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Article

Effects of Bt+CpTI transgenic cotton on the performance of *Tetranychus turkestani* (Acari: Tetranychidae)

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Abstract

Transgenic cotton is very effective in controlling targeted pests such as cotton bollworm (*Helicoverpa armigera*). However, increases in spider mite (Acari: Tetranychidae) populations have been reported in fields of transgenic cotton. The objectives of our laboratory experiments were (i) to determine host plant preference (transgenic or non-transgenic cotton) of *T. turkestani* females and (ii) to compare the life table parameters of *T. turkestani* females reared on transgenic or non-transgenic cotton. Enzyme-linked immunosorbent assays indicated that *T. turkestani* females reared on transgenic cotton leaves contained 14.9±0.23 µg Bt toxic protein per gram fresh weight, about 57.4% toxin in the transgenic cotton leaves. Results of dual-choice disc tests showed that *T. turkestani* females preferred to feed and lay eggs on non-transgenic cotton. Food source (transgenic or non-transgenic cotton) had no significant effect on the life table parameters of *T. turkestani* females. We conclude that increases in the population of spider mites in fields of transgenic cotton cannot be attributed to host plant preference or to the effects of Bt toxic protein on the non-targeted arthropods life cycle. Additional studies should be done to determine if the increases are due to less insecticide application or less competition with primary insects in transgenic cotton fields.

Key words: Transgenic cotton, ELISA, Host plant preference, Life table

Introduction

Spider mites are one of the most destructive herbivorous pests in cotton field worldwide (Luttrell *et al*. 1994). The mites generally feed on the lower surface of cotton leaves by piercing mesophyll cells with their stylets. Yellowish-white spots appear on damaged leaves shortly after infestation. Several days later, reddish brown patches form as the number of spots increases. Finally the cotton leaves begin to roll. Mite injury always causes a reduction in leaf chlorophyll content, resulting in severe declines in cotton yield and quality (Park and Lee, 2002). In northwest China, the spider mite population consists of several species, including *Tetranychus turkestani* (Ugarov et Nikolskii), *Tetranychus truncatus* Ehara, *Tetranychus dunhuangensis* Wang, and *Tetranychus cinnabarinus* Boisduval. Among these species, *T. turkestani* is a damaging and important pest (Guo *et al*. 2013), also it causes the most damage to cotton in the northern part of the Xinjiang Uyghur Autonomous Region (Lu, 1990). Spider mite populations have increased since the widespread adoption of transgenic cotton (Wu and Guo, 2005; Zhao *et al*. 2011). In some cases, spider mites have become the primary pest of transgenic cotton.

Transgenic cotton expressing Bt toxic protein such as Cry1Ab and Cry1Ac derived from *Bacillus thuringiensis* has been effectively used to control targeted pests such as cotton bollworm (Shelton *et al*. 2002; Cattaneo *et al*. 2006; Yves *et al*. 2006; Wu *et al*. 2008; Tabashnik *et al*. 2010; Hutchison *et al*. 2010). As a result, total pesticide expenditures by farmers have declined by 87% (Liu and Huang, 2012) since 1997 when transgenic cotton was first commercially planted in China. The planting area of transgenic cotton has dramatically increased with time. Now there is growing concern that transgenic cultivars may threaten non-targeted organisms, including other pests (Li *et al*. 2010; Mota *et al*. 2013) or beneficial insects.

Many field and laboratory studies have been conducted to evaluate the potential effects of genetically modified crops on non-targeted insects. Enzyme-linked immunosorbent assay (ELISA) has detected Bt toxic protein in both herbivorous pests and their natural enemies (Alvarez-Alfageme *et al.* 2008; Torris and Ruberson. 2008; Li *et al.* 2011; García *et al.* 2012; Tian *et al.* 2013). A resurgence of secondary pests such as aphids, spider mites, and mirid bugs has also been reported in transgenic crops (Wu *et al*. 2002; Lu *et al*. 2010; Zhao *et al*. 2011). The effect of transgenic crops on the life histories of non-targeted insects has been observed under laboratory conditions, but the results are inconsistent. Most studies indicated that transgenic crops had no adverse effects on either *Aphis gossypii* or *Adelphocoris suturalis* (Lawo *et al*. 2009; Li *et al*. 2010). Beneficial insects such as predatory mites (Obrist *et al*. 2006; de Castro *et al*. 2012), green lacewings (*Chrysoperla carnea)* (Romeis *et al*. 2004; Li *et al*. 2008) and beetles (Ferry *et al*. 2007) were also unaffected. In contrast, some researchers observed high mortality rates among the larvae of ladybird beetle (*Adalia bipunctata*) reared on diets with Bt toxic protein (Hilbeck *et al*. 2012).

