Impacts of the transgenic *CrylAc* and *CpTI* insect-resistant cotton SGK321 on selected soil enzyme activities in the rhizosphere

Y.J. Zhang, M. Xie, C.Y. Li, G. Wu, D.L. Peng

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

ABSTRACT

Transgenic *CrylAc* and *CpTI* insect-resistant cotton SGK321 is widely adopted for many years in several regions of China, however the understanding of its potential effects on soil enzyme activities is not studied. The impacts of transgenic cotton SGK321 on dehydrogenase, urease and phosphatase activities in rhizosphere soil were investigated in a two-year field study in Northern China. Rhizosphere soil enzyme activities between transgenic cotton SGK321 and its non-transgenic parental cotton Shiyuan 321 were found to differ at senescence. However compared to the plant growth stages and cotton cultivar, the impacts of the transgenic trait were minor or transient. The principal component analysis also showed no significant or minor difference in the activities of dehydrogenase, urease and phosphatase in the rhizosphere soil of transgenic cotton SGK321 and its counterpart. Our results indicated that the transgenic cotton SGK321 has no apparent impact on dehydrogenase, urease and phosphatase activities in rhizosphere soil.

Keywords: toxic proteins; dehydrogenase; urease; phosphatase

Soil enzymes play an essential role in catalyzing reactions of organic matter decomposition and nutrient cycling, involved in energy transfer, environmental quality and crop productivity, and often are used as indices of microbial activity and soil fertility (Karaca et al. 2011). Previous studies showed that the *Bt* proteins bind rapidly onto the surface-active particles in soil and become less accessible to microbial degradation but retain insecticidal activity (Vettori et al. 2003, Stotzky 2004). If these toxin proteins accumulate in soil, they may influence soil biological processes and microbial community. Some studies have showed the significant accumulation and persistence of active *Bt* toxins in soil of the transgenic *Bt* corn but not of the transgenic *Bt* cotton (Saxena and Stotzky 2000, Saxena et al. 2004). On the other hand, the transgenic *Bt* cotton instead of transgenic *Bt* corn resulted in the significant accumulation and persistence of active *Bt* toxins in soil (Sun et al. 2007, Gruber et al. 2012). For the selected enzyme activities, most of studies did not find a significant difference between the transgenic *Bt* cotton or rice and their counterparts (Shen et al. 2006, Sun et al. 2007, Liu et al. 2008).

Transgenic cotton SGK321, expressing both *Cry1Ac* and *CpTI* toxic proteins, was introduced in 1999 and was largely planted in Northern China (Guo et al. 1999). The content of *Bt* toxin in rhizosphere soil of SGK321 was reported to be higher

than its non-transgenic parental cultivar during the mid- period of cotton developmental growth, and then returned to the level of its non-transgenic parental cultivar with the senescence of cotton (Rui 2005). The understanding about impacts of the transgenic cotton SGK321 on soil biological processes is limited, especially soil enzyme activities. In this study, we evaluated the effects of the transgenic cotton SGK321 on soil enzyme activities over a period of two years during 2010–2011 in Northern China under field conditions.

MATERIAL AND METHODS

Plant materials. We used the transgenic cotton SGK321 (that expresses both *Cry1A* and *CpTI* gene) and its non-transgenic parental cotton Shiyuan 321, and a standard control cotton Simian 3. All cotton seeds were provided by the Institute of Plant Protection, Chinese Academy of Agricultural Sciences.

