

# Microbial properties, enzyme activities and the persistence of exogenous proteins in soil under consecutive cultivation of transgenic cottons (*Gossypium hirsutum* L.)

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## ABSTRACT

One *Bacillus thuringiensis* (*Bt*) and two stacked *Bt* and cowpea trypsin inhibitor (*Bt* + *CpTI*) cottons and their non-transgenic isolines were consecutively cultivated to investigate the soil persistence of *CryIAC* and *CpTI* proteins and their effects on microbial properties and enzyme activities involving C, N, P, and S cycling in soil. Results showed that there were the persistence of *CryIAC* and *CpTI* proteins in soil under 4-year consecutive cultivation of transgenic cottons. *CryIAC* proteins varied from 6.75 ng/g to 12.01 ng/g and *CpTI* proteins varied from 30.65 to 43.60 ng/g. However, neither of these two proteins was detected in soil under non-transgenic cottons. Soil microbial biomass carbon, microbial activities, and soil enzyme activities (except urease and phosphodiesterase) significantly decreased in soil under transgenic cottons. Correlation analysis showed that most of microbial properties and enzyme activities in soil had a negative relationship with *CryIAC* content, while most of them had a positive relationship with *CpTI* content. Our data indicate that consecutive cultivation by genetically modified cottons with *Bt* and *CpTI* genes can result in persistence of *CryIAC* and *CpTI* proteins and negatively affect soil microbial and biochemical properties.

**Keywords:** genetically modified plants; pest-resistant cotton; soil biological properties; insecticidal toxin; anti-nutritional factor

In China, the *CryIAC* gene produced from *Bt* and the *CpTI* gene produced from cowpea, both were transformed into the cotton (*Gossypium hirsutum* L.) to resist cotton bollworms (*Helicoverpa armigera*) (Zhang et al. 2004). These lines were planted on a large scale, 3.80 million ha in 2008, accounting for 68% of the total cotton area in China. Soil persistence of insecticidal *Cry* proteins derived from cultivation of these crops might have adverse impacts on microbe-mediated functions and processes in soil (Icoz et al. 2008, Icoz and Stotzky 2008a, Hu et al. 2009).

Previous studies revealed that *Cry* proteins were soluble in water (Douville et al. 2005, Icoz et al. 2009), and others claimed that they could be adsorbed or bound on clay particles, humic components, or organic-mineral complexes (Tapp and Stotzky 1995, Crecchio and Stotzky 2001). These traits of *Cry* proteins could cause different

concentrations between rhizosphere and bulk soil. Icoz and Stotzky (2008a) determined that continuous release of *Cry* proteins via root exudates led to higher concentrations of the proteins in the rhizosphere than in bulk soil. Thus, numerous field studies were carried out to investigate the persistence of *Cry* proteins in rhizosphere soil and its potential risk to rhizosphere soil or the soil-litter interactive zone (Icoz and Stotzky 2008b, Hu et al. 2009, Wang et al. 2009). Among these studies, a great variation of *Cry* protein amounts in rhizosphere soil was determined. For example, Head et al. (2002) detected that there was no *CryIAC* protein in soils where transgenic cotton was cultivated for three to six consecutive years. Hopkins and Gregorich (2003) claimed that much of the endotoxin in *Bt* maize residue was highly labile and quickly decomposed in soils except for a small fraction in relatively recalcitrant residues. Icoz et

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al. (2008) found no detectable *Cry3Bb1* protein in rhizosphere soil of *Bt* maize after four consecutive years of cultivation. However, Zwahlen et al. (2003) found that *Cry1Ab* protein in maize residue could still be detected after 240 and 200 days under tillage and no-tillage soil. In disagreement with *Cry3Bb1* protein, Icoz et al. (2008) found that *Cry1Ab* protein was still detectable in rhizosphere soil of *Bt* maize after four years of consecutive cultivation under field conditions. Wang et al. (2009) reported that *Cry* proteins still could be detected in soil after corn residue incorporation for 180 days, and the degradation of *Cry* protein varied with straw return patterns and transgenic varieties. Moreover, recent investigation by Icoz et al. (2009) even found that the *Cry1Ab* protein might be taken up by plants from soils that had been previously planted with *Bt* corn.

