

## Dynamics of microbial population size in rhizosphere soil of Monsanto's *Cry1Ac* cotton

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### ABSTRACT

Monsanto's *Bt*-cotton NC 33B, planted in northern China for more than one decade, effectively controls cotton bollworms; however the understanding of its potential effects on soil microorganisms is limited. The dynamics of eubacterial, fungal and actinomycetes population sizes in rhizosphere soil of the *Bt* cotton were analysed by real-time PCR (qPCR) at the different growth stages under field conditions during 2009 to 2011. Results showed that the population sizes (microbial rDNA gene copies) of eubacteria, fungi and actinomycetes in rhizosphere soil were markedly affected by natural variations in the environment related to the year, cotton growth and cultivar. However, there was no significant difference in eubacterial, fungal and actinomycetes population size in rhizosphere soil between the *Bt*-cotton NC 33B and its near-isogenic comparator DP 5415. In general, the *Bt*-cotton NC 33B did not show evident effects on the population sizes of eubacteria, fungi and actinomycetes in rhizosphere soil under field conditions after three-year cultivation.

**Keywords:** *Bt* protein; soil ecosystem; microbial community; *Gossypium hirsutum* L.

Microorganisms are an important component of soil, tightly related to soil fertility and function, and often considered as sensitive indicators reflecting changes of the soil ecosystem (Visser and Parkinson 1992). Monitoring the changes of microbial community in soil will increase our understanding of the potential risks of introduction of exogenous genes. Ecological impacts of transgenic *Bt* crops on soil microorganisms have been studied since 1995 (Donegan et al. 1995). Several studies have shown that the repeated and large-scale use of transgenic *Bt*-crops could lead to significant accumulation or persistence of *Bt*-toxins (Saxena

and Stotzky 2000, Stotzky 2004, Icoz et al. 2008), change the microbial population size (Donegan et al. 1995, Pindi and Sultana 2013) and moreover alter the microbial community in soil (Castaldini et al. 2005, Singh et al. 2013). By contrast, other studies have indicated that *Bt*-crops cause no or only minor changes in the microbial population size and community structure (Donegan et al. 1996, Shen et al. 2006, Wu et al. 2009, Na et al. 2011, Barriuso et al. 2012, Wei et al. 2012).

*Bt*-cotton, most widely cultivated transgenic crop in the world, was planted on  $3.9 \times 10^6$  ha, representing over 80% of the total cotton grow-

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ing area in China (James 2014). The concerns about its impacts on soil microorganisms had been raised for a long time. Monsanto's NC 33B, expressing the *CryIAc* 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 its commercial release in 1997 (Wu et al. 2003). No significant changes in the eubacterial, fungal and actinomycetes population size (expressed as the CFUs by the selective cultivation method) was found in rhizosphere soil of NC 33B (Zhang et al. 2015). However, the results based on the cultivation presented should be considered only preliminary because a culture-based technique detects only a small portion of the microbial community. In this study, we employed real-time PCR (qPCR) to investigate the influence of the transgenic *Bt*-cotton NC 33B on the population size of three kinds of soil microorganisms (eubacteria, fungi and actinomycetes) in rhizosphere soil under field conditions during three consecutive years.

## MATERIAL AND METHODS

**Field design.** Field trials were conducted on the experimental farm of the Chinese Academy of Agricultural Sciences, located at Cuizhuang town (39°30'N, 116°36'E), Langfang, Hebei province, China, in 2009–2011. The field sites in the north temperate zone with continental monsoon climate, annual mean temperature of 11.8°C and annual mean rainfall of 570.3 mm. The soil is a clay loam type soil with the following properties (on a dry mass basis): pH (soil:water ratio 1:2.5) 8.4, organic matter 15.6 g/kg, organic C 9.0 g/kg, total N 1.0 g/kg, total P 0.96 g/kg, total K 19.8 mg/kg, available N 65.9 mg/kg, available P 10.25 mg/kg, available K 177.2 mg/kg. The field was originally planted with the conventional maize, then sown beginning in 2009 with the tested cotton cultivars. The Monsanto's transgenic cotton NC 33B (that contains a *CryIAc* protein), its near-isogenic comparator DP 5415, and a non-transgenic conventional cotton Simian 3 were grown in a randomized block design consisting of four plots (each plot 6 m × 10 m) per cultivar. Seeds were sown on April 29, 2009, May 7, 2010 and May 11, 2011, respectively. The growth of cotton extended from May to November annually. Cotton was maintained

in accordance with normal agronomic practices in northern China. Animal waste was used as the base fertilizer at one ton per acre, then urea was applied twice at the seedling stage (375 kg/ha) and at the budding stage (750 kg/ha). Chemical pesticide acetamiprid was used for aphid control at the seedling stage and hand-weeding was done as needed by manpower. In addition, the field lay fallow from November to the following April.

