

Effects of repeated cultivation of transgenic Bt cotton on functional bacterial populations in rhizosphere soil

Hai-Yan Hu · Xiao-Xia Liu · Zhang-Wu Zhao ·
Jian-Guang Sun · Qing-Wen Zhang ·
Xing-Zhong Liu · Yong Yu

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Abstract The impact of multiple-year (0–5 years) cultivation of transgenic *Bacillus thuringiensis* (Bt) cotton on the functional bacterial populations in rhizosphere soil was investigated. The transgenic Bt + CpTI cotton line SGK321 and a non-Bt cotton line Shiyuan321 were planted in four fields in which Bt cotton had been continuously cultivated for 0, 1, 3, and 5 years. Rhizosphere soil samples were collected at the seedling, squaring, flower and boll, and boll-opening stages of cotton. Numbers of bacteria involved in nitrogen-fixing, organic phosphate-dissolving, inorganic phosphate-dissolving, and potassium-dissolving were measured with cultivation-dependent approaches. The data presented here showed no consistent statistically significant differences in the numbers of different groups of functional bacteria between rhizosphere soil of Bt and non-Bt cotton in the same field, and no obvious trends in

the numbers of the various group of functional bacteria with the increasing duration of Bt cotton cultivation. These studies suggest that multiple-year cultivation of transgenic Bt cotton may not affect the functional bacterial populations in rhizosphere soil.

Keywords Functional bacteria · Repeated cultivation · Rhizosphere soil · Transgenic Bt cotton

Introduction

A transgenic approach to crop protection was realized in the mid 1990s with the commercial introduction of genetically modified insect-resistant crops. Field and laboratory studies showed that transgenic plants expressing *Bacillus thuringiensis* Berliner (Bt) Cry proteins afford effective resistance to the larvae of a number of Lepidoptera species. For example, Bt cotton plants are protected against the cotton bollworm, *Helicoverpa armigera* (Hübner) (Zhao et al. 1998; Shelton et al. 2002; Deng et al. 2003), thus reducing the requirement for multiple insecticide treatments (Mascarenhas and Luttrell 1997) and the risk of pollution from chemical insecticide applications. Cowpea Trypsin Inhibitor (CpTI), a broad-spectrum insect resistant protein, could form an enzyme-inhibitor complex in the midgut of insect when ingested, inhibiting the hydrolyzing activities of the digestive enzymes of insect (Bi et al. 2006). Many other crops have been genetically modified to express insecticidal toxins from various subspecies of *B. thuringiensis* (Llewellyn et al. 1994; Waage 1996; Federici 1998). According to the International Service for the Acquisition of Agri-Biotech Applications (ISAAA), worldwide production of genetically modified crops has increased 67-fold, from 1.7 million ha in 1996 to

H.-Y. Hu · X.-X. Liu · Z.-W. Zhao · Q.-W. Zhang (✉)
College of Agronomy and Biotechnology, China Agricultural
University, Beijing 100094, China
e-mail: zhangqingwen@263.net

H.-Y. Hu · J.-G. Sun
Department of Agro-microbial Resource and Utilization,
Institute of Agricultural Resources and Regional Planning,
Chinese Academy of Agricultural Sciences, Beijing 100081,
China
e-mail: haiyanhyy@163.com

X.-Z. Liu (✉)
Institute of Microbiology, Chinese Academy of Sciences,
Beijing 100101, China
e-mail: liuxz@sun.im.ac.cn

Y. Yu
Institute of Mountain Hazards and Environment, Chinese
Academy of Sciences, Chengdu 610041, China

114.3 million ha in 2007 (James 2007), and is predicted to increase even more in the future. However, the large-scale commercial release of Bt crops is a public concern, because of the danger to natural and agricultural ecosystems (Williamson 1992; Hails 2000; Stotzky 2000, 2002, 2005).

Inevitably, Bt toxin will be introduced to soil in root exudates throughout the growth of the transgenic plant (Saxena et al. 1999, 2002b; Saxena and Stotzky 2000; Icoz and Stotzky 2007), through pollen deposition during tasseling, e.g., maize (Losey et al. 1999; Hansen Jesse and Obrycki 2000), and by incorporation of plant residues after harvest (Zwahlen et al. 2003; Stotzky 2002, 2005). There is no consensus about the persistence of the Cry proteins in soils. Some studies have shown that repeated and large-scale use of transgenic Bt crops could lead to the accumulation and persistence of Bt proteins in soil (Tabashnik 1994; Tapp and Stotzky 1995, 1998; Crecchio and Stotzky 1998; Saxena and Stotzky 2001; Saxena et al. 2002a, b; Zwahlen et al. 2003; Muchaonyerwa et al. 2005; Stotzky 2005; Icoz et al. 2008). Saxena and Stotzky (2002) showed that the toxin released in root exudates and from the biomass of Bt corn rapidly binds to surface-active particles in soil and remains larvicidal activity for at least 180 days. Tapp and Stotzky (1995, 1998) reported that when purified Cry proteins from *B. thuringiensis* subsp. *kurstaki* (Btk) was added to soil, the insecticidal activity was still detected after 234 days. These small, cumulative changes could lead to large differences in soil over time. It is therefore necessary to evaluate toxin persistence and associated changes in agricultural fields where Bt crops have been cultivated for multiple, successive years.