*CpTI* is a small polypeptide belonging to the Bowman-Birk type of double-headed serine protease inhibitors. As the analogue of enzymes, *CpTI* and *Cry1Ac* proteins released from transgenic crops might compete with soil enzymes for binding sites of soil particles, interacting with soil enzymes and each other. On the other hand, the changes in soil microbial population and crop metabolism under transgenic crop cultivation may also influence the activity of soil enzymes by affecting their origins. Yet, little information about the fate of *CpTI* and *Cry1Ac* proteins in soil ecosystem had been collected under cotton cultivation in China, and whether or not the proteins pose a risk to the area where the root is scarce, especially to soil microbial properties and enzyme activities in soil under transgenic cottons consecutive cultivation, needs further investigation.

Dehydrogenase and catalase activities can be used as indicators of microbial activities in soil (Achuba and Peretiemo-Clarke 2008). Activity of fluorescein diacetate (FDA) hydrolysis has also been suggested as a possible indicator for measuring overall microbial activity because the ubiquitous lipase, protease, and esterase enzymes are involved in the hydrolysis of FDA (Schnurer and Rosswall 1982, Green et al. 2006). However, conflict results were obtained in previous culture or short-term field experiments about *Bt* crops on soil dehydrogenase, catalase activities, and other enzyme activities involved in nutrient cycling (Icoz and Stotzky 2008b); and there was no study that report the effect of *Bt* crops on FDA hydrolysis in soil.

In our previous work, we showed that *Bt* toxin could be persistent in soil and had negative effect on soil enzyme activities in a shorter incuba-

tion test (Sun et al. 2007). However, a long-term experiment is necessary to confirm our results obtained from the short-term test. In this study, we collected soils from a 4-year pot experiment with transgenic *Bt* (variety ZM30) and *Bt* + *CpTI* (varieties ZM41 and sGK321) cottons expressing *Cry1Ac* and *CpTI* proteins to determine the effects of transgenic cotton plantation on the persistence of *Cry1Ac* and *CpTI* proteins, and responses of soil microbial properties and soil enzyme activities involved in nutrient cycling to consecutive cultivation of transgenic cottons.

## MATERIALS AND METHODS

**Soil and cotton lines.** The soil for the pot experiment was collected from the top layer (0–20 cm) of a maize field at the experimental site of Shenyang Agricultural University (40°48'N, 123°33'E), Liaoning Province of China, where no transgenic plants had ever been grown. The soil was classified as Luvisols according to WRB-1998 with 21% clay, 56% silt, and 23% sand. The basic soil characteristics were 8.48 g/kg organic C, 1.22 g/kg total N, 0.49 g/kg total P, 106.37 mg/kg extractable K, and pH 5.84.

Three pairs of cottons, *Bt* (transgenic *Bt* cotton ZM30; non-transgenic *Bt* cotton with its isoline ZM16), *CpTI*+ (transgenic *Bt* + *CpTI* cotton ZM41; non-transgenic *Bt* + *CpTI* cotton with its isoline ZM23), and *CpTI*++ (transgenic *Bt* + *CpTI* cotton sGK321; non-transgenic *Bt* + *CpTI* cotton with its isoline Shiyuan321) were used. ZM30 and ZM41 were developed by the Chinese Academy of Agricultural Sciences, and sGK321 were developed by Shijiazhuang Agricultural Academy of Sciences together with Chinese Academy of Agricultural Sciences (Zhang et al. 2004). All cotton lines were supplied by the National Medium-term Gene Bank of Cotton Germplasm Resources, China.

**Pot experiment and soil sampling.** The experiment started in the greenhouse of the Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang, China in 2004, which included six replicates of each treatment in a completely randomized design.

