Effects of Bt Cotton on *Thrips tabaci* (Thysanoptera: Thripidae) and Its Predator, *Orius insidiosus* (Hemiptera: Anthocoridae)

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ABSTRACT Laboratory studies were conducted to investigate tritrophic transfer of insecticidal Cry proteins from transgenic cotton to an herbivore and its predator, and to examine effects of these proteins on the predator's development, survival, and reproduction. Cry1Ac and Cry2Ab proteins from the bacterium *Bacillus thuringiensis* (Bt) produced in Bollgard-II (BG-II, Event 15985) cotton plants were acquired by *Thrips tabaci* Lindeman (Thysanoptera: Thripidae), an important sucking pest of cotton, and its generalist predator, *Orius insidiosus* (Say) (Hemiptera: Anthocoridae). The average protein titers in BG-II cotton leaves were 1,256 and 43,637 ng Cry1Ac and Cry2Ab per gram fresh leaf tissue, respectively. At the second trophic level, larvae of *T. tabaci* reared on BG-II cotton for 48–96 h had 22.1 and 2.1% of the Cry1Ac and Cry2Ab levels expressed in leaves, respectively. At the third trophic level, *O. insidiosus* that fed on *T. tabaci* larvae had 4.4 and 0.3% of the Cry1Ac and Cry2Ab protein levels, respectively, expressed in BG-II plants. *O. insidiosus* survivorship, time of nymphal development, adult weight, preoviposition and postoviposition periods, fecundity, and adult longevity were not adversely affected owing to consumption of *T. tabaci* larvae that had fed on BG-II cotton compared with non-Bt cotton. Our results indicate that *O. insidiosus*, a common predator of *T. tabaci*, is not harmed by BG-II cotton when exposed to Bt proteins through its prey. Thus, *O. insidiosus* can continue to provide important biological control services in the cotton ecosystem when BG-II cotton is used to control primary lepidopteran pests.

KEY WORDS Cry1Ac, Cry2Ab, cotton, biosafety, nontarget effect

Transgenic cotton producing insecticidal crystal (Cry) proteins from *Bacillus thuringiensis* (Bt) for control of Lepidoptera was planted on 24.3 million ha in 15 countries in 2012, with India having the largest area of 10.8 million ha (James 2012). Cotton is attacked by a large complex of insect pests but the most damaging is the bollworm complex, primarily *Helicoverpa zea* (Boddie), *Helicoverpa armigera* (Hübner), *Helicoverpa punctigera* (Wallengren), *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuiidae), and *Pectinophora gossypiella* Saunders (Lepidoptera: Gelechiidae). The Cry proteins produced in Bt cotton target these and additional Lepidoptera but do not affect a number of other cotton pest species (Naranjo et al. 2008).

Initially, Bt cotton varieties producing only a single Cry protein were cultivated, but these have largely been replaced in most areas of the world by cotton producing two Cry proteins, such as Bollgard-II (BG-II), producing Cry1Ac and Cry2Ab (Naranjo et al. 2008). The Cry proteins in Bt cotton are produced throughout the entire growing season, and so target and nontarget arthropods have ample opportunity for exposure to these proteins. These include predators and parasitoids that may be exposed when they feed on arthropods that have consumed plant tissue containing Bt proteins (Harwood et al. 2005; Obrist et al. 2005, 2006; Torres et al. 2006, 2008; Meissle and Romeis 2009). Such tritrophic interactions may have consequences for insect pest management and should be examined as part of an environmental risk assessment.