**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). Five plants were removed from the soil in a plot. After lightly shaking, the roots with adhering soil were cut into pieces, and combined as a composite sample. Three composite samples from each cultivar were processed further. For recovery of the rhizosphere soil, 10 g of roots were 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. The resulting pellets of soil samples were frozen at –80°C until the further analysis.

**DNA extraction and real-time quantitative PCR.** Soil genomic DNA was extracted with an E.Z.N.A.<sup>TM</sup> soil DNA kit (Omega Bio-Tek, Georgia, USA) according to the instruction of manufacturer. The genomic DNA was diluted with TE buffer to about 5 ng/μL for PCR reaction. Real-time quantitative PCR (qPCR) amplification was performed to quantify the abundance of internal transcribed spacer (ITS) rDNA gene copy number using the primer pairs of Eub338/Eub518, 5.8s/ITS1f (Fierer et al. 2005) and 517F and Act704R (Xiao et al. 2010) for the eubacteria, fungi and actinomycetes, respectively. The amplifications were carried out in a final volume of 25 μL containing 2 × SYBR Green PCR master mix (Omega Bio-Tek, Georgia, USA) using an ABI 7500 PCR (Applied Biosystems, Foster City, USA). The reaction mixture comprised of 12.5 μL master mix, 10 pmol each of primer and 50 ng DNA template. The PCR conditions were initial denaturation at 94°C for 15 min, followed by 40 cycles of 94°C for 1 min, annealing at 53°C (eubacteria and fungi) or 55°C (actinomycetes) for 30 s and extension at 72°C for 30 s. The amplified products of the ITS rDNA gene were purified using the PCR purification kit (Omega Bio-Tek, Georgia, USA), ligated into pEASY by A-T clon-