Before the experiments, a total of 15 kg of the soil was mixed thoroughly with 0.25 g urea, 0.30 g  $\text{NH}_4\text{H}_2\text{PO}_4$  and 1.00 g  $\text{K}_2\text{SO}_4$  per 1 kg soil and placed into pots, then the soils were saturated with water and equilibrated for two weeks before sowing cotton seeds. In April, six germinating cotton seeds were planted in each pot. After 15 days, the

weak seedlings were removed and three seedlings were kept in each pot. The soils were watered with tap water to keep soil moisture appropriate for cotton growth and all were equally fertilized with 500 mL urea solution (10 g/L) at flowering and budding. No insecticide was applied to the plants for control of bollworms and pink bollworms.

At harvest of the first two years of cultivation, the above-ground plant residues were cut and removed from the pots, but the soil was kept. The soil remains in the pots were fertilized the same as 2004 for additional cultivation of the same cotton variety in the following years. The management of the cotton plants in the pots was the same for all years. In 2007 (after four years of consecutive cotton cultivations), non-rhizosphere soil samples (~ 150 g) were taken and mixed well after the cotton was harvested, parts of soil samples were stored at -20°C for the determination of *CryIAc* and *CpTI* proteins within a week. Other subsamples were kept at 4°C for use in measuring soil microbial properties and enzyme activities.

**Analysis of basic soil characteristics.** Soil organic C and total N measured with an elemental analyzer (elementar Vario EL). Soil particle size distribution, soil moisture contents, total P, extractable K and pH were analyzed according to Ryan et al. (2001).

**Analysis of *CryIAc* and *CpTI* protein concentrations in soil.** The concentrations of *CryIAc* and *CpTI* proteins were measured according to the procedure of Envirologix Inc. and Rui et al. (2004). Briefly, 2 mL extraction buffer (from the kit) was added to 3 g soil samples, then the mixture was shaken for 10 min and incubated at 4°C for 4 h, and centrifuged (16 000 × g) at 4°C. *CryIAc* and *CpTI* proteins in the supernatant were determined by ELISA with 0.4 ppm *CryAb/CryAc* and 1.0 ppm *CpTI* proteins as the positive control, respectively.

**Measurement of soil microbial properties and soil enzyme activities.** Soil microbial biomass carbon (MBC) was estimated from 15.0 g of soil using the fumigation-extraction technique, and calculated as the difference between the C content of fumigated and unfumigated samples extracted with 0.5 mol/L K<sub>2</sub>SO<sub>4</sub>, with a mineralization constant ( $k_{EC}$ ) of 0.41 (Parkinson and Paul 1982). Activities of dehydrogenase (DHA), FDA hydrolysis, and catalase were detected as described by Tabatabai (1994), Adam and Duncan (2001), and Trasar-Cepeda et al. (1999), respectively.

Urease, acid phosphomonoesterase, phosphodiesterase, arylsulfatase, and β-glucosidase were detected according to Tabatabai (1994). Nitrate

reductase activity was measured by the colorimetric method (Kandeler 1996). The determination of protease activity was adapted from Ladd and Butler (1972).

**Statistical analysis.** All results are expressed on a soil oven dry (105°C) weight basis. Data shown are the arithmetic means (± standard deviation) of six replicates of each treatment. Statistical analyses were performed with SPSS 10.0 for Windows. Comparisons among means were evaluated by Student-Newman-Keuls test at  $P = 0.05$  level with a one-way ANOVA. Relationships between varieties were based on Pearson correlations.

## RESULTS AND DISCUSSION

**The persistence of *CryIAc* and *CpTI* proteins.** The amount of *CryIAc* protein in soil under *Bt* cotton (12.01 ng/g dry soil) was significantly higher than that in the two transgenic *Bt + CpTI* cottons (7.86 and 6.75 ng/g dry soil) ( $P < 0.05$ ). There was no significant difference in the amount of *CryIAb* protein in soil between the two transgenic *Bt + CpTI* cottons. The amount of *CpTI* proteins in soil under the two transgenic *Bt + CpTI* cottons (30.65 and 43.60 ng/g dry soil) was significantly different ( $P < 0.05$ ); there was a significantly higher amount in *CpTI++* treatment. No *CryIAc* or *CpTI* protein was found in soil under the non-transgenic isolines (Table 1). It suggests that *CryIAc* and *CpTI* proteins persisted in soil under transgenic cottons which were consecutively cultivated.

