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Bt corn plants and their straw have no apparent impact on soil microbial communities

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Abstract Polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE) and sequences of the 16S and 18S rRNA genes were used to access the effects of actively growing *Bacillus thuringiensis* (*Bt*) corn Pioneer 34B24 and Nongda 1246*1482, and plant straw (leaves plus stalks) of *Bt* hybrid Pioneer 34B24 and Nongda 61 on soil bacterial and fungal communities. Two-way indicator species analysis (TWINSPAN®), and detrended correspondence analysis (DCA) of the DGGE data indicated that neither the actively growing *Bt* corn nor its straw had any constant apparent effect on the soil bacteria and fungi community structure. The age of the growing plants, or the timing of plant straw decomposition may have more effect on the microbial community than other factors, i.e., the presence of Cry protein, plant hybrid and variety.

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Introduction

Genetically modified (GM) crop plants for resistance to pests represent a potential promising tool with which to decrease the amount of chemical pesticides used in agriculture. Cry-proteins from *Bacillus thuringiensis* (*Bt*) are by far the most common insecticidal proteins that have been engineered into plants. *Bt cry* genes have been engineered into a large number of plant species, such as corn, cotton, potato, tomato, eggplant, rice, etc. (see, e.g., Icoz et al. 2008; Sanvido et al. 2007). *Bt* corn is grown on about 11.3 million ha worldwide and is, by far, the most widely grown *Bt* crop in the world (James 2005). The "first generation" Cry proteins engineered into corn were Cry1Ab, and Cry9C. Monsanto's YieldGard® corn, transformation event MON810, expressing Cry1Ab toxic to some lepidopteran insect pests, is the most widely grown *Bt* crop today (Benedict and Ring 2004; Icoz and Stotzky 2008).

In China, *Bt* corn has not yet been approved for commercial cultivation, although evaluation of the environmental risk of some released *Bt* corn hybrids [e.g., Pioneer 34B24 (Mon810) from Monsanto, expressing Cry1Ab toxin, and Nongda 61 and Nongda 1246*1482 from China Agricultural University, expressing Cry1A protein] in the North East Plain and North China Plain has lasted for more than 10 years (1998–2008). The aim of introducing *Bt* corn to China is to control the damage of Asian corn borer *Ostrinia furnacalis* (Guenee) and reduce pesticide input in corn production. The delayed commercial introduction of *Bt* corn in China results mainly from the following: firstly, cultivating *Bt* corn may lead to development of resistance of the target pests (i.e., *Ostrinia furnacalis* [Guenee] and *Helicoverpa armigera* [Hubner]) of *Bt* cotton. The North East and North China Plains are the main cultivated *Bt* cotton belt, as well as being the most suitable region for corn cultivation in China. By 2006, *Bt* cotton had been grown intensively in every cotton-growing area, and had reached 4 million ha, accounting for 70% of the total growing area (Wu 2007). The conventional corn fields in these regions had provided a natural refuge for management of the resistance to *Bt* cotton by *H. armigera* (Hubner). Secondly, the release of *Bt* corn remains a long-term concern because of the presumed potential ecological and environmental risks, e.g., effects on non-target organisms, effects on soil ecosystems, etc. (Icoz et al. 2008; Saxena et al. 2004; Sanvido et al. 2007).

To date, laboratory and field studies have demonstrated that Cry protein expressed in *Bt* corn enters the soil system mainly via root exudation throughout the growth of the plant, in pollen released during tasseling, and from crop residues after harvest (e.g., Icoz and Stotzky 2007; Icoz et al. 2008; Lehman et al. 2008; Saxena et al. 1999, 2002a; Saxena and Stotzky 2000; Zscheischler et al. 1984; Zwahlen et al. 2003). As a toxin, Cry protein released to soil from *Bt* corn has been shown to degrade slowly and to accumulate in soil (Tapp and Stotzky 1998; Saxena et al. 2002a; Zwahlen et al. 2003). Organisms in soil will come into contact with transgenic Cry proteins when the proteins are released from *Bt* corn in root exudates or from decomposing plant tissue, thus posing a potential risk for nontarget organisms, including microorganisms (e.g., Icoz et al. 2008; Saxena et al. 1999; Saxena and Stotzky 2000; Zwahlen et al. 2003).

When assessing the ecological risks of transgenic plants, their impact on soil microbes should be considered, because soil microbial communities carry out complex processes that are of major ecological and agricultural significance (e.g., for biogeochemical cycles and soil fertility; Icoz et al. 2008; Saxena et al. 1999). Any changes, however small, in the composition of the microbial community should be considered as an early warning indicator for risk assessment. There have been a number of studies, using different parameters and techniques, on the effects of *Bt* corn hybrids on the soil microbial community. Most studies have indicated that *Bt* corn causes no or only minor changes in microbial community structure, and that these changes are often transient in duration (Blackwood and Buyer 2004; Brusetti et al. 2004; Devare et al. 2004; Flores et al. 2005; Icoz and Stotzky 2008; Lang et al. 2006; Icoz et al. 2008; Saxena and Stotzky 2001a; Zwahlen et al. 2007). For example, Saxena and Stotzky (2001a) found no significant differences in the number of colony-forming units of culturable bacteria (including actinomycetes) and fungi or in the numbers of nematodes and protozoa between rhizosphere soil of *Bt* and non-*Bt* corn or between soil amended with biomass of *Bt* and non-*Bt* corn. A 4-consecutive-year study (2003–2006) of corn cultivation reported no constant statistically significant differences in the numbers of different groups of microorganisms, the activities of enzymes, or the pH between soils planted with *Bt* and non-*Bt* corn (Icoz et al. 2008). The numbers and types of microorganisms and enzyme activities differed with the season and with the variety of corn, but these differences were not related to the presence of Cry proteins in the soil (Icoz et al. 2008). Zwahlen et al. (2007) found no major changes in the decomposition of *Bt* corn residue or in the composition of the soil organism community. No constant significant differences in bacterial counts were detected either in a greenhouse study comparing *Bt*- and non-*Bt*-corn (Brusetti et al. 2004). As expected, major differences were present in the bacterial community in the bulk soil compared to the rhizosphere; these differences were, however, unrelated to the specific hybrid. In the study of Brusetti et al. (2004), differences were detectable only when using molecular profiling techniques, whereas conventional culturing techniques reveal no differences.

