Eliminating host-mediated effects demonstrates Bt maize producing Cry1F has no adverse effects on the parasitoid Cotesia marginiventris

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Eliminating host-mediated effects demonstrates Bt maize producing Cry1F has no adverse effects on the parasitoid *Cotesia marginiventris*

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Abstract The fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), is an important pest of maize in the United States and many tropical areas in the western hemisphere. In 2001, Herculex I® (Cry1F) maize was commercially planted in the United States to control Lepidoptera, including *S. frugiperda*. In 2006, a population of *S. frugiperda* was discovered in Puerto Rico that had evolved resistance to Cry1F maize in the field, making it the first well-documented case of an insect with field resistance to a plant producing protein from *Bacillus thuringiensis* (Bt). Using this resistant population, we conducted tri-trophic studies with a natural enemy of *S. frugiperda*. By using resistant *S. frugiperda*, we were able to overcome possible prey-mediated effects and avoid concerns about potential differences in laboratory- or field-derived Bt resistance. We used the Cry1F-resistant *S. frugiperda* to evaluate effects of Cry1F on *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae), a larval endoparasitoid of *S. frugiperda,* over five generations. Our results clearly demonstrate that Cry1F maize does not affect development, parasitism, survivorship, sex ratio, longevity or fecundity of *C. marginiventris* when they parasitize Cry1F maize-fed *S. frugiperda.* Furthermore, the level of Cry1F protein in the leaves was strongly diluted when transferred from Bt maize to *S. frugiperda* and was not detected in larvae, cocoons or adults of *C. marginiventris.* Our results refute previous reports of *C. marginiventris* being harmed by Bt proteins and

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suggest that such results were caused by prey-mediated effects due to using Bt-susceptible lepidopteran hosts.

Keywords Cry1F · Biosafety · *Spodoptera frugiperda* · Non-target effects · Study design

Introduction

Since the first transgenic insect-resistant crops were commercially grown in Australia, Mexico and the United States in 1996, they have become widely adopted globally. The present insect-resistant crops (maize and cotton) express proteins from *Bacillus thuringiensis* (Bt) and were grown on nearly 70 million ha in 27 countries in 2012 (James 2012). Although Bt crops have provided many benefits to the economy, human health and the environment (Shelton et al. 2002), the potential effect of Bt crops on non-target organisms continues to be debated. Most of these debates focus on natural enemies, which play an important role in pest control (Kennedy 2008; Romeis et al. 2008). To date, numerous studies have been conducted to evaluate the potential effects of Bt crops on natural enemies, including predators and parasitoids. Most studies have demonstrated that Bt crops do not harm natural enemies (Romeis et al. 2006; Marvier et al. 2007; Wolfenbarger et al. 2008; Naranjo 2009). However, a few reports have claimed Bt crops have negative effects on natural enemies, especially parasitoids (Lövei et al. 2009). In those studies, natural enemies were exposed to Bt proteins by feeding on Bt-susceptible prey or hosts, thus compromising their quality (Naranjo 2009; Shelton et al. 2009a, b). Therefore, much of the debate about the effect of Bt proteins on natural enemies has focused on whether any purported negative effects are, in fact, due to the Bt protein or quality of the host or prey on which the natural enemy fed (Romeis et al. 2006; Shelton et al. 2009a, b). One of the best ways to eliminate the potential impact of host or prey quality is to use a Bt-resistant host or prey that can develop well on the Bt crop, and thereby transfer a high concentration of the Bt protein to the host or prey and eventually the natural enemy (Romeis et al. 2011).

The fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), is an agricultural pest in tropical–subtropical regions that feeds on more than 60 plant species (Luginbill 1928). It has become one of the most serious pests of corn throughout the Americas (Ashley et al. 1989; Kumar and Mihm 1996). In 2001, Herculex I ® (Cry1F) maize was commercially planted in the United States and targeted Lepidoptera, including *S. frugiperda* (Hellmich et al. 2008). Cry1F maize has been shown to substantially reduce losses by *S. frugiperda* in many areas (Buntin et al. 2004; Siebert et al. 2008). However, *S. frugiperda* resistance to Cry1F maize was documented in Puerto Rican fields in 2006 (Matten et al. 2008; Tabashnik et al. 2009; Storer et al. 2010). Thus, *S. frugiperda* was the first well-documented crop pest to have evolved resistance to Bt plants in the field. This resistance afforded us an opportunity to use it for studies on tri-trophic interactions with natural enemies of *S. frugiperda*. By using Cry1F-resistant *S. frugiperda*, we could overcome prey-mediated effects (Romeis et al. 2011).