Because the structure of the soil microbial community is an important component of soil quality and health, soil microbiological properties could be early and sensitive indicators of anthropogenic effects on soil ecology in both natural and agricultural ecosystems (Visser and Parkinson 1992). In the last decade many reports on potential impacts of transgenic crops on the structure and functioning of the soil microbial community have been published. Two of three transgenic Bt cotton lines caused a transient increase in total bacterial and fungal population levels; in contrast, neither the third transgenic Bt cotton line nor the purified Bt toxins affected the total numbers of bacteria and fungi (Donegan et al. 1995). There were no significant differences in the numbers of culturable bacteria, actinomycetes, fungi, protozoa, and nematodes in the rhizosphere of Bt vs. non-Bt corn or in soil amended with biomass of Bt vs. non-Bt corn (Saxena and Stotzky 2001). Despite the detection of Cry1Ab protein in the rhizosphere soil of MON810 maize, the bacterial community structure was less affected by the Cry1Ab protein than by other environmental factors, such as plant age or field heterogeneity (Baumgarte and Tebbe 2005).

Most studies, however, have concentrated on assessing the accumulation and persistence of Bt proteins in soil in which Bt crops have been continuously cultivated for several years (Head et al. 2002; Ahmad et al. 2005; Dubelman et al. 2005; Icoz et al. 2008). To date, the community structure of functional bacteria in rhizosphere soil after multiple years of transgenic cotton cultivation has not been evaluated under field conditions. The relationship between functional groups of microorganisms involved in C, N, and P cycling and their influence on plant growth are potential indicators of the impacts of disturbance on the soil environment (Ferreira et al. 2003). Therefore, in the present study we evaluated levels of functional bacteria in soil in which transgenic Bt cotton producing Cry protein had been grown for 0–5 successive years; in all cases the crop residues were incorporated into soil by post-harvest tillage. The data from this study will contribute to the assessment of environmental risks of transgenic crops.

Materials and methods

Transgenic plant lines

The transgenic cotton cultivar SGK321, carrying both *cryIA* and *CpTI* genes and developed by the Agri-Biotechnology Research Institute of the Chinese Academy of Agricultural Sciences (CAAS) (Guo et al. 1999; Zhang et al. 2004), was used. Shiyuan321, the parental cultivar of SGK321 and a major commercial cotton cultivar originally used in northern China (Liu et al. 2005), was used as the conventional cotton. Seeds of both cotton cultivars were provided by the Shijiazhuang Academy of Agricultural Sciences.

Field design and sampling

Experiments were performed in 2006 at Baoding, Hebei Province, northern China. Four fields were selected; in three fields transgenic Bt cotton had been grown for the previous 1, 3, or 5 consecutive years, and in the fourth field transgenic Bt cotton had never been planted. The fields are hereafter termed zero-, one-, three-, and five-year fields. All fields had been cultivated with standardized farming management practices. Seeds of both the transgenic cotton SGK321 and the non-transgenic cotton Shiyuan321 were sown in every field with a randomized block design in three blocks each on 30 × 20 m for each cultivar. Each year, all the fields were cultivated once during the growing season, and the plant stalks were tilled into the soil using disk plows immediately following harvest of the cotton lint and seeds.

Soil chemical and physical properties are listed in Table 1. Soil samples were collected at the seedling,

Table 1 Physicochemical characteristics of the soil in the experimental fields used in the study

Bt cotton cultivation (years)	N (mg kg ⁻¹)	P (mg kg ⁻¹)	K (mg kg ⁻¹)	Organic matter (g kg ⁻¹)	pH
0	37.62	9.35	152.48	18.15	7.35
1	40.70	9.97	118.68	19.07	7.41
3	51.90	8.15	166.66	23.45	7.59
5	53.53	7.24	230.85	23.70	7.55

squaring, flower and boll, and boll-opening stages of cotton development. Rhizosphere soil was defined as the soil still attached to the roots after the roots were shaken by hand (Bruseti et al. 2005). For each sampling, rhizosphere soil from 10 randomly selected plants per block was mixed and used as a composite rhizosphere soil sample. The soil samples were stored at 4°C until analysed, usually within 1 month of sampling.