Spider mites are not only one of the most significant cotton pests but also the prey for many insect species. It is important to determine whether transgenic cotton has any effect on this non-targeted pest. The objectives of this laboratory study were (i) to determine whether the spider mite (*T. turkestani*) has host preference or oviposition preference for transgenic cotton and its corresponding isogenic cultivar and (ii) to verify whether transgenic cotton affects the life table parameters of *T. turkestani*.

Materials and methods

Spider mites

A colony of *T. turkestani* was started in the laboratory using individuals originally obtained from a cotton field near Huayuan, Shihezi City, Xinjiang Province, P. R. China in 2010. The colony was maintained on young, potted sword bean (*Semen canavaliae* Gladiatae) plants in a climate chamber. The temperature in the chamber was 28±1°C, the relative humidity was 70±10%, and the photoperiod was 16 h light: 8 h dark. Before they were used in the experiment the spider mites were reared on non-transgenic cotton for several generations.

Plant material

The transgenic cotton cultivar ‘SGK321’ and its corresponding non-transformed isoline, ‘Shiyuan321’, were used in this experiment. ‘SGK321’ is obtained by transforming Shiyuan 321 with Cry1Ac+CpTI (Guo, 1995). Both Cry1Ac and CpTI can express toxic proteins targeted for Lepidoptera pests. These cultivars were provided by the Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China.

The two cotton cultivars were sown in experiment stations of Shihezi University in late April 2013, and maintained with standard agronomic practices of the region, but no pesticides were applied to the plants. A new batch of cotton was planted each week to ensure that there were enough plants at the 4–8 leaf stage for the experiment.

Bt-toxin detection

Transgenic cotton and its near isoline cotton leaves were collected when the cotton plants reached the 4–8 leaf stage. Adult *T . turkestani* reared with cotton leaves at the 4–8 leaf stage were also collected. The leaves and spider mites were put into separate microreaction tubes and stored at -20 ºC before ELISA tests.

The concentration of Bt toxic protein in the leaves and spider mites was measured using a double antibody sandwich enzyme-linked immunosorbent assay from Agdia (Elkhart, IN, USA). Before conducting the ELISA tests, 1g leaf sample or 40-50 mg of spider mites material were ground in 1× phosphate-buffered saline, 0.1% Tween 20 (PBST) wash buffer at a 1:10 ratio (w/v) and then centrifuged for 15 min at 12000 g. The supernatants were used in the ELISA tests according to the instructions of the manufacturer.

Host preference and oviposition preference test

To prepare the dual-choice disc tests, we used a 30 mm diameter punch to cut round discs from SGK321 and Shiyuan321 leaves collected when plants were at the 4–8 leaf stage. Each disc was then cut in half. One half-disc of SGK321 and one half-disc of Shiyuan321 were put side by side on filter paper in 9 mm petri dishes. Moist sponges beneath the filter paper kept the filter paper damp. Four *T .turkestani* females were transferred onto each half disc. The position of both the spider mites and their eggs were recorded every 12 hours for three days. The eggs were removed from the discs after each observation. *T .turkestani* females that were dead, missing, or at the boundary between the two half discs were not included in the count. The experiment was conducted at a temperature of 28±1 ºC, a relative humidity of 75%±5%, and a photoperiod of 16 h light: 8 h dark. There were 30 replications in this experiment. The relative position of the SGK321 and Shiyuan321 discs (i.e., left or right side) was alternated between replications.

Laboratory performance of *T. turkestani* on different cotton cultivars

Development of *T. turkestani* on different cotton cultivars

Flat leaves were collected from SGK321 and Shiyuan321 plants and then washed with water. The leaves were put upside down on filter paper in 90 mm diameter petri dishes. Moist sponges beneath the filter paper kept the paper damp. The edges of the leaves were surrounded by strips of wet cotton to prevent the escape of the spider mites. Each leaf was divided into four rearing units using wet cotton strips. One female *T. turkestani* was transferred into each unit. The spider mites and all the eggs except one were removed 12 h later. 120 replications were set for each cotton cultivar. The development of each egg was observed every 24 h until the larvae reached the adult stage. The cotton leaves were replaced every several days to ensure that the leaves were fresh.