Cotesia marginiventris (Cresson) (Hymenoptera: Braconidae) is a parasitoid wasp that has a wide host range that includes many Noctuid species and is an important natural enemy of *S. frugiperda* (Ashley 1979). Adult females usually deposit one egg into a young host larva (first to second instar). The egg hatches and the parasitoid larva develops through three instars by feeding on hemolymph and other tissues, killing the host. The larva then emerges from the host to pupate and form a cocoon (Boling and Pitre 1970). Several studies on the non-target effects of Bt crops have used *C. marginiventris*. Vojtech et al. (2005) reported that survival, developmental times and cocoon weights of *C. marginiventris* developing inside susceptible Bt maize-fed *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) were significantly negatively affected, compared with those that developed on non-Bt maize-fed hosts. In a second study, the developmental time, adult size, and fecundity of *C. margeniventris* were significantly negatively affected if it fed on susceptible *S. frugiperda* larva that had consumed Cry1Ab (Ramirez-Romero et al. 2007). Both studies used susceptible hosts as the carrier to deliver the Bt proteins to *C. marginiventris* and, therefore, could not rule out that the effect was indirect and host-quality mediated. Without recognition of the potential for this indirect effect, one could conclude that the natural enemy was harmed by the Bt protein, rather than the poor quality of the parasitoid's host. This mistaken reasoning attributes the hazard to the Bt

protein rather than the host quality and may inappropriately influence the environmental risk assessment (Shelton et al. 2009a, b; Romeis et al. 2013).

In the present study, Cry1F-resistant *S. frugiperda* were used to eliminate host quality effects and to evaluate the direct effects of Cry1F maize on *C. marginiventris*. Development time, success of parasitism, survivorship, sex ratio (% females), adult longevity and fecundity of *C. marginiventris* were evaluated. Furthermore, assessments were conducted over five generations to address possible long-term effects of Cry1F maize on *C. marginiventris* and to provide additional certainty to the risk assessment.

Materials and methods

Plants

Seeds of hybrid Bt maize (Mycogen 2A517) producing Cry1F protein, and the non-transformed near-isoline hybrid (Mycogen 2A496) were obtained from Dow AgroSciences (Indianapolis, IN). Bt maize and non-Bt maize hybrids were grown simultaneously in the same greenhouse at Cornell's New York State Agricultural Experiment Station in Geneva, NY. Plants were grown in Ray Leach Cone-tainer Cells (diam. 3.8 cm; depth 21 cm; vol. 164 ml) (Stuewe and Sons, Tangent, OR) at LD 16:8, 21 ± 3 °C. Seeds were planted every week and plants were used for the experiment at the 4–5 leaf stage (~4 week).

Insects

A Cry1F-resistant strain of *S. frugiperda* was obtained from Dow AgroSciences in 2010 and maintained on artificial diet (Bio-Serv, Frenchtown, NJ). This strain developed resistance to Cry1F maize in Puerto Rico (Storer et al. 2010) and can complete its development on Cry1F maize (Tian et al. 2012). Newly hatched *S. frugiperda* were fed Bt maize or non-Bt maize for 4 days before being presented to parasitoids. A colony of *C. marginventris* was obtained from Mike Strand (Department of Entomology, University of Georgia) where it had been maintained on non-Bt maize-fed *S. frugiperda* for many generations. The insect colonies were maintained in an environmental chamber (LD 13:9, 25 ± 1 °C, 50 ± 10 % RH).

