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Full Length Research Paper

Long-term effects of transgenic *Bacillus thuringiensis* cotton on the non-target *Aphis gossypii* (Homoptera: Aphididae) maintained for multiple generations

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The effects of transgenic *Bacillus thuringiensis* (Bt) cotton on non-target *Aphis gossypii* were assessed by comparing the life-table parameters of aphids that fed on Bt vs. non-Bt cotton cultivars for the first and 37th generations. The enzyme-linked immunosorbent assay (ELISA) method was used to detect the transmission of the Bt protein from Bt cotton to *A. gossypii* and their honeydew. We found that the life-table parameters of *A. gossypii* that fed on Bt cotton for the first and 37th generations did not differ significantly from those of the non-Bt-fed individuals. However, the Bt protein was detected by ELISA in the Bt cotton leaves, and the content varied significantly at different growth stages. Furthermore, trace amounts of the Bt protein were detected in some of the Bt-fed aphids, and the honeydew of the Bt-fed aphids contained over 10 ng/g Bt protein. These results indicate that although trace amounts of the Bt protein were ingested, the Bt cotton had no significant negative impacts on *A. gossypii* in either the short or long term.

Key words: Transgenic *Bacillus thuringiensis* cotton, *Aphis gossypii*, *Bacillus thuringiensis* detection, life-table parameters, multiple-generation, non-target effects.

INTRODUCTION

Since the first commercial release of transgenic crops containing *Bacillus thuringiensis* (Bt) genes, their use has increased substantially worldwide (James, 2011). Despite the fact that Bt sprays are considered safer and transgenic Bt crops are more friendly to natural enemies and non-target pests over conventional broad-spectrum insecticides (Hilbeck, 2001; Way and van Emden, 2000; Meissle and Lang, 2005; Naranjo, 2009; Romeis et al., 2006), there are still concerns about their potential impacts on the environment and on non-target organisms (O'Callaghan et al., 2005; Virla et al., 2010; Duan et al.,2010). In particular, the continuous expression of the insecticidal protein in most tissues of transgenic Bt plants during the growing season has raised concerns regarding the possible effects on various groups of non-target organisms with ecological and economic values (Wolfenbarger et al., 2008; Howald et al., 2003; Romeis et al., 2008; Meissle and Romeis, 2009; Li and Romeis, 2010). There have been several reports of significant quantities of Cry1Ab endotoxin present in non-target herbivores that have fed on transgenic Bt plants (Dutton et al., 2002; Harwood et al., 2005, Harwood and Obrycki 2006; Howald et al., 2003; Obrist et al., 2005, 2006; Burgio et al., 2011). Furthermore, biological modifications such as longevity (Ponsard et al., 2002) and survival, developmental times and larval weights (Vojtech et al.,

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2005) in non-target insects have been reported as a result of exposure to Bt cultivars. However, the general opinion is that Bt crops have no direct effects on non-targets (Romeis et al., 2006).

Aphids play important role in agricultural systems since they serve as prey or host to a number of predators and parasitoids and their honeydew is an important energy source for several arthropods. Analyses of the impact of transgenic plants expressing Cry toxins on aphids gave variable results ranging from minor negative effects on aphid survival and fecundity to significant beneficial effects on aphid populations (Ashouri, 2004a, b; Ashouri et al., 2001; Burgio et al., 2007; Faria et al., 2007; Lawo et al., 2009; Mellet and Shoeman, 2007; Raps et al., 2001).

The cotton aphid, *Aphis gossypii* Glover (Homoptera: Aphididae), is a key pest on cotton that causes severe damage to crops worldwide, and the reported effects of Bt cotton on *A. gossypii* are not consistent.