Adoption of Bt cotton has changed the traditional pest complex in adopting countries such as India. There has been a dramatic decline in the pest status of bollworms, but sap feeders, including aphids, leafhoppers, mirids, and mealybugs, are emerging as serious pests (Vennila 2008). In addition, a traditionally minor pest, the onion thrips, *Thrips tabaci* Lindeman (Thysanoptera: Thripidae), has become a serious pest on Bt cotton in India (Sharma and Pampapathy 2006, Sarode et al. 2009). This species overwinters in plowed soil, plant debris, and perennial weeds and becomes active in the spring. With its rapid life cycle and high reproductive capacity, it has become a perennial and serious pest of seedling to mid-season cotton in many cotton regions in India (Gupta et al. 1997, Khan et al. 2008). *T. tabaci* has a unique feeding method in which it rasps leaf surface cells and consumes their liquid contents,

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thus reducing the photosynthetic capacity of the plant. Previous reports have confirmed that thrips species acquire Cry proteins when feeding on Bt plants (Dutton et al. 2004; Obrist et al. 2005; Torres et al. 2006, 2008; Lawo et al. 2009; Meissle and Romeis 2009). This phenomenon has raised concern that predators of *T. tabaci* may ingest Cry proteins and be harmed. In India, most of the predators of *T. tabaci* are generalists that rely on *T. tabaci* as a food source early in the season, the time when Cry protein expression in the cotton plant is high (Kranthi et al. 2005, Bakhsh et al. 2012). Similar nontarget pest issues with different pest species have been observed in China, Australia, and the United States (Naranjo et al. 2008).

Worldwide, cotton supports large and diverse arthropod natural enemy communities, and there is ample evidence to suggest that these natural enemies can have a significant impact on cotton pest population dynamics (see review by Naranjo et al. 2008). One genus in particular, *Orius* spp. (Hemiptera: Anthocoridae), contains well-known omnivorous and generalist predators that feed on various arthropods including thrips, spider mites, leafhoppers, aphids, whiteflies, and lepidopteran eggs and young larvae (McMurtry et al. 1970, Coll and Bottrell 1991, Kohno and Kashio 1998, Wang 1998, Lattin 2000). Members of this genus are important biological control agents in many crop ecosystems including cotton (Lattin 1999). *Orius insidiosus* (Say) is a widely distributed species that has proven to be an important biological control agent in greenhouse and field situations and which also feeds on plant tissues (e.g., pollen grains and young leaves) to supplement its diet (Naranjo and Gibson 1996). Previous studies have confirmed that it can acquire Cry proteins expressed in Bt plants (Obrist et al. 2005; Torres et al. 2006, 2008; Meissle and Romeis 2009). Thus, *O. insidiosus* has the capacity to acquire Cry proteins from feeding on hosts that have consumed Bt plant tissue, as well as feeding on Bt plant tissues directly.

In the current study, we investigated if Cry proteins expressed in Bt cotton move from plants to *T. tabaci* and subsequently to *O. insidiosus.* Specifically, we 1) documented the amount of Cry1Ac and Cry2Ab protein in BG-II cotton; 2) determined the uptake of Cry1Ac and Cry2Ab by *T. tabaci;* 3) determined whether *O. insidiosus* could acquire Cry1Ac and Cry2Ab when it fed on *T. tabaci* that had fed on cotton expressing Cry1Ac and Cry2Ab; and 4) determined if the survival, development, and reproduction of *O. insidiosus* were affected by consuming *T. tabaci* that had fed on cotton expressing Cry1Ac and Cry2Ab.

Materials and Methods

Predator. Eggs of *O. insidiosus* were obtained from the U.S. Department of Agriculture–Agricultural Research Service (USDA-ARS), Maricopa, AZ (SEN laboratory), where they had been reared on eggs of *P. gossypiella* and *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae), and green bean pods for over 20 yr, with yearly introduction of wild stock. The insects were maintained in a climatic chamber at 27°C, 65% relative humidity (RH), and a photoperiod of 16:8 (L:D) h at Cornell University's Department of Entomology at the New York State Agricultural Experiment Station, Geneva, NY. Adults of *O. insidiosus* were reared on eggs of diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae), provided with green bean pods as a source of moisture and a substrate for egg laying. The ends of the green beans were dipped in paraffin wax to keep them fresh for a longer period. Prey eggs (*P. xylostella*) and green beans were replenished every 3–4 d. Sections of the green beans from adult rearing were saved and used to provide immature insects for bioassays.