Tri-trophic bioassay with *C. marginventris*

Newly emerged female and male *C. marginventris* adults were paired in 0.5 L clear soda plastic bottle whose bottom was cut and covered with cotton gauze. Parasitoids were supplied honey and a 10 % sugar water solution-saturated cotton wick. After allowing 48 h for mating, ten 4-days Cry1F maize-fed or non-Bt maize-fed *S. frugiperda* larvae were presented to paired wasps for 24 h. These larvae were exposed to parasitoids by placing them in a Petri dish (5 cm diam.) with six 5-cm Cry1F maize or non-Bt maize leaf clippings. Water-saturated Bounty® white paper towels were placed at the bottom of each Petri dish to provide moisture. After the 24 h exposure period, the *S. frugiperda* larvae were individually transferred into 50-ml cups and supplied with a 13 cm Cry1F maize leaf or non-Bt maize leaf and wetted filter paper. A second batch of ten 4-days Cry1F maize-fed or non-Bt maize-fed *S. frugiperda* larvae were then exposed to the same *C. marginvertris* for another 24 h in the same manner and were then transferred individually into 50-ml cups. Maize leaves in the cup were changed when necessary. *S. frugiperda* larvae were checked twice per day (9 am and 9 pm) and the time when parasitoid cocoons formed was recorded. Cocoons were individually transferred to a clear 50-ml cup and checked twice per day (9 a.m. and 9 p.m.) until adults emerged. Ten pairs of *C. marginvertris* were utilized for both Bt maize and non-Bt maize treatments. The offspring of *C. marginiventris* underwent another 4 generations of testing, as described above. For the 1st, 2nd, and 4th generations, *C. marginiventris* were allowed to parasitize for 2 days as described above; for the 3rd and 5th generations they were provided hosts for their entire lifespan (10 *S. frugiperda* larvae per day). These two generations were then used to estimate adult parasitoid longevity and total fecundity.

Transfer of Cry1F through tri-trophic levels

Another 20 pairs of *C. marginiventris* for the Bt maize and non-Bt maize treatments were set up during the 5th generation study, as described in the tri-trophic bioassays above. For both treatments, 100 *S. frugiperda* larvae were dissected 8 days after they were

Table 1 Tri-trophic effects of Cry1F maize on life table parameters of *C. marginiventris* (1st generation) when parasitized Cry1F-resistant *S. frugiperda* were reared on Cry1F maize or non-Bt maize [means ± SE (n)]

Parameters	Cry1F maize	Non-Bt isoline	*t* value (*P*)
Development time (days)			
Male eggs to cocoons	10.9 ± 0.2 (10)	10.9 ± 0.1 (9)	0.41 (0.68)
Female eggs to cocoons	11.1 ± 0.2 (9)	11.1 ± 0.2 (9)	0.25 (0.73)
Male cocoons to adults	5.0 ± 0.1 (10)	5.1 ± 0.1 (9)	1.44 (0.17)
Female eggs to cocoons	5.3 ± 0.1 (9)	5.3 ± 0.1 (9)	0.09 (0.93)
Success of parasitism (%)	93.0 ± 2.3 (10)	92.6 ± 3.0 (10)	0.17 (0.87)
Cocoon-adult survivorship (%)	84.1 ± 1.6 (10)	88.1 ± 1.8 (10)	0.78 (0.44)
Sex ratio (% females)	68.8 ± 4.7 (10)	52.8 ± 4.8 (10)	1.78 (0.09)

Ten pairs of *C. marginiventris* were utilized for both Bt-maize and non-Bt maize treatments

parasitized and 20 larvae of *C. marginiventris* were collected as one replicate. Another 20 cocoons and resulting adults of *C. marginiventris* were also collected as one replicate, respectively. After the larvae of *C. marginiventris* emerged from the their hosts, 20 *S. frugiperda* mummies (the hosts which were killed by *C. marginiventris*) were collected as one replicate. For all different insect samples, three replicates were conducted. Three replicates of Bt and non-Bt maize leaves (50 mg as one replication) were also collected. The Cry1F toxin concentrations in maize leaves and insects were measured by enzyme-linked immunosorbent assays (ELISA) using Cry1F detection kits from Agdia (Elkhart, IN). Prior to analysis, all insects were washed with phosphate buffered saline + tween 20 (PBST) four times to remove any Bt toxin from the surface. Maize leaf samples were diluted at a rate of 1:2,000 (mg sample: µl PBST buffer) and fully ground by mortar and pestle. Insect samples were diluted at a rate of 1:10 (mg sample: µl PBST buffer) in 1.5 ml centrifuge tubes, and ground by hand using a plastic pestle. ELISA was performed according to the manufacturer's instructions.