Field design and sampling. Field trials were conducted during 2010–2011 on the experimental farm of the Institute of Plant Protection of the Chinese Academy of Agricultural Sciences, located at Cuizhuang town (39°30'N, 116°36'E), Langfang, Hebei province, China. The study area falls under the North Temperate Zone with a continental monsoon climate, with 11.8°C and 570.3 mm annual temperature and rainfall, respectively. The soil is a clay loam type 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. This field was previously planted with the conventional maize for 15 years. Since 2005, the field had been in cultivation under the transgenic insect-resistant cotton Monsanto's NC 33B expressing *Cry1Ac* prior to be used for the present study in 2010–2011. The cotton plants were grown in a randomized block design in triplicates (each plot 6 m × 10 m) per cultivar. Seeds of all the cotton cultivars were sown on May 7, 2010 and May 11, 2011, respectively. The cotton growing season extended from May to November annually. After cotton crop harvest, the field was fallow between November to next April. Cotton was maintained in accordance with typical 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. Sampling was carried out at six crop growth stages of the plants each year from 2010 to 2011, namely seedling, budding, full flowering, boll formation, boll opening and senescence. For each sampling, rhizosphere soils from five randomly selected plants per plot were mixed and used as a composite sample. Plant and root residues were removed from soil samples with forceps, followed by sieving (2-mm mesh size). Soil sample were frozen and stored at –20°C until further analysis.

Soil enzymatic assays. Soil dehydrogenase activity was analyzed by the reduction of triphenyl tetrazolium chloride (TTC) to triphenyl formazan (TPF) as described by Guan (1986). Five grams of fresh soil was incubated for 24 h at 37°C in 5 mL of a TTC solution (5 g TTC in 0.2 mol/L Tris-HCl buffer, pH 7.4). Two drops of concentrated sulfuric acid were added immediately after the incubation to end the reaction. The sample was then blended with 20 mL of methanol and shaken for 1 h at 200 rpm, followed by filtering to extract TPF. The optical density of the filtrate was measured at 485 nm in a UV-4802 double beam UV/Vis spectrophotometer (Unico, Shanghai, China). Soil dehydrogenase activity was expressed as the μg TPF/g dry soil/24 h.

Soil urease activity was analyzed by the method described by Guan (1986). Five grams of dry soil was carefully transferred into a 25 mL measuring flask and 1 mL of toluene was added. After standing for 15 min, 10 mL of 10% urea solution and 20 mL of citric acid buffer (pH 6.7) were incorporated. The soil sample and the added solutions were mixed evenly and incubated at 37°C for 24 h, followed by filtering. Three milliliter of the filtrate was mixed with 20 mL of distilled water, 4 mL of 12.5% (w/v) potassium sodium tartrate and 3 mL of hypochlorous acid sodium solution (0.9% active chlorine). Color was allowed to develop for 20 min, then the solution was diluted to 50 mL and the absorbance of the diluted solution was measured at 578 nm in a UV-4802 double beam UV/Vis spectrophotometer. Soil urease activity was expressed as the mg NH_4^+-N/g dry soil/24 h.

Soil neutral phosphatase activity was analyzed by the disodium phenyl phosphate method described

by Guan (1986). Five grams of dry soil was carefully transferred into a 100 mL measuring flask and 2.5 mL of toluene was added. After standing for 15 min, 0.05 g of disodium phenyl phosphate and 20 mL of 1 mmol citric acid buffer (pH 7.0) were incorporated. The soil sample and the added solutions were mixed evenly and incubated at 37°C for 24 h. Then, 100 mL of 0.3% aluminum sulfate solution was added and filtered. Three milliliter of the filtrate was transferred into another 25 mL measuring flask, and 5 mL of 0.0625 mmol boric acid buffer (pH 9.6) and 2.5 mg of 2,6-dibromo-quinone chloride ($C_6H_2Br_2ClNO$) were added and carefully mixed. Color was allowed to develop for 30 min, then the solution was diluted to 50 mL and the absorbance of the diluted solution was measured at 660 nm in a UV-4802 double beam UV/Vis spectrophotometer. Soil neutral phosphatase activity was expressed as the mg phenol/g dry soil/24 h.

