Available online at www.sciencedirect.com
ScienceDirect
Crop Protection 26 (2007) 953–962
Crop Protection
www.elsevier.com/locate/cropro

Impact assessment of Bt-maize on a moth parasitoid, *Cotesia marginiventris* (Hymenoptera: Braconidae), via host exposure to purified Cry1Ab protein or Bt-plants

Ricardo Ramirez-Romero[a,*], Julio S. Bernal[b], Josette Chaufaux[c], Laure Kaiser[a,1]

[a]*INRA, Laboratoire de Neurobiologie Comparée des Invertébrés, F 91440 Bures-sur-Yvette, France*
[b]*Department of Entomology, Biological Control Laboratory, Texas A&M University, College Station, TX 77843-2475, USA*
[c]*INRA, Unité Génétique Microbienne et Environnement, La Minière 78285 Guyancourt Cedex, France*

Received 15 February 2006; accepted 1 September 2006

Abstract

Addressing whether Cry1Ab protein produced by Bt-maize affects non-target insects, including parasitoids, is a necessary component in the risk assessment of this crop protection alternative. This study assessed host-mediated effects of Cry1Ab protein on the parasitoid *Cotesia marginiventris* Cresson (Hymenoptera) via two delivery methods: delivery of purified Cry1Ab protein via artificial diet, and delivery of Cry1Ab protein via Bt-maize plant tissue. In the first case, lethal and sublethal effects of purified Cry1Ab protein on the host, *Spodoptera frugiperda* (Lepidoptera), were evaluated prior to evaluating effects on the parasitoid. Unparasitized host larvae were exposed to one of three Cry1Ab concentrations, 0.46 (C1), 9.13 (C2), and 182.6 (C3) μg Cry1Ab/ml diet. The C3 concentration proved highly toxic to host larvae, so only host-mediated effects of C1 and C2 concentrations on the parasitoid *C. marginiventris* were studied. As expected, purified Cry1Ab affected survival, developmental times, and growth rates of *S. frugiperda* larvae at all three Cry1Ab concentrations. In contrast, host-mediated effects of purified Cry1Ab protein on *C. marginiventris* were not evident at the two concentrations that were evaluated, C1 and C2. However, several host-mediated effects on *C. marginiventris* were detected when Cry1Ab protein was delivered via Bt-maize tissue. Exposure to Cry1Ab protein via Bt-maize tissue affected parasitoid developmental times, adult size, and fecundity. Though effects on parasitoids of direct exposure (i.e. not mediated by the host) to Cry1Ab protein were not evaluated, the results of the present study suggested a direct effect of the protein, delivered via host feeding on Bt-maize, on *C. marginiventris*.

Keywords: Cry1Ab; Transgenic crop; Bt-maize; Parasitoid; Non-target

1. Introduction

The area planted with genetically modified (GM) crops in 2004 was ~81 million ha worldwide, an increase of almost 20% relative to 2003 (James, 2004). Similarly, the area planted to GM corn in 2004, ~19.3 million ha (23% of global GM area), increased by ~25% relative to 2003 (James, 2004). Such increased utilization of GM crops raises the need for examining any impacts of these crops on populations of non-target insects. Assessing any impacts on non-target insects is an important component of ecological risk assessments of GM crops (Andow and Hilbeck, 2004), and is particularly necessary in light of the documented deleterious effects on some natural enemies when Bt-susceptible or sublethally damaged herbivores have been used as prey or hosts (Romeis et al., 2006). Commercially available Bt-maize hybrids (events MON810, Bt11, and 176) express *Bacillus thuringiensis* Cry1Ab protein, which is active primarily against *Ostrinia nubilalis* (H.) (Lepidoptera: Crambidae) (Chaufaux et al.,

*Corresponding author. Present address: Instituto de Ecología, A.C. Apartado Postal 63, 91000, Xalapa, Veracruz, México.
Tel.: +52 228 842 1841; fax: +52 228 842 4115.
E-mail address: ricardo.ramirez@inecol.edu.mx (R. Ramirez-Romero).
[1]Present address: Institut Fédératif de Neurobiologie Alfred Fessard, CNRS, Avenue de la Terrasse, Bât 32/33, 91198 Gif-sur-Yvette Cedex, France.

