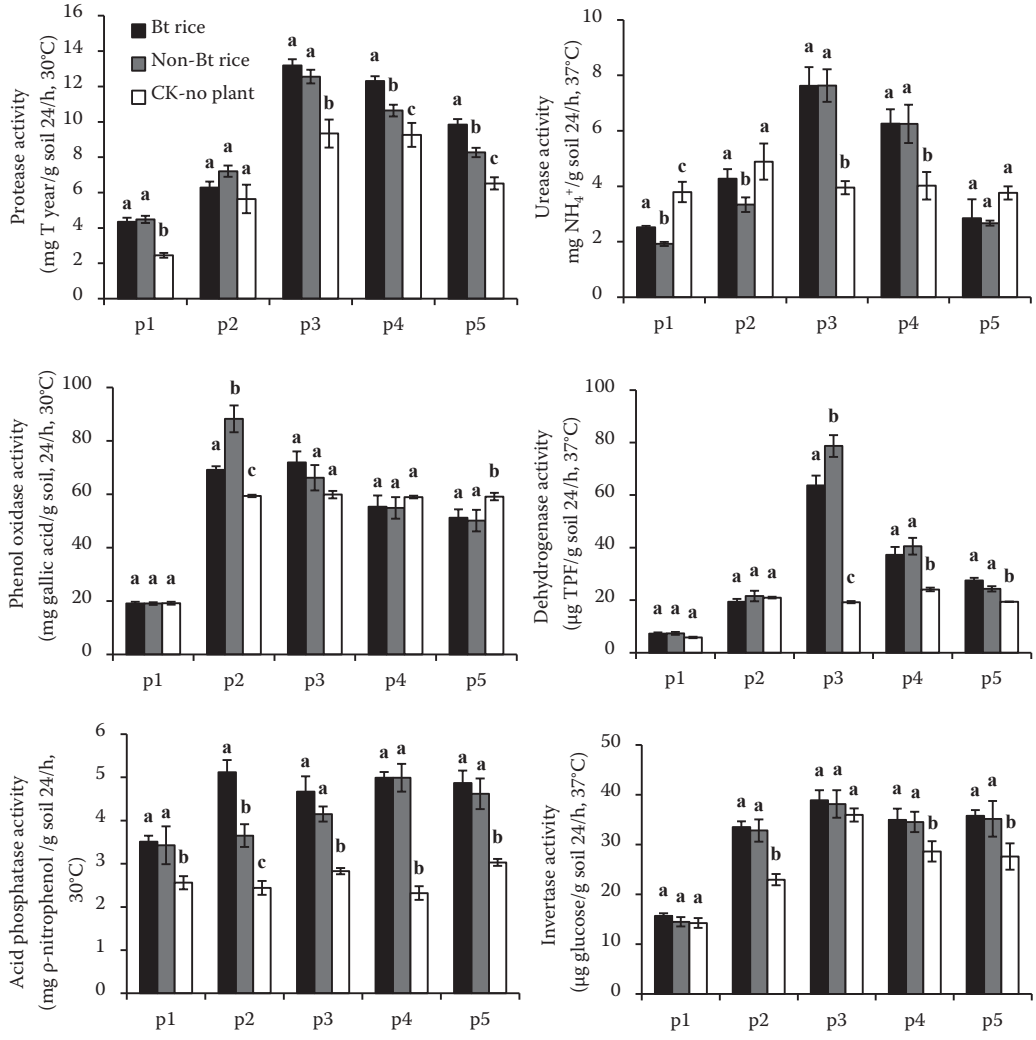

Figure 1. Activities of proteases, ureases, acid phosphatases, invertase, dehydrogenases and phenol oxidases in rhizosphere soil of a Bt rice and its non-Bt near-isogenic counterpart at different growth periods, and in control (CK) soil incubated without a growing rice plant. P1 – seedling; P2 – tillering; P3 – booting; P4 – heading; P5 – maturing periods. Different letters (a, b, c) at the same growth stages indicate a significant difference at $P < 0.05$. Vertical bars indicate the standard error of the means

ilar during the whole development cycle (Figure 1). Enzyme activities increased firstly after the incorporation, reached their highest rates at tillering or booting stages, and decreased thereafter. Our results indicated that changes of soil enzyme activities were mainly related with development period, and Bt rice had little effect on soil enzyme activities. Previous researches also found that there was no persistent difference in soil enzyme activities between Bt and non-Bt rice (Wu et al. 2004a,b, Liu et al.2008).