By contrast, some studies have indicated that *Bt* corn affects microbial communities, the activities of some enzymes, and microbe-mediated processes and functions in soil (e.g., Dinel et al. 2003; Castaldini et al. 2005; Griffiths et al. 2005, 2006; Icoz et al. 2008; Turrini et al. 2004; Xue et al. 2005). Xue et al. (2005) found that the ratio of Gram-positive to Gram-negative bacteria was lower in soil with *Bt* corn than in soil with near-isogenic non-*Bt* corn. Turrini et al. (2004) reported that root exudates of *Bt* corn (event 176) significantly reduced presymbiotic hyphal growth of the arbuscular mycorrhizal fungus, *Glomus mosseae*, compared with root exudates of another *Bt* hybrid (event *Bt*11) and non-*Bt* corn. Constant significant differences were also detected between *Bt* corn (*Bt*11 and *Bt*176) and non-*Bt* corn plants in both total and metabolically active 16S rRNA fractions of culturable rhizosphere heterotrophic bacteria by denaturing gradient gel electrophoresis (DGGE) and in mycorrhizal colonization by microscopy (i.e., a significantly lower level of *G. mosseae* was detected in roots of *Bt* corn; Castaldini et al. 2005). Moreover, plant residues of transgenic corn affected soil respiration, bacterial community, and mycorrhizal establishment by indigenous endophytes (Castaldini et al. 2005).

From the abundance of data available, it is clear that the effect of transgenic plants on the soil microbial community, and consequently on the evolution of potential adverse effects, depends strongly on the particular plants, techniques, protein and conditions considered (Brusetti et al. 2004). It is therefore important to have reliable tests to evaluate these effects. Some methodological approaches, including the use of molecular biological techniques, show some promise in helping to understand the impact of GM crops on soil microbial ecology (Bruinsma et al. 2003; Sanvido et al. 2007). These molecular techniques, including polymerase chain reaction (PCR)-dependent approaches, yield fingerprint-type data, which allow the complexity of a microbial community to be described.

In this study, we carried out a greenhouse experiment with the aim of gaining knowledge for the evaluation of the environmental impact of *Bt* corn on soil ecosystems. We assessed the effects on the bacterial and fungal community structures in soils amended with growing plants of *Bt* corn (Pioneer 34B24 and Nongda 1246*1482) at different stages of the life cycle by using (1) PCR-DGGE of 16S and 18S rRNA genes, and (2) cloning and determination of the nucleotide sequence of nearly complete 16S and 18S rRNA genes amplified by PCR. In addition, the effects of plant residues (leaves plus stalks) of *Bt* corn (Pioneer 34B24 and Nongda 61) on the microbial community were further investigated using the same molecular parameters.

Materials and methods

Soils

Soils were collected from the top layer (0–20 cm) of a conventional sweetcorn field at the Agricultural Experiment Station of South China Agricultural University, Guangzhou, China. The soil was air-dried at room temperature, homogenized by sieving (2-mm mesh), and stored at 4°C before use. The soil at this site was a clay loam, containing 18.2 gkg^{-1} organic matter, 0.9 gkg^{-1} total N, 1.6 gkg^{-1} total P, and 20.1 gkg^{-1} total K (K_2O), with a pH (H_2O) of 6.16 and a cation exchange capacity (CEC) of 9.54 cmol kg^{-1}.

Experimental design

Rhizospheric microbial community structure in soils with growing Bt corn

Two transgenic *Bt* corn hybrids [Pioneer 34B24 (event Mon810, expressing Cry1Ab protein) from Pioneer International USA, and Nongda 1246*1428 (Cry1A) from China Agricultural University] and their near-isolines (Pioneer 34B23, and Nongda 3138) were used in the experiment. Seeds of each hybrid were planted at 75 cm spacing into a 3.0 m× 3.4 m plot (a total of four plots) in a greenhouse on 12 October 2002, watered every 3 days and fertilized five times (on 28 October; 11, 21, 30 November; and 20 December) as top spray 20 g compound fertilizer per time per pot.

Sampling (three replicates) of the rhizospheric soils from each plot was performed after 25 (nine leaves per plant), 39 (stem elongation phase), 53 (tasseling), and 82 (flowering, anthesis) days of plant growth. Plants were gently removed and the rhizospheric soils were collected by gently shaking the roots to dislodge

small clumps of soil adhering to the roots. Soil samples were stored immediately at −80°C before assay.

Effect of plant residues on soil microbial communities

Seeds of Bt-transgenic corn [Pioneer 34B24, and Nongda 61 (Cry1A) from China Agricultural University] and their non-Bt isolines (Pioneer 34B23, and Nongda 3138) were planted in 31 cm×31 cm pots (one seed per pot) on 9 June 2002, following the method described above. In October 2002, after the corn had reached physiological maturity, plants from pots representing the four hybrids were removed. Straw (leaves plus stalks) was cut into 2–4 cm length pieces, freeze-dried, ground, and sieved through a 2-mm mesh. General characteristics of the straw are listed in Table 1.

Soil samples (250 g) were mixed thoroughly with 19.05 g straw from each corn hybrid, and saturated with distilled water. Three replicates were prepared for each treatment. The blended soils were incubated in an artificial growth chamber followed Sims and Holden's protocol (1996), with a relative humidity of 80% at 25°C. On day 15 and day 75 after incubation, the soils (three replications per treatment per time) were ground, sieved through a 2-mm sieve, and stored at −80°C until use.

Polymerase chain reaction–denaturing gradient gel electrophoresis

Isolation of soil microbial DNA

Total soil DNA was extracted using soil DNA isolation kits according to the manufacturer's instructions (FASTDNA SPIN Kit for Soil, BIO 101, Vista, CA; Borneman et al. 1996). The final concentration of each DNA sample was adjusted to 10 ng μL^{-1} for all PCR.

