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

Y.-J. Zhang, M. Xie, D.-L. Peng, J.-J. Zhao, Z.-R. Zhang

State Key Laboratory for Biology of Plant Diseases and Insect Pests/Key Laboratory for Biological Control of Ministry of Agriculture, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, P.R. China

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 3.9×10^6 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 *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 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 *Cry1Ac* 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™ 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-

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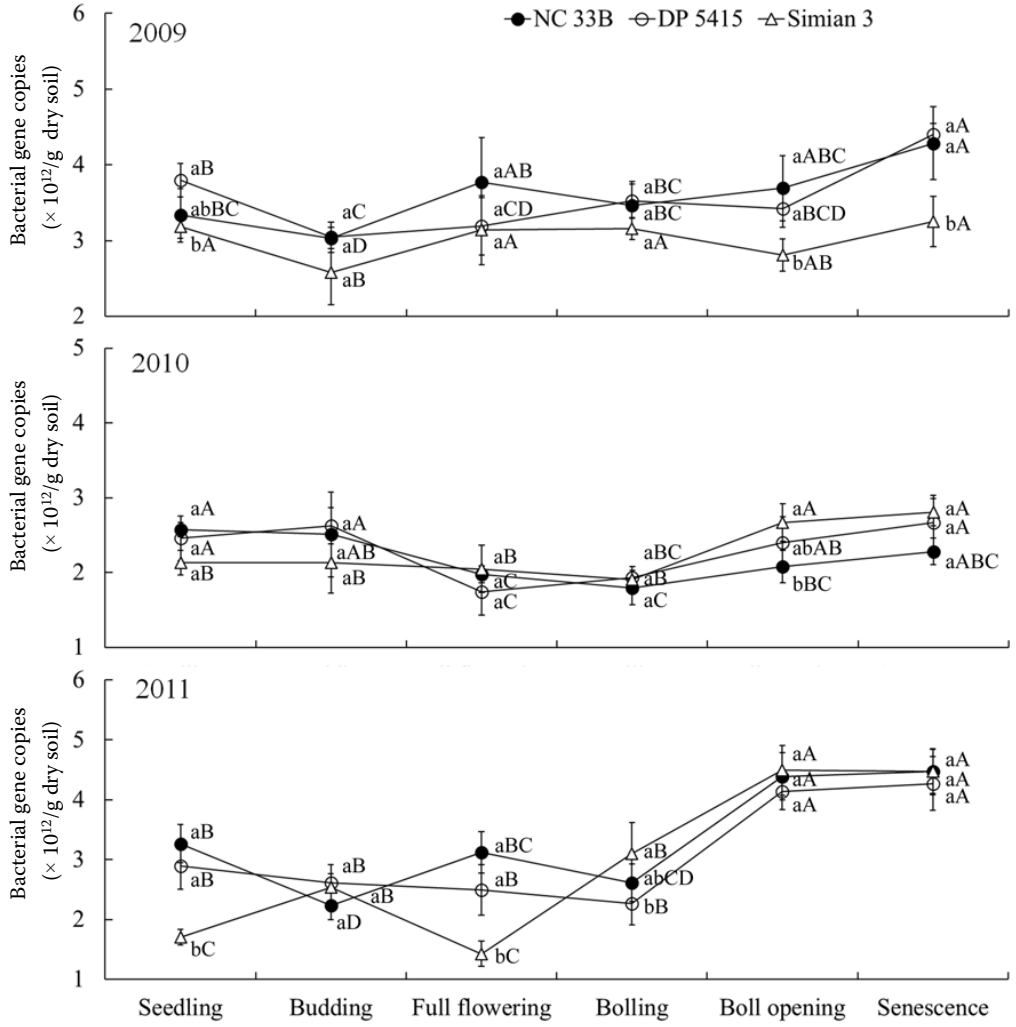

Figure 1. The eubacterial population size in rhizosphere soil of cotton. Error bars indicate standard errors (n = 4). Different lowercase or uppercase letters above bars indicated statistically significant differences between cultivars (at the same growth stage each year) or growth stages (for the same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