Effect of Bt rice on functional diversity of microbial communities. Biolog EcoPlates, as a rapid and community-level method to characterize microbial metabolic diversity, were successfully used to evaluate potential risk of Bt cotton on functional diversity of microbial communities (Shen et al. 2006). In the present study, although some significant differences between Bt and non-Bt rice were found in AWCD curves at seedling, booting and heading periods, there was a little difference at the maturing stage (Figure 2). Additionally, a little significant difference was found among the Shannon, Simpson, McIntosh and Evenness indices of Bt, non-Bt and control (Table 1). These results were also confirmed by PCA analysis. Although PCA analysis revealed a significant discrimination of soil microbial community functional diversity

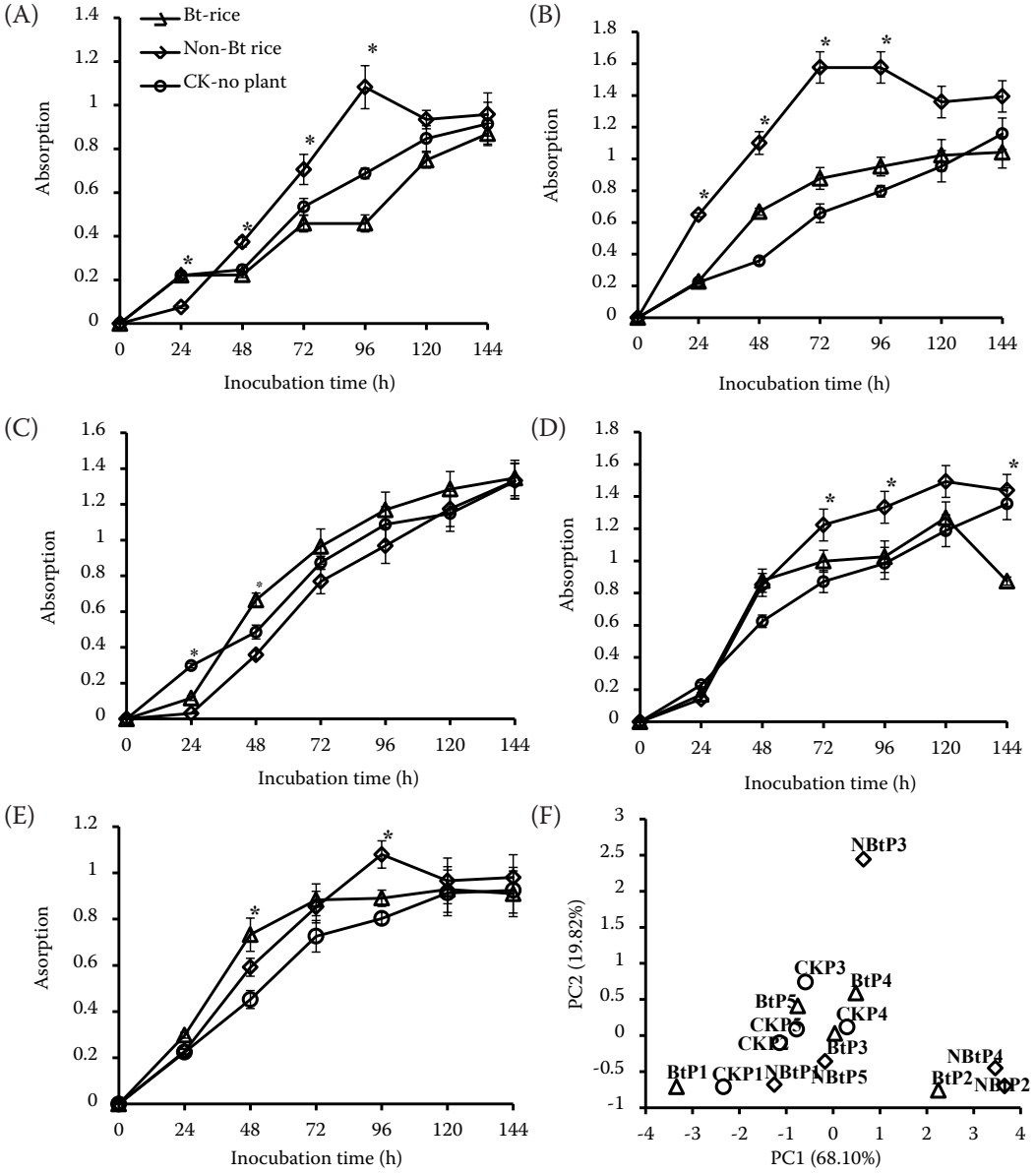

Figure 2. Average well color development (AWCD) and principal component analysis (PCA) based on Biolog EcoPlates by rhizosphere microflora of Bt rice, non-Bt rice and CK at different development stages. AWCD at (A) seedling, (B) tillering, (C) booting, (D) heading, and (E) maturing periods. (F) PCA. Bt – Bt rice; NBt – non-Bt rice; CK – control–no plant. P1 – seedling; P2 – tillering; P3 – booting; P4 – heading; P5 – maturing periods. *$P < 0.05$ between Bt rice and non-Bt rice. Vertical bars indicate standard error of the means

Table 1. Diversity and evenness indices based on substrate utilization patterns on Biolog EcoPlates and denaturing gradient gel electrophoresis (DGGE) band patterns