A previous greenhouse study addressing the performance of *A. gossypii* on Bt and non-Bt cotton plants showed a variation in some life-table parameters among three consecutive generations (Liu et al., 2005). On the other hand, Lawo et al. (2009) concluded that plant transformation did not have any influence on aphid performance and none of the aphid life-table parameters was influenced by the expression of the Bt protein; other studies comparing aphid populations in Bt and non-Bt cotton fields gave inconsistent results. Some studies recorded no difference in aphid populations (Bambawale et al., 2004; Mellet and Shoeman, 2007; Zhang et al., 2008), others found either increased (Cui and Xia, 2000; Deng et al., 2003) or decreased aphid densities (Wu and Guo, 2003) in the Bt cotton. Men et al. (2004) have indicated that the effects of Bt cotton on *A. gossypii* were inconsistent between years in the fields. It is still unclear if the Bt protein caused the observed effects or if they were caused by the used variety.

In the present study, we aimed to assess the effects of Bt cotton on the non-target *A. gossypii* with regard to its development, reproduction and life-table parameters in both the short and long term. We also quantified the levels of Bt toxin in *A. gossypii* and their honeydew using immunological tests (ELISA) to confirm the direct exposure of *A. gossypii* to Bt protein.

MATERIALS AND METHODS

Cotton cultivars and planting

Seeds of the transgenic Bt cotton cv., GK12, containing the 1824-bp GFM *Cry1A Bt* gene (fusion gene of the *Cry1Ac* and *Cry1Ab* genes) and expressing the Cry1Ab/Ac fusion protein (Guo et al., 1995) were provided by Liangshan Cotton Seed Company (Liangshan, Shandong Province, China). Seeds of Simian3, the non-transgenic parental cultivar of GK12, were provided by Siyang Cotton Raw Material Farm (SIYANG, JIANGSU PROVINCE, CHINA).

The aforementioned two cotton cultivars were planted individually in plastic pots (8 cm in diameter, 11 cm in height) with composted garden soil (garden soil : nutrient solution : vermiculite = 1 : 1 : 1) and kept in a glasshouse under the conditions of 25 ± 1°C, 60% to 70% r.h. and under a natural photoperiod. The youngest fully expanded leaves of the cotton plants at the 2-cotyledon, 4 to 5 true-leaf and boll stages were analyzed for the presence of the Bt protein (see below). The seedlings at the 2-cotyledon stage were used for the life-table parameter tests of *A. gossypii*.

Insects

The clones of the cotton aphid, *A. gossypii*, were collected from non-transgenic cotton plants in greenhouses and reared on caged cotton plants of either Simian3 or GK12 cultivar. Both populations were maintained for more than 30 generations to compare the long-term effects of Bt-cotton on *A. gossypii* in the greenhouse.

Life-table study of cotton aphids on Bt and non-Bt cotton

Using a completely randomized design, two experiments were conducted to compare the development, reproduction and other life-table parameters of *A. gossypii* feeding on GK12 and Simian3.

The 1st generation experiment

Forty adults were randomly selected from Simian3 and were individually transferred to GK12 seedlings using a fine paintbrush. After 12 h, one newly born nymph was left on each cotton seedling. Forty cotton seedlings were randomly and evenly allocated into two cages (0.6 × 0.50 × 0.50 m, constructed of a metal frame and a fine-mesh cover), which were kept in the laboratory at 25 ± 1°C, 60 to 70% r.h. and under a natural photoperiod. The aphids were checked daily for survival and development until the start of their reproductive cycle. The number of offspring produced by each aphid was then recorded daily, and all of the nymphs produced were removed. Observations were continued until the adults died. As a control, aphids feeding on non-transgenic cotton cv. Simian3 were observed in the same manner.

The multiple-generation experiment

Following 36 generations of *A. gossypii* on GK12, the reproducing adults were transferred to the seedlings of GK12, and the same procedures as described above were conducted. Both experiments were replicated three times.

Bt toxin protein analysis

Sample collection and preparation

The leaves of each cotton cultivar were analyzed to verify the presence of the Bt protein. For each growth stage (the 2-cotyledon, 4 to 5 true leaf and boll stage), 20 leaves were collected. To quantify the Bt protein in *A. gossypii*, samples consisting of different quantities of adults (10, 100, 200 or 600 individuals) were collected and kept in 1.5-ml centrifuge tubes. For each sample, 5 to 10 batches were collected and the weight was quantified (for details, Table 2).