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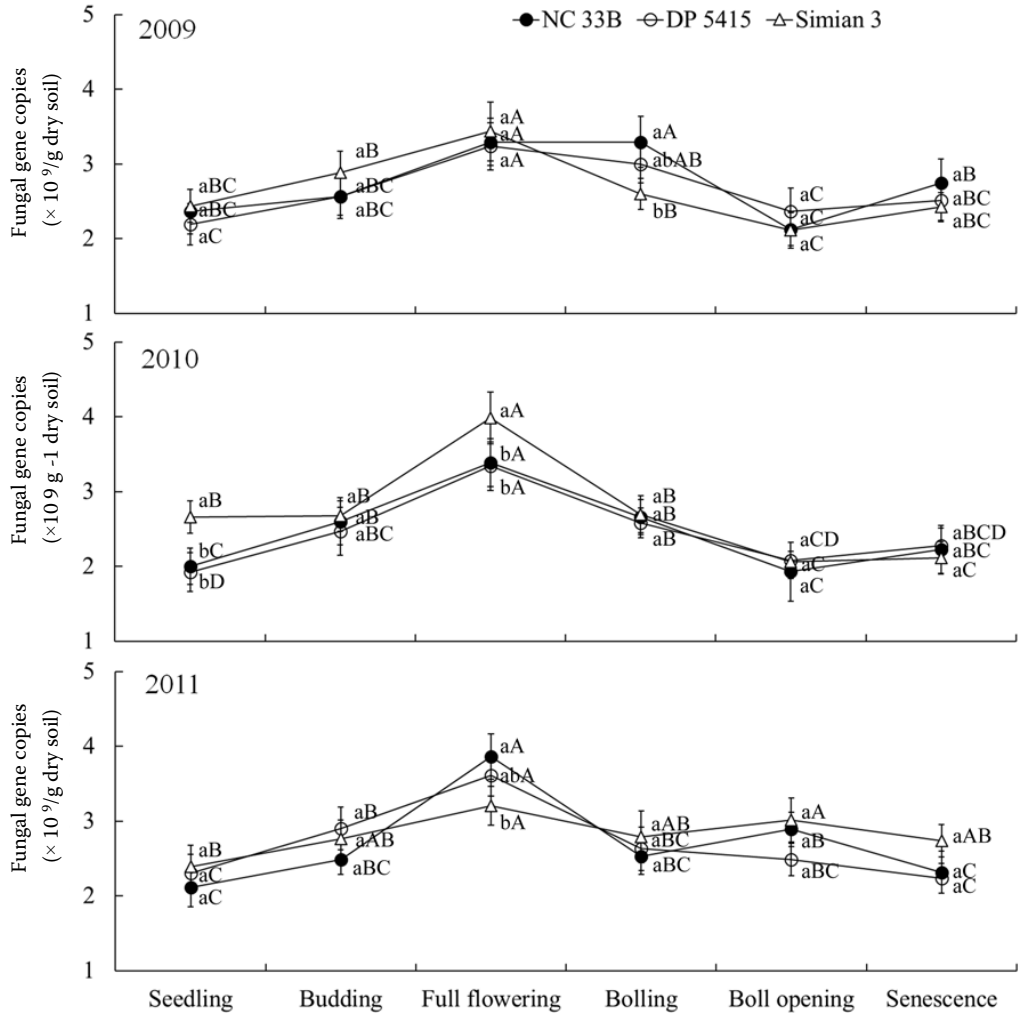

Figure 2. The fungal population size in rhizosphere soil of cotton. For explanations, see Figure 1