Our previous study found that the concentrations of *CryIAc* and *CpTI* proteins in rhizosphere soil after the consecutive plantation of transgenic cotton (data not shown) were higher than that in

Table 1. *CryIAc* and *CpTI* protein concentration in soil

Treatments		<i>CryIAc</i>	<i>CpTI</i>
		(ng/g dry soil)	
<i>Bt</i>	non-transgenic	*ND	ND
	transgenic	12.01 ± 2.61 <sup>a</sup>	ND
<i>CpTI+</i>	non-transgenic	ND	ND
	transgenic	7.86 ± 1.16 <sup>b</sup>	30.65 ± 5.51 <sup>b</sup>
<i>CpTI++</i>	non-transgenic	ND	ND
	transgenic	6.75 ± 1.52 <sup>b</sup>	43.60 ± 7.06 <sup>a</sup>

Values are means ± standard deviation ( $n = 6$ ). Values followed by the same superscript letter in each column do not differ at the  $P_{0.05}$  level according to a one-way ANOVA. \*ND – not detected

the present study. In our previous incubation test, the concentration of *Cry1Ac* protein in the same soil released from *Bt* transgenic cotton residues amendment maintained 14.19 and 22.69 ng/g dry soil by the end of incubation (Sun et al. 2007). The different concentrations of the *Cry1Ac* protein between these experiments suggest that more insecticidal crystal proteins are introduced into soil under transgenic cotton residues incorporation than under transgenic cotton cultivation. As investigated by many authors, the origin of transgenic proteins and their persistence in soil depend on many aspects (e.g. release from living root cells and from lysed cells, adsorption on soil colloids, microbial utilization, proteolysis, soil characteristic, and so on) (Tapp and Stotzky 1998, Crecchio and Stotzky 2001). Desorption of *Cry1Ac* and *CpTI* proteins from rhizosphere soil colloids and their diffusion in soil solution might

be responsible for persistence of *Cry1Ac* and *CpTI* proteins in soil in the present study. Studies on the fate of *Cry3Bb1* protein found that its persistence was mainly dependent both on the type and amount of clay mineral present and on the pH of the soils. In general, a higher amount of kaolinite and lower pH value led to a higher concentration of *Cry* protein in soil (Icoz and Stotzky 2008a). Further study should be carried out to warrant which aspects dominate the persistence of transgenic proteins in this ecosystem.

The previous studies on transgenic *Bt* + *CpTI* cotton cultivar sGK321 showed that *Cry1Ac* and *CpTI* proteins were both detectable in the rhizosphere after harvest, but they could rapidly degraded (Rui et al. 2005, 2007). Our present study showed that there was the persistence of *CpTI* protein in non-rhizosphere soils after four years of consecutive cultivation of two transgenic *Bt* +