PCR for amplification of microbial 16S rRNA gene fragments

Partial 16S rRNA genes were amplified from the extracted genomic DNA by PCR using a PTC 100 thermal cycler (MJ Research, Watertown, MA). Each PCR mixture (a total volume of 50 μL) contained 50 ng genomic DNA, 200 μM each deoxynucleotide triphosphate, 50 pmol universal primers, 2.0 mM MgCl$_2$, 5 μL 10×PCR buffer, and 5 units Taq DNA polymerase. The 16S rRNA genes of the bacteria were amplified with the 40-nucleotide guanine-cytosine (GC)-clamp primer PRBA338F-GC [5′-(CGCCCGCCGCGCGCGGCGGGCGGGGCGGGG GCACGGGGGG) ACTCCTACGGGAGGCAGCAG-3′] and the PRUN518R (5′-ATTACCGCGGCT GCTGG-3′; Nakatsu et al. 2000) using a thermocycling program consisting of a 5-min initial denaturation at 94°C, 30 cycles of 92°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by 15 min at 72°C. For amplification of fungal 18S rRNA genes, the universal primers were FR1-GC [5′-(CCCCCGCCGCGCGCGG CGGGCGGGGCGGGGGCGCGGGCC) AI*CCAT TCAATCGGTAIT-3′] and FF390 (5′-CGATAA CGAACGAGACCT-3′; Vainio and Hantula 2000) with the following cycling conditions: denaturation at 95°C for 8 min, 30 cycles of 95°C for 30 s, 50°C for 45 s, and 72°C for 2 min, followed by a final extension for 10 min at 72°C. All PCR products were incubated at 4°C until processed further.

Denaturing gradient gel electrophoresis

DGGE was performed on a D-Gene apparatus (Bio-Rad, Hercules, CA). Samples containing approxi-

Table 1 General chemical properties of the straws used in this study. Different lowercase letters indicated statistically significant differences between groups of materials (Duncan's multiple range tests, $P<0.05$). Bt Bacillus thuringiensis

Sample	Total C (%)	Total N (%	Total P (%)	Total K (%)	C/N
Pioneer 34B24 (Bt)	57.64±33.11b	1.87±0.15c	0.44±0.18a	3.36±0.84a	30.80
Pioneer 34B23 (non-Bt)	51.12±24.92b	1.95±0.45bc	0.31±0.12b	2.39±0.31b	26.23
Nongda 61 (Bt)	74.93±51.02a	2.11±0.26a	0.32±0.12b	2.77±1.36b	35.46
Nongda 3138 (non-Bt)	55.60±38.32b	2.01±0.26b	0.34±0.12b	2.62±1.05b	27.43

mately equal amounts of PCR amplicons were loaded onto 8% (w/v) polyacrylamide gels (37.5:1, acrylamide:bisacrylamide) in 0.53 Tris-acetate-EDTA (TAE) with a denaturing gradient ranging from 40% to 60% denaturant for bacterial 16S rRNA genes [100% denaturant contains 7 M urea and 40% (v/v) formamide in 0.53 TAE], or ranging from 45% to 60% for fungal PCR products. Electrophoresis was performed at a constant 80 V for 14 h (bacteria) or at 50 V for 18 h (fungi) at 65°C. Following electrophoresis, the gels were rinsed and stained for 20 min in an ethidium bromide solution (0.5 mg L^{-1}), followed by 1 min of destaining in water repeated three times. The DGGE profile images were digitally captured and recorded (Gel Doc^{TM} XR170-8170 Molecular Imager System, Bio-Rad).

DGGE data analysis

DGGE fingerprints were converted to binary data on the basis of the migration and presence of bands using the BioNumerics 2.0 software (Applied Maths, Sint-Martens-Latem, Belgium). The micro-community of soils inferred from DGGE profiles was classified using Two-Way Indicator Species Analysis (TWINSPAN®; Hill, 1979). Detrended correspondence analysis (DCA; Hill and Gauch 1980) was used to check the delimitation defined by TWINSPAN and to study interrelationships. All statistical analyses were implemented in TWINSPAN and the Canoco for Windows v.4 software package (ter Braak and Šmilauer 1998).

Sequence analysis of the16S and 18S rRNA genes

Recovery of bands from DGGE gels

DGGE bands were picked up using sterile tips, placed in a 1.5 mL microcentrifuge tube with 20 µL sterile distilled water, and incubated overnight at 4°C. To check the purity of the extracted products, PCR was performed with the primers noted above (i.e., PRBA338F-GC and PRUN518R for bacterial genes, and FR1-GC and FF390 for fungal genes), and DGGE was carried out using previous PCR products as molecular weight markers. Only those extracted products whose reamplicons presented a single band with the same migration distance as the

marker were chosen for nested PCR. The nested universal primers with non-GC-clamp [PRBA338F (5′-ACTCCTACG GGAGGCAGCAG-3′) and PRUN518R for bacterial 16S rRNA gene, and FR1(5′-AICCATTCAATCGGTAIT-3′) and EF390 for fungi 18S rRNA gene)] were applied using the same protocol as described above.

Cloning and sequencing

The nested PCR products were purified with a PCR purification kit (Guoling Company, China) and ligated into the cloning vector pGEM-T (Promega) following the manufacturer's instructions. Ligated DNA was transformed into competent *Escherichia coli* DH5α cells. Plasmid inserts were extracted by the alkaline lysis method (Sambrook et al. 1989). Three to five positive clones from each DGGE band were selected randomly for sequencing with an automated ABI 3100 sequencer using a T7 primer.

Nucleotide sequences obtained in this study were compared with sequences from the GenBank database using the BLASTn program. To reduce the size of the dataset, only those reference sequences with highest similarity were selected for analysis.