Survival and reproduction of *T. turkestani* on different cotton varieties

The sex of the spider mites was determined within 24 h of reaching the adult stage. Males and females were paired and placed together on one quarter of a cotton leaf as described above. The pairs were checked every day and the following parameters were recorded: pre-oviposition period, daily fecundity, post-oviposition period, and survival rate. Eggs were removed each day. In total, we observed 47 *T. turkestani* pairs reared on SGK321 cotton and 37 *T. turkestani* pairs reared on Shiyuan321.

The experiment was conducted in climate chambers to ensure that spider mites were reared under stable conditions. The experiment was conducted at a temperature of 28±1 ºC, a relative humidity of 75%±5%, and a photoperiod of 16 h light: 8 h dark.

Statistical analyses

An age-specific female life table was constructed according to the method developed by Zhao and Zhou (1984). Survival rate (l_x , percentage of spider mites still living at age x) and daily fecundity (m_x, mean number of female eggs laid per adult female at age x) were calculated based on the number of female individuals. Population parameters were calculated according to the following formulae:

$$Ro=\sum l_x m_x$$
$$r_m=\ln Ro/T$$
$$T=\sum(x l_x m_x)/\sum l_x m_x$$
$$Dt=\ln 2/r_m$$
$$\lambda=\exp(r_m)$$

where R_o is the net reproductive rate; r_m is the intrinsic rate of increase; λ is the finite rate of increase; T is the mean generation time; and Dt is the doubling time.

In the calculation of population parameters, 120 individuals were divided into four groups. The homoscedasticity of variance for each parameter was verified before performing *T*-tests using SPSS 17.0. The difference in survival rates between *T. turkestani* reared on SGK321 cotton and Shiyuan321were compared using Kaplan-Meier method and analyzed with log-rank test in SPSS. The m_x were analyzed using Microsoft Excel software.

In the host preference and oviposition preference test Wilcoxon signed rank test was adopted to analyze data of both series of experiments.

Results

Bt-toxin concentrations in cotton and *T.turkestani*

The concentration of Bt toxic protein in the transgenic cotton leaves averaged 26.0±0.77 µg/g fresh weight (Fig. 1). The concentration of Bt toxic protein in *T .turkestani* reared on transgenic

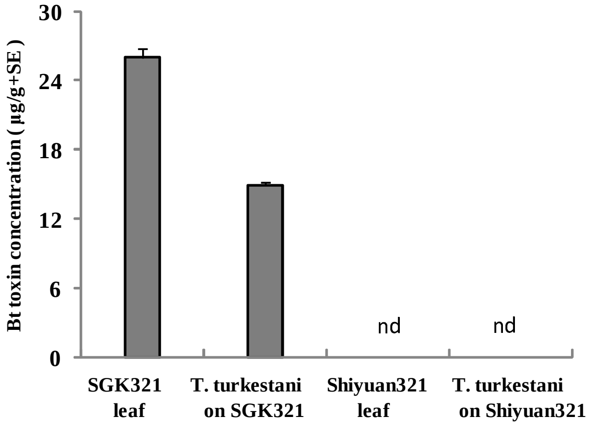

FIGURE 1. Concentration of Bt toxic protein (mean±SE) in transgenic (SGK321) cotton, in its corresponding non-transformed isoline (Shiyuan321), and in *T .turkestani* females reared on the leaves of these two cultivars. nd: not detectable.

cotton leaves averaged 14.9±0.23 µg/g fresh weight, 42.6% less than in cotton. No Bt toxic protein was detected in the non-transgenic cotton leaves or in *T .turkestani* reared on non-transgenic cotton leaves.

Feeding preference of *T. turkestani*

There was no significant difference in the percentage of *T. turkestani* females on leaf discs of transgenic or non-transgenic cotton during the first 24 h of the dual-choice disc tests (Fig. 2). In contrast, a larger percentage of spider mites was observed on leaf discs of non-transgenic cotton 36 to 72 h after the start of the tests. The differences were significant at 36, 48, and 72 h (*T*-tests, $P<0.05$). At 60 h, number of spider mites found on Shiyuan321 (mean ± SD) were also higher than that of SGK321, though no significant difference was seen. There is a tendency that more and more spider mites preferred to live on the Shiyuan321 as the time passed by.