Prey. *T. tabaci* were maintained on onion plants in a climatic chamber at 20.3°C, 60% RH, and a photoperiod of 16:8 (L:D) h. Fresh cabbage leaves were provided to adult thrips for egg laying. On hatching, first-instar *T. tabaci* were transferred to either Bollgard-II cotton or non-Bt cotton leaves (see Plants, below). After feeding for 2–4 d, *T. tabaci* larvae were supplied to *O. insidiosus* as food. Before initiation of the bioassay, the protein uptake by different stages of *T. tabaci* was confirmed (see ELISA Procedures, below).

Plants. Seeds of Bollgard-II (Event 15895) producing Cry1Ac and Cry2Ab, and the corresponding nontransformed near-isoline Stoneville 474, were obtained from Monsanto Company (St. Louis, MO). The two cotton varieties were grown simultaneously in the same growth chambers. Plants were individually grown in 6-liter plastic pots filled with Cornell mix soil (Boodley and Sheldrake 1977). Small leaves from the upper canopy were used to conduct the bioassays with *T. tabaci* and *O. insidiosus.*

Bioassay Conditions. All bioassays were conducted in an environmental chamber at 27°C, 65% RH, and a photoperiod of 16:8 (L:D) h. In the bioassays, *O. insidiosus* were fed either larvae (mixed stages) of *T. tabaci* reared on non-Bt cotton leaves, or larvae reared on BG-II cotton. Newly hatched *O. insidiosus* nymphs were released into a plastic 0.5-ml microcentrifuge tube (Laboratory Product Sales, Rochester, NY) provided with a small section of a Bt or non-Bt cotton leaf with *T. tabaci* larvae, depending on the treatment. From our preliminary tests, we determined that *O. insidiosus* used 5–10 *T. tabaci* larvae per day. To ensure there was a sufficient number of prey, each tube contained a leaf with 10–20 *T. tabaci* larvae that were replaced daily. After the second molt, *O. insidiosus* nymphs were transferred to a 30-ml plastic cup and provided with a Bt or non-Bt leaf with *T. tabaci*, and leaves were replaced daily. The number of *T. tabaci* larvae on a cotton leaf provided to *O. insidiosus* was increased by 5 after each molt, based on our preliminary studies. The bioassays began with 30 individually kept, newly hatched *O. insidiosus* nymphs each for BG-II and non-Bt cotton. Nymphs of *O. insidiosus* were observed daily, and their survival and development were recorded. When the adults emerged, they were weighed and their gender was determined. A male and a female from the same treatment were kept

Table 1. Concentration of Cry proteins (ng) in Bollgard-II cotton leaves, *T. tabaci* (prey), and *O. insidiosus* (predator)

Sample	Expression of Cry proteins[a]	
	Cry1Ac (ng/g FW)	Cry2Ab (ng/g FW)
Cotton leaves	1,256 ± 88 (1,131–1,426)	43, 637 ± 1, 663 (41, 749–46, 952)
T. tabaci (larvae)	277 ± 7 (263–287)	916 ± 72 (802–1, 049)
O. insidiosus (adults)	55 ± 2 (53–59)	119 ± 4 (111–126)

[a] Means ± SEM (range).

in a 30-ml plastic cup and allowed to mate. All pairs of adult *O. insidiosus* from both treatments were fed 50–60 their respective *T. tabaci* larvae per day, reared on BG-II or non-Bt cotton. Leaves and prey were replaced daily. The duration of pre- and postoviposition periods as well as fecundity and longevity were measured. Progeny from these adults were then used to examine nymphal survival and development for a second generation using the methods described above.

Concentration of Cry1Ac and Cry2Ab in the Cotton Plant, Prey, and Predator. Leaf samples (leaf discs of 5 mm diameter) were collected from five different Bt and non-Bt cotton plants at regular intervals during the assays. Each sample was obtained from a leaf in the upper third of a BG-II or control plant. All samples were weighed and placed into 1.5-ml centrifuge tubes, respectively, and stored at −20°C until Cry protein measurements using enzyme-linked immunosorbent assays (ELISA; see below) were made.