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

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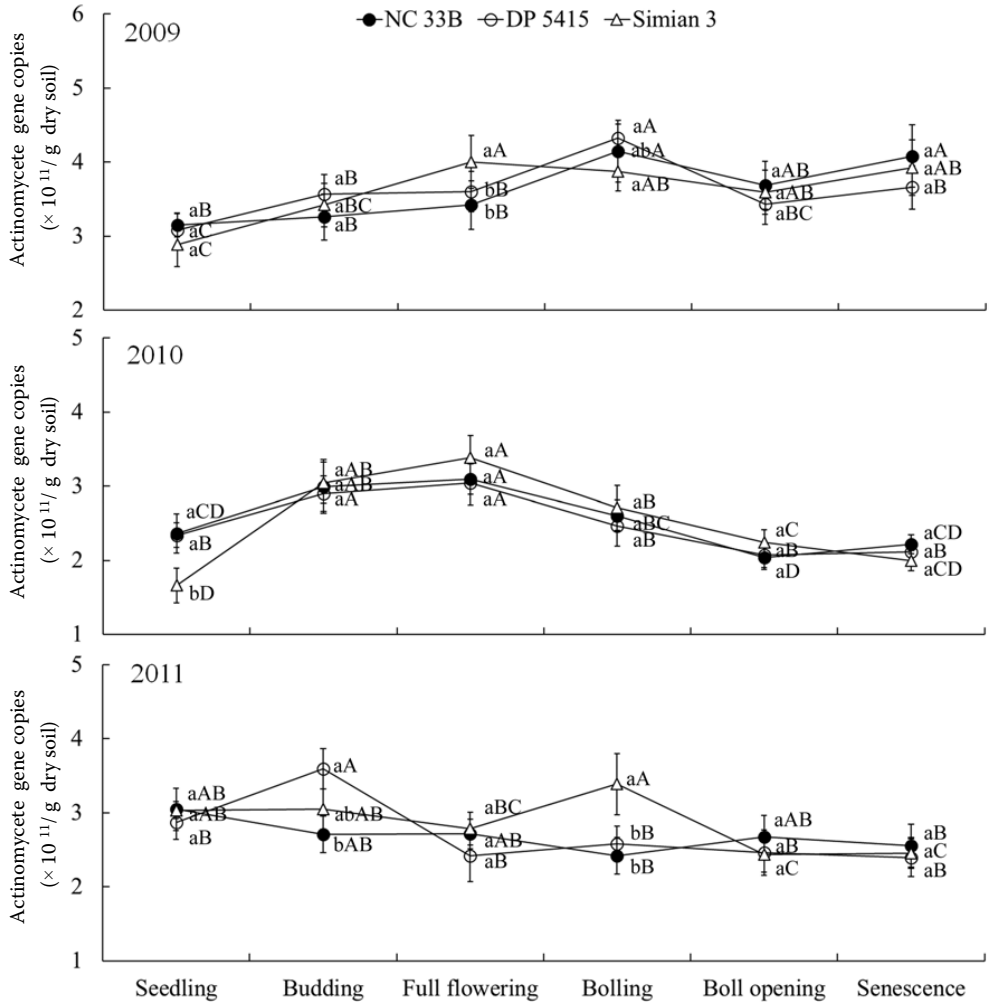

Figure 3. The actinomycetes population size in rhizosphere soil of cotton. For explanations see Figure 1

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 (*df* 2)		Growth stage (*df* 5)		Cultivar (*df* 2)		*Bt*-comparator (*df* 1)	
	F-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-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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REFERENCES

Barriuso J., Valverde J.R., Mellado R.P. (2012): Effect of *Cry1Ab* protein on rhizobacterial communities of *Bt*-maize over a four-year cultivation period. PloSONE, 7: e35481.

Castaldini M., Turrini A., Sbrana C., Benedetti A., Marchionni M., Mocali S., Fabiani A., Landi S., Santomassimo F., Pietrangeli B., Nuti M.P., Miclaus N., Giovannetti M. (2005): Impact of *Bt* corn on rhizospheric and soil eubacterial communities and on beneficial mycorrhizal symbiosis in experimental microcosms. Applied and Environmental Microbiology, 71: 6719–6729.

Donegan K.K., Palm C.J., Fieland V.J., Porteous L.A., Ganio L.M., Schaller D.L., Bucao L.Q., Seidler R.J. (1995): Changes in levels, species and DNA fingerprints of soil microorganisms associated with cotton expressing the *Bacillus thuringiensis* var. *kurstaki* endotoxin. Applied Soil Ecology, 2: 111–124.

Donegan K.K., Shaller D.L., Stone J.K., Ganio L.M., Reed G., Hamm P.B., Seidler R.J. (1996): Microbial populations, fungal species diversity and plant pathogen levels in field plots of potato plants expressing the *Bacillus thuringiensis* var. *tenebrionis* endotoxin. Transgenic Research, 5: 25–35.

Fierer N., Jackson J.A., Vilgalys R., Jackson R.B. (2005): Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. Applied and Environmental Microbiology, 71: 4117–4120.

Hannula S.E., de Boer W., van Veen J. (2012): A 3-year study reveals that plant growth stage, season and field site affect soil fungal communities while cultivar and GM-trait have minor effects. PloSONE, 7: e33819.

Icoz I., Saxena D., Andow D.A., Zwahlen C., Stotzky G. (2008): Microbial populations and enzyme activities in soil *in situ* under transgenic corn expressing Cry proteins from *Bacillus thuringiensis*. Journal of Environmental Quality, 37: 647–662.

James C. (2014): Global Status of Commercialized Biotech/GM crops: 2014. ISAAA Brief No. 49. Ithaca, International Service for the Acquisition of Agri-biotech Applications.