Samples		Shannon index	Shannon evenness	McIntosh index	McIntosh evenness	Simpson's index
BtP1	Biolog ECO	3.148 ± 0.105^{a}	0.853 ± 0.012^{a}	5.554 ± 0.039^{a}	0.945 ± 0.003^{a}	0.952 ± 0.001^{a}
	DGGE	3.259 ± 0.022^{a}	0.836 ± 0.041^{a}	3.146 ± 0.068^{a}	0.941 ± 0.002^{a}	0.914 ± 0.043^{a}
NBtP1	Biolog ECO	3.362 ± 0.123^{a}	0.932 ± 0.087^{a}	5.124 ± 0.076^{b}	0.947 ± 0.007^{a}	0.958 ± 0.010^{a}
	DGGE	3.266 ± 0.001^{a}	0.918 ± 0.054^{a}	3.058 ± 0.062^{a}	0.935 ± 0.012^{a}	0.949 ± 0.002^{a}
CKP1	Biolog ECO	3.234 ± 0.033^{a}	0.842 ± 0.002^{a}	5.234 ± 0.041^{b}	0.948 ± 0.012^{a}	0.951 ± 0.021^{a}
	DGGE	3.232 ± 0.072^{a}	0.875 ± 0.002^{b}	3.814 ± 0.032^{b}	0.937 ± 0.024^{a}	0.934 ± 0.009^{a}
BtP2	Biolog ECO	3.252 ± 0.000^{a}	0.837 ± 0.002^{a}	6.528 ± 0.015^{a}	0.952 ± 0.008^{a}	0.958 ± 0.022^{a}
	DGGE	3.064 ± 0.091^{a}	0.892 ± 0.021^{a}	3.676 ± 0.021^{a}	0.952 ± 0.021^{a}	0.924 ± 0.014^{a}
NBtP2	Biolog ECO	3.337 ± 0.011^{b}	0.839 ± 0.013^{a}	6.621 ± 0.023^{b}	0.949 ± 0.004^{a}	0.915 ± 0.031^{a}
	DGGE	3.193 ± 0.052^{a}	0.843 ± 0.024^{b}	3.623 ± 0.022^{a}	0.956 ± 0.016^{a}	0.942 ± 0.012^{ab}
CKP2	Biolog ECO	3.284 ± 0.021^{b}	0.839 ± 0.044^{a}	6.602 ± 0.016^{b}	0.948 ± 0.013^{a}	0.951 ± 0.013^{a}
	DGGE	3.407 ± 0.003^{b}	0.881 ± 0.008^{a}	3.111 ± 0.014^{b}	0.959 ± 0.013^{a}	0.957 ± 0.005^{b}
BtP3	Biolog ECO	3.164 ± 0.009^{a}	0.865 ± 0.012^{a}	7.822 ± 0.025^{a}	0.954 ± 0.009^{a}	0.952 ± 0.006^{a}
	DGGE	3.162 ± 0.047^{a}	0.847 ± 0.003^{a}	2.978 ± 0.021^{a}	0.948 ± 0.007^{a}	0.944 ± 0.005^{a}
NBtP3	Biolog ECO	3.145 ± 0.012^{a}	0.848 ± 0.006^{b}	7.729 ± 0.058^{b}	0.952 ± 0.006^{a}	0.951 ± 0.004^{a}
	DGGE	3.209 ± 0.032^{a}	0.855 ± 0.005^{a}	3.237 ± 0.018^{a}	0.952 ± 0.017^{a}	0.943 ± 0.004^{a}