T. tabaci larvae, used as prey for *O. insidiosus* after feeding on BG-II or non-Bt plants, were collected in microcentrifuge tubes. They were grouped and weighed in a batch of ≈20 mg per replication, and then stored at −20°C until the Cry level could be determined (see below). Similarly, *O. insidiosus* adults at the end of a bioassay were collected and stored in microcentrifuge tubes. They were also grouped and weighed in a batch of ≈20 mg per replication, and then stored at −20°C until assayed using ELISA. For all ELISA samples, five replications were used.

ELISA Procedures. Bt protein concentrations in plants and insects, including the non-Bt treatments, were measured using sandwich ELISA using Cry1Ab/Cry1Ac and Cry2Ab detection kits from EnvironLogix (Portland, ME; Tian et al. 2014). Kits were identified as QualiPlate Kit for Cry1Ab/Cry1Ac—AP 003 CRBS and QuantiPlate Kit for Cry2A—AP 005. Before analysis, all insects were washed in phosphate-buffered saline with Tween-20 buffer (provided in the kit) to remove any Bt protein from their outer surface. After adding phosphate-buffered saline with Tween-20 to the samples at a ratio of at least 1:200/1:100/1:20 (plant/*T. tabaci*/*O. insidiosus* for Cry1Ac) and 1:5,000/1:200/1:50 (plant/*T. tabaci*/*O. insidiosus* for Cry2Ab) in 1.5-ml centrifuge tubes, the samples were ground by hand using a plastic pestle. After centrifugation and appropriate dilution of the supernatants, ELISA was performed according to the manufacturer's instructions. Spectrophotometric measurements were taken using a UV-visible recording spectrophotometer (UV160U, Shimaszu, Columbia, MD). Because no purified Cry1Ab/Cry1Ac protein was provided in the Cry1Ab/Cry1Ac kit, Cry1Ab was purchased from Department of Biochemistry, Case Western Reserve University (Cleveland, OH). Purified Cry1Ab protein samples at concentrations of 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 ng/g were used as calibrators. We ran the negative controls (samples from non-Bt treatment), and absorbance readings and values double the negative control were considered positive.

Statistical Analysis. Data on survival of *O. insidiosus* were analyzed using the Wilcoxon test for homogeneity. Data on other life history parameters of *O. insidiosus* reared on thrips fed on BG-II and non-Bt cotton were compared using Student's paired *t*-test. Before analysis, all percentage data were arcsine or square root transformed, as necessary, but untransformed means are presented. All statistical analyses were performed with SAS version 9.1 (SAS Institute 2001). For all tests, $\alpha = 0.05$.

Results

Cry1Ac and Cry2Ab in Plants, Prey, and Predators. In the Bt treatment, Cry1Ac and Cry2Ab proteins were detected in all three trophic levels (Table 1). In contrast, no Bt protein was detected at any trophic level in the non-Bt treatment. The average Bt protein titers from BG-II cotton leaves used for rearing the prey and predators were 1,256 and 43,633 ng Cry1Ac and Cry2Ab per gram of fresh leaf tissue, respectively. From this quantity of Cry1Ac and Cry2Ab proteins expressed in leaves of BG-II cotton, 277 and 916 ng (22.1 and 2.1% of the total expressed in BG-II cotton leaves) per gram fresh weight (FW), respectively, were detected in *T. tabaci* larvae reared on BG-II cotton leaves for 2–4 d at the second trophic level. This confirmed the flow of the protein from BG-II cotton to the nontarget herbivore. Subsequently, at the third trophic level, 55 and 119 ng per gram FW of Cry1Ac and Cry2Ab, respectively, were detected (4.4 and 0.3% of the total expressed in BG-II cotton leaves) in *O. insidiosus* fed on *T. tabaci* larvae reared on BG-II cotton leaves for 2–4 d. This confirms the acquisition of Cry1Ac and Cry2Ab by *T. tabaci* larvae from BG-II cotton and its further uptake by *O. insidiosus* after feeding on *T. tabaci* larvae.