Results

Microbial community in rhizosphere soils

Bacterial community

The results provided by TWINSPAN and DCA from the DGGE dataset of bacterial communities from rhizosphere soils are shown in Fig. 1. A total of 48 samples were classified into four groups by TWINSPAN analysis. However, none of the groups included all the samples from the same sampling time or the same plant hybrid or variety. Separation between *Bt* and non-*Bt* rhizosphere soils was not obviously detected by the DCA, showing that there was no great variation between samples from *Bt* and non-*Bt* hybrids. However, a small amount of variation was seen between plant individuals (replicates), indicating the spatial heterogeneity of the microenvironment. The first two axes of the DCA ordination of DGGE data described 42.2% of the total variation in bacterial community structure.
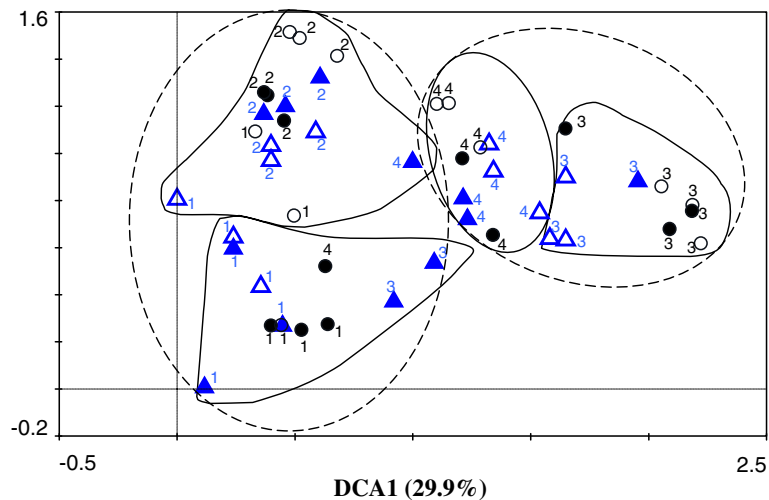
Fig. 1 Detrended correspondence analysis (DCA) of 16S rDNA denaturing gradient gel electrophoresis (DGGE) profiles of bacterial communities of rhizosphere soils. Groups are identified according to two-way indicator species analysis (TWINSPAN) results. The numbers *1*, *2*, *3*, and *4* adjacent to each point represent the sampling times 25 days, 39 days, 53 days, and 82 days, respectively. Sampling with three replicates was implemented. ● Pioneer 34B24(*Bt*), ○ Pioneer 334B23(non-*Bt*), ▲ Nongda 1246*1482 (*Bt*), △ Nongda 3138 (non-*Bt*)

A total of 38 operational taxonomic units (OTUs) were detected, represented by 20 DGGE bands derived from the 16S rDNA gene. Using the program BLAST, sequences with most similarity to reference strains were found in the GenBank database (Table 2). The most represented bands (i.e., those present in all samples over the sampling time) were B2 and B20 (data not shown, see Table 2 for their phylogenic affiliations). No hybrid was detected to have unique DGGE band (i.e., present only in a single hybrid). Some bands were observed in *Bt* or its non-*Bt* isoline samples only at some stages; however, the case was not constant across all of four sampling time. At some growth stages, differences in the presence of B7, B8, B9, B11, B13, B14, and B16, B17, and B19 were observed between Pioneer 34B24 and Pioneer 34B23. In addition, B6, B7, B11, B19, and B21 were differentially present in Nongda 1246*1482 and Nongda 3138 (Table 2).

Fungal community

Using TWINSPAN analysis, 48 samples were identified to four groups (Fig. 2). Similar to the results obtained for bacterial communities from rhizosphere soils, no obvious separation between *Bt* and non-*Bt* rhizosphere soil samples was detected by DCA. The first two axes of the DCA ordination described 37.0% of the total variation in the fungal community.

By means of sequencing the 18S rRNA genes isolated from 24 DGGE bands of rhizosphere soils, a total of 41 OTUs were determined. Band F2 was found in all samples from hybrids Pioneer 34B24 and Pioneer 34B23 across the four growth stages (data not shown, see Table 3 for their phylogenic affiliations). However, no ubiquitous band was shared by all samples of Nongda 1246*1482 and Nongda 3138 across the sampling time. At some stages, differences in the occurrence of bands F3, F5, F9, and F14 were observed between Pioneer 34B24 and Pioneer 34B23, whereas F5, F7, and F18 were found differentially present in Nongda 1246*1482 and Nongda 3138.

Microbial community in soils associated with plant residues

Bacterial community

TWINSPAN analysis of bacterial communities in soils associated with plant residues distinguished two main groups differentiated by sampling time but not by plant hybrid (Fig. 3). At some sampling times, an obvious distinction between *Bt* and non-*Bt* hybrids was detected by DCA. The first two axes of the DCA ordination described 43.5% of the total variation in bacterial community structure.

Table 2 Closest relatives of partial of 16S rDNA sequences derived from denaturing gradient gel electrophoresis (DGGE) bands

DGGE band	Closest relatives Microorganisms (phylogenic affiliations)	Accession number	Similarity (%)[b]
B1	*Lactobacillus*	CP001617, etc.	97
	Uncultured bacterium	GQ074078, etc.	97
B2-a[a]	Uncultured bacterium	EU431756, etc.	99
	Uncultured Flavobacteria bacterium	FJ535183, etc.	99
B2-b	*Sphingobacterium*	AB461769, etc.	98
	Uncultured bacterium	DQ279610, etc.	98
B2-c	Uncultured bacterium	FJ869168, etc.	98
	Uncultured *cyanobacterium*	AY948066, etc.	96
B3-a	Uncultured *Flavobacterium*	EU097206, etc	99
B3-b	*Bacillus*	EU363701, etc.	100
	Uncultured bacterium	GQ113455, etc.	100
B3-c	Uncultured bacterium	EU542365, etc.	100
	Uncultured Bacteroidetes bacterium	EF179855, etc.	99
B4-a	Lactobacillales	FJ429984, etc.	98
B4-b	Uncultured bacterium	DQ798748, etc.	92
	Erysipelothrix	EF494749	
B4-c	Uncultured bacterium	FJ228784, etc.	98
	Uncultured Flavobacteria bacterium	EF061021, etc.	96
B5-a	Uncultured bacterium	FM956796	98
B5-b	Uncultured bacterium	AY920020, etc.	100
B6-a	*Acinetobacter*	FJ897486, etc.	100
	Uncultured bacterium	GQ115526, etc.	100
B6-b	Uncultured bacterium	EU560857, etc.	99
	Enterobacteriaceae	FJ890897, etc.	98
B6-c	Uncultured bacterium clone	EU803802, etc.	92
B7	*Pseudomonas*	GQ303714, etc.	98
	Uncultured bacterium	GQ114844, etc.	98
B8-a	Uncultured Bacteroidetes bacterium	EF179859, etc.	97
B8-b	*Bacillus*	GQ284474, etc.	100
	Geobacillus stearothermophilus	FJ581462	100
B8-c	*Pseudomonas*	GQ284545, etc.	99
	Uncultured bacterium	GQ106274, etc.	99
B9-a	Uncultured bacterium	FJ228812, etc.	99
	Uncultured *Sphingobacterium*	FJ756565, etc.	98
B9-b	*Acinetobacter*	GQ284485, etc.	100
	Uncultured Gammaproteobacteria	CU921160	100
B10-a	Uncultured bacterium	EU083359, etc.	99
	Sphingobacterium	EF122436, etc.	93
B10-b	Uncultured bacterium	AY990703, etc.	100
	Clostridium	NR_026103, etc.	99
B10-c	*Brevundimonas*	FJ950569, etc.	100
	Uncultured bacterium	FJ893991, etc.	100
B11	*Bacillus*	GQ284474, etc.	98
B12-a	*Pseudomonas*	GQ284542, etc.	100
	Uncultured bacterium	AB482903, etc.	100
B12-b	Uncultured bacterium	EU284503, etc.	97