To collect the aphid honeydew, 20 cotton plants (both the GK12 and Simian3 cultivars) at the 2-cotyledon stage were arranged with a clip-cage on each plant. The clip-cage was made of two plastic rings with a sponge on one edge to prevent the plant tissue from

being damaged and gauze on the other edge to prevent the escape of the insects; 20 apterous *A. gossypii* adults were present in each cage. A pre-weighed sheet of silver paper (0.035 m in diameter) was placed on the bottom of each clip-cage for collection of the honeydew. After 10 days, the silver papers were reweighed, and the honeydew collected was dissolved in 120 µl of extraction buffer. All of the samples were kept at -20°C for less than one month before the ELISA analysis, upon which the samples were weighed with an electronic balance (Sartorius, Germany, BS224S, max = 220 g, d = 0.1 mg), frozen in liquid nitrogen, homogenized immediately with extraction buffer and centrifuged for analysis.

Enzyme-linked immuno-sorbent assay (ELISA)

The Bt-Cry1Ab/Cry1Ac PathoScreen Kit (catalog # PSP 06200) was used to determine the Bt toxin concentration, and the spectrophotometric measurements were performed using a microtiter plate reader (BIO-RAD MODEL 680) at 650 nm. For protein extraction, Bt leaves material and aphid samples was frozen in liquid nitrogen in 1.5 ml centrifugal tube, grinded with a mortar pestle match 1.5 ml centrifugal tube. The 1×PBST (provided in the kit) was added to the samples at a ratio of at least 1:10 (g sample: ml buffer) (Table 2). The glass rod was washed with the buffer in the end. The homogenate was then centrifuged at 15,000 × g for 15 min at 4°C and the supernatant was transferred into a new tube. For ELISA, 100 µL of the protein extract was used. The ELSA was conducted following the instructions of the manufacturer. Results of the assay were visualized with a color development step. Each sample colour was spectrophotometrically measured, thus obtaining an optical density (OD) for each sample. The absorbance values of the negative controls were subtracted from the values of the test wells. If the result was positive, this sample was entered into the calculation using a standard curve (provided in the kit) to extrapolate the concentration of the Bt protein, which was converted into ng/g for the concentration of Bt protein contained in each sample by multiplying by the dilution factor.

Data analyses

The life-table parameters were calculated using the equations:

$$R_0 = \sum l_x m_x \quad (1)$$

$$T = \sum x l_x m_x \ / \ \sum l_x m_x \quad (2)$$

$$r_m = (\ln R_0) / T \quad (3)$$

$$I = S_{L1} \times S_{L2} \times S_{L3} \times S_{L4} \times S_A \times P \quad (4)$$

$$t = \ln 2 / r_m \quad (5)$$

$$\lambda = e^{r_m} \quad (6)$$

In the above equations, the age-specific survival rate (l_x) is the proportion of individuals in initial cohort alive at age x time (day), and the age-specific fecundity (m_x) is the mean number of female progeny produced per female alive at the age interval x day. S_{L1}…S_{L4}, S_A is the survival rate of the 1st to the 4th instar larvae and normal adults, respectively and P is the fecundity per adult. R_0 is the net reproductive rate; T is the average generation lifespan; r_m is the intrinsic rate of natural increase; I, is the population growth exponents; t, is the time of population doubling and λ is the finite rate of increase.

For the Bt vs. non-Bt cotton, the data were expressed as the means ± SEM. To detect significant differences of the life-table parameters of *A. gossypii* due to the different feeding treatments and Bt protein concentration in the different plant tissues of GK12 and Simian3, analysis of variance (ANOVA) and a subsequent post hoc Duncan test (P = 0.05) were employed using SPSS 12 software (SPSS Institute, Chicago, IL, USA). The figures were displayed using SigmaPlot 9.0 software.