Data analysis. The dehydrogenase, urease, and neutral phosphatase activities were expressed as the means ± standard deviation. The statistical differences among the data were determined by the Fisher's least significant difference (*LSD*) test at the 5% significance level. The principal component analyses (PCA) for effects of the cultivar or growth stages on selected soil enzymatic activities were conducted. All statistical analyses were performed with SPSS 19.0 (SPSS Inc., Chicago, USA).

RESULTS AND DISCUSSION

The activities of dehydrogenase, urease and phosphatase in the rhizosphere soil of the same cotton among different growth stages were different in two successive years (Figures 1–3). PCA analyses showed that the plant growth stage was the strongest explanatory factor for the differences in enzymatic activities in the rhizosphere soil (Figure 4). There were no significant differences in the activities of urease in the rhizosphere soil between the transgenic cotton SGK321 and its non-transgenic parent at all the growth stages, however significant differences in dehydrogenase and phosphatase activities at senescence were observed (Figures 1 and 3). PC scores of the transgenic cotton SGK321 were significantly higher or lower than its non-transgenic parent (Figure 4). However compared to the cropping year, plant growth stages and cotton cultivars, the transgenic trait had no or minor significant effect on dehydrogenase, urease and phosphatase activities in the rhizosphere soil. As far as we know, there are no reports of the impacts of the double-gene transgenic cotton SGK321 on soil enzymatic activities, but several reports of mono-transgenic *Bt* cotton cultivars (Shen et al. 2006, Sun et al. 2007). Shen et al. (2006) reported that the activities of urease, phosphatase, dehydrogenase in rhizosphere soil of the transgenic *Cry1A* cotton
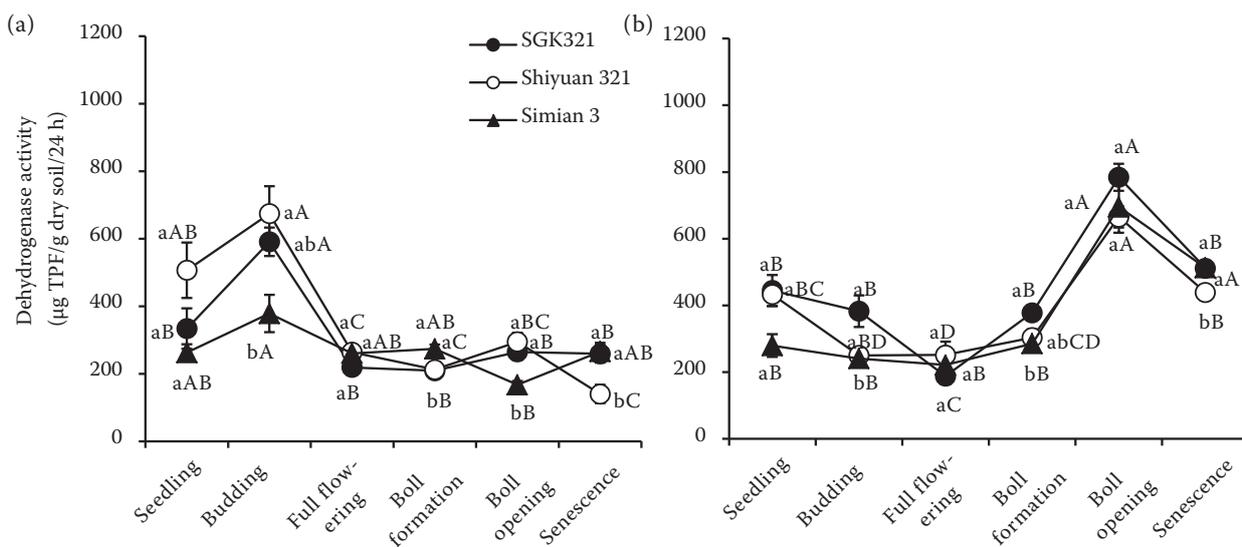