Quantification of functional bacteria in rhizosphere soil

Total nitrogen-fixing bacteria, organic phosphate-dissolving bacteria, inorganic phosphate-dissolving bacteria, and potassium-dissolving bacteria were enumerated using a 10-fold dilution plate technique. Soil (10 g) from the various treatments was suspended in 100 ml sterile water, shaken for 20 min at 250 rev min⁻¹, and 10-fold serially diluted. The colony-forming units (c.f.u.) of functional bacteria in each sample were determined by spreading 100 µl of the diluted sample on appropriate culture media in Petri plates, with three replicate plates per dilution. The following media were used to assay for different bacteria types (l⁻¹ in each case): nitrogen-fixing bacteria (10.0 g glucose, 0.2 g KH₂PO₄, MgSO₄·7H₂O, NaCl, 5.0 g CaCO₃, 0.1 g CaSO₄·2H₂O, 15 g agar, pH 7.0; Xu and Zheng 1986); organic phosphate-dissolving bacteria (10.0 g peptone, 5.0 g beef, 5.0 g NaCl, 15 g agar, pH 7.0–7.2, and 60 ml yolk:0.85% NaCl [1:1] solution); inorganic phosphate-dissolving bacteria (10.0 g glucose, 0.5 g yeast extract, 0.1 g CaCl₂, 0.3 g MgSO₄·7H₂O, 15 g agar, and a combined solution of 200 ml 10% CaCl₂ and 20 ml 10% K₂HPO₄ added immediately before use, pH 7.0; Li et al. 1996); and potassium-dissolving bacteria (10.0 g sucrose, 5.0 g CaCO₃, 0.5 g K₂HPO₄, 0.5 g (NH₄)₂SO₄, 0.2 g MgSO₄·7H₂O, 15 g agar, pH 7.2–7.4; Rui et al. 2005). Plates were incubated at 28°C for 2 days for assay of organic phosphate-dissolving bacteria, 3 days for potassium-dissolving bacteria, and 7 days for nitrogen-fixing and inorganic phosphate-dissolving bacteria. Colonies were counted visually and expressed as c.f.u. g⁻¹ dry soil. Both organic and inorganic phosphate-dissolving bacteria produce colonies with transparent peripheries. Potassium-dissolving bacteria form colonies with the shape of a fluid drop.

Statistical analysis

Statistical differences between numbers of bacteria (log₁₀ c.f.u. g⁻¹ dry rhizosphere soil) from transgenic Bt and non-Bt cotton grown in the same field at each developmental stage were determined by independent-sample *t* tests at the 5% significance level. Comparisons of the mean log₁₀ c.f.u. g⁻¹ dry soil between fields within each sampling stage were made with the Tukey honestly significant difference (HSD) test at the 5% significance level. The cultivar, field, and cultivar * field interaction effects on the numbers of nitrogen-fixing bacteria, organic phosphate-dissolving bacteria, inorganic phosphate-dissolving bacteria, and potassium-dissolving bacteria were analysed using a generalized linear model (GLM) procedure. All statistical analyses were performed with SPSS 11.0.

Results

Nitrogen-fixing bacteria

The analysis of data for all fields combined showed no significant effect of cultivar, field, and cultivar * field interaction for numbers of nitrogen-fixing bacteria (Table 2). When the data for each sample time were analysed separately, field effects were not significant at the squaring stage but were significant at the other three developmental stages. There were significantly fewer nitrogen-fixing bacteria in the one-year cotton field than in the other three fields at the seeding stage. Significantly more nitrogen-fixing bacteria were detected in the three-year field than in the other three cotton fields at the flower and boll stage. At the boll-opening stage, the number of nitrogen-fixing bacteria was significantly higher in the three-year field than in the zero-year cotton field (Fig. 1a).

The numbers of nitrogen-fixing bacteria in rhizosphere soil of Bt and non-Bt cotton showed similar trends over time and did not differ significantly in the zero-year field (Fig. 2a). In the one-year field, there were significantly more nitrogen-fixing bacteria in rhizosphere soil of Bt cotton than non-Bt cotton at the boll-opening stage, but not at any other sampling stages (Fig. 2b). In the three-year field, there were no significant differences in the numbers of nitrogen-fixing bacteria in the rhizosphere soil of Bt and

Table 2 Generalized linear model results of the overall effects of cotton cultivar (SGK321 and Shiyuan321) and field (four fields in which transgenic Bt cotton had been grown previously for 0–5 years) on the number of nitrogen-fixing bacteria, organic phosphate-dissolving bacteria, inorganic phosphate-dissolving bacteria, and potassium-dissolving bacteria in rhizosphere soil

Source of variation (by bacterial type)	df	F	P
Nitrogen-fixing bacteria			
Cultivar	1	2.050	0.156
Field	3	1.650	0.183
Cultivar * field	3	0.843	0.474
Organic phosphate-dissolving bacteria			
Cultivar	1	1.663	0.201
Field	3	4.560	0.005
Cultivar * field	3	0.314	0.815
Inorganic phosphate-dissolving bacteria			
Cultivar	1	3.812	0.054
Field	3	1.147	0.335
Cultivar * field	3	0.335	0.800
Potassium-dissolving bacteria			
Cultivar	1	4.866	0.030
Field	3	2.311	0.082
Cultivar * field	3	2.054	0.112

non-Bt cotton at the seeding and squaring stages; however, there were significantly more nitrogen-fixing bacteria in rhizosphere soil of Bt cotton than non-Bt cotton at the flower and boll stage and boll-opening stage (Fig. 2c). In the five-year field, no differences were found in the numbers of nitrogen-fixing bacteria in rhizosphere soil of Bt and non-Bt cotton except at the seeding stage, when there were significantly more nitrogen-fixing bacteria with Bt than with non-Bt cotton (Fig. 2d).