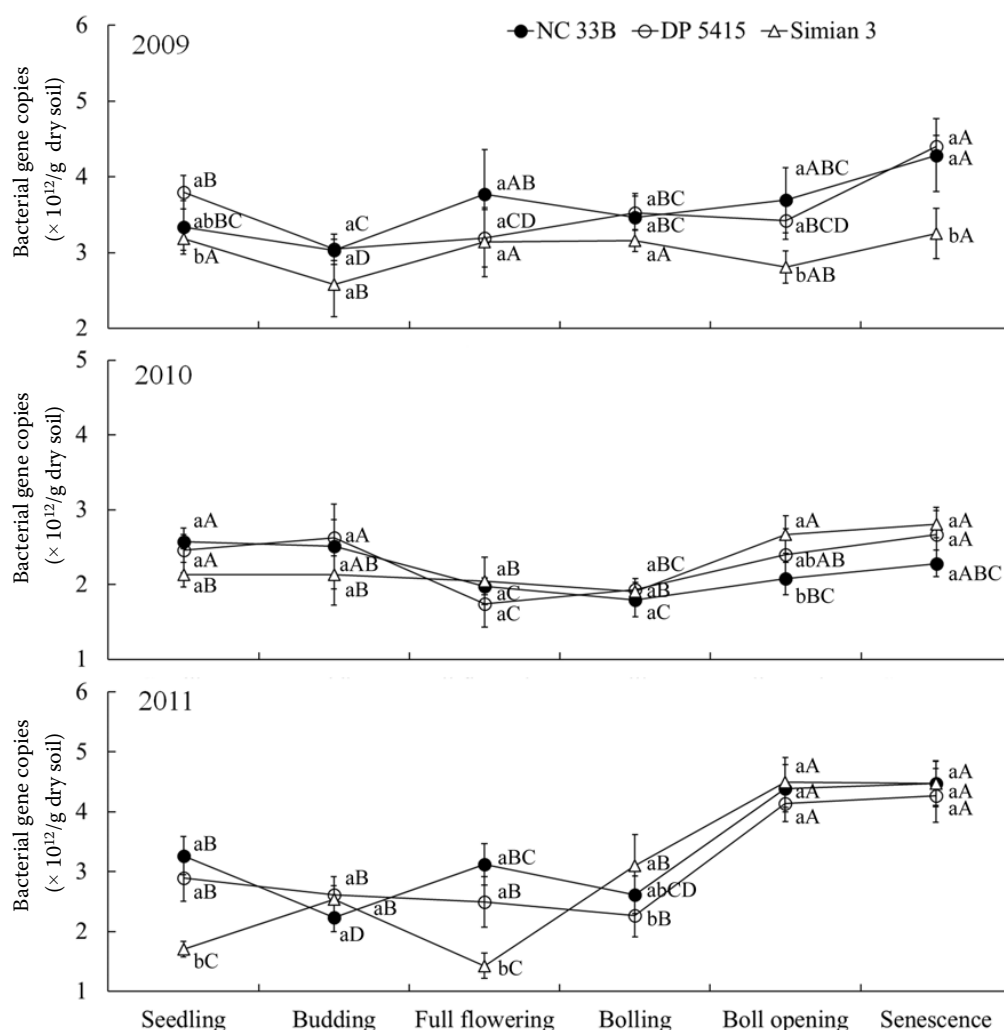


Figure 1. The eubacterial population size in rhizosphere soil of cotton. Error bars indicate standard errors ( $n = 4$ ). Different lowercase or uppercase letters above bars indicated statistically significant differences between cultivars (at the same growth stage each year) or growth stages (for the same cultivar each year), respectively (*HSD*,  $P < 0.05$ )

ing (TranGen Biotech, Beijing, China) and cloned into *Escherichia coli* Top 10 (TranGen Biotech, Beijing, China). The standards were prepared from recombinant *E. coli* plasmid containing the targeted gene. Ten-fold serial dilutions ( $10^{-1}$  to  $10^{-7}$ ) were used to construct the standard curve. The standard curves gave the linear relationships for eubacteria ( $y = -3.22x + 38$ ,  $R^2 = 0.998$ ), fungi ( $y = -3.18x + 44$ ,  $R^2 = 0.99$ ) and actinomycetes ( $y = -3.15x + 42$ ,  $R^2 = 0.994$ ) over the range of DNA concentrations examined. The detection limits of these assays were determined to be about 10 pg of genomic DNA per reaction.

**Data analysis.** Multivariate analysis of variance (MANOVA) with a linear mixed effect model was used to compare the population size and Shannon

index data. The year, growth stage, cultivar and *Bt*-trait were used as fixed factors and the block was set as the random factor. Differences among treatments were compared by a post hoc Tukey's honestly significant difference (*HSD*) test at the 5% significance level with SPSS 19.0 (SPSS Inc., Chicago, USA).

## RESULTS AND DISCUSSION

Real-time quantitative PCR-based results showed significant differences in the population size of eubacteria, fungi and actinomycetes in rhizosphere soil among growth stages each year or among cotton cultivars each growth stage (Figures 1, 2 and 3). MANOVA analysis also showed that the population