Figure 1. Dehydrogenase activity in the rhizosphere of transgenic cotton SGK 321; non-transgenic parental cv. Shiyuan 321, and conventional cv. Simian 3 at different crop stages in 2010 (a) and 2011 (b). Significant differences between cotton or growth stages are indicated by lowercase or uppercase letters (*LSD*, *P* < 0.05). TPF – triphenyl formazan
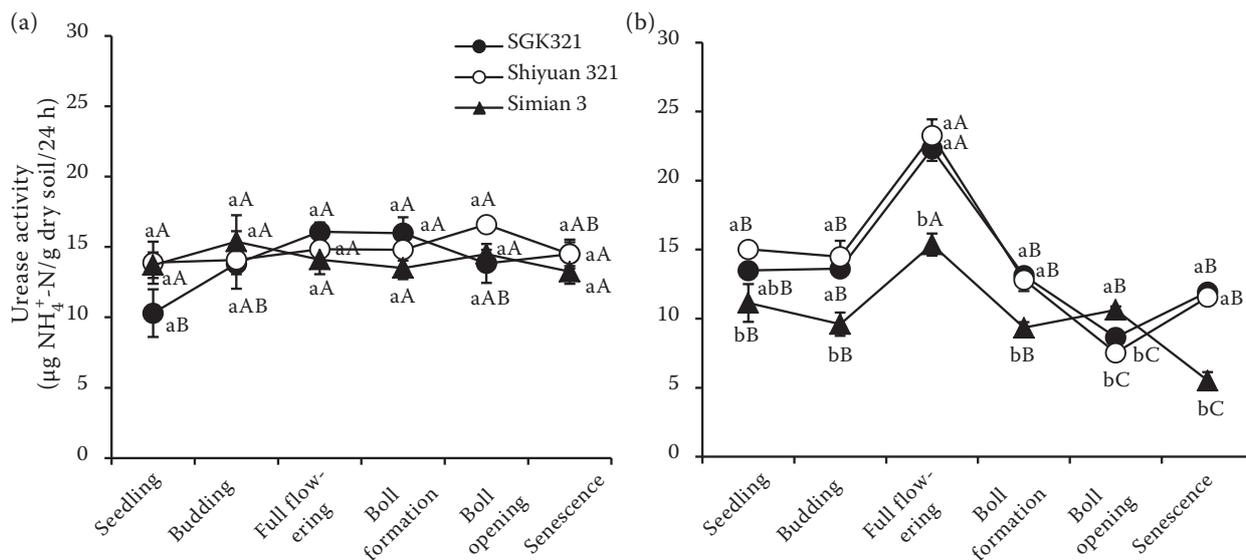
Figure 2. Urease activity in the rhizosphere of transgenic cotton SGK 321, non-transgenic parental cv. Shiyuan 321; and conventional cv. Simian 3 at different crop stages in 2010 (a) and 2011 (b). For explanations see Figure 1

Sukang 103 were not significantly different from its non-transgenic parental cotton at any of growth stages and after the period of harvest. Although the tested transgenic cotton cultivar in our study had two different introduced genes, our results also showed minor effect of transgenic cotton on the selected enzyme activities, consistent with the results obtained by Shen et al. (2006). However, the activities of soil urease were stimulated by the addition of transgenic *Cry1Ac* cotton tissues (Sun et al. 2007). Sun et al. (2007) thought that cotton tissue probably stimulated microbial activity in soil, and as a consequence, enzyme activities of soil were generally increased. Experimental methods