Statistical analyses

Data on life table parameters of *C. marginiventris* and data on Cry1F protein levels in plants and insects were analyzed using Student's *t*-test. All percentage data were converted to arcsine square-root values prior to analysis, but untransformed means are presented. All statistical calculations were performed with SAS version 9.1 (SAS Institute 2001).

Results

Tri-trophic effects of Cry1F maize on *C. marginiventris* for the first and second day of parasitism

Nine to twelve days after parasitism, *C. marginiventris* larvae emerged from *S. frugiperda* and formed cocoons. Adults emerged 4–6.5 days later. For the 1st generation, there were no significant differences detected for any of the life table parameters measured for *C. marginiventris* (Table 1).

Similar results were found for the 2nd, 3rd, 4th and 5th generations. No significant differences were found for any life table parameters between the Cry1F maize treatment and control maize treatment (only data from the 3rd and 5th generations are shown) (Tables 2, 3).

Tri-trophic effects of Cry1F maize on fecundity and longevity of mated female *C. marginiventris*

For the 3rd and 5th generations, *S. frugiperda* were continually supplied to *C. marginiventris* pairs until the female died. For the 3rd generation, fecundity of *C. marginiventris* in the Bt maize and non-Bt control treatment means (±SE) were 52.2 ± 6.7 and 51.1 ± 3.8, respectively; these differences were not significant ($t = 0.37$, $P = 0.36$, $n = 10$). Also, longevity of mated female *C. marginiventris* from the Bt maize and non-Bt maize treatments were not significantly different, 7.7 ± 0.7 days and 7.3 ± 0.3 days, respectively ($t = 0.62$, $P = 0.27$, $n = 10$).

For the 5th generation, fecundity and longevity of *C. marginiventris* were lower than those for the 3rd

Table 2 Tri-trophic effects of Cry1F maize on life table parameters of *C. marginiventris* (3rd generation) when parasitized Cry1F-resistant *S. frugiperda* were reared on Cry1F maize or non-Bt maize [means ± SE (n)]

Parameters	Cry1F maize	Non-Bt isoline	*t* value (*P*)
Development time (days)			
Male eggs to cocoons	10.2 ± 0.1 (10)	10.5 ± 0.1 (10)	1.66 (0.11)
Female eggs to cocoons	10.3 ± 0.1 (10)	10.5 ± 0.2 (10)	1.53 (0.14)
Male cocoons to adults	5.2 ± 0.1 (10)	5.4 ± 0.1 (10)	1.39 (0.18)
Female eggs to cocoons	5.3 ± 0.1 (10)	5.3 ± 0.1 (10)	0.07 (0.94)
Success of parasitism (%)	93.0 ± 1.6 (10)	93.0 ± 2.6 (10)	0.001 (0.99)
Cocoon-adult survivorship (%)	85.2 ± 2.2 (10)	89.6 ± 2.1 (10)	1.45 (0.16)
Sex ratio (% females)	66.7 ± 4.3 (10)	53.3 ± 5.2 (10)	1.97 (0.06)

Ten pairs of *C. marginiventris* were utilized for both Bt-maize and non-Bt maize treatments

Table 3 Tri-trophic effects of Cry1F maize on life table parameters of *C. marginiventris* (5th generation) when parasitized Cry1F-resistant *S. frugiperda* were reared on Cry1F maize or non-Bt maize [means ± SE (n)]