0261-2194/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.cropro.2006.09.001

2001). In susceptible insects, Cry1 crystal proteins are dissolved and activated by specific proteases after ingestion, and are then attached to specific receptors in the midgut epithelium to form a toxic lesion leading to insect death (Knowles and Dow, 1993).

Spodoptera frugiperda (Smith) (Lepidoptera: Noctuidae), commonly known as fall armyworm, is an important pest of maize in Central America and Mexico (Kumar and Mihm, 1996). Miranda et al. (2001) showed that Cry1Ab protoxins are cleaved in the *S. frugiperda* midgut, thus releasing the active toxin components, i.e. fragments of 62 and 60 kDa. Furthermore, Aranda et al. (1996) found that, compared to other Cry1 toxins, activated Cry1Ab toxin binded weakly on the epithelial brush border membrane of *S. frugiperda* larvae, so causing low mortality. Such observations explain in part why currently available Bt-maize expressing Cry1Ab protein is partially effective against this species, although it is not the primary target (e.g., Lynch et al., 1999; Bokonon-Ganta et al., 2003).

Cotesia marginiventris (Cresson) (Hymenoptera: Braconidae) is a solitary, larval endoparasitoid, and important natural enemy of *S. frugiperda* (Ashley, 1979). Adults oviposit into 1st- and 2nd-instar host larvae; young parasitoid larvae feed primarily on host hemolymph, while the last instar kills the host by feeding on tissues and emerges from the host body to pupate (Ashley et al., 1982). Receptors for Cry1Ab protein are presently unknown in Hymenoptera, thus these toxins are not expected to have direct lethal or sublethal effects in parasitoid hymenopterans. Thus, some earlier studies showed that Bt biopesticides, which deliver inactive protoxins, had no effect when ingested by adult parasitoids (Blumberg et al., 1997; Chilcutt and Tabashnik, 1999). However, host-mediated effects can occur when parasitoids develop on hosts exposed to Bt biopesticides or Bt-plants expressing Cry proteins (reviewed in Obrycki et al., 2004). In the specific case of *C. marginiventris*, recent research with Bt-plants suggested some host-mediated effects. Thus, Baur and Boethel (2003) showed that *C. marginiventris* developing in *Pseudoplusia includens* (Walker) fed transgenic cotton expressing Cry1Ac protein, suffered reductions in longevity and number of oocytes. Vojtech et al. (2005) showed that *C. marginiventris* survival, developmental times, and cocoon weights were significantly affected when the non-target host *Spodoptera littoralis* (Boisduval) fed on a mixture of Bt-maize leaves and stems.

The present study relied on two different approaches for assessing lethal and sublethal host-mediated effects of Cry1Ab protein, the protein produced by Bt-maize, on the parasitoid *C. marginiventris*. Both approaches delivered Cry1Ab protein to *C. marginiventris* via *S. frugiperda* larvae. The first approach consisted of delivering purified Cry1Ab protein, at three concentrations, via artificial diet. The second approach consisted of delivering Cry1Ab protein via tissue from Bt-maize plants. Whereas the first approach offered technical advantages (e.g., several, precise Cry1Ab protein concentrations could be tested at one time) over the second, the second approach offered more realistic conditions. We report herein the rates of parasitism, host suitability, developmental periods, offspring longevity, size and sex ratio of *C. marginiventris* after host-mediated exposure to Cry1Ab protein delivered via artificial diet or Bt-maize tissue.

2. Material and methods

2.1. Cry1Ab protein

The Cry1Ab protein used in this study, purchased from Case Western Reserve University, Department of Biochemistry (Cleveland, Ohio, USA), had a molecular size of 131 kDa and was isolated from the crystals of a clone carrying the Cry1Ab gene from *Bt kurstaki* HD-1 (Wabiko et al., 1986). Protoxin toxicity was checked on *O. nubilalis* using a CL_{50} protocol described by Chaufaux et al. (2001), and was estimated at 2.5 ng/cm^2 (CL 95: 1.9–3.3) of artificial diet (Poitout and Bues, 1970).