Organic phosphate-dissolving bacteria

There was a significant overall field effect but no effect of cultivar or cultivar * field interaction on number of organic phosphate-dissolving bacteria (Table 2). With the exception of the seeding stage, the differences in numbers of organic phosphate-dissolving bacteria in rhizosphere soil among the four fields at the other three stages were not significant. At the seeding stage, the number of organic phosphate-dissolving bacteria was significantly less in the one-year field than in the other three fields, and higher in the five-year field than in the other three fields (Fig. 1b).

No consistent significant differences in the numbers of organic phosphate-dissolving bacteria between rhizosphere soil of Bt and non-Bt were observed during the four sampling times in the four fields. The only significant differences in the numbers of organic phosphate-dissolving bacteria were in the following combinations of field and

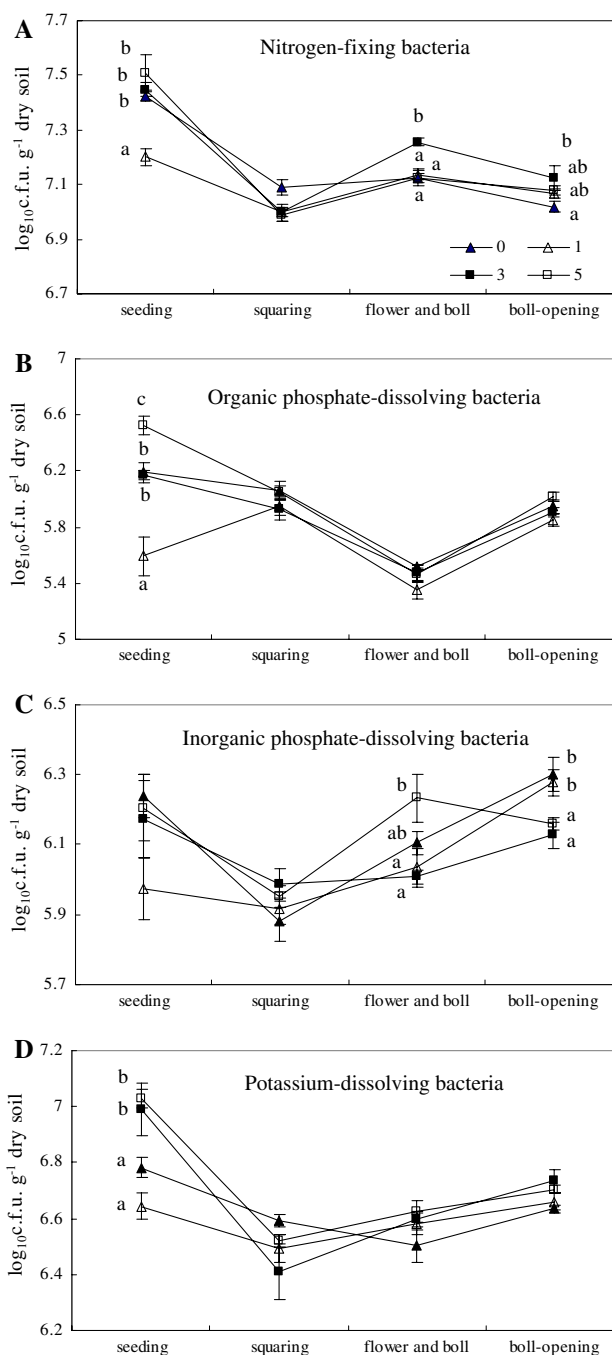


Fig. 1 Numbers of bacteria (log₁₀ c.f.u. g⁻¹ dry soil) in fields where transgenic Bt cotton was previously grown for 0 year (▲), 1 year (△), 3 years (■), and 5 years (□). Values are averages of data from Bt and non-Bt cotton. (a) nitrogen-fixing bacteria, (b) organic phosphate-dissolving bacteria, (c) inorganic phosphate-dissolving bacteria, and (d) potassium-dissolving bacteria. Different letters at the same sample date indicate significant differences at P = 0.05

sampling stage: zero-year field and seedling stage, one-year field and flower and boll stage, three-year field and squaring stage, five-year field and seeding stage, with significantly higher numbers in rhizosphere soil of Bt cotton than non-Bt cotton (Fig. 3a–d).