Parameters	Cry1F maize	Non-Bt isoline	*t* value (*P*)
Development time (days)			
Male eggs to cocoons	10.1 ± 0.1 (10)	10.3 ± 0.1 (10)	1.52 (0.15)
Female eggs to cocoons	10.2 ± 0.1 (10)	10.5 ± 0.1 (10)	1.66 (0.11)
Male cocoons to adults	5.3 ± 0.2 (10)	5.2 ± 0.1 (10)	0.99 (0.34)
Female eggs to cocoons	5.5 ± 0.1 (10)	5.2 ± 0.1 (10)	1.93 (0.06)
Success of parasitism (%)	91.0 ± 3.5 (10)	89.1 ± 5.2 (10)	0.29 (0.77)
Cocoon-adult survivorship (%)	91.1 ± 1.9 (10)	86.6 ± 0.3 (10)	1.21 (0.24)
Sex ratio (% females)	64.4 ± 4.1 (10)	52.2 ± 7.4 (10)	1.78 (0.09)

Ten pairs of *C. marginiventris* were utilized for both Bt-maize and non-Bt maize treatments

generation, but there were no significant differences between the Bt maize and non-Bt maize treatments for fecundity [44.0 ± 6.9 (Bt), 39.1 ± 3.3 (control); $t = 0.64$, $P = 0.26$, n = 10] or for longevity [6.9 days ± 0.8 (Bt), 6.3 days ± 1.2 (control); $t = 1.12$, $P = 0.14$, n = 10].

Cry1F protein levels in Cry1F maize, *S. frugiperda* and *C. marginiventris*

For the Bt maize treatment, 5th-leaf stage leaves contained a mean of 3.21 μg/g Cry1F per fresh weight (FW) (Table 4). The average of Cry1F protein in *S. frugiperda* was 0.12 μg/g Cry1F per FW, which was significantly lower than those in Bt maize leaves ($t = 11.88$, $P < 0.001$, n = 3). Levels of Cry1F protein were below the detection limits in larvae, cocoons or adults of *C. marginiventris*.

As expected, no Cry1F protein was detected in maize leaves, *S. frugiperda* and *C. marginiventris* from the control non-Bt maize treatment.

Discussion

Studying potential impacts of insect-resistant genetically-engineered plants on beneficial non-target arthropods is an important component of the environmental risk assessment. The initial steps of risk assessment for many regulatory agencies include early-tier laboratory studies that expose test species, or their surrogates, to a high dose of the biologically active insecticidal compound (Romeis et al. 2008). Herbivores that have consumed tissues from Bt crops, when used as prey or hosts for a natural enemy, provide a realistic exposure pathway. However, Bt proteins will affect Bt-susceptible herbivores and

Table 4 Cry1F protein levels in Cry1F maize, *S. frugiperda* and *C. marginiventris*

Sample	Cry1F per fresh weight (μg/g)
Maize (5 leaf stage)	3.21 ± 0.23 a
S. frugiperda mummies	0.12 ± 0.01 b
C. marginiventris larvae	Not detectable
C. marginiventris cocoons	Not detectable
C. marginiventris adults	Not detectable

Means (±SE) within a column followed by different letters are significantly different (Student's *t*-test, $P < 0.05$); N = 3

consequently affect their quality as a resource for natural enemies. Such 'host/prey-quality mediated effects' have been observed in numerous tri-trophic feeding studies with Bt crops (Romeis et al. 2006; Naranjo 2009) and have erroneously been interpreted as direct toxic effects of Bt proteins (Shelton et al. 2009a, b). An excellent way to avoid the impact of 'host/prey-quality mediated effects' in tri-trophic study systems is to use Bt-resistant herbivores as a Bt protein carrier (Romeis et al. 2011). To date, Bt-resistant strains of Lepidoptera have been used to assess the impact of Bt crops on several natural enemies (Romeis et al. 2011), but these have primarily been with predators that generally consume multiple species of prey over their lifetimes. In contrast, a parasitoid depends on a single host to develop and therefore could be more susceptible to the effects of a Bt protein.