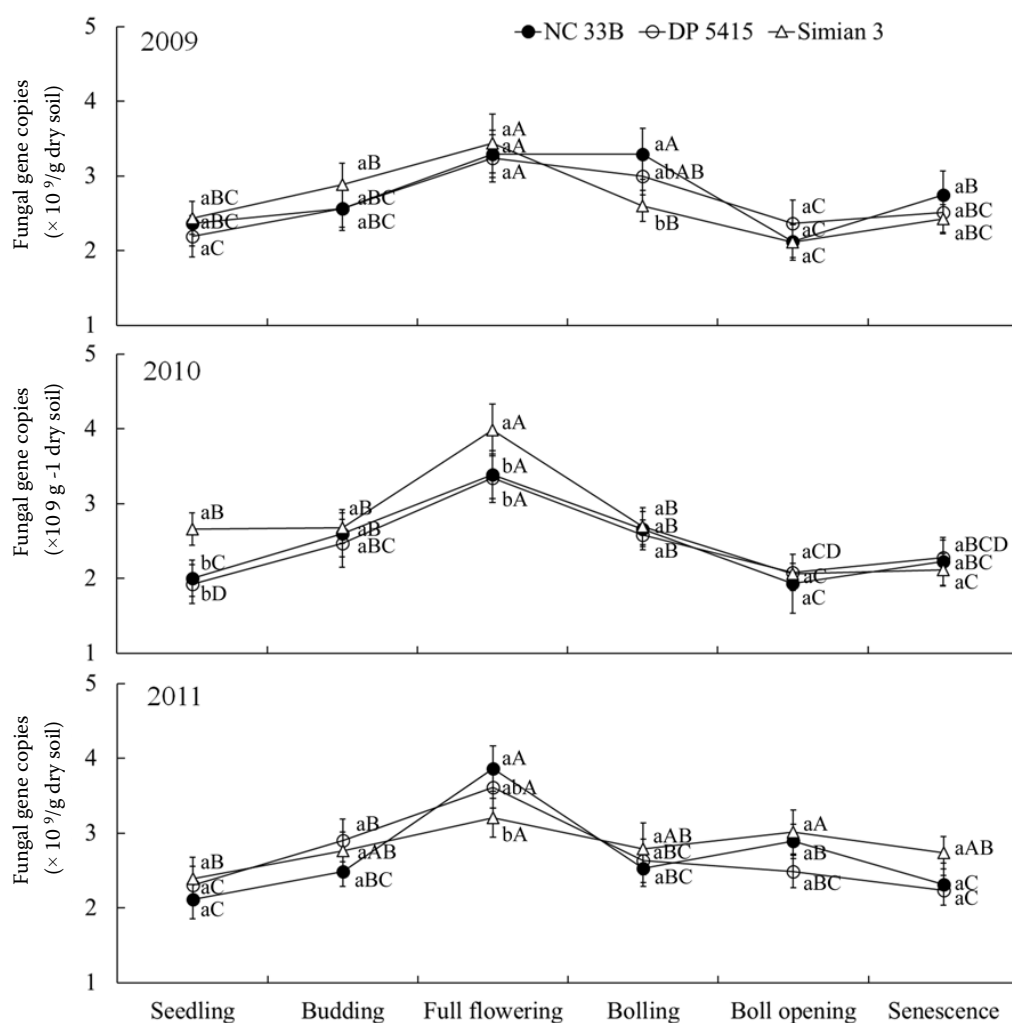


Figure 2. The fungal population size in rhizosphere soil of cotton. For explanations, see Figure 1

size of these microorganisms in rhizosphere soil was significantly affected by the year, growth stage and cultivar (Table 1). However there were no significant differences in the population size of these microorganisms in rhizosphere soil between the transgenic *Bt*-cotton NC 33B and its near-isogenic comparator DP 5415 (Table 1), with the exception of actinomycetes at budding in 2011 (Figure 3).

Most studies proved that transgenic *Bt* crops had no significant effects on microbial population sizes in soil, or only had the transient effects (Donegan et al. 1995, Saxena and Stotzky 2002, Li et al. 2011). Our results were in accordance not only with the above studies that the transgenic trait had no significant or minor effects on microbial population size in rhizosphere soil, but also a recent report that the transgenic cotton NC 33B has no apparent impact on the population size of microorganisms in soil

(expressed as the CFUs by the selective cultivation method) (Zhang et al. 2015). By contrast, another study reported that the *Bt*-trait had significant effects on the population size of actinomycetes (16S rDNA gene copies detected by qPCR) in rhizosphere soil of eggplant (Singh et al. 2013). Different crops or environmental factors may be the reason for the above different results.

Although the experimental methods or technologies were discriminated, most of researches had revealed no or minor effect of *Bt*-traits on microbial communities in soil (Zhang et al. 2013). In our previous study, no significant differences in eubacterial, fungal and actinomycetes community structure (reflected by DGGE profiles) were detected in the presence of the transgenic *Bt*-cotton NC 33B. Another *Bt*-crops (eg. cotton, maize and rice, etc.) also had no significant effect on the