Fig. 2 Numbers of nitrogen-fixing bacteria (\log_{10} c.f.u. g^{-1} dry soil) in the rhizosphere soil of Bt (●) and non-Bt (○) cotton cultivars in fields where Bt cotton had been previously grown for 0 year (a), 1 year (b), 3 years (c), and 5 years (d). Asterisks indicate significant differences at $P = 0.05$

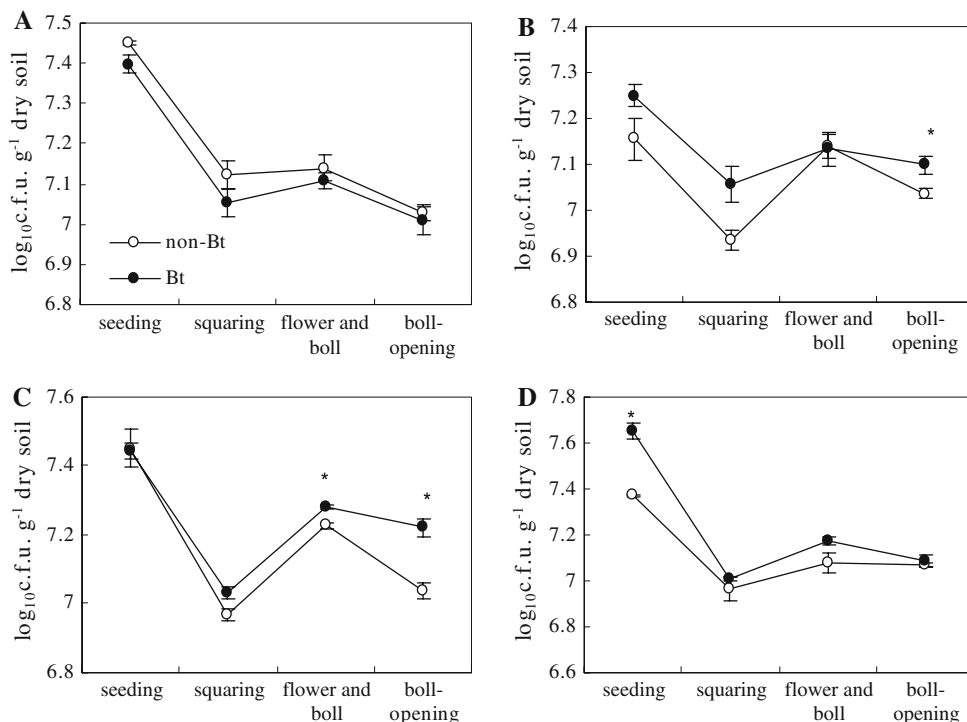
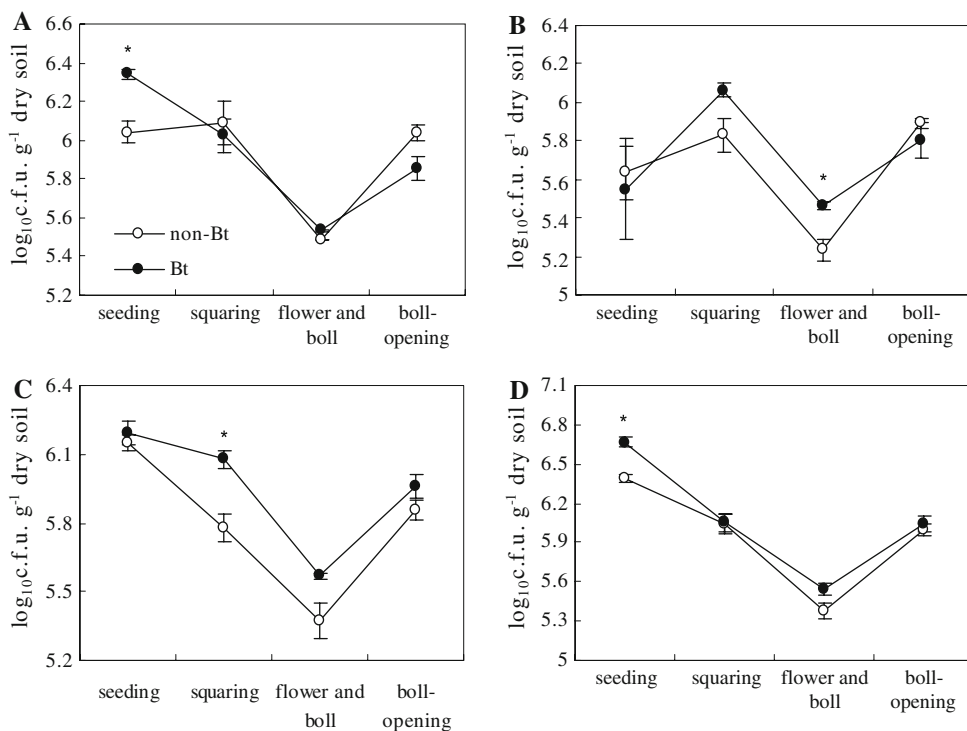