or technologies, used in the soil microbial ecology, are various and discriminated, which can reflect the status of soil microbial ecology from different sides. However most of researches revealed no or minor effect of *Bt* traits on soil microbial communities (Zhang et al. 2013). No significant differences of the number of culturable bacteria, fungi or actinomycetes in rhizosphere soil between the *Bt* and non-*Bt* corn or cotton were found by counting colony forming units (CFU) (Saxena et al. 2002, Li et al. 2011). There were no significant differences in rhizosphere soil microbial community structure and diversity between *Bt* and non-*Bt* crops using the Biolog system (Shen et al. 2006),
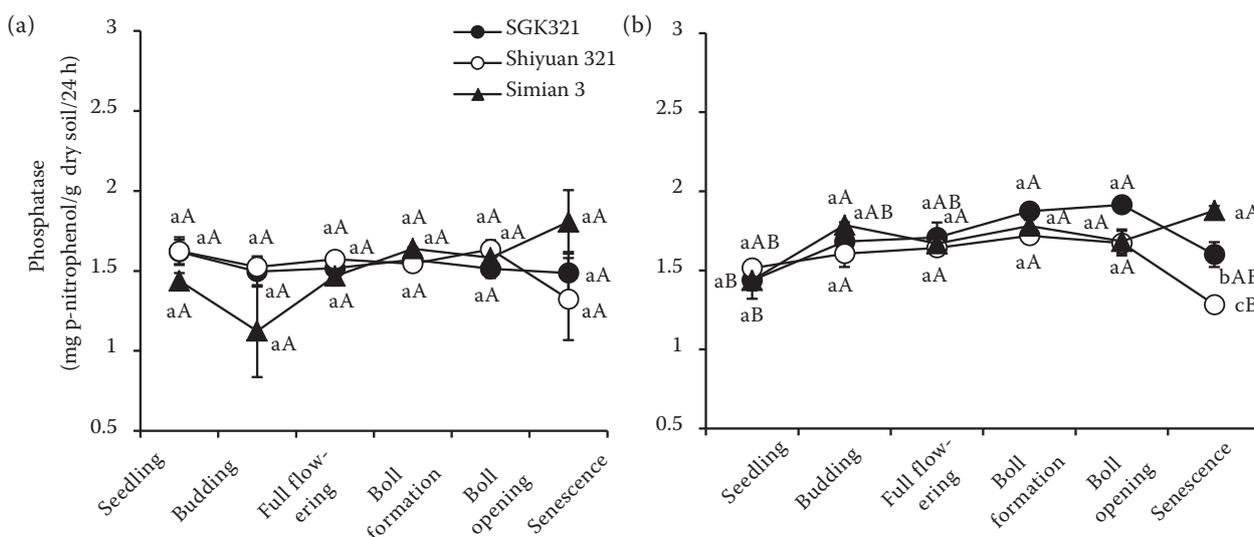