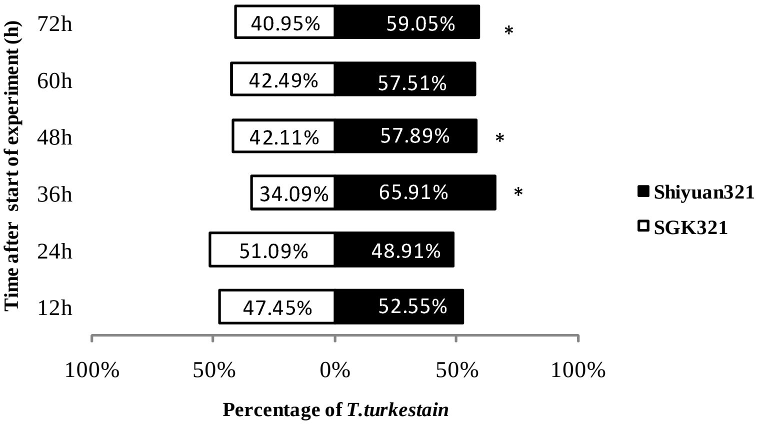

FIGURE 2. Percentage of *T. turkestani* females observed on leaf half-discs of transgenic (SGK321) cotton and its corresponding non-transformed isoline (Shiyuan321) during a 72 h trial. Asterisks indicate that the percentage of *T. turkestani* females were significantly different on Shiyuan 321 than on SGK321 ($P<0.05$).

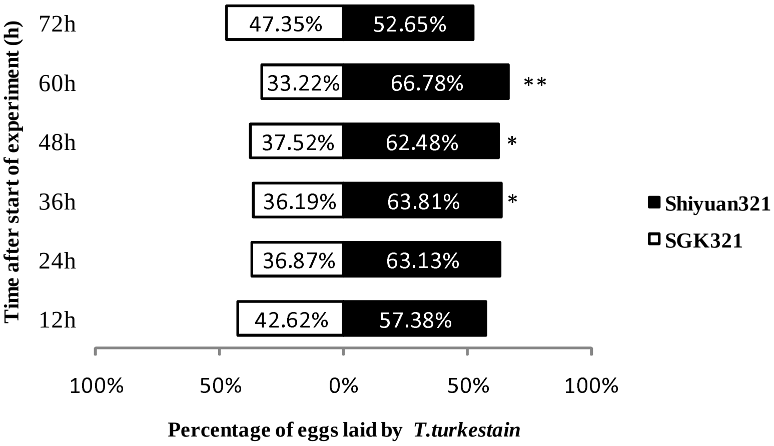

FIGURE 3. Percentage of *T. turkestan* eggs on leaf half-discs of transgenic (SGK321) cotton and its corresponding non-transformed isoline (Shiyuan321) cotton during a 72 h trial. Single and double asterisks indicate that the percentage of *T. turkestani* eggs were significantly different on Shiyuan321 than on SGK321 at the $P<0.05$ and $P<0.01$ levels of signficance.

Oviposition preference of *T. turkestani*

The percentage of *T. turkestani* eggs on leaf discs of non-transgenic cotton was always greater than the percentage of eggs on leaf discs of transgenic cotton (Fig. 3). The differences in oviposition were not significant at 12 h (*P*=0.656) or 24 h (*P*=0.054). *T. turkestani* showed significant oviposition preference from 36 to 60 h after the start of the test. Furthermore, the differences in oviposition were highly significant at 60 h (*P*=0.001). These results indicate that number of *T. turkestani* females who chose to lay eggs on non-transgenic cotton leaves increased with time.

Life table parameters of *T. turkestani*

No significant differences were observed in the developmental parameters (i.e., eggs, larvae, protonymph, deutonymph, larva-adult, generation and adult) of *T. Turkestan* (Table 1, P>0.05). The fecundity parameters (pre-oviposition, oviposition, post-oviposition, and adult longevity) of *T. turkestani* females reared on transgenic or non-transgenic cotton were also similar (Table 2, P>0.05). Furthermore, no significant differences were observed in the total number of eggs per female or in the number of eggs per female per day (Table 2, P>0.05). For these reasons, it was not surprising that the population parameters (i.e., R_0, T, r_m, D_t, and λ) of *T. turkestani* females reared on SGK321 were not differ from those reared on Shiyuan321.

TABLE 1. Developmental period parameters (mean±SE) of *T. turkestani* females reared on transgenic cotton (SGK321) and its corresponding non-transformed isoline (Shiyuan321). Data are mean±SE. n is the number of *T. turkestani* at each life stage. P values were obtained by independent *T*-tests (*P*<0.05).