We demonstrate in our present study that *C. marginiventris* is not affected by plant-produced Cry1F protein when it was present in the parasitoid's host. Our ELISA analyses confirmed the presence of the Cry protein in *S. frugiperda* larvae. However, Cry protein levels (per FW sample) were less than 4 % of those measured in the maize leaves. These toxin levels in the plants and in *S. frugiperda* larvae were comparable to those reported by Tian et al. (2012, 2013). ELISA analyses of *C. marginiventris* larvae, cocoons and adults did not detect any Cry1F toxin. These results are similar to ELISA studies conducted by Vojtech et al. (2005) for the same parasitoid and *S. littoralis* as the host. In our study, the *S. frugiperda* larva died before a *C. marginiventris* larva left the host to pupate. This suggests that, although the newly hatched parasitoid larva fed on hemolymph that contained little Bt protein, they continued to feed on other host tissues and could have been exposed to the host's gut contents where Bt proteins would be present. Nonetheless, Cry1F was not detected in *C. marginiventris*. By using a Cry1F-resistant host in this realistic tri-trophic study, it is clear that *C. marginiventris* is not harmed when it feeds on a host that has ingested Cry1F.

We are aware of only two other studies that have used a Bt resistant host (*Plutella xylostella* L.) (Lepidoptera: Plutellidae) (Schuler et al. 2004; Chen et al. 2008) to study the effects on a parasitoid, but there are no commercialized Bt crops on which this insect feeds. Thus, the present study represents the first case of using a commercialized Bt crop to study its effect on an important parasitoid without the influence of reduced host quality.

To avoid the problem of confounding host-quality mediated effects, we used Cry1F-resistant *S. frugiperda* as the Bt protein carrier. Although no study was conducted to clarify the mechanisms of resistance to Cry1F protein of *S. frugiperda*, the midgut binding site (including cadherin, alkaline phosphatase and aminopeptidases N) modification to Cry proteins were the most probable mechanism (Ferré and Van Rie 2002, Jurat-Fuentes et al. 2011, Tiewsiri and Wang 2011) that would not substantially alter the other characters of the resistant strain. Our previous studies had shown that Cry1F-resistant *S. frugiperda* were not affected by Cry1F when they fed on Cry1F maize, even though they contained a high dose of bioactive Cry1F protein (Tian et al. 2012, 2013). Furthermore, in those studies we conducted a tri-trophic bioassay that showed the predator *Coleomegilla maculata* (De Geere) (Coleoptera: Coccinellidae) and the predator *Chrysoperla rufilabris* (Neuroptera: Chrysopidae) were not affected by feeding on resistant *S. frugiperda* that had consumed Cry1F maize. The present study with the parasitoid, *C. marginiventris*, clearly shows that Cry1F-producing maize does not affect development, parasitism, survivorship, sex ratio, longevity and fecundity of *C. marginiventris* when they parasitize Cry1F maize-fed *S. frugiperda*. Furthermore, we also observed no chronic long-term effects of Cry1F over five generations of continuous exposure. This provides additional assurance of Cry1F maize's safety to *C. marginiventris*, an important parasitoid species of many economically important noctuid caterpillars (Miller 1977; Kunnalaca and Mueller 1979; Marsh 1979; McCutcheon et al. 1990; Ruberson et al. 1994).

There is a large body of literature on the potential effects of Bt crops on non-target organisms, and the overwhelming evidence is that Bt crops are safe to natural enemies (Naranjo 2009). The preservation of natural enemies is critical because they help control primary and secondary pests not controlled by the Bt crop. Furthermore, recent modeling work (Onstad et al. 2013) has suggested natural enemies can also delay the evolution of resistance to the Bt plants by the targeted pest. Recent work with *P. xylostella*, Bt broccoli and the generalist predator, *C. maculata*, demonstrated that this natural enemy could delay the evolution of resistance in *P. xylostella* to Bt broccoli expressing Cry1Ab protein (AMS, unpublished). These data suggest that natural enemies could play an important role in diminishing the likelihood of resistance evolution by a pest species to a Bt crop. This study shows conclusively that direct long-term exposure to Cry1F through its host over multiple generations does not affect the biology of an important parasitoid species. Our results clearly indicate that previous non-target studies (Vojtech et al. 2005; Ramirez-Romero et al. 2007) on *C. marginiventris* and Bt proteins that showed harm to this important parasitoid suffered from an inability to take into account host-quality mediated effects.

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