CKP3	Biolog ECO	3.153 ± 0.002^{a}	0.857 ± 0.021^{ab}	7.758 ± 0.024^{b}	0.956 ± 0.014^{a}	0.949 ± 0.002^{a}
	DGGE	3.189 ± 0.011^{a}	0.829 ± 0.001^{b}	3.059 ± 0.014^{c}	0.956 ± 0.026^{a}	0.938 ± 0.005^{a}
BtP4	Biolog ECO	3.274 ± 0.009^{a}	0.854 ± 0.010^{a}	6.613 ± 0.015^{a}	0.934 ± 0.008^{a}	0.964 ± 0.007^{a}
	DGGE	3.043 ± 0.045^{a}	0.887 ± 0.005^{a}	2.026 ± 0.019^{a}	0.937 ± 0.015^{a}	0.934 ± 0.003^{a}
NBtP4	Biolog ECO	3.325 ± 0.051^{a}	0.892 ± 0.012^{b}	6.611 ± 0.003^{a}	0.940 ± 0.003^{a}	0.961 ± 0.005^{a}
	DGGE	3.304 ± 0.073^{b}	0.901 ± 0.013^{a}	2.011 ± 0.021^{a}	0.941 ± 0.005^{a}	0.953 ± 0.004^{a}
CKP4	Biolog ECO	3.284 ± 0.032^{a}	0.876 ± 0.018^{ab}	6.656 ± 0.014^{b}	0.947 ± 0.011^{a}	0.964 ± 0.004^{a}
	DGGE	3.431 ± 0.024^{c}	0.877 ± 0.022^{a}	2.017 ± 0.008^{a}	0.938 ± 0.006^{a}	0.964 ± 0.031^{a}
BtP5	Biolog ECO	3.378 ± 0.002^{a}	0.859 ± 0.007^{a}	5.546 ± 0.011^{a}	0.951 ± 0.007^{a}	0.964 ± 0.004^{a}
	DGGE	3.365 ± 0.006^{a}	0.941 ± 0.005^{a}	2.648 ± 0.009^{a}	0.952 ± 0.023^{a}	0.956 ± 0.008^{a}
NBtP5	Biolog ECO	3.386 ± 0.009^{a}	0.869 ± 0.004^{a}	5.535 ± 0.015^{a}	0.948 ± 0.013^{a}	0.961 ± 0.001^{a}
	DGGE	3.387 ± 0..012^{a}	0.932 ± 0.006^{a}	2.631 ± 0.008^{a}	0.949 ± 0.021^{a}	0.962 ± 0.009^{a}
CKP5	Biolog ECO	3.376 ± 0.002^{a}	0.859 ± 0.002^{a}	5.532 ± 0.009^{a}	0.946 ± 0.012^{a}	0.963 ± 0.007^{a}
	DGGE	3.422 ± 0.007^{b}	0.896 ± 0.004^{b}	2.609 ± 0.004^{b}	0.942 ± 0.008^{a}	0.947 ± 0.006^{a}

Bt – Bt rice; NBt – non-Bt rice; CK – control–no plant. P1 – seedling; P2 – tillering; P3 – booting; P4 – heading; P5 – maturing periods. Different letters (a, b, c) at the same growth stages indicate a significant difference at $P < 0.05$

among Bt, non-Bt rice and control at different growth stages (Figure 2F), Bt, non-Bt rice and control were clustered together at the maturing stage.