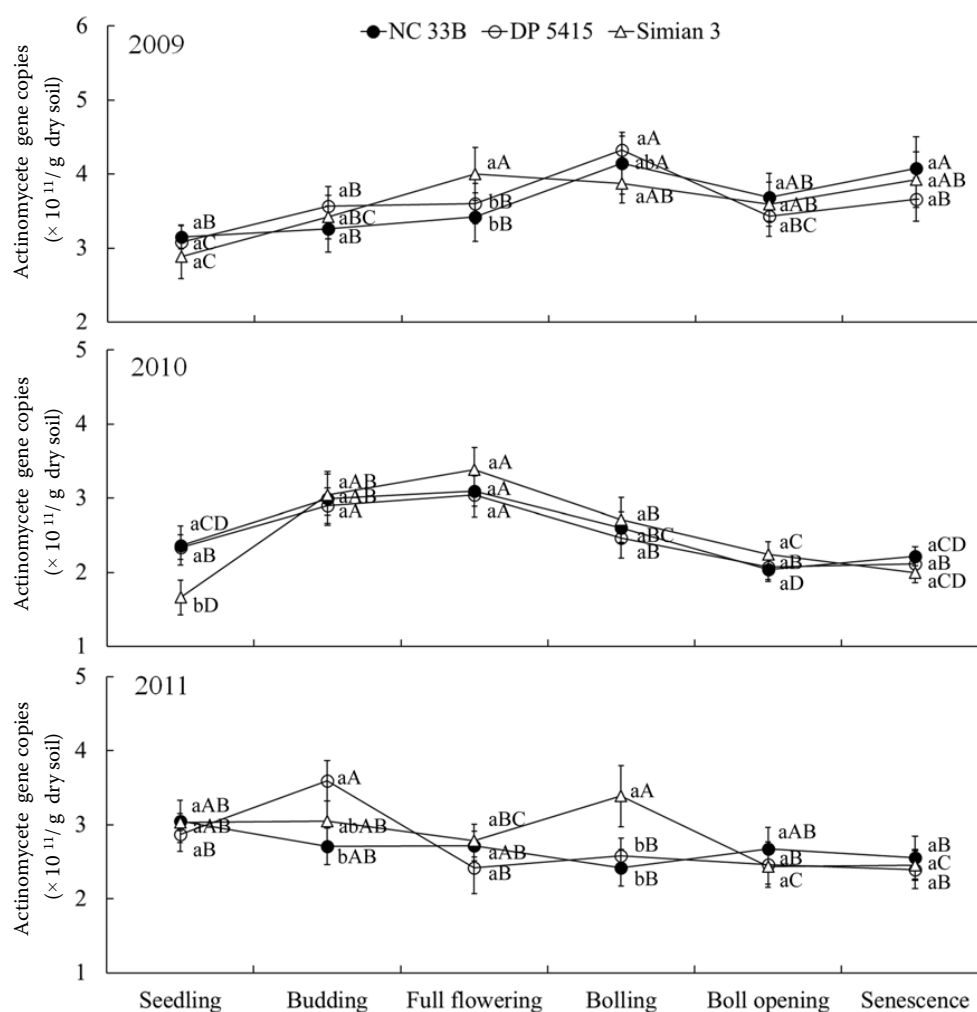


Figure 3. The actinomycetes population size in rhizosphere soil of cotton. For explanations see Figure 1

richness and diversity of microbial communities in soil compared to near-isogenic cultivar using the Biolog system (Shen et al. 2006), DGGE (Na et al. 2011), next generation sequence (Barriuso et al. 2012) and RFLP (Singh et al. 2014), but the significant changes in the microbial community were detected in soil during the growing season (Hannula et al. 2012).

In conclusion, the differences of eubacterial, fungal and actinomycetes population size in rhizosphere soil between the transgenic cotton NC 33B and its near-isogenic comparator were either transient or absent. The knowledge obtained from the present study will help in understanding the natural changes of the microbial population size in rhizosphere soil of *Bt*-cotton.

Table 1. Multivariate analysis of variance (MANOVA) on the differences of microbial population sizes in rhizosphere soil of cotton

Parameter	Year ( <i>df</i> 2)		Growth stage ( <i>df</i> 5)		Cultivar ( <i>df</i> 2)		<i>Bt</i> -comparator ( <i>df</i> 1)	
	<i>F</i> -value	<i>P</i> -value	<i>F</i> -value	<i>P</i> -value	<i>F</i> -value	<i>P</i> -value	<i>F</i> -value	<i>P</i> -value
Bacteria	36.85	0.0001	7.50	0.0001	4.76	0.0077	1.24	0.2912
Fungi	9.34	0.001	5.65	0.0061	2.42	0.0281	0.64	0.3558
Actinomycetes	168.99	0.0001	8.59	0.0001	4.35	0.003	2.09	0.1512



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