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A 3-year field investigation of impacts of Monsanto's transgenic Bt-cotton NC 33B on rhizosphere microbial communities in northern China

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ABSTRACT

Monsanto's Bt-cotton NC 33B, planted in northern China for more than one decade, effectively controls cotton bollworms and decreases the use of chemical insecticides. Because of the concern about undesirable ecological side-effects of transgenic Bt-cottons, it is important to assess Bt-cotton NC 33B's effects on soil microorganisms in this zone. Microbial communities in the rhizosphere soil of Bt and non-Bt cottons were monitored under field conditions by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) fingerprints of eubacteria, fungi and actinomycetes at six growth stages after three-year cultivation. Results showed that the population sizes and community structures of eubacteria, fungi and actinomycetes in rhizosphere soil were markedly affected by natural variations in the environment related to cotton growth stages. However, there was no significant difference in eubacterial, fungal and actinomycete population size and community structures in rhizosphere soil between NC 33B and its non-transgenic parent. In general, Bt-cotton NC 33B did not show evident effects on microbial communities in the rhizosphere soil under field conditions after three-year cultivation. This study provides a theoretical basis for environmental impact monitoring of transgenic Bt cottons.

1. Introduction

Modified and/or truncated genes of *Bacillus thuringiensis* encoding insecticidal proteins have been genetically engineered into many crops, such as maize, cotton, rice and poplar, for the purpose of pest insect control (James, 2011). Although Bt crops have a great potential in integrated pest management (IPM) programs, it is concerned that Bt crops may pose a hazard to natural or agricultural ecosystems due to the release and persistence of larvicidal Cry proteins from *B. thuringiensis* in soil (Saxena et al., 1999; Saxena and Stotzky, 2000, 2001; Saxena et al., 2002). Microorganisms play a key role in agriculture because they are important for plant growth and health, turnover of organic materials, and maintenance of ecosystem functions. Plant species have been reported to influence microbial communities in the rhizosphere (Smalla et al., 2001). The soil microbial community is often considered as an early and sensitive indicator reflecting changes in the soil ecosystem (Visser and Parkinson, 1992). The effects of transgenic Bt cottons on the development and population dynamics of such target lepidopteran pests and non-target arthropods have been largely studied (Wu et al., 2008; Lu et al., 2010), however, the potential ecological effects of transgenic Bt cottons on soil microbial communities are still poorly understood.

Ecological impacts of transgenic Bt crops on soil microorganisms may be direct (toxicity of introduced gene products), indirect (effects via unexpected changes in the metabolism of crops) or caused by changes in the management regime used with Bt crops (Birch et al., 2007). Bt-toxins are introduced to soil in root exudates throughout the growth of Bt crops (Saxena et al., 1999) and by incorporation of plant residues after harvest (Zwahlen et al., 2003). Even small accumulation of Bt-toxins could lead to large differences in agricultural soil ecosystem over time. Some studies have shown that the repeated and large-scale use of Bt-crops could lead to the significant accumulation and persistence of Bt-toxins in soils (Saxena and Stotzky, 2000; Stotzky, 2005; Icoz et al., 2008), and significant differences in soil microbial communities (Donegan et al., 1995; Castaldini et al., 2005). By contrast, neither the significant accumulation/persistence of Bt-toxins in soil (Head et al., 2002; Rui 2005), nor the significant changes of soil microbial communities (Saxena et al., 2002; Baumgarte and Tebbe, 2005;

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Hu et al., 2009) were observed in other studies. Thus the impact of transgenic crops on soil microbial ecology is very complex relating with many factors, e.g. crop species, introduced genes, and genetic transformation, etc.

Bt-cotton, the most widely cultivated transgenic crop in the world, was planted on 3.9×10^6 ha^2, representing over 80% of the total cotton growing area in China (James, 2011). The concerns about their impacts on soil microorganisms were raised for a long time. A few preliminary have been reported examining the effect of Bt-cotton on microbial community composition and numbers. Eubacterial species and number in rhizosphere of Bt-cotton significantly changed, but those of fungi and actinomycetes did not (Shen et al., 2004; Tang et al., 2007). The significant differences of *Mucor* spp. or *Penicillium* spp. number in the rhizosphere of Bt-cotton were detected at some plant growth stages (Duan et al., 2008). However the community-level understanding of impacts of transgenic insect-resistant cottons on soil microorganisms was limited.

Monsanto's NC 33B, expressing the Cry1Ac insecticidal protein from *B. thuringiensis* Berliner sp. Kurstaki, was the first introduced transgenic Bt cotton in China, and had been adopted in northern China since it's commercial release in 1997 (Wu et al., 2003). To our knowledge, the understanding of NC 33B's potential effect on soil microorganisms is lack. In this study, we employed PCR-DGGE to monitor the rhizosphere soil microbial communities after three-year cultivation of NC 33B in northern China. The results will make clear the effects of NC 33B on microbial communities in the rhizosphere soil, and help explore the potential environmental risk assessment of Bt cottons on the soil ecosystem.