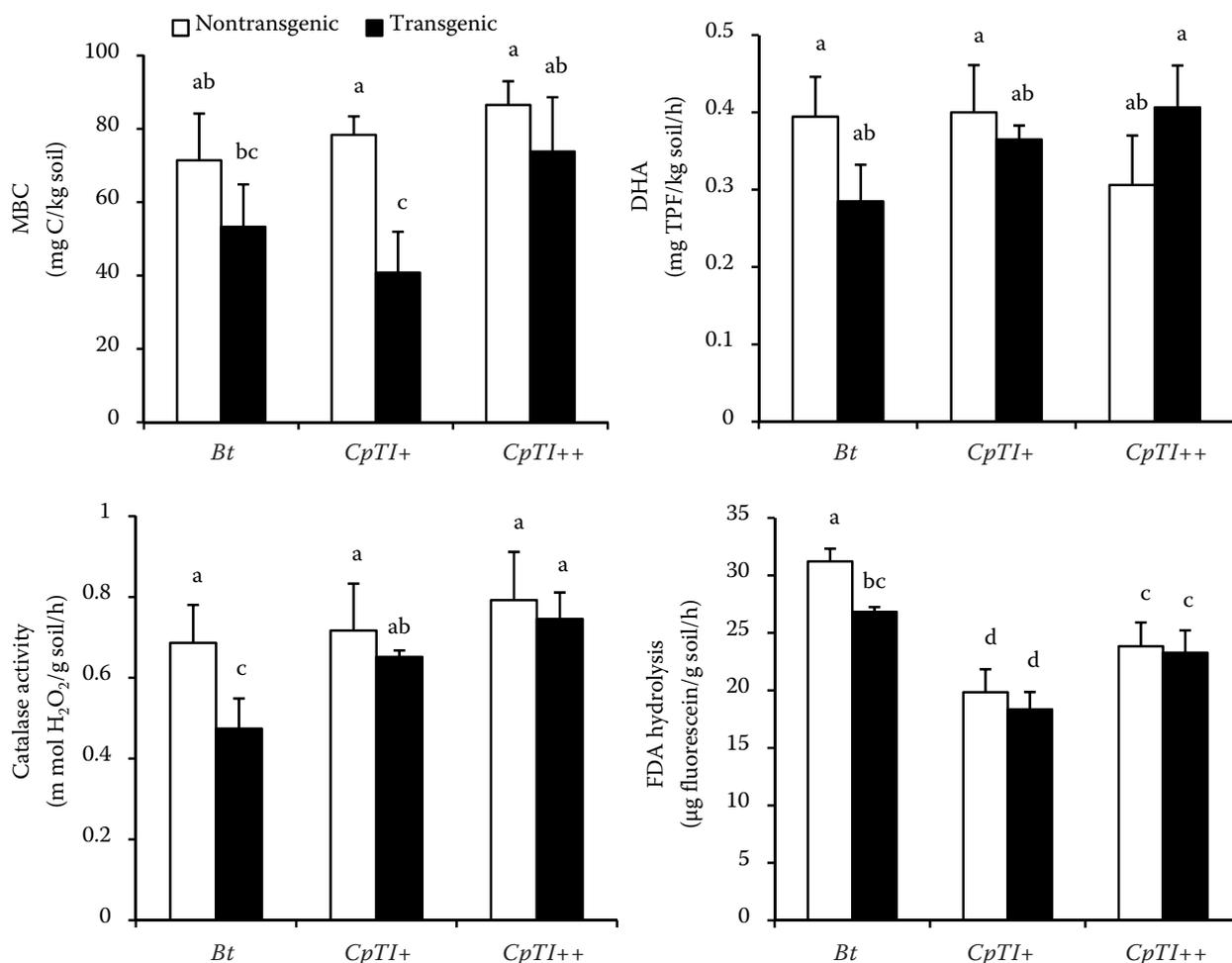


Figure 1. Soil microbial properties under transgenic cottons and their non-transgenic isolines. For each property, values sharing the same letter are not significantly different ( $P < 0.05$ ) according to a one-way ANOVA. Vertical bars represent standard deviation ( $n = 6$ ). Cotton varieties are indicated by *Bt* (Z30 and its isolate Z16), *CpTI+* (Z41 and its isolate Z23), and *CpTI++* (sGK321 and its isolate Shiyuan321)