Parameter	Shiyuan321	n1	SGK321	n2	*P-values*
Egg	4.09±0.03	96	4.14±0.04	86	0.311
Larvae	2.15±0.04	93	2.03±0.04	77	0.094
Protonymph	1.36±0.06	84	1.49±0.06	73	0.127
Deutonymph	1.87±0.06	77	1.87±0.05	68	0.976
Larva-adult	9.36±0.15	77	9.45±0.12	68	0.639
Generation	10.72±0.12	73	10.50±0.09	66	0.151
Adult	16.04±0.70	47	15.81±0.84	37	0.829

TABLE 2. Fecundity parameters (mean±SE) of *T. turkestani* reared on transgenic cotton (SGK321) and its corresponding non-transformed isoline (Shiyuan321). Data are mean±SE. n is the number of *T. turkestani* females at each life stage. P values were obtained by independent *T*-tests (*P*<0.05).

Parameter	Shiyuan321	n1	SGK321	n2	*P-values*
Pre-oviposition	1.13±0.07	75	1.01±0.07	63	0.248
Oviposition	13.26±0.56	46	14.27±0.77	37	0.278
Post-oviposition	2.68±0.30	25	1.83±0.30	12	0.090
Eggs/adult/day	6.49±0.25	47	5.92±0.24	37	0.104
Adult longevity	24.98±0.73	47	25.24±0.83	37	0.811
Total eggs	82.60±2.99	47	79.97±3.45	37	0.566

TABLE 3. Population parameters of *T. turkestani* females reared on transgenic cotton (SGK321) and its corresponding non-transformed isoline (Shiyuan321). Data are mean±SE. P values were obtained by independent *T*-tests ($P<0.05$).

Parameters	Shiyuan321	SGK321	*P-values*
Net reproductive rate (R_o)	44.03±5.83	39.15±5.88	0.578
Mean generation time (T)	16.40±0.25	16.39±0.39	0.980
Intrinsic rate of increase (r_m)	0.23±0.01	0.22±0.01	0.505
Doubling time (D_t)	3.04±0.11	3.13±0.09	0.533
Finite rate of increase (λ)	1.26±0.01	1.25±0.01	0.503

The survival rate of *T. turkestani* females reared on non-transgenic cotton was more or less the same with that of females reared on transgenic cotton,no significant difference was observed between them (Fig. 4; log-rank test, χ^2=0.951, df=1, p=0.330). The number of eggs/female/day increased from about 1 egg/female/day at the start of the oviposition period to a peak of 8–9 eggs/ female/day about one week later. The fecundity then decreased gradually for about three weeks. No eggs were observed 26 d after the start of oviposition. The fecundity of *T. turkestani* females reared on SGK321 was similar to that of females reared on Shiyuan321 (Fig. 5).

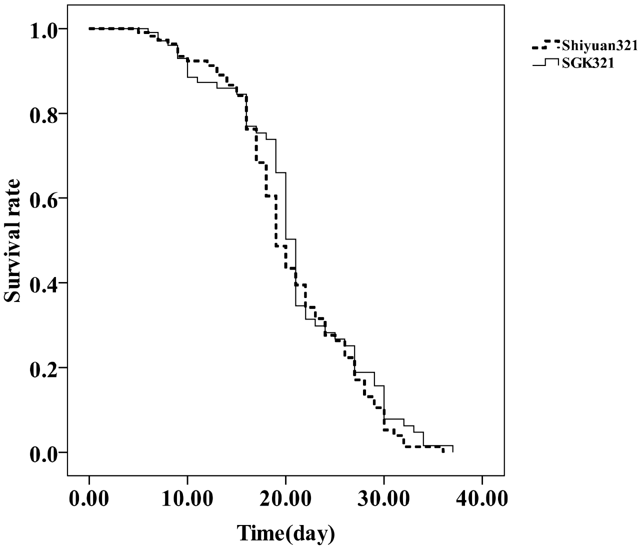

FIGURE 4. Survival rates of *T. turkestani* females reared on transgenic cotton (SGK321) and its corresponding non-transformed isoline (Shiyuan321).