2.2. Artificial diets

Cry1Ab protein was dissolved in micro-tubes with Na_2CO_3 buffer (50 mM, pH 10) using a dilution factor of 0.125 mg/ml. This solution was added to artificial diet (Poitout and Bues, 1970, for effects on *S. frugiperda*; Martinez et al., 1988, for host-mediated effects on *C. marginiventris*), and subsequently the micro-tube was washed with Na_2CO_3 buffer, which was incorporated into the artificial diet until reaching a final buffer concentration of 2.5% (v:v). The final concentrations of Cry1Ab tested were 0.456 μg Cry1Ab/ml diet (hereafter C1), 9.13 μg Cry1Ab/ml (C2), and 182.6 μg Cry1Ab/ml (C3). Control artificial diet contained only Na_2CO_3 buffer at 2.5% (v:v). The C1 concentration is similar to concentrations found in leaves of Bt-maize event MON810 (Bokonon-Ganta et al., 2003; Vojtech et al., 2005).

2.3. Maize plants

Bt- and conventional maize plants were grown in planters (65 cm × 30 cm × 25 cm) in a greenhouse with natural light and ambient temperature never exceeding 35 °C. Bt-plants were of a hybrid (Pioneer 35N05) expressing a Cry1Ab endotoxin gene (MON810), while conventional plants were of a second, near-isogenic hybrid (Pioneer 3567), which does not express a Bt endotoxin gene. Two rows of each hybrid were sown per planter in an equal mixture of moist vermiculite and potting soil (SunGro Horticulture Inc., Vancouver, Canada). Plants were 10–14 d old when used in experiments, were not fertilized, and were watered daily. For all experiments, plants were individually transplanted in 148 ml plastic vials (49 mm × 85 mm, BioQuip Products Inc., Gardena, CA) perforated at the bottom for drainage.

2.4. Host-mediated effects of Cry1Ab protein on Cotesia marginiventris

The first step in this approach was to assess the effects of Cry1Ab protein on *S. frugiperda* larvae from a culture maintained at Le Magneraud INRA, Surgères, France. This culture was maintained at 25±2 °C, 50–70% RH and a 14:10 (L:D) lighting regime. Under these conditions, newly laid *S. frugiperda* eggs were incubated in plastic bottles (2 cm diam., 3 cm tall) and neonate larvae were placed in plastic boxes (25 cm × 25 cm × 8 cm) containing artificial diet (Poitout and Bues, 1970) until pupation, or used after 3 d in experiments. Use of neonate larvae in experiments was avoided because of the high mortality due to handling and other factors, such as death due to drowning in condensed droplets of water in diet cups or on plants, death due to desiccation of larvae falling off plants, and others.

Experimental conditions were set at 25±2 °C, 50–70% RH and a 14:10 (L:D) lighting regime. When *S. frugiperda* larvae were 3 d old they were transferred to plastic cups (2 cm × 3.5 cm × 1.5 cm) (one larva per cup) containing ~4 g of C1, C2, C3, or control diet. Each cup was covered with filter paper and a plastic lid, with two perforations (1 mm diam.) for aeration and held in place with elastic bands. Cups were placed upside down to facilitate collection of frass, and diet was replenished as needed. Respectively, 110, 111, 96, and 107 *S. frugiperda* larvae were assigned to control, C1, C2, and C3 diets.