RESULTS

Effects of Bt cotton on the survival, development and fecundity of *A. gossypii*

Neither the survival rate nor the development period of *A. gossypii* was negatively affected by the Bt cotton, for either the 1st or the 37th generation (Figure 1). Furthermore, there were no significant differences in the survival rates of *A. gossypii* nymphs from the 1st instar to the 4th instar, in the entire nymphal stage between the different cotton cultivars or between the different generations (1st instar: $F_{3, 8}$ = 0.465, P = 0.714; 2nd instar: $F_{3, 6}$ = 1.8, P = 0.247; 3rd instar: $F_{3, 6}$ = 0.847, P = 0.517; 4th instar: $F_{3, 5}$ = 1.065, P = 0.442; entire nymphal stage: $F_{3, 5}$ = 2.179, P = 0.209; Figure 1). The survival rates of the four larval stages and the entire nymphal stage of the *A. gossypii* feeding on both Simian3 and GK12 were up to 95% for the 1st and 37th generations; the survival rates of *A. gossypii* that fed on GK12 for the 1st or 37th generation did not differ significantly. There were no significant differences in the development period of the different instars between the GK12-fed and Simian3-fed aphids, even for the 37th generation (1st instar: $F_{3, 291}$ = 1.24, P = 0.295; 2nd instar: $F_{3, 285}$ = 1.441, P = 0.231; 3rd instar: $F_{3, 266}$ = 0.661, P = 0.577; 4th instar: $F_{3, 230}$ = 0.658, P = 0.579; entire nymphal stage: $F_{3, 258}$ = 1.982, P = 0.117; Figure 1).

The Bt cotton did not significantly affect the reproduction of *A. gossypii* (Table 1). There was no significant difference in reproductive duration between GK12-fed adults and Simian3-fed adults (for both the 1st and 37th generations), Furthermore, the differences among the treatments of cotton cultivars and aphid generations did not differ significantly (reproductive duration: $F_{3, 7}$ = 1.329, P = 0.342; total number of offspring: $F_{3, 7}$ = 0.203, P = 0.891), and there was no significant difference for the GK12-fed aphids between the 1st and 37th generations.

Effects of Bt cotton on the life-table parameters of *A. gossypii*

The net reproductive rate ($F_{3,8}$ = 0.465, P = 0.715), development duration ($F_{3,8}$ = 1.585, P = 0.267) and intrinsic rate of natural increase ($F_{3,8}$ = 1.846, P = 0.217) of the *A. gossypii* feeding on the Bt cotton for the 1st and

Table 1. Fecundity and life-table parameters of *A. gossypii* feeding on Bt vs. non-Bt cotton for different generations.

Biological parameter[a]	Simian3	GK12	Simian3	GK12
	1st generation	1st generation	37th generation	37th generation
Reproduction duration (days)	12.8 ± 0.4[a]	11.1 ± 1.8[a]	10.9 ± 1.6[a]	8.4 ± 1.3[a]
Total number of embryos (individuals)	32.0 ± 18.6[a]	29.2 ± 15.4[a]	23.7 ± 4.7[a]	20.5 ± 4.8[a]
Net reproductive rate (individuals•female^{-1})	33.9 ± 12.5[a]	22.8 ± 10.0[a]	32.7 ± 5.7[a]	23.4 ± 3.2[a]
Developmental duration (days)	10.5 ± 1.2[a]	9.7 ± 0.6[a]	14.0 ± 1.0[a]	12.5 ± 1.1[a]
Intrinsic rate of natural increase (individuals•female^{-1}•d^{-1})	0.97 ± 0.41[a]	0.69 ± 0.35[a]	0.25 ± 0.00[a]	0.20 ± 0.06[a]
Population growth exponents	24.3 ± 8.6[a]	20.3 ± 9.6[a]	27.5 ± 5.0[a]	21.5 ± 7.2[a]
Finite rate of increase	3.05 ± 0.94[a]	2.27 ± 0.87[a]	1.28 ± 0.01[a]	1.23 ± 0.02[a]
Time of population doubling	1.5 ± 1.1[a]	1.3 ± 0.1[a]	2.8 ± 0.1[a]	2.7 ± 0.1[a]

[a]Means within a row followed by the same letters were not significantly different (post hoc Duncan test with P = 0.05).

Table 2. ELISA analysis for different samples.