Fig. 3 Numbers of organic phosphate-dissolving bacteria (\log_{10} c.f.u. g^{-1} dry soil) in the rhizosphere soil of Bt (●) and non-Bt (○) cotton cultivars in fields where Bt cotton had been previously grown for 0 year (a), 1 year (b), 3 years (c), and 5 years (d). Asterisks indicate significant differences at $P = 0.05$

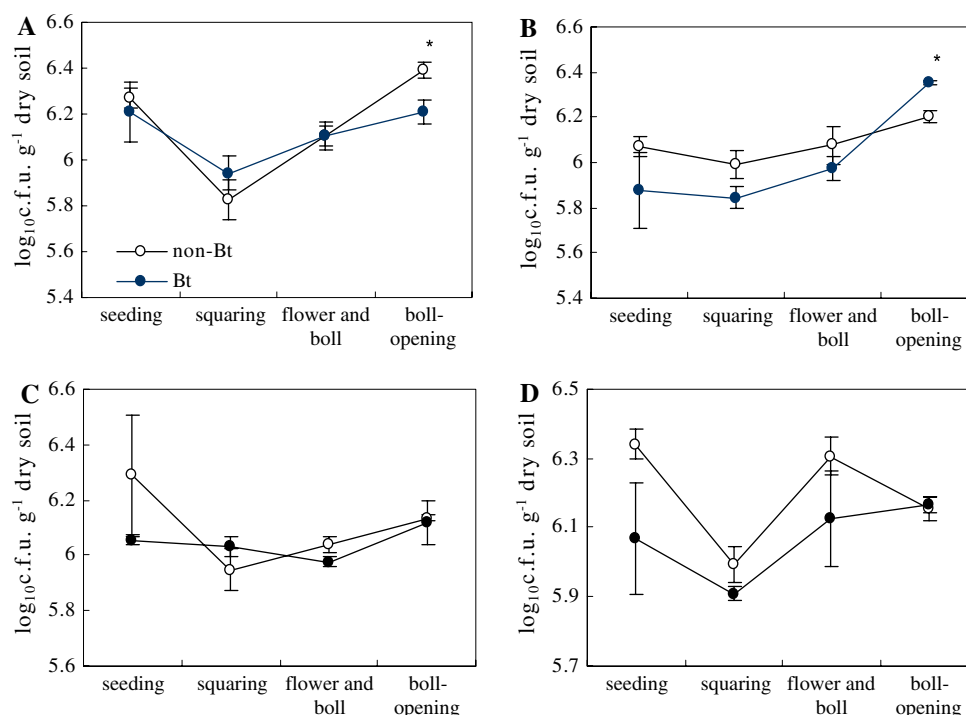


Inorganic phosphate-dissolving bacteria

The analysis of all cotton fields combined revealed no significant effects of cultivar, field, or cultivar * field interaction for inorganic phosphate-dissolving bacteria (Table 2). There was a significant field effect at the flower

and boll stage and boll-opening stage, but the differences between fields at the seeding and squaring stages were not significant. No obvious increase or decrease in numbers of inorganic phosphate-dissolving bacteria was detected with increasing duration of transgenic Bt cotton cultivation (Fig. 1c).

Fig. 4 Numbers of inorganic phosphate-dissolving bacteria (\log_{10} c.f.u. g^{-1} dry soil) in the rhizosphere soil of Bt (●) and non-Bt (○) cotton cultivars in fields where Bt cotton had been previously grown for 0 year (a), 1 year (b), 3 years (c), and 5 years (d). Asterisks indicate significant differences at $P = 0.05$



In the zero-year field, significantly fewer inorganic phosphate-dissolving bacteria were detected in Bt than in non-Bt rhizosphere soil at the boll-opening stage, but the differences were not significant at the other stages (Fig. 4a). In the one-year field, significantly more inorganic phosphate-dissolving bacteria were detected in Bt than in non-Bt rhizosphere soil at the boll-opening stage; differences at the other stages were not significant (Fig. 4b). In both the three- and five-year fields, no significant differences were found in the numbers of inorganic phosphate-dissolving bacteria in Bt and non-Bt rhizosphere soil at the four sampling stages (Fig. 4c, d).

Potassium-dissolving bacteria

The analysis of all fields combined indicated a significant cultivar effect but no field or cultivar * field interaction effects for potassium-dissolving bacteria (Table 2). The cultivar effect was due to the presence of significantly more bacteria in Bt than in non-Bt rhizosphere soil at six instances in the four fields. A separate analysis by sampling stage showed that the field effect was significant at the seeding stage but not at the other three stages. At the seeding stage, significantly more potassium-dissolving bacteria were detected in the three- and five-year fields than in the zero- and one-year fields (Fig. 1d).