Survival and frass production of *S. frugiperda* larvae were recorded following 2 d of exposure (5 d-old larvae), and ~30 larvae per treatment were removed from artificial diets, weighed to the nearest 0.1 mg, and the head capsule widths (from the outer margin of one compound eye to the other) measured to the nearest 0.08 mm using a stereomicroscope fitted with an ocular micrometer. The filter paper lining the cup lids was replaced at this time, and larvae were returned to their respective diets after weighing and measuring. This procedure was repeated at 6 and 10 d of exposure (9- and 13-d old larvae, respectively). Mean frass production per larva was estimated for each period and compared among diets by using a Kruskal–Wallis test and non-parametric multiple comparisons tests when appropriate (Zar, 1998). Proportions of survivors at each observation period and proportion of larvae forming pupae were compared among treatments using χ^2 tests; in case of significant differences, multiple comparison for proportions were conducted (Zar, 1998). Mean head capsule widths, larval development times, and larval and pupal weights were compared among diets, at each observation, using a one-way analysis of variance (ANOVA) followed by Tukey's tests, as warranted (Zar, 1998).

The second step consisted of assessing host-mediated effects of Cry1Ab protein on *C. marginiventris*. Life history parameters were compared among parasitoids developing on *S. frugiperda* larvae fed C1, C2, or control diet; C3 diet effects on parasitoids were not evaluated due to excessive *S. frugiperda* mortality (see Section 3). *S. frugiperda* larvae from a culture maintained at the Biological Control Laboratory, Texas A&M University, USA, were used in this portion of the study. *C. marginiventris* were reared by exposing 4 d-old *S. frugiperda* larvae for 1 h to mature female *C. marginiventris* at a 3:1 host:parasitoid ratio in glass vials (8.5 cm diam., 13 cm tall). Exposed larvae were then placed individually in small plastic cups containing ~5 g of artificial diet (Martinez et al., 1988) until they yielded parasitoid cocoons (or moth pupae), which were collected and placed individually in glass vials (12 mm × 35 mm) plugged with cotton. Parasitoid cocoons were incubated at 30±1 °C, 50–70% RH, and a 14:10 (L:D) lighting regime until adults emerged. Adult parasitoids were offered honey:water solution (20% v:v) immediately upon emergence, allowed to mate, and females were used in experiments when they were 1–2 d-old.

Experimental conditions were set at 30±1 °C, 50–70% RH, and a 14:10 (L:D) lighting regime. Neonate *S. frugiperda* larvae were fed artificial diet for 3 d, and then were individually exposed to 1–2 d-old *C. marginiventris* females in glass vials (25 mm × 95 mm) until they were parasitized by the female; each female was allowed to parasitize 5–10 larvae. Following exposure to a parasitoid, host larvae were placed individually in glass vials (1.5 ml) containing an artificial diet treatment and plugged with cotton. Survivorship of *S. frugiperda* larvae was checked daily until formation of parasitoid cocoons (or moth pupae). The number of days to the appearance of cocoons was scored, and cocoons were transferred to individual glass vials (1.5 ml) plugged with cotton where parasitoid adults emerged. The gender and number of days to adult emergence were scored for each parasitoid adult. All adult parasitoids were offered 20% honey solution, which was replenished as needed, and adult survivorship was monitored daily. Upon their death, female size was estimated by measuring the length of their left hind tibia (hereafter HTL) to the nearest 0.001 mm using a stereomicroscope fitted with an ocular micrometer. Successful parasitism (= hosts yielding adult parasitoids/hosts exposed to parasitoid attack) and cocoon to adult survivorship (= cocoons yielding adult parasitoids/total number of cocoons) rates were estimated.

Percentages of successful parasitism, egg-to-cocoon and cocoon-to-adult survival were compared among diet treatments using log-likelihood ratio tests (Zar, 1998). In addition, egg-to-cocoon and egg-to-adult developmental times, longevity of adult parasitoids, and HTL were compared among treatments, within sexes, via a Kruskal–Wallis single-factor analysis of variance (Zar, 1998) because data did not meet the assumptions of normality. Parasitoid sex ratios (% males) were compared against a 1:1 ratio using a χ^2 goodness-of-fit test, and were compared between control and treatments using χ^2-tests (Zar, 1998). All statistical analyses were performed using Systat® software (SPSS, 2000).