Analyzed sample	Number of replicates	Amount of material (mg)	Positive (%)	Buffer (ml)	ppb[a]	Bt protein [(b) (c)]
Leaf tissue						
Simian3 (2-cotyledons stage)	10	66.1 ± 8.6	0	0.3	n.d.	n.d.
GK12 (2-cotyledons stage)	20	80.0 ± 20.0	100	0.3	11.31 ± 1.09	44.84 ± 2.79[ac]
Simian3 (4-true leaves stage)	20	65.8 ± 22.1	0	0.5	n.d.	n.d.
GK12 (4-true leaves stage)	20	81.3 ± 25.4	100	0.77	8.94 ± 4.80	86.03 ± 12.60[ab]
Simian3 (boll stage)	20	99.6 ± 38.6	0	0.5	n.d.	n.d.
GK12 (boll stage)	20	89.9 ± 21.9	100	0.5	1.26 ± 1.07	8.81 ± 1.91c
Cotton aphids fed on Bt cotton and non-Bt cotton						
adult (on GK12)	20	0.4 ± 0.1	0	0.13	n.d.	n.d.
adult (on Simian3)	20	0.7 ± 0.1	0	0.13	n.d.	n.d.
adult (on GK12)	5	9.3 ± 1.6	0	0.13	n.d.	n.d.
adult (on GK12)	6	21.6 ± 7.4	33.3	0.13	0.22 ± 0.07	1.44 ± 1.04
adult (on Simian3)	5	21.5 ± 1.9	0	0.13	n.d.	n.d.
adult (on GK12)	7	57.0 ± 14.5	42.9	0.18	0.14 ± 0.09	0.36 ± 0.22
adult (on Simian3)	4	63.2 ± 11.5	0	0.18	n.d.	n.d.
Honeydew						
Simian3	20	0.5 ± 0.1	0	0.13	n.d.	n.d.
1st-2nd generation (on GK12)	20	0.9 ± 0.3	50	0.13	0.11 ± 0.08	13.73 ± 8.13

[a]ppb indicates the concentration of Bt protein in the buffer solution; n.d. indicates 'not detectable'; [b] n.d. means not detectable. [c] Means within a column followed by the same letters were not significantly different (post hoc Duncan test with *P* = 0.05).

37th generations were not significantly influenced by the Bt cotton. The time of population doubling ($F_{3, 6}$ = 2.56, P = 0.151), population growth exponents ($F_{3, 8}$ = 0.306, P = 0.821) and finite rate of increase ($F_{3, 8}$ = 1.838, P = 0.218) did not differ between the Bt-fed *A. gossypii* and the non-Bt fed individuals (Table 1).

Bt protein detection and quantification

The Bt protein content in the leaves varied significantly at different growth stages ($F_{2, 43}$ = 20.37, P < 0.001) (Table 2). The leaves at the 4 true-leaf stage contained the highest average concentration of Bt protein (86.03 ng/g), the 2-cotyledon stage contained a moderate level (44.84 ng/g), and the lowest level was found at the boll stage (8.81 ng/g).

Among the Bt cotton cv. GK12-fed aphids, trace amounts of Bt protein was detected in some of the aphid samples and the honeydew samples. The Bt protein could only be detected in one-third of the aphid samples with body weights over 20 mg.