Table 2 (continued)

DGGE band	Closest relatives Microorganisms (phylogenic affiliations)	Accession number	Similarity (%)[b]
B13-a	*Sinorhizobium*	GQ184204, etc.	100
	Chryseobacterium	AB461658, etc.	100
B13-b	Uncultured bacterium	EF019235, etc.	94
	Bdellovibrio	DQ302728, etc.	94
B13-c	*Sphingomonas*	AB201254, etc.	100
	Altererythrobacter	FN397680, etc.	100
	Uncultured bacterium	AM697304, etc.	100
B14	*Bacillus*	GQ280069, etc.	99
	Geobacillus	FJ581462,, etc.	99
B15	Uncultured *Janthinobacterium*	GQ129895	100
	Phenanthrene-degrading bacterium	AY177375	100
	Oxalobacteraceae	AB488284, etc.	99
B16-a	*Bacillus*	GQ199766, etc.	99
B16-b	*Agrobacterium*	FJ607997, etc.	100
	Rhizobium	GQ258493, etc.	100
	Uncultured bacterium	AB485298, etc.	100
B17	Clostridiaceae	AB298755, etc.	100
B18	*Sphingomonas*	FM999997, etc.	100
	Uncultured bacterium	EU786143, etc.	100
B19	Enterobacteriaceae	GQ280118, etc.	100
	Uncultured bacterium	GQ264458, etc.	100
B20	Rhizobiaceae	FJ607997, etc.	99
	Uncultured bacterium	AB485298, etc.	99

[a] Lowercase letters indicates that more than one operational taxonomic unit (OTU) was detected from the same DGGE band

[b] Similarity (%) = no. of identical nucleotides/ total no. of nucleotides

The bands that were present in all hybrids at both sampling times were B10, B14 B15, B16, B17, and B18, B19, and B20 (data not shown, see Table 2 for their phylogenic affiliations). At some sampling times, differences in the presence of some bacteria strains were revealed between Pioneer 34B24 and Pioneer 34B23, or between Nongda 61 and Nongda 3138, respectively (B1, B4, B5, B6, B7, B8, B9, B10, B14, B21, and B22).

Fungal community

The analysis of fungal communities in soils amended with plant residues by TWINSPAN and DCA led to the distinguishing of two major groups representing samples from the same sampling time (Fig. 4). The first two axes of the DCA ordination described 59.0% of the total variation in fungal community structure. Soil samples associated with Nongda 61 at 15 days were notably distinct from the others (Fig. 4).

Bands F3 and F8 were present in all samples from soils amended with corn straw (see Table 3). At some sampling times, differences in the presence of some bands (F2, F3, F7, F8, F11, and F12) were found between Pioneer 34B24 and Pioneer 34B23 (Table 3), whereas bands F5, F6, F7, F12, F19 were differentially present between Nongda 61 and Nongda 3138.

Discussion

Soils are home to a diverse range of life and are complex and dynamic biological systems. Therefore, it is often difficult to determine the composition of microbial communities in soils and their response to perturbations of their ecosystem (Icoz and Stotzky 2008). Recent methodological advances, especially molecular techniques, are helping researchers to understand the soil communities. In this work, we assessed the impact of

Fig. 2 DCA of 18S rDNA DGGE profiles for fungal community of rhizosphere soils. Groups are identified according to TWINSPAN results. The numbers *1, 2, 3,* and *4* adjacent to each point represent the sampling times 25 days, 39 days, 53 days, and 82 days, respectively. Sampling with three replicates was implemented. ● Pioneer 34B24(*Bt*), ○ Pioneer 34B23(non-*Bt*), ▲ Nongda 1246*1482 (*Bt*), △ Nongda 3138 (non-*Bt*)
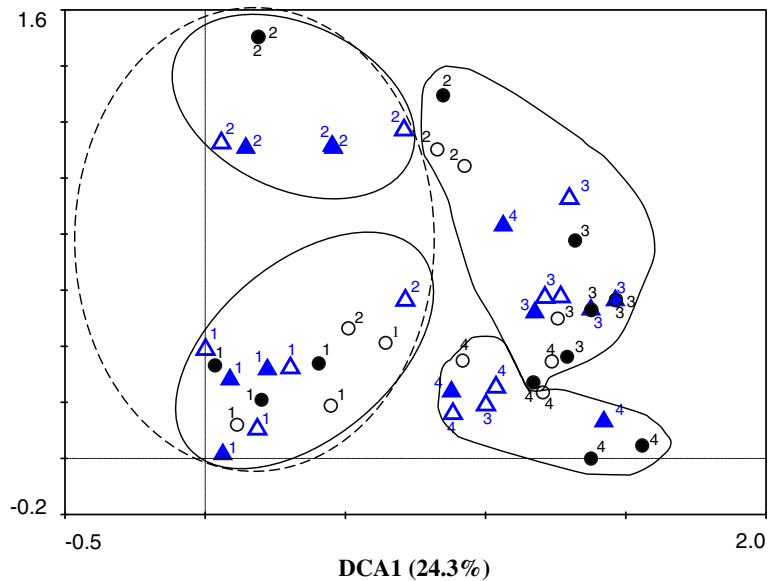
growing *Bt* corn (Pioneer 34B24, and Nongda 1246*1482), and the straw of *Bt* corn (Pioneer 34B24, and Nongda 61) and the processes of decomposition on soil bacterial and fungi communities, using PCR-DGGE and sequencing of 16S and 18S rDNA. Our results proved these molecular techniques useful in revealing the diversity of microbial communities by separating different sequence variants and for identifying the affected microbial groups in the amended soils.