Discussion

Results from the dual-choice disc tests indicated that *T. turkestani* females preferred to feed and lay eggs on non-transgenic cotton. Interestingly, these preferences were not observed until 36 h after the start of the tests. The preference increased with time, especially oviposition preference. This is consistent with the hypothesis that because insects and spider mites cannot immediately perceive host plant quality in small-scale experiments, they require feeding experience on the host plant before adjusting their preference (Egas, 2003). Previous studies reported conflicting results about the preference of insects regarding transgenic cultivars or their non-transformed isolines. For example, Rovenska (2005) demonstrated that *T. urticae* preferred transgenic eggplant (*Solanum melongena* L.) to non-transgenic eggplant. However, the same species (*T. urticae*) had greater preference for non-transgenic potato (*Solanum tuberosum* Solanaceae) (Rovenska and Zemek, 2006). This indicated that in regard to a choice between transgenic or non-transgenic plant materials, the preference of an insect and spider mites may vary depending on plant species. Bakonyi (2006) reported that *Folsomia candida* preferred non-transgenic maize (*Zea mays* L.) to Bt-maize (producing Cry1Ab toxin) as a food source, whereas *Heteromurus nitidus* and *Sinella coeca* preferred Bt-maize. This indicates that non-targeted arthropods species react differently to the same pair of transgenic and non-transgenic plants. Additional studies need to be conducted to determine if insect preference can be attributed to differences in plant morphology or secondary metabolite content after genetic transformation.

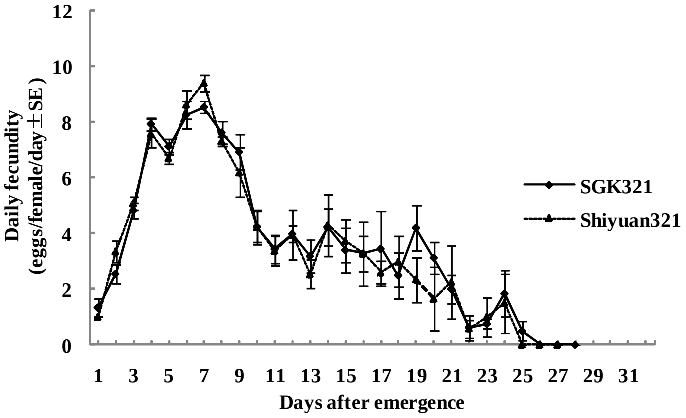

FIGURE 5. Daily fecundity (eggs/female/day, mean±SE) of *T. turkestani* females reared on transgenic cotton (SGK321) and on its corresponding non-transformed isoline (Shiyuan321) (n=37-47).

Because of their preference for non-transgenic cotton, we predicted that that *T. turkestani* females reared on Shuyuan321 would develop more rapidly and have higher survival rates than females reared on SGK321. This would be in accordance with the oviposition-preference offspring-performance hypothesis (Thompson, 1988). However, our results indicated females reared on transgenic or non-transgenic cotton showed no difference in either development or fecundity. Similarly, transgenic crops (cotton and maize) in two others studies had no effect on the development, survival, or reproduction of *T. urticae* (Esteves, 2010; Li and Romeis, 2010). Dutton (2002) and Lozzia *et al*. (2000) reported no difference in the intrinsic rate of natural increase of *T. urticae* reared on Bt or non-transgenic plants. Our results as well as most of the above studies indicated that although spider mites reared on transgenic plants had significant concentrations of Bt toxic protein, but the protein apparently had no effect on the development and reproduction of either *T. turkestani* or *T. urticae*.

Our results and those of previous studies indicated that transgenic cotton was effective in controlling targeted pests, but had no negative impact on the survival or development of non-targeted arthropods such as spider mites. Some researchers have reported that spider mite populations were 25 to 44% higher in transgenic cotton fields than in non-transgenic cotton fields (Cui and Xia, 1998; Xia, 1999; Zhao, 2011). In our study, we were surprised that *T. turkestani* females preferred non-transgenic to transgenic cotton. Bt toxic protein had no side effect on the life parameters of *T. turkestani*. In contrast, field observations indicated that spider mite populations were significantly higher in SGK321 than those in Shiyuan321. One reason might be that systemic insecticide use was lower in the fields of transgenic cotton fields. Alternatively, weak biological control by natural enemies or reduced interspecific competition between spider mites and the primary pests accounts for this phenomenon (Qiu *et al*. 2006; Yong and Ding, 2011).

T. turkestani is one of the three most important pests in cotton fields of Xinjiang Province. It is also a food source for many natural enemies including ladybird beetles, green lacewings, predatory thrips and predatory mites. As an important member in the food web, *T. turkestani* plays a vital role in the environment risk assessment of transgenic crops. In order to completely evaluate the effects of transgenic crops on *T. turkestani,* additional studies should be conducted to determine if increased spider mite populations in fields of transgenic cotton are due to reduction in insecticide application, less competition with primary insects, or weak biological control by natural enemies.

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