2. Materials and methods

2.1. Field design

Field trials were conducted on the experimental farm of the Institute of Plant Protection of the Chinese Academy of Agricultural Sciences, located at Cuizhuang town (39° 30′ N and 116° 36′ E,), Langfang, Hebei Province, China, in 2009–2011. This field site is in the North Temperate Zone with a continental monsoon climate. The annual mean temperature is 11.8 °C, and the annual mean rainfall is 570.3 mm. Soil was a clay loam with the following properties (on a dry mass basis): pH 8.4, organic matter 15.6 g kg^{-1}, organic C 9.0 g kg^{-1}, total N 1.0 g kg^{-1}, total P 0.96 g kg^{-1}, total K19.8 mg kg^{-1}, available N 65.9 mg kg^{-1}, available P 10.25 mg kg^{-1}, available K 177.2 mg kg^{-1}. The field was originally planted with the conventional maize, then sown beginning in 2009 with the transgenic insect-resistant cotton Monsanto's NC 33B.

The transgenic cotton cultivar NC 33B (that contains a Cry1Ac protein), its non-transgenic parental cotton cultivar DP 5415, and a non-transgenic conventional cotton cultivar Simian 3 were grown in a randomized block consisting of triplicate plots (each plot 6 m × 10 m) per cultivar. The cotton growing season extended from May to November annually. Cotton was maintained in accordance with normal agronomic practices in northern China. Fertilizer was applied at the seedling stage and at the budding stage. Only the chemical pesticide acetamiprid was used for aphids control at the seedling stage and weeding was done by manpower. In addition, the field lay fallow from November to the following April.

2.2. Sampling and sample processing

Sampling occurred at six developmental stages in 2011, namely seedling, budding, full flowering, bolling, boll opening and senescence, as described by Munger et al. (1998). Rhizosphere soil was defined as the soil still attached to roots after shaking twice by hand (Brusetti et al., 2005). Five plants per plot were carefully removed from the soil. After the plants were shaken, the roots with adhering soil were combined, cut into pieces, and treated as a composite sample. Three composite samples of each cultivar were processed further. For recovery of the rhizosphere soil, 10 g of root material was transferred into a sterile 150 ml Erlenmeyer flask containing 30 ml Milli-Q water and shaken for 30 min at 220 rpm on a shaker. This homogenization step was repeated three times, and the combined suspensions were collected in two 50-ml tubes. The first tube was centrifuged for 15 min at 4 °C and 10,000 g, the supernatant was discarded, and the tube was filled with the contents of the second tube prior to another centrifugation. The resulting pellets containing the root-associated microorganisms were divided into two parts. One part was stored at 4 °C for no longer than one month before plate counts analysis. The other part was frozen at −80 °C until DNA was extracted.

2.3. Quantification of eubacteria, fungi, and actinomycetes

Eubacteria, fungi and actinomycetes were enumerated using a 10-fold dilution plate technique. Half gram of pellets from each sample were suspended in 50 ml sterile water, shaken for 30 min at 220 rpm, and 10-fold serially diluted. The colony forming units (CFU) of eubacteria, fungi, and actinomycetes in each sample were determined by spreading 100 μl of the diluted sample on appropriate culture media in Petri plates, with five replicate plates per dilution. The following media were used to assay for different microorganism types (Shen et al., 2004): eubacteria (NB agar: 10.0 g peptone, 3.0 g beef extract, 5.0 g NaCl, 15 g agar, 1000 ml distilled water, pH 7.0–7.2); fungi (Martin's Rose Bengal streptomycin agar: 10.0 g glucose, 5.0 g peptone, 1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 33 mg Rose Bengal, 1000 ml distilled water, and 3.0 ml 1% streptomycin added immediately before use); actinomycetes (Gauze No. 1 agar: 20.0 g soluble starch, 1.0 g KNO_3, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g NaCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 1000 ml distilled water, and 3.3 ml 3% potassium dichromate added immediately before use). Plates were incubated at 30 °C for 3 d for the assay of eubacteria, 25 °C for 5 d for fungi, and 30 °C for 7 d for actinomycetes. Colonies were counted visually and expressed as CFU g^{-1} dry soil. All the results are expressed on the basis of oven-dried soil.

2.4. DNA extraction

For extraction of DNA, 0.5 g of pellets obtained from 10 g root material was used. Genomic DNA of soil samples were extracted with an E.Z.N.A^{TM} soil DNA kit (Omega Bio-Tek Georgia, USA) according to the instructions of manufacturer. The quality of genomic DNA was checked by use of a 0.8% (wt/vol) agarose gels photographed under UV light after ethidium bromide staining. For PCR amplification reaction, the genomic DNA was diluted with TE buffer to 5 ng μl^{-1}.

2.5. Primers and PCR amplifications

The primer pairs F341-gc/R534 or F243/R513-gc, specific for the 16S rRNA fragment, were used for amplifying eubacteria or actinomycetes in rhizosphere soil samples respectively (Muyzer et al., 1993; Heuer et al., 1997). The primer pairs ITS1F/ITS4 and ITS1F-gc/ITS2 were performed for the amplification of fungal internal transcribed spacer (ITS) fragment (Anderson et al., 2003). Primers were synthesized by Sangon (Shanghai, China) and purified by high-performance liquid chromatography. Lyophilized primers were dissolved in water to a concentration of 10 μM and stored at −20 °C.