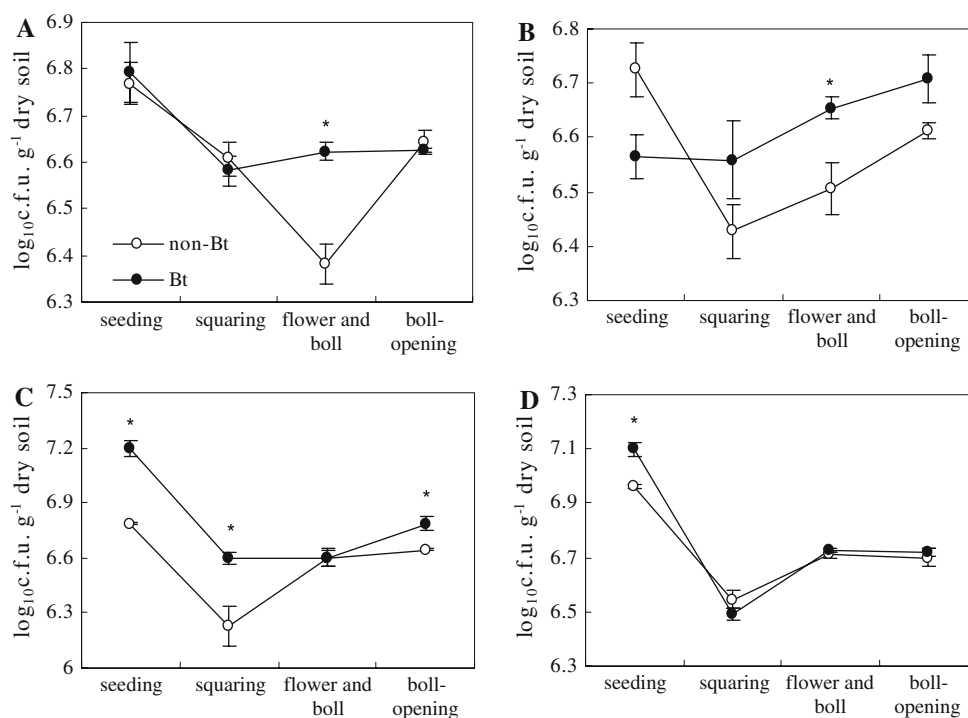
In the zero- and one-year fields, significantly more potassium-dissolving bacteria were detected in Bt than in non-Bt rhizosphere soil at the flower and boll stage; differences were not significant at any of the other stages

(Fig. 5a, b). In the three-year field, numbers of potassium-dissolving bacteria were significantly higher in Bt than in non-Bt rhizosphere soil at the seeding, squaring and boll-opening stages, but not at the flower and boll stage (Fig. 5c). At the seeding stage in the five-year field, significantly more potassium-dissolving bacteria were detected in Bt than in non-Bt rhizosphere soil, but numbers did not differ at the other three sampling stages (Fig. 5d).

Discussion

The present study showed no consistent statistically significant differences between rhizosphere soil of Bt and non-Bt cotton in the numbers of culturable nitrogen-fixing bacteria, bacteria that dissolve organic and inorganic phosphates and potassium-dissolving bacteria during the four sampling stages in the four fields. Some differences between Bt and non-Bt cotton rhizosphere soil in the numbers of the various group of functional bacteria were observed in 16 instances (the total number of instances was 4 fields \times 4 sampling dates \times 4 types = 64), but these differences were not consistent from one sampling stage to the next. These results are consistent with those of Icoz et al. (2008), who reported that after 4 consecutive years of corn cultivation, there were, in general, no consistent statistically significant differences in the numbers of different groups of microorganisms, the activities of the enzymes, and the pH between soils planted with *Bt* and non-*Bt* corn. Similarly, Saxena and Stotzky (2001) also did not find any

Fig. 5 Numbers of potassium-dissolving bacteria (\log_{10} c.f.u. g^{-1} dry soil) in the rhizosphere soil of Bt (●) and non-Bt (○) cotton cultivars in fields where Bt cotton had been previously grown for 0 year (a), 1 year (b), 3 years (c), and 5 years (d). Asterisks indicate significant differences at $P = 0.05$



significant differences in the numbers of culturable bacteria, actinomycetes, fungi, protozoa, and nematodes in the rhizosphere of transgenic Bt and non-transgenic maize. In addition, Blackwood and Buyer (2004) assessed the effects of two lines of transgenic corn expressing different Cry endotoxins on the soil microbial community structure in three soil types in a growth chamber experiment; the bacterial community structure was less affected by transgenic Bt corn than by the soil type. Muchaonyerwa et al. (2005) reported that larvicidal proteins produced by different *B. thuringiensis* subspecies and Bt maize could persist in tropical soils as a result of adsorption on soil clays, but that there were no observable effects on the soil microbial biomass carbon or counts of culturable bacteria and fungi. Brusetti et al. (2005) detected no differences in the rhizosphere bacterial communities between transgenic Bt 176 maize and its non-transgenic counterpart.