Effects of *Bt* corn on the microcommunity in rhizosphere soils

In this study, neither the bacterial nor the fungal DGGE profiles showed any obvious difference between *Bt* hybrid Pioneer 34B24 and non-*Bt* hybrid Pioneer 34B23, or between Nongda 1246*1482 (*Bt*) and its non-*Bt* isoline Nongda 3138 in the rhizosphere soils throughout the four growth stages (Figs. 1, 2). The results indicated that the root exudate of *Bt* corn did not constantly affect the microecosystem of the rhizosphere soil. Similar results have been reported in studies of soil microbial community structure with Cry proteins and products of other transgenic plants, e.g., *Bt* cotton (*Gossypium hirsutum* L.), *Bt* potato (*Solanum tuberosum* L.), transgenic tobacco (*Nicotiana tabacum* L.) expressing protease inhibitor I, transgenic alfalfa (*Medicago sativa* L.) expressing α-amylase or lignin peroxidase (Donegan et al. 1995, 1996, 1997, 1999). Conversely, constant significant

differences have been detected in other studies of soil microbial community structure between non-transgenic and transgenic crops, such as *Bt* corn (Castaldini et al. 2005), *Bt* cotton (Gupta et al. 2002), *Bt* potato, etc. These conflicting results are probably due to differences in the type of Cry protein, plant variety, experimental methods used, soil type, and environmental factors, etc. (Icoz et al. 2008).

Plants can alter the composition and diversity of soil microbial communities in a selective manner (Nehl et al. 1997). The type of microbial community that results from plant-selective pressure differs with plant species, indicating that plant type and root exudates influence the microorganisms that colonize their rhizosphere (Icoz et al. 2008; Smalla et al. 2001). Indeed *Bt* toxin can be utilized as a carbon and nitrogen resource by soil microbes, even though its binding on clays reduced the degration. Laboratory studies by Stotzky and coworkers have indicated that the intact Cry1Ab protein binds rapidly to soil particles, maintains its insecticidal property, and persists in various soils for at least 234 days, the longest time evaluated (Crecchio and Stotzky 1998, 2001; Tapp and Stotzky 1995, 1998). Some studies reported that soil bacterial communities were more strongly influenced by plant species and different hybrids than by other environmental factors like soil type and agricultural practices (Gomes et al. 2001; Heuer et al. 2002). However, Koskella and Stotzky (2002) confirmed that several Cry toxins from *B.*

Table 3 Closest relatives of partial of 18S rDNA sequences derived from DGGE bands

| DGGE band	Closest relatives Microorganisms (Phylogenic affiliations)	Accession number	Similarity (%)[b]
F1-a[a]	*Rhizopus*	EU710835, etc.	99
	Amylomyces rouxii	AY228093, etc.	99
	Uncultured fungus	EF628992, etc.	99
F1-b	*Eukaryota*	EZ070696, etc.	100
F2	*Rhizophlyctis rosea*	AY635829	99
	Uncultured fungus	EF628917, etc.	99
F3	*Geotrichum*	AB000652, etc.	99
F4	Uncultured fungus	EF628808, etc.	98
	Mortierella sp.	EU710842	98
	Zygomycete sp.	EU428773	98
	Eimeriidae	FJ716244, etc	98
F5-a	*Mortierella indohii*	EU688965	99
	Uncultured fungus	EF628808, etc.	
F5-b	*Eurotium*	EU263606, etc.	100
	Aspergillus glaucus	AY083218, etc.	100
	Edyuillia athecia	AB002082	100
F6-a	*Candida*	DQ471323, etc.	99
	Lodderomyces elongisporus	EF120591	99
	Uncultured fungus	EF628774, etc.	99
F6-b	*Pyxidiophora arvernensis*	FJ176839	97
	Chalara hyalina	AF222509	96
F6-c	*Trinema enchelys*	AJ418792	98
	Cercomonadida	EF024983, etc.	97
	Uncultured fungus	EF024218, etc.	97
F7	*Asterotremella humicola*	FJ153130	93
F8-a	*Powellomyces*	AF164245, etc.	97
	Spizellomycete	DQ536477, etc.	97
	Entophlyctis confervae-glomeratae	EF014367	97
F8-b	Agaricomycetes	AF026569, etc.	98
	Uncultured fungus	EU733570, etc.	98
F9	Uncultured cercozoan	AM114812	96
F10	Uncultured eukaryote	FJ592308, etc	99
F11-a	Uncultured fungus	EF071900, etc	97
	Ustilaginaceae	DQ832229, etc.	97
F11-b	*Rhizopus*	EU710835, etc.	98
	Uncultured fungus	EF628992, etc.	97
	Amylomyces	AF157120, etc.	97
F12-a	*Chaetomium sp.*	EU710831	99
	Uncultured fungus	EF628920, etc.	99
12-b	*Hypocrea koningii*	EU722404	100
	Trichoderma	EF672343, etc.	100
	Ephelis	AB114631, etc.	100
F13	Uncultured fungus	EF628802, etc.	96
F14-a	*Chaetomium sp.*	EU710831	99
	Uncultured fungus	EF628636, etc.	99
F14-c	Uncultured fungus	EF628683	99

Table 3 (continued)

DGGE band	Closest relatives Microorganisms (Phylogenic affiliations)	Accession number	Similarity (%)[b]
	Cercomonadida	EF024163	99
	Olpidium brassicae	EF024163	99
F15-a	*Oryza sativa*	AP009051	98
	Zea mays	EZ070696	98
F15-b	Uncultured fungus	EF628683, etc.	96
	Uncultured rhizosphere chytridiomycete	AJ506018	96
	Cercomonadida	EF024163, etc.	96
	Olpidium brassicae	DQ322624	96
F16-a	Clavicipitaceae	FM178243, etc.	100
F16-b	*Geotrichum*	AB000652, etc.	99
F17-a	*Zea mays*	EZ070696	98
	Oryza sativa	AP009051	98
F17-b	Uncultured eukaryote	EF025015, etc.	97
	Acanthamoeba	EF023782, etc.	97
	Eimeriidae	EF023659, etc.	97
F18-a	*Hypocrea*	EU722404, etc.	100
	Trichoderma	EF672343, etc.	100
	Ephelis	AB113828	100
F18-b	Uncultured fungus	EF629191, etc.	97
F19-a	Hypocreales	EU722404, etc.	98
F19-b	Uncultured eukaryote	DQ865665, etc.	100
F19-c	*Issatchenkia*	DQ438180, etc.	99
	Candida	M55528, etc.	99
	Saccharomyces	DQ345283, etc	99
F20-a	*Zea mays*	EZ070696	99
	Oryza sativa	AP009051	99
F20-b	Uncultured fungus	EF628546	99
	Amylomyces rouxii	AF157120	95
	Rhizopus sp.	EU710835	94
F21-a	*Entophlyctis confervae-glomeratae*	EF014367	98
	Powellomyces	AF164245, etc.	98
	Spizellomycete	DQ536490, etc.	98
F21-b	Onygenales	AY177297, etc.	92
F22-a	*Trinema*	AJ418792	98
F22-b	Entophlyctis	EF014367, etc.	98
	Powellomyces	AF164242, etc.	98
F25	Uncultured fungus clone	DQ157217, etc.	99
	Actinomucor elegans	AF157119	99
	Mucor	AY054699, etc.	99
F26	*Trinema enchelys*	AJ418792	98
	Cercomonadidae	EF024859, etc.	97