Li X.G., Liu B., Cui J.J., Liu D.D., Ding S., Gilna B., Luo J.Y., Fang Z.X., Cao W., Han Z.M. (2011): No evidence of persistent effects of continuously planted transgenic insect-resistant cotton on soil microorganisms. Plant and Soil, 339: 247–257.

Munger P., Bleiholder H., Hack H., Hess M., Stauss R., van den Boom T., Weber E. (1998): Phenological growth stages of the cotton plant (*Gossypium hirsutum* L.): Codification and description according to the BBCH scale. Journal of Agronomy and Crop Science, 180: 143–149.

Na R.S., Yu H., Yang D.L., Zhao J.N., Li G., Na B.Q., Liu L. (2011): Effect of plantation of transgenic Bt cotton on the amount of rhizospheric soil microorganism and bacterial diversity in the cotton region of Yellow River basin. Chinese Journal of Applied Ecology, 22: 114–120.

Pindi P.K., Sultana T. (2013): Bacterial and fungal diversity in rhizosphere soils of *Bt* and non-*Bt* cotton in natural systems. Bulgarian Journal of Agricultural Science, 19: 1306–1310.

Saxena D., Flores S., Stotzky G. (2002): *Bt* toxin is released in root exudates from 12 transgenic corn hybrids representing three transformation events. Soil Biology and Biochemistry, 34: 133–137.

Saxena D., Stotzky G. (2000): Insecticidal toxin from *Bacillus thuringiensis* is released from roots of transgenic *Bt* corn *in vitro* and *in situ*. FEMS Microbiology Ecology, 33: 35–39.

Shen R.F., Cai H., Gong W.H. (2006): Transgenic *Bt* cotton has no apparent effect on enzymatic activities or functional diversity of microbial communities in rhizosphere soil. Plant and Soil, 285: 149–159.

Singh A.K., Singh M., Dubey S.K. (2013): Changes in actinomycetes community structure under the influence of *Bt* transgenic brinjal crop in a tropical agroecosystem. BMC Microbiology, 13: 122.

Singh A.K., Singh M., Dubey S.K. (2014): Rhizospheric fungal community structure of a *Bt* brinjal and a near isogenic variety. Journal of Applied Microbiology, 117: 750–765.

Stotzky G. (2004): Persistence and biological activity in soil of the insecticidal proteins from *Bacillus thuringiensis*, especially from transgenic plants. Plant and Soil, 266: 77–89.

Visser S., Parkinson D. (1992): Soil biological criteria as indicators of soil quality: Soil microorganisms. American Journal of Alternative Agriculture, 7: 33–37.

Wei M., Tan F., Zhu H., Cheng K., Wu X., Wang J., Zhao K., Tang X. (2012): Impact of *Bt*-transgenic rice (SHK601) on soil ecosystems in the rhizosphere during crop development. Plant, Soil and Environment, 58: 217–223.

Wu K.M., Guo Y.Y., Lv N., Greenplate J.T., Deaton R. (2003): Efficacy of transgenic cotton containing a *cry1Ac* gene from *Bacillus thuringiensis* against *Helicoverpa armigera* (Lepidoptera: Noctuidae) in northern China. Journal of Economic Entomology, 96: 1322–1328.

Wu W.X., Liu W., Lu H.H., Chen Y.X., Medha D., Janice T. (2009): Use of ^{13}C labeling to assess carbon partitioning in transgenic and nontransgenic (parental) rice and their rhizosphere soil microbial communities. FEMS Microbiology Ecology, 67: 93–102.

Xiao Y., Zheng G.M., Yang Z.H., Ma Y.H., Huang C., Xu Z.Y., Huang J., Fan C.Z. (2010): Changes in the actinomycetal communities during continuous thermophilic composting as revealed by denaturing gradient gel electrophoresis and quantitative PCR. Bioresource Technology, 102: 1383–1388.

Zhang Y.J., Xie M., Peng D.L. (2013): Effects of transgenic crops on soil microorganisms: A review. Chinese Journal of Applied Ecology, 24: 2685–2690.

Zhang Y.J., Xie M., Wu G., Peng D.L., Yu W.B. (2015): A 3-year field investigation of impacts of Monsanto's transgenic *Bt*-cotton NC 33B on rhizosphere microbial communities in northern China. Applied Soil Ecology, 89: 18–24.

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Corresponding author:
Dr. Ming Xie, Chinese Academy of Agricultural Sciences, Institute of Plant Protection (South Campus), No. 12, Zhong-Guan-Cun Nan-Da-Jie, Haidian, 100 081 Beijing, P.R. China; e-mail: xiem406@126.com