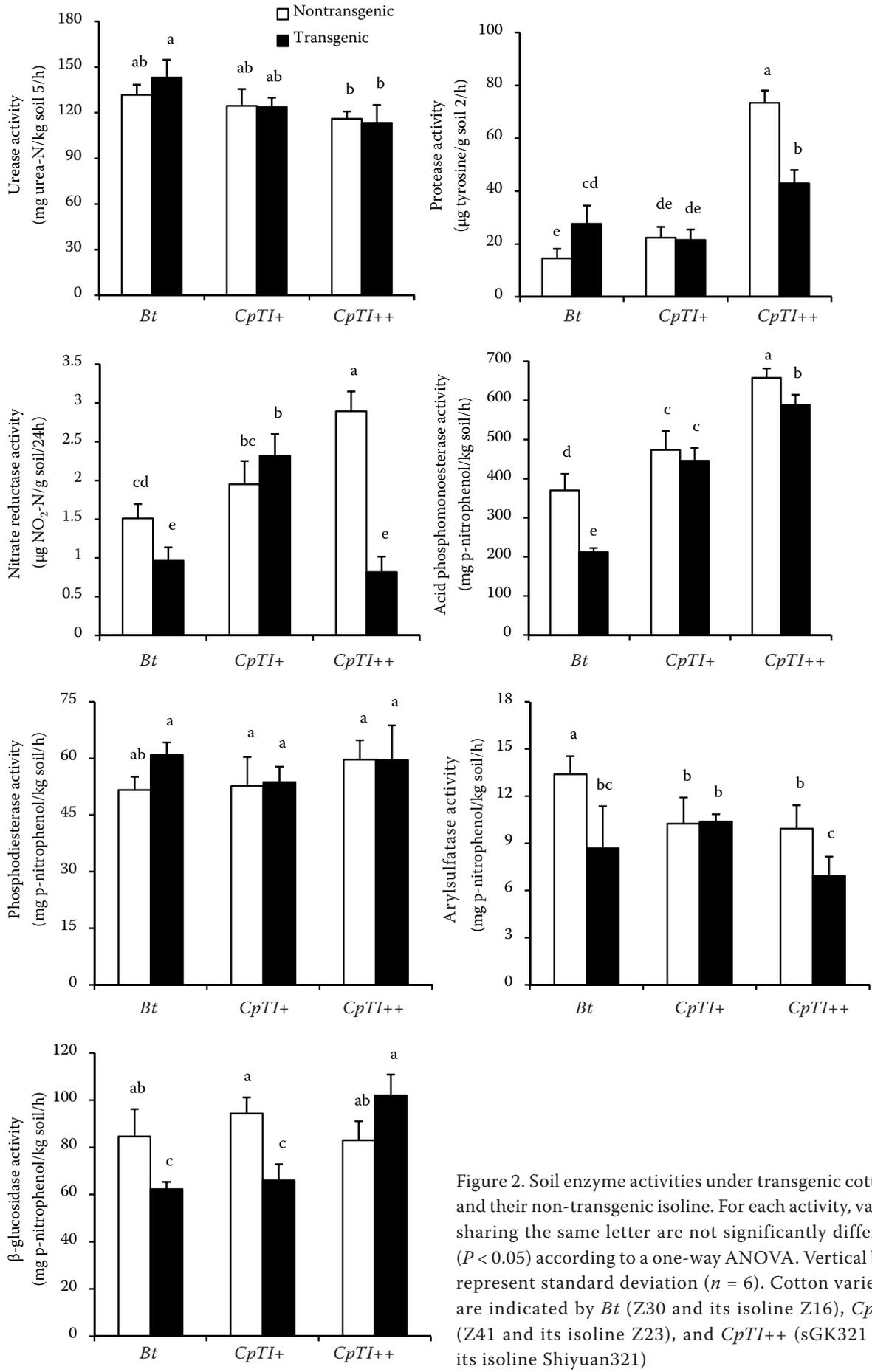


Figure 2. Soil enzyme activities under transgenic cottons and their non-transgenic isoline. For each activity, values sharing the same letter are not significantly different ( $P < 0.05$ ) according to a one-way ANOVA. Vertical bars represent standard deviation ( $n = 6$ ). Cotton varieties are indicated by *Bt* (Z30 and its isoline Z16), *CpTI+* (Z41 and its isoline Z23), and *CpTI++* (sGK321 and its isoline Shiyuan321)

*CpTI* cottons. Different amount of these proteins in soil between Rui et al. (2005, 2007) and our study might be due to differences of environmental factors (Icoz and Stotzky 2008b). Moreover, our data showed different amount of transgenic proteins in soil under different transgenic cotton cultivars (Table 1). *CryIAC* protein in soil under the cotton with single gene was significantly higher than those under cottons with stacked genes. *CpTI* proteins under two stacked transgenic cottons were also significantly different. It suggests that different amount of proteins might be introduced into soil under different transgenic cotton cultivars.