Effect of Bt rice on bacterial, fungal and actinobacterial community composition. DGGE patterns were slight variation among Bt, non-Bt and control at the same growth stage, whereas the same dominant bands were found (Figure 3). Additionally, there was no significant difference among diversity indices (H', D and U), Shannon and McIntosh Evenness of Bt, non-Bt rice and control based on DGGE patterns (Table 1). Cluster analysis revealed that the effect of rice development was stronger than the effect of Bt rice plants on the soil microbial communities (Figure 4). These results revealed that Bt-transgenic rice had little effect on dominant microorganisms essential for long-term sustainability of soil ecosystems. Wu et al. (2009) studied soil microbial communities in the rhizosphere of Bt and non-Bt rice using

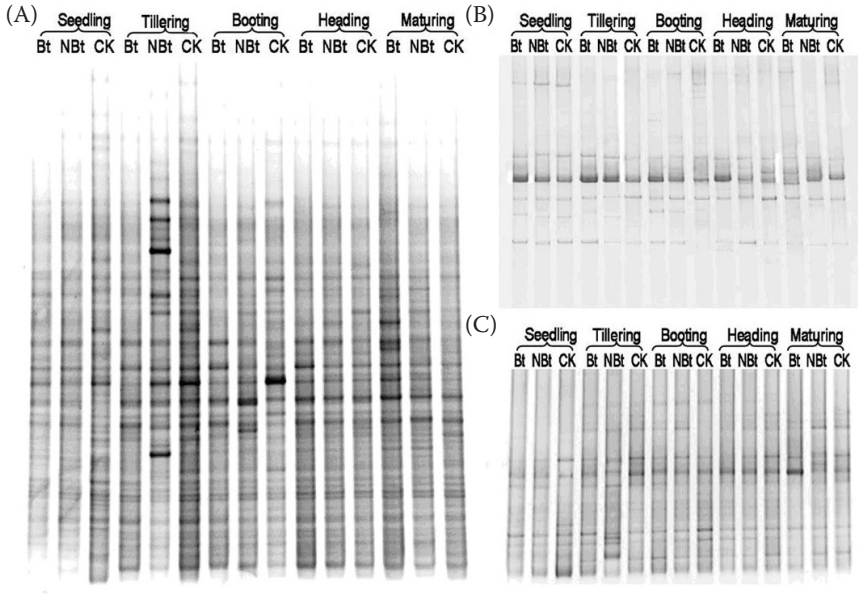

Figure 3. Denaturing gradient gel electrophoresis (DGGE) profiles of bacteria (A), fungi (B) and actinobacteria (C) from rhizosphere soil of Bt rice, non-Bt rice and CK at different development stages. Bt – Bt rice; NBt – non-Bt rice; CK – control–no plant

phospholipid fatty acid analysis, and found that Bt rice had no persistent effect on microbial community composition in the rhizosphere. Other studies showed that the effects of GM plants on microbial communities are subject more to seasonal variations or to other environmental factors than to expression of Cry or other proteins (Fang et al. 2005, Icoz et al. 2008).

Although different effects, ranging from no effect to minor and significant effects, of different transgenic Bt plants on microbial communities and soil enzyme activities were reported (Gupta and Watson 2004, Rui et al. 2005), most studies indicated that Bt rice have no or only minor effects, and effects are often transient in duration (Wu et al. 2004b, Icoz et al. 2008, Liu et al. 2008). It was shown that transgenic rice produced less Bt toxic proteins and they degraded at a faster rate than purified Bt protein in the same soil (Clark et al. 2005, Wang et al. 2006) – this seems to explain