[a] Lowercase letters indicates that more than one OTU was detected from the same DGGE band

[b] Similarity (%) = no. of identical nucleotides/ total no. of nucleotides

Fig. 3 DCA of 16S rDNA DGGE profiles for bacterial community of soils associated with corn straw. Groups are identified according to TWINSPAN results. The numbers *1* and *2* adjacent to each point represent sampling times 15 days and 75 days, respectively. Sampling with three replicates was implemented. ● Pioneer 34B24 (*Bt*), ○ Pioneer 34B23(non-*Bt*), ▲ Nongda 61 (*Bt*), △ Nongda 3138 (non-*Bt*)
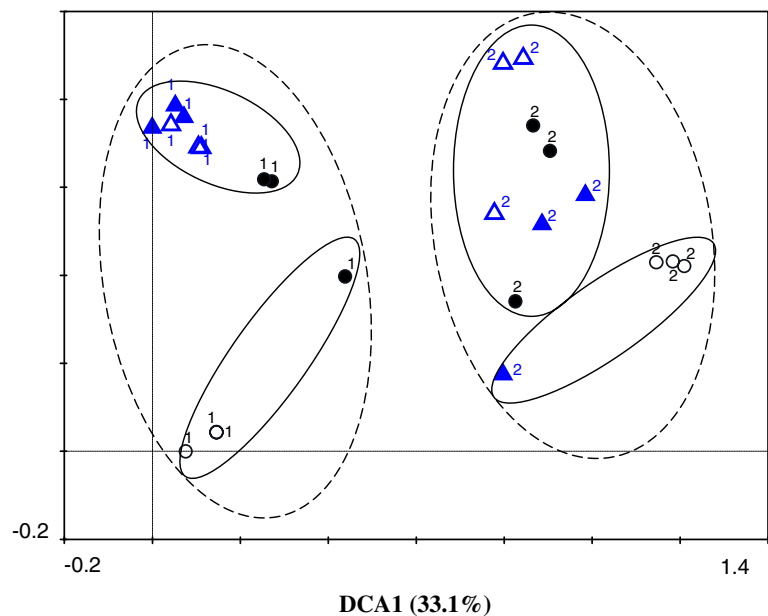

thuringiensis have no microbicidal or microbiostatic activities against selected bacteria. Donegan et al. (1995) found that the Cry1Ab and Cry1Ac proteins, both purified and expressed in transgenic plants, had no direct effect on soil microorganisms and that the effects observed, which were related to the plant varieties used, may have been caused by unexpected changes in plant characteristics that resulted from genetic manipulation or tissue culturing of the engineered plants. Molecular data showed that both different corn lines and actively growing *Bt* corn plants did not reduce rhizospheric bacterial species richness, as assessed by DGGE fragments (Castaldini et al. 2005). In the present study, DCA of both bacterial and fungal communities from rhizosphere soils did not reveal any large variation between *Bt* lines Pioneer 34B23 with the truncated Cry1Ab gene (MON810), and Nongda 1246*1482 with the inserted Cry1A gene (Figs. 1, 2) throughout the different growth stages, indicating that the type of Cry protein, and the genetic manipulation of the engineered plants had no significant effect on soil microbial community structure.

TWINSPAN analysis inferred that plant growth stage may have a more selective effect on the microbial community than other factors, i.e., the presence of Cry protein, the type of Cry protein, and varieties used in this study (Figs. 1, 2). This result was in agreement with previous studies showing that the effects of transgenic plants on microbial commu-

nities in soil are dependent on the age of the plants. Baumgarte and Tebbe (2005) reported that, despite the detection of Cry1Ab protein in the rhizosphere of MON810 corn, the bacterial community structure was less affected by the Cry1Ab protein than by other environmental factors, e.g., the age of the plants or field heterogeneities, which was detected by single-stranded conformational polymorphism (SSCP) gel electrophoresis of 16S rRNA genes. Microbial plate counting and DGGE confirmed that differences between *Bt* and non-*Bt* corn were affected more by season (sampling time) and plant varieties, and that these differences were transient (Icoz et al. 2008). Brusetti et al. (2004) compared the rhizosphere bacterial community associated with *Bt* corn expressing the Cry1Ab protein and near-isogenic non-*Bt* corn using PCR-based automated ribosomal intergenic spacer analysis (ARISA), and found that the community structure differed with the age of the plants, suggesting that root exudates could select different bacterial communities. The relative low variability observed by ARISA in the rhizosphere samples of each plant type at 30 days could be due to the active root exudation of young plants, which could have selected a bacterial community with only a few dominant strains (Brusetti et al. 2004).

Soil type has previously been shown to be the major determinant of soil microbial community structure in agricultural systems (Girvan et al. 2003) or growth experiments (Buyer et al. 1999, 2002). It