Development and Reproduction of *O. insidiosus* Fed on *T. tabaci* Larvae Reared on BG-II and non-Bt Cotton Plants. There were no significant differences ($P > 0.05$) in survival and nymphal development for the first or second generation when *O. insidiosus* fed on *T. tabaci* larvae reared on leaves of BG-II compared

Table 2. Development and survival of *O. insidiosus* fed on *T. tabaci* reared on Cry1Ac/Cry2Ab-expressing Bt cotton leaves (Bollgard-II) or its non-Bt isoline

Parameter	Generation			
	1[a]		2[a]	
	BG-II	Non-Bt	BG-II	Non-Bt
Survival (%)	96.7a	96.7a	100a	100a
Development time (d)				
Instar I	2.2 ± 0.1a	2.0 ± 0.1a	2.1 ± 0.1a	2.1 ± 0.1a
Instar II	1.8 ± 0.1a	1.9 ± 0.1a	1.8 ± 0.1a	1.6 ± 0.1a
Instar III	2.9 ± 0.1a	2.7 ± 0.1a	2.7 ± 0.1a	2.8 ± 0.1a
Instar IV	1.9 ± 0.1a	2.0 ± 0.1a	1.9 ± 0.1a	1.8 ± 0.1a
Instar V	3.5 ± 0.1a	3.3 ± 0.1a	3.1 ± 0.1a	3.2 ± 0.1a
Nymphal duration (d)	11.8 ± 0.1a	11.4 ± 0.1a	11.9 ± 0.1a	11.2 ± 0.1a

Means (±SEM) followed by the same letter between columns, within a generation and the same instar, are not significantly different (Survival: Wilcoxon test, $P < 0.05$; other parameters: Student's *t*-test, $P < 0.05$).

[a] $n = 30$.

with non-Bt cotton (Table 2). Furthermore, in the first generation, there were no significant differences ($P > 0.05$) in preoviposition, oviposition periods, fecundity, fertility, and adult longevity of adult *O. insidiosus* that had fed as both nymphs and adults on larvae of *T. tabaci* in the two treatments (Table 3).

Discussion

Genetically modified crops producing Bt proteins may pose a risk to a nontarget organism if they are susceptible to the protein and if the organism is exposed. The hazard posed by the protein can be determined in Tier 1 laboratory studies in which the organism is subjected to a high dose by feeding directly on the protein in a diet or on a plant producing the proteins (Romeis et al. 2011). Determining the level of exposure of a nontarget, natural enemy to a Bt protein is more complex. It must be shown that its prey has acquired the protein from the plant and that the natural enemy can in turn acquire it from the prey. Thus, the concentration of the Cry protein contained in the food source has to be determined (Dutton et al. 2002, 2003). Leaves of BG-II are known to have the highest concentrations of Cry proteins compared with other plant parts (Li et al. 2011) and in this study we found high levels of both Cry1Ac and Cry2Ab proteins in plant leaves.

T. tabaci is an important pest of cotton worldwide and attacks the cotton crop during the earlier part of the season (Khan et al. 2008) when populations of the other sucking pests are low or absent. *O. insidiosis* is a generalist predator that feeds on all stages of thrips during its life cycle, and the uptake of Cry1Ac and Cry2Ab by the pest and predator was confirmed in the current study. Previous studies have confirmed the flow of Cry proteins from plant to herbivore and their predators in Bt cotton and Bt maize, the two widely cultivated Bt crops worldwide (Dutton et al. 2002, Harwood et al. 2005, Obrist et al. 2005, Torres et al. 2008, Meissle and Romeis 2009). Meissle and Romeis (2009) and Obrist et al. (2005, 2006) reported that predatory heteropterans in the field will likely contain detectable amounts of Bt proteins, but note this depends on the amount and types of prey consumed, and the capacity of the prey to acquire and concentrate the protein.