Figure 3. Phosphatase activity in the rhizosphere of transgenic cotton SGK 321; non-transgenic parental cv. Shiyuan 321, and conventional cv. Simian 3 at different crop stages in 2010 (a) and 2011 (b). For explanations see Figure 1
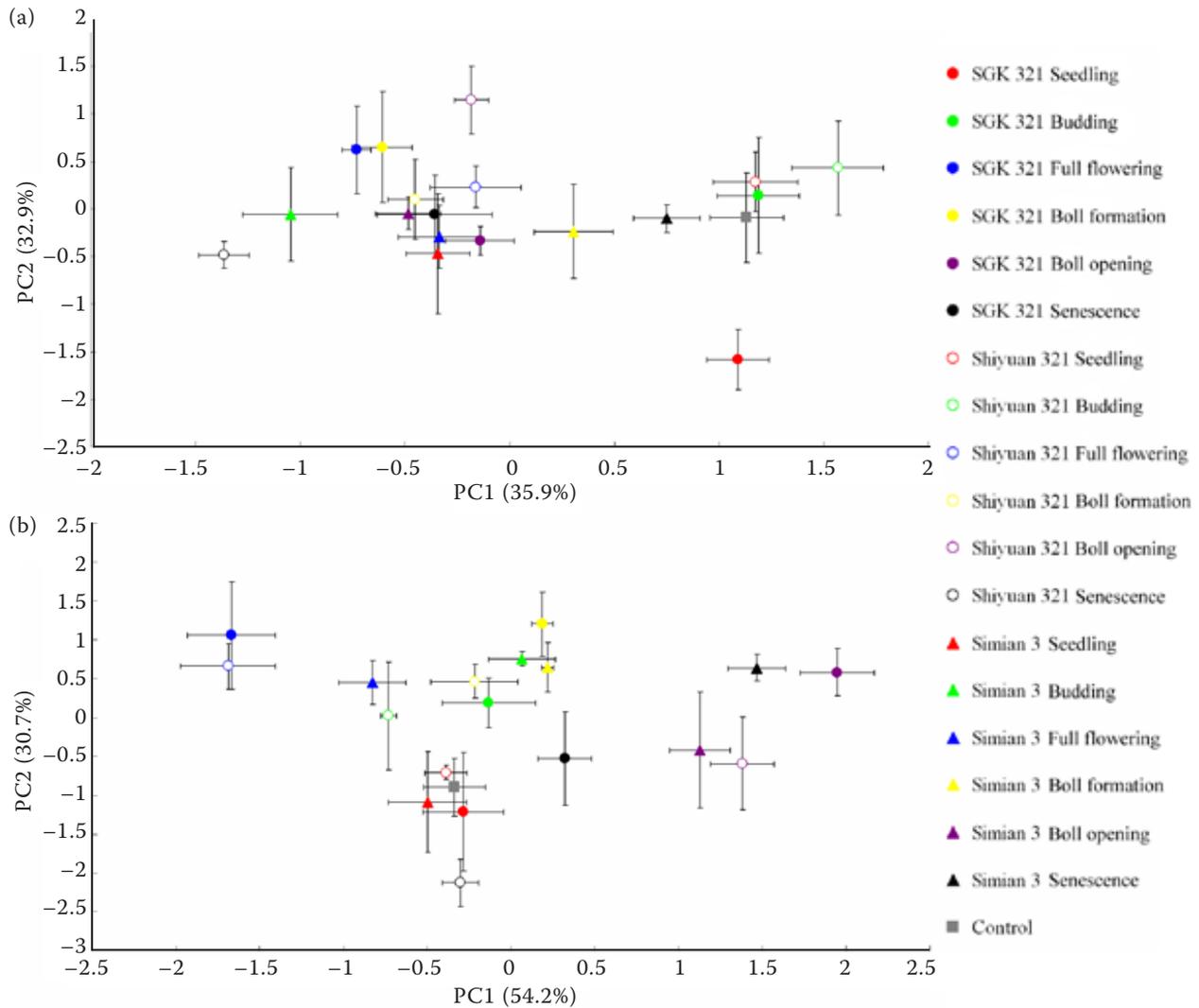
Figure 4. Principal component analysis of enzymatic activities from rhizosphere soil of the transgenic cotton SGK 321, its non-transgenic parental cotton cv. Shiyuan 321 and conventional cotton cv. Simian 3 at different stages in 2010 (a) and 2011 (b). Pre-cropping samples are used as control. The level of variation explained by each principal component is indicated in parentheses

phospholipid fatty acid analysis with ^{13}C labeling (Wu et al. 2009), denaturing gradient gel electrophoresis (Na et al. 2011), terminal restriction fragment length polymorphism (Hannula et al. 2012), next generation sequencing (Barriuso et al. 2012). Only one report stated that the structures of soil bacterial communities of transgenic *Bt* maize, as determined by the single strand conformation polymorphism (SSCP), showed a significant difference (Baumgarte and Tebbe 2005).

In conclusion, the transgenic cotton SGK321 had no evident effects on the selected enzyme activities in rhizosphere soil. However, the results presented here should be considered preliminary because the enzyme activities are only one aspect of soil

microbial ecology. Therefore, more work has to be done using more recent and fine technologies to further evaluate the effects of the transgenic cotton SGK321 on the soil microbial community structure and function.

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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, Beijing, 100 081, P.R. China
phone/fax: + 86 10 8210 9568, e-mail: xiem406@126.com