**Soil microbial properties and enzyme activities.** MBC, activities of catalase, and FDA hydrolysis in soil were inhibited under *CpTI+* and *Bt* cottons compared with their non-transgenic controls. No significant difference was found in DHA activity between transgenic cottons and their non-transgenic controls (Figure 1).

Activities of nitrate reductase, acid phosphomonoesterase, arylsulfatase, and  $\beta$ -glucosidase in soil were inhibited under *Bt* cotton compared with its non-transgenic isolate; activities of nitrate reductase, protease, acid phosphomonoesterase, and arylsulfatase in soil were inhibited under *CpTI++* cotton compared with its non-transgenic isolate; and activity of  $\beta$ -glucosidase in soil was inhibited under *CpTI+* cotton compared with its non-transgenic isolate. There were no significant differences in urease and phosphodiesterase activities under transgenic cottons compared with their non-transgenic isolines. The data indicate

that there were some significant negative effects on soil MBC, activities of soil microorganisms, enzyme activities under consecutive cultivation of transgenic *Bt* or *Bt + CpTI* cottons (Figure 2).

The previous study in our laboratory showed that *Bt* cotton residues incorporation could not distinguish the positive effect of cotton residues on soil enzyme activities from the negative effect of *Cry* proteins on soil enzyme activities (Sun et al. 2007). This pot experiments confirmed the negative effects of consecutive cultivation of transgenic crops on soil microbial properties and enzyme activities. Studies claimed that MBC in rhizosphere soil of transgenic *Bt* cotton was equivalent to (Wan 2007) and even higher (Sarkar et al. 2009) than its non-*Bt* cotton at different growth stages. Shen et al. (2006) showed that transgenic *Bt* cotton had no apparent effect on dehydrogenase, urease, phosphatase, phenoloxidase, and protease in rhizosphere soil. There is no previous information about MBC and enzyme activities in soil under consecutive transgenic cotton cultivation. The test soil in our study had higher organic carbon, more silt, longer cultivation years, and lower pH than previous studies on transgenic cottons in China, and these factors might cause more *CryIAC* protein to accumulate in soil. Further analysis found that, in contrast to *CpTI*, most soil microbial properties and enzyme activities had a negative correlation with *CryIAC* (Table 2). This suggests that *CryIAC* protein might be the origin of the negative effects on soil microbial properties and soil enzyme activities in soil under transgenic cottons. On the one hand, the

Table 2. Correlation between proteins (*CryIAC* and *CpTI*) concentrations and soil microbial properties and enzyme activities

	Coefficient of correlation ( <i>r</i> value)	
	<i>CryIAC</i> ( <i>n</i> = 18)	<i>CpTI</i> ( <i>n</i> = 12)
Microbial biomass C	-0.536	0.268
Dehydrogenase	-0.407	0.630
Catalase	0.240	0.434
FDA hydrolysis	-0.783**	0.396
Urease	0.410	-0.147
Nitrate reductase	-0.286	-0.585
Acid phosphomonoesterase	-0.781**	0.536
Phosphodiesterase	-0.066	0.350
Arylsulfatase	-0.246	-0.759**
$\beta$ -glucosidase	-0.458	0.642*
Protease	-0.356	0.526

\*indicates significant correlation at  $P_{0.05}$  level, and \*\*indicates significant correlation at  $P_{0.01}$  level

possible reason was that consecutive cultivation of transgenic cotton increased the accumulation of *Bt* and *CpTI* proteins in soils in the present study; on the other hand, the planting density in our pot experiment was larger than that in the field condition which might contribute to gather exogenous proteins from transgenic cottons and increase their effects on soil microbial and enzyme activities. More investigations, especially in fallow stages between two cotton-growing seasons, should be considered to determine whether these effects on soil microbial properties and enzyme activities were transient.

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