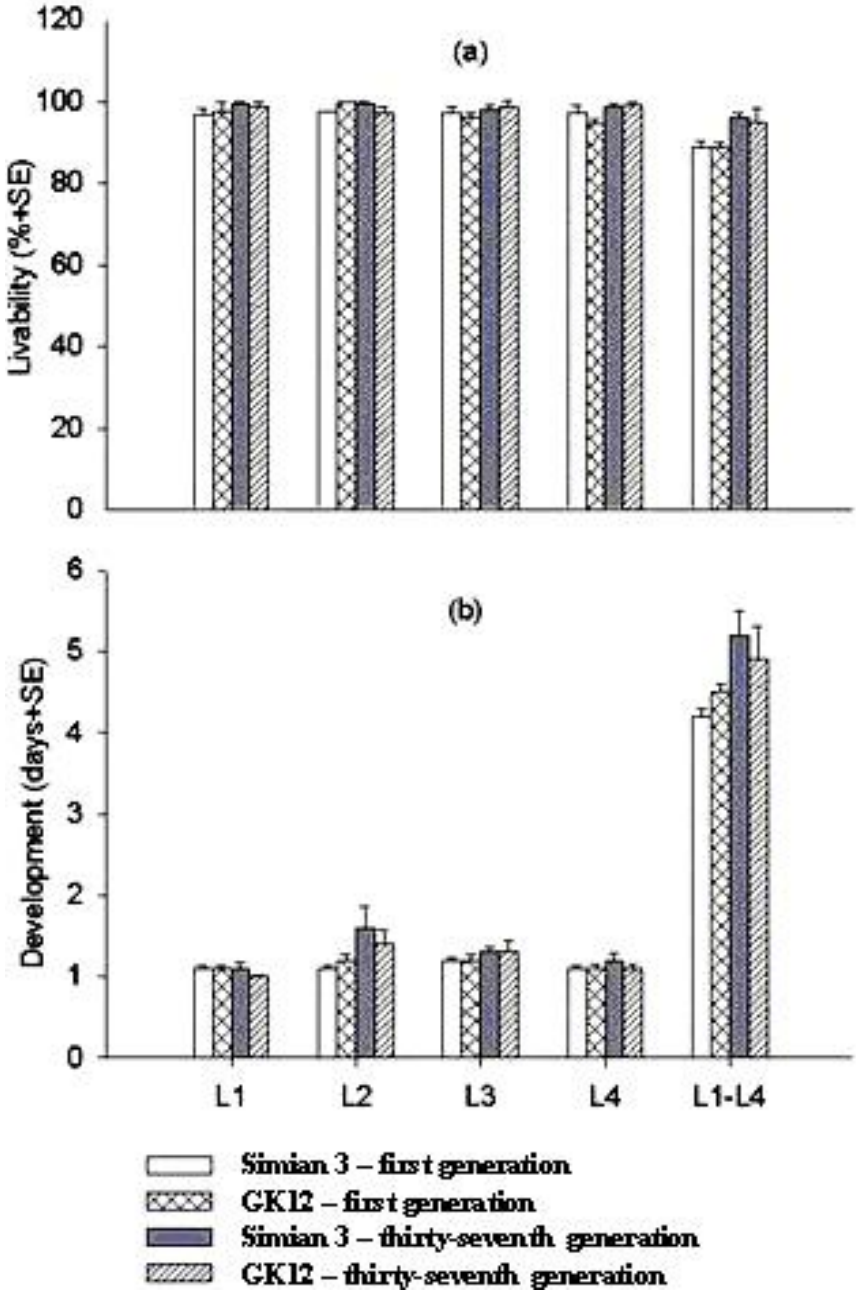

Figure 1. (a) Survival rate and (b) development of the five larval stages of *A. gossypii* feeding on non-Bt cotton (Simian 3) and Bt cotton (GK12) for different generations. L1, L2, L3, and L4 indicate the four larval stages, and L1-L4 indicates the entire larval period.

DISCUSSION

In the present study, the effects of Bt cotton on non-target cotton aphids were assessed. The results indicate that Bt cotton had no significant effects on the life-table parameters of cotton aphids even though they fed on Bt cotton for 37 generations, which was in accordance with previous reports by Dutton et al. (2002) and Ramirez-Romero et al. (2008) that Bt maize did not affect non-target aphids. In contrast, Belén et al. (2004) have reported that Bt maize affected the development, reproduction and intrinsic rate of increase (r_m) of the first generation offspring of alate and apterous *Rhopalosiphum padi* adults, but those indices were not affected after

several generations (3 weeks, 10 weeks, and 18 weeks). A laboratory study of *A. gossypii* fed on Bt cotton for three generations has indicated that the Bt cotton did not affect the r_m of *A. gossypii* in the first and second generations, yet the potential maximum fecundity was larger in all three of the generations observed (Liu et al., 2005).