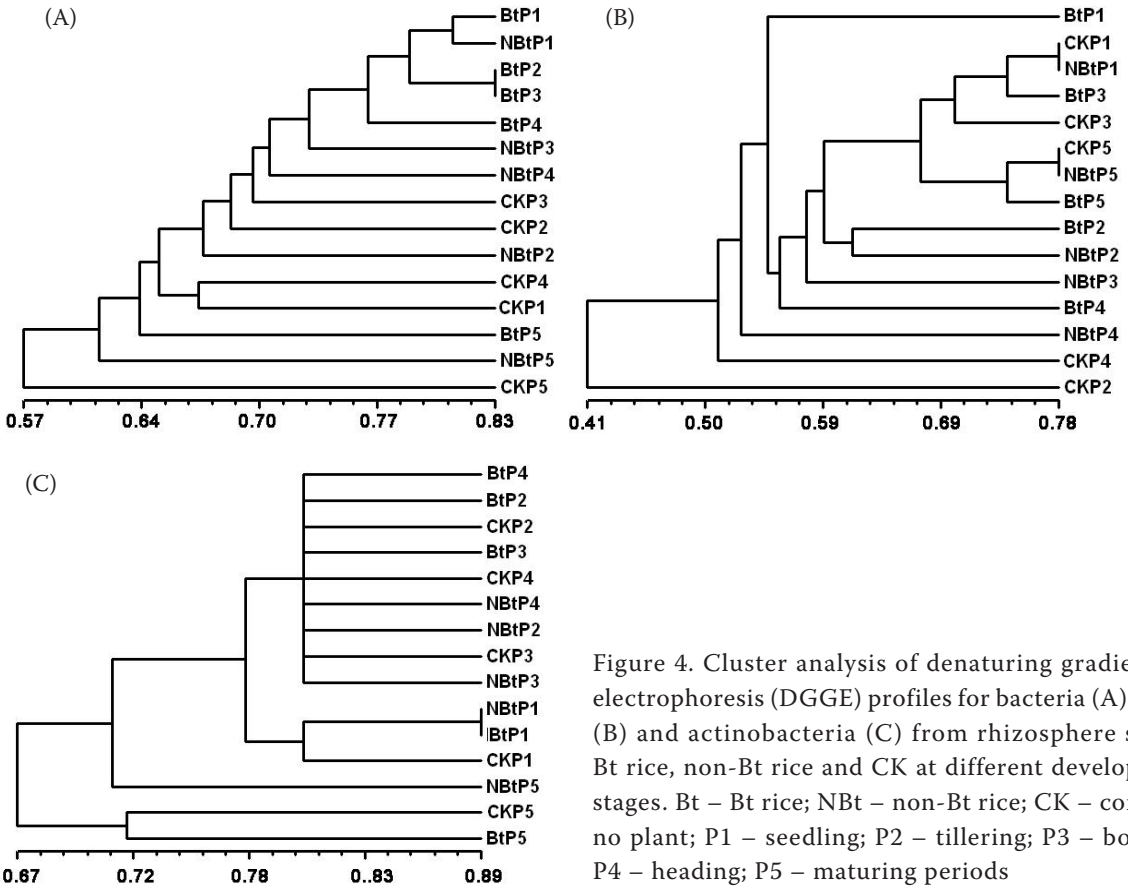

Figure 4. Cluster analysis of denaturing gradient gel electrophoresis (DGGE) profiles for bacteria (A), fungi (B) and actinobacteria (C) from rhizosphere soil of Bt rice, non-Bt rice and CK at different development stages. Bt – Bt rice; NBt – non-Bt rice; CK – control–no plant; P1 – seedling; P2 – tillering; P3 – booting; P4 – heading; P5 – maturing periods

several study results in which transgenic Bt rice had no apparent effect on soil enzymes and microbial communities (Wu et al. 2004a,b), consistent with our results.

The present study revealed minor or little effects of Bt rice (SHK601) on soil enzyme activities, microbial community composition and functional diversity. Such studies are important to determine the potential risks associated with the release of Bt rice. This is the first relatively comprehensive study on risk assessment of Bt rice on rhizosphere soil ecosystems.

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Received on October 28, 2011

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