Fig. 4 DCA of 18S rDNA
DGGE profiles for fungal
communities of soils asso-
ciated with corn straw.
Groups are identified
according to TWINSPAN
results. The numbers *1* and
2 adjacent to each point
represent the sampling times
15 days and 75 days, re-
spectively. Sampling with
three replicates was imple-
mented. ● Pioneer 34B24
(*Bt*), ○ Pioneer 34B23(non-
Bt), ▲ Nongda 61 (*Bt*), △
Nongda 3138 (non-*Bt*)
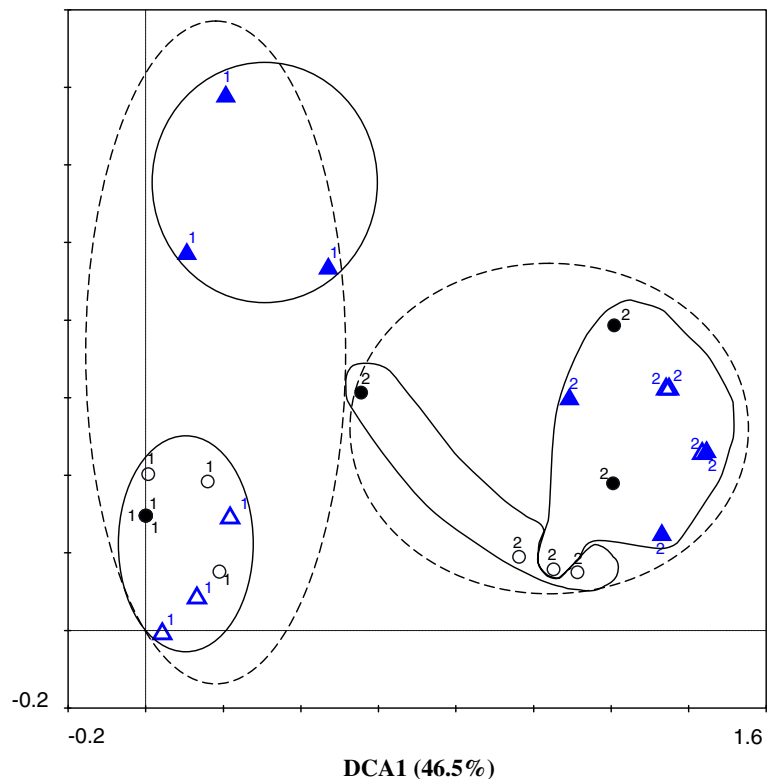
probably affects both the initial community composi-
tion and the conditions for growth during an exper-
iment. Clay has been reported to increase the retention
of Cry protein in soil, allowing for an extended period
of exposure to microbes in the rhizosphere of higher
clay soil (Saxena et al. 2002b; Blackwood and Buyer
2004). In this study, only one soil type, a clay roam,
was implemented in all treatments. Further study
comparing different soil types (e.g., clay loam, sandy
clay loam, and loamy sand) will be necessary to
account for whether the few significant effects from
Bt corns were due to the high clay content.

Effects of plant residues of *Bt* corn on the soil
microcommunity

Plant residues (e.g., leaves, stalks, and roots) in the
corn soil represent the major reservoir of the transgenic
product after harvesting, and are the primary source of
carbon in soil. Therefore, any change to the quality of
crop residues could modify the dynamics of the
composition and activity of organisms in soil (Icoz et
al. 2008). Several authors have proposed that if some
microbial communities have the potential to degrade

Cry1Ab, this extra protein in the environment may
cause their proliferation, leading to a faster decom-
position of *Bt* versus non-*Bt* maize (Blackwood and
Buyer 2004; Baumgarte and Tebbe 2005; Zwahlen et
al. 2007). However, biodegradation of the biomass of
Bt canola, cotton, potato, rice, and tobacco was found
to be significantly lower than that of the biomass of
near-isogenic non-*Bt* plants, but the lignin content of
these plant species, which was considerably lower
than that of corn, was not significantly different
between *Bt* and non-*Bt* biomass (Stotzky 2004).

Our previous study on the degradation of *Bt* protein
in soils (clay loam) amended with plant straw (leaves
plus stalks) of four *Bt* corn hybrids (Pioneer 34B24,
NK58-D1, R×601 RR/ YG and Nongda 61) demon-
strated a rapid decline of *Bt* protein in soils within the
initial 14 days, and thus only a low concentration of *Bt*
protein was detected in the soil during subsequent days
(21, 30, 43, 60 and 134 days; Wang and Feng 2005).
Thus, we selected 15 days and 75 days as sampling
times in this study. TWINSPAN analysis of the DGGE
profiles from residue-amended soil showed two major
groups according to sampling time and not to the plant
hybrids or varieties (Figs. 3, 4), which probably

represented the result of the dynamics of degradation of plant residues (Heuer et al. 2002; Dunfield and Germida 2003; Castaldini et al. 2005).

Obvious distinction of *Bt* and the corresponding non-*Bt* hybrid detected by DCA were revealed at some sampling times (Figs. 3, 4); however, the effect was transient. Castaldini et al. (2005) found no significant differences in total soil bacterial communities affected by plant residues between *Bt* (*Bt*11, *Bt*176) and non-*Bt* lines, as inferred from the UPGMA analysis of DGGE pattern of the 16S rRNA gene. Naef et al. (2006) revealed no direct effect of the Cry1Ab protein in corn residues on the pathogen *Fusarium graminearum*, and on the biocontrol agent *Trichoderma atroviride*, and showed that some *Bt* and their near-isogenic non-*Bt* corn counterparts differed more in the chemical composition of the corn tissue as a result of different environmental conditions, such as drought-stress, which can affect the saprophytic growth of fungi on crop residues, than from the Cry protein content alone.

The effects of crop residue composition and the importance of several indices of residue quality—such as the C to N ratio, lignin content, lignin to N ratio, initial N content, initial soluble C concentrations of the residue—on decomposition and N mineralization have been extensively examined under greenhouse or field conditions (e.g., Fernandes et al. 1997; Saxena and Stotzky 2001b; Motavalli et al. 2004; Blackwood and Buyer 2004). Tarkalson et al. (2008) showed there were differences in total C, total N, biomass fractions, and C:N ratios between initial *Bt* (Pioneer 34N44 *Bt* and NC+ 4990 *Bt*) and non-*Bt* (Pioneer 34N43 and NC+ 4880) corn residues, and between companies (NC+and Pioneer); however, these differences did not result in differences in decomposition rates over time. The order of C:N ratio from the initial plant parts was leaves < stalks < cobs. Nevertheless, plant parts differed in decomposition rate where leaves > stalks > cobs. In our study, significant higher total C, total N, and C:N ratios were found in Nongda 61 (*Bt*) (Table 1), but this difference did not have any durable effect on soil microbial community structure (Figs. 3, 4).

In summary, PCR-DGGE and sequences of 16S rRNA and 18S rRNA genes in this study showed that *Bt* corn plants and their straw had no apparent lasting effect on soil bacterial and fungal communities. The age of the actively growing plants, or the time required for plant straw decomposition may have more effect on the microbial community than other factors, e.g., the presence of Cry protein, plant hybrid and variety. Although PCR-DGGE is a convenient and useful tool to track microbial communities, this method cannot provide precise quantitative information. Further studies using other experimental methods, and also more soil types, are necessary to evaluate the long-term impact of *Bt* corn on soil microbial communities.

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