Comparing these reports with our results indicates that the biological responses of non-target aphids to Bt crops depend on the aphid species tested and the Bt plant evaluated. We speculate that the reasons for this differential effect of Bt plants on aphids must be other than those linked to the expression of the Bt toxin because Bt proteins are not transported in the phloem (Romeis and Meissle, 2011).

Factors affecting the process of aphid settlement or retention on plants, such as host attraction or plant structure, should also be considered. The insertion of new genes into plants through transgenic technology and the selection and breeding process that is necessary to develop a newly transformed plants into stable and robust agricultural products with the desired properties could inadvertently change the nutritional quality and the secondary compounds of the plant itself (Chen et al., 2004) or the plant structure (Saxena and Stotzky, 2001), which could, in turn, affect non-target pest populations or the biochemical quality. However, our results showed no adverse impacts on the life-table parameters of the cotton aphids after long-term (37 generations) rearing on Bt cotton plants as all parameters were similar to the control cultivar and to the values in the first generation.

Our current study demonstrated that only low concentrations of Bt protein were sporadically detected in *A. gossypii* adults feeding on the Bt cotton cv., GK12: Bt protein could only be detected in one-third of the aphid samples with body weights over 20 mg, and the Bt protein was not detected in aphid samples weighing less than 10 mg. Similarly, Lawo et al. (2009) collected *A. gossypii* from glasshouse grown Cry1Ac-expressing cotton plants and obtained a positive signal in enzyme-linked immunosorbent assays (ELISAs) for 11 out of 12 samples. Dutton et al. (2002) have reported that only trace amounts of Cry1Ab were detected in *R. padi* feeding on transgenic Bt maize and Ramirez-Romero et al. (2008) also reported that no Cry1Ab protein was present in *Sitobion avenae* nymphs developing on either Bt or conventional maize. Aphids feed on the phloem sap (Douglas, 2003), which has been shown to contain no or very small amounts of Bt protein (Raps et al., 2001; Burgio et al., 2007), with the consequence that aphids do not ingest the Bt protein (Head et al., 2001; Raps et al., 2001; Dutton et al., 2002, 2004). Thus, the concentrations detected in aphids were most likely false positives due to contamination with faeces of thrips that entered the samples during the aphid collection. On the other hand, aphids could come in contact with the Cry protein when inserting their stylets in search for the phloem system. Depending on the probing behavior, some aphid species may ingest measurable amounts of Bt protein by this way.

Although, no or little Bt protein was detected in aphids, much higher concentration of the Bt protein was found in the 50% of honeydew samples. Since Homopteran sap-sucking insects digest little or no protein in their gut (Stoger et al., 1999), most of the ingested protein is excreted; thus, the Bt protein ingested by *A. gossypii* may have been excreted in the honeydew. The relatively high concentrations detected in honeydew were most likely due to contamination with Cry-containing material, like faeces of thrips that entered the samples during the honeydew collection. In an earlier study with Bt maize it was found that the faeces of thrips contained about 10-fold higher Cry1Ab concentrations than the fresh plant material (Obrist et al., 2005). Moreover, cotton plants are highly susceptible to infestation by thrips, spider mites and other herbivores.

We proposed three explanations of why the life-table parameters of *A. gossypii* were not affected by the Bt protein. First, the cotton aphids ingested little or no Bt protein, and the protein sucked in was excreted into the honeydew. Secondly, the Bt protein had no impact on the compounds that conferred resistance to the aphids in the leaves at the cotyledon stage (Zhang et al., 2002). Third, the nutritive component in the phloem sap at the cotyledon stage was not affected by the Bt protein. Therefore, we suggest that the impacts of the Bt cotton on *A. gossypii* due to the potential food quality changes were negligible. In conclusion, *A. gossypii* sporadically obtained, if any, the Bt protein by feeding on the cotton leaves when probing plants, but the resulting Bt content in the *A. gossypii* body was very low and not consistently detectable, and the life-table parameters of *A. gossypii* feeding on Bt cotton were not affected in either the short term or the long term. The Bt protein was excreted in the aphid honeydew, and Bt protein concentrations in the honeydew was higher than in the insects. This may come not only from Bt protein-containing thrips, spider mites and other herbivorous species, but also from their faeces.

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