Cry proteins present in transgenic crops (Bt cotton and Bt corn) have been detected in nonsusceptible pests such as spider mites, thrips, and leafhoppers feeding on Bt corn and Bt cotton (Dutton et al. 2004, Torres et al. 2008, Meissle and Romeis 2009), but not in *Aphis gossypii* (Glover) (Torres et al. 2006) and *Rhopalosiphum padi* (L.) or *Rhopalosiphum maidis* (Fitch) (all Hemiptera: Aphididae) (Dutton et al. 2002). Herbivores feeding on Cry1A-expressing Bt corn or Bt cotton revealed large differences in the quantity of ingested proteins among species (Obrist et al. 2006, Torres et al. 2006). In Bt cotton, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) exhibited 0.73 times the protein level in its body than was expressed in Bt cotton plants (Torres and Ruberson 2008), but even higher absolute concentrations of the Cry protein were detected in thrips *Frankliniella tenuicornis* (Uzel) collected from Bt maize (Obrist et al. 2006). From plants, relatively higher uptake of Bt protein concentrations has been recorded in *Tetranychus urticae* (Trombidiformes: Tetranychidae), *F. occidentalis*, and *Spodoptera exigua*, than could be detected in the predators *Geocoris punctipes* (Say) (Hemiptera: Lygaeidae), *Podius maculiventris* Say (Hemiptera: Pentattomidae), *O. insidiosus* (Say) (Hemiptera: Anthocoridae), and *Nabis roseipennis* Reuter (Hemiptera: Nabidae) (Torres and Ruberson 2008). In our study, *T. tabaci* reared on

Table 3. Preoviposition and oviposition periods, fecundity, fertility, and longevity of adult *O. insidiosus* fed *T. tabaci* larvae reared on cotton leaves of Bollgard-II and its non-Bt isoline

Life history trait[a]	*n*	Cotton variety Bollgard-II	*n*	Non-Bt isoline
Preoviposition (d)	8	4.9 ± 0.3a	9	5.1 ± 0.3a
Oviposition (d)	8	17.5 ± 0.5a	9	16.5 ± 0.4a
Fecundity (total eggs)	8	69.2 ± 3.8a	9	71.2 ± 3.5a
Fertility (%)	8	71.5 ± 2.1a	9	71.9 ± 1.3a
Adult longevity (d)	8	19.3 ± 0.1a	9	20.0 ± 0.1a

[a] Means (±SEM) for each life history trait, followed by the same letter between columns, are not significantly different (Student's *t*-test, $P < 0.05$).

BG-II cotton contained 24 and 2.2% of the amount of Cry1Ac and Cry2Ab protein, respectively, expressed in BG-II cotton plant leaves, while lower amounts (4.4 and 0.3%) were found in *O. insidiosus* when it fed on larvae of *T. tabaci* reared on BG-II cotton.

Some studies have suggested that Cry proteins conveyed through nontarget herbivores to natural enemies may negatively affect aspects of the natural enemy's life history (Hilbeck et al. 1999, Dong et al. 2003, Vojtech et al. 2005, Lövei et al. 2009, Schmidt et al. 2009). However, such effects can usually be traced back to the poor quality of the host used and do not demonstrate any toxicity to the natural enemy by the Bt protein itself (Shelton et al. 2009, Romeis et al. 2013). In the current study, acquisition of Cry1Ac and Cry2Ab by *T. tabaci*, a nontarget pest on BG-II cotton, and the further transmission of the proteins to *O. insidiosus*, were confirmed. However, the life history parameters of *O. insidiosus* were not affected in this tritrophic interaction, similar to that shown in Bt maize with *O. insidiousus* (Al-Deeb et al. 2001).

Based on a realistic exposure route with confirmed transfer of Bt proteins through the food chain, our results demonstrate no biological effects of BG-II cotton containing Cry1Ac and Cry2Ab on an important predator. Our findings support those of other laboratory studies using other Bt proteins and are consistent with the lack of effects observed on populations of this predator in the field (Head et al. 2005, Torres and Ruberson 2005, Naranjo 2009).

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