Other authors, however, have reported minor to significant effects of Cry proteins and transgenic Bt crops on microbial community structure in soil. Petras and Casida (1985) observed slight increases in populations of bacteria, actinomycetes, fungi, and nematodes after the addition of *B. thuringiensis* subsp. *kurstaki* to the soil; Petras and Casida (1985) inferred that the crystal proteins were used as a substrate. A significant but transient increase in the populations of culturable bacteria and fungi was observed in soil with leaves of Bt cotton (*Gossypium hirsutum* L.) expressing the Cry1Ac protein (Donegan et al. 1995). Rui et al. (2005) found higher numbers of functional bacteria in the rhizosphere soil of Shiyuan321 (non-Bt cotton) than

NuCOTN99^B (Bt cotton); after adding pure Bt toxin to soil, Rui et al. (2005) also indicated that Bt toxin may not directly affect the numbers of functional bacteria in the rhizosphere.

Previous studies revealed that qualitative and quantitative differences in root exudation could strongly affect the structure of microbial communities in the rhizosphere (Maloney et al. 1997; Mansouri et al. 2002; Oger et al. 1997, 2000; Savka and Farrand 1997). Based on principal component analysis of automated ribosomal intergenic spacer data, bacterial communities differed when collected from hydroponic solutions in which transgenic Bt 176 vs. non-transgenic maize had been grown, indicating that root exudates may select for different bacterial communities (Brusetti et al. 2005). Early studies by Neal et al. (1970, 1973) found that a simple genetic substitution in spring wheat (*Triticum aestivum* L.) through traditional plant breeding resulted in obvious changes in the numbers and types of rhizosphere microorganisms. In addition, Yan et al. (2007) found that the roots of transgenic Bt cotton secreted more organic acids and less amino acids and soluble sugars than wild-type cotton roots in the full-nutrient solution. With respect to this study, we could not yet conclude that the transient increases in the number of functional bacteria in rhizosphere soil of transgenic Bt cotton are related to changes in root exudation and root characteristics. The nature of root exudates from transgenic Bt crops and the effects of the exudates on rhizosphere microorganisms requires further investigation.

Some studies have shown that transgenic Cry protein is introduced into soil through root exudates and plant

residues (Saxena et al. 1999, 2002b; Saxena and Stotzky 2000; Zwahlen et al. 2003; Stotzky 2005). Therefore, several studies have examined whether the Bt proteins will accumulate and persist in soil where transgenic Bt crops have been grown in successive years. Using both an insect bioassay and ELISA, Head et al. (2002) reported that no Cry1Ac protein was detected in any soil sample collected from fields experiencing 3–6 years continuous cultivation of Bt cotton. Similarly, Dubelman et al. (2005) found no evidence of persistence and accumulation of Cry1Ab protein in soils after 3 years of sustained Bt corn cultivation. In addition, Ahmad et al. (2005) showed that the Cry3Bb1 protein released from root exudates or decaying plant residues of transgenic Bt corn (event MON863) was rapidly degraded and did not persist in soil for three consecutive seasons under field conditions. Conversely, Icoz et al. (2008) reported that the Cry1Ab protein was detected in most soils with Bt corn expressing the *cry1Ab* gene during the 4 consecutive years of corn cultivation, whereas the Cry3Bb1 protein was not detected in soils of Bt corn (event MON863) expressing the *cry3Bb1* gene.

Whether Bt proteins are present or not in the soil—the important consideration is if it has an impact on the soil microbial community due to their functional significance. The soil microbial community structure is an early and sensitive indicator of anthropogenic effects on soil ecology. In the present study, we evaluated the functional bacterial population in soil in which transgenic Bt cotton had been cultivated for 0–5 years. When the data for individual sampling stages were analysed for each kind of functional bacteria, differences in the numbers of functional bacteria among the field treatments were significant in seven instances (the total number of instances was 4 sampling dates \times 4 types = 16). However, these differences were temporary, transient and varied among the 4 sampling stages with no obvious trend in numbers of functional bacteria associated with increasing duration of Bt-transgenic cotton cultivation.

In conclusion, differences in the functional bacteria population between rhizosphere soil of Bt and non-Bt cotton in the same field or among the four fields were either transient or absent. The major conclusions from this study are: (1) repeated cultivation of transgenic Bt cotton expressing Cry protein did not result in significant change in the overall numbers of functional bacteria; and (2) transgenic Bt cotton had no clear effect on the number of functional bacteria in the rhizosphere within one growing season. These results suggest that cultivation of Bt crops over multiple years probably poses little ecological or environmental risk. However, the results presented here should be considered preliminary because we used a culture-based technique (which detects only a small portion of the microbial community) and because we evaluated only a few functional types of bacteria.

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