2.5. Host-mediated effects of Bt-maize plants on Cotesia marginiventris

Life history parameters of *C. marginiventris* were compared between parasitoids developing on *S. frugiperda* fed Bt- or conventional maize plants. Insect rearing, experimental conditions, host larvae exposure to *C. marginiventris* females and recorded data were as described above for Cry1Ab protein delivered via artificial diet, with differences noted below to accommodate the use of living plants versus artificial diet. Neonate larvae were placed on Bt- or conventional plants and exposed to female parasitoids at 3-d of age, and then returned to the corresponding plant, and transferred to new plants as necessary until formation of parasitoid cocoons (or moth pupae). A 1-cm layer of white sand (PlaySand, Quikrete®, Atlanta, GA) was spread over the soil surface surrounding the base of plants to facilitate recovery of parasitoid cocoons (or moth pupae). Plants were enclosed by 148 ml (49 mm × 85 mm) plastic vials with their bottoms replaced with fine-mesh metal screen; pots and enclosures were joined with their corresponding lids, which had a ~40 mm diam. perforation to allow passage of the plant from the pot to the enclosure. Recovered cocoons and adult parasitoids handled as indicated above. Percentages of successful parasitism per female parasitoid were arc-sine $\sqrt{}$x-transformed and compared between treatments via a *t*-test (Zar, 1998). Proportions of egg to cocoon and cocoon-to-adult survival were compared between treatments via log-likelihood ratio tests with Yates' correction for continuity (Zar, 1998). Mean egg-to-cocoon and egg-to-adult developmental times, mean longevities and HTLs were compared between treatments via *t*-tests (Zar, 1998), separately for male and female parasitoids. Parasitoid sex ratios (% males) were compared against a 1:1 ratio using a χ^2 goodness-of-fit test, and sex ratios on Bt- and conventional maize treatments were compared using a χ^2-test (Zar, 1998).

Separately, an experiment was conducted to compare the relationships between adult parasitoid size and egg load in the Bt- and conventional maize treatments. Two hundred and forty 4 d-old *S. frugiperda* larvae were exposed in groups of twelve to twenty individual *C. marginiventris* females inside glass vials (25 mm × 95 mm) for 6 h; 160 larvae were assigned to the Bt-maize treatment, which yielded 25 *C. marginiventris* females, and 80 to the conventional maize treatment, which yielded 23 *C. marginiventris* females. All female parasitoids from each treatment were dissected in physiological saline solution (7.5 g of NaCl in 1 l of distilled water) within 24 h of their emergence, and their egg loads (= ovarian eggs) and HTLs measured using a stereomicroscope fitted with an ocular micrometer. The relationships between adult size (= HTL) and egg load of female parasitoids on Bt- and conventional maize were assessed via linear regression analyses, and slopes were compared via *t*-tests (Zar, 1998). In addition, mean egg loads and HTLs were compared between treatments using *t*-tests (Zar, 1998). Finally, the relationships between adult parasitoid size (= HTL) and host size (= weight in mg), were compared via linear regression analyses involving 58 and 46 female parasitoids emerging from hosts in the Bt- and conventional maize treatments, respectively, followed by slope comparison via a *t*-test (Zar, 1998). In addition, mean host weights and HTLs were compared between treatments using *t*-tests (Zar, 1998).

Finally, the presence of Cry1Ab in *S. frugiperda* larvae fed Bt-maize tissue was assessed placing single 4-d old larvae on individual maize seedlings and allowing them to feed during 4 d. After this period, larvae were analyzed with DAS ELISA tests (AGDIA Inc., Elkhart, IN, USA). In every case, larvae were removed from maize seedlings ~30 min prior to assays and kept in plastic vials at ca. 8 °C until used.

3. Results

3.1. Host-mediated effects of Cry1Ab delivered via artificial diet on Cotesia marginiventris

3.1.1. Effects on S. frugiperda larvae

Survivorship of *S. frugiperda* larvae after 2 d of exposure (i.e. 5 d-old larvae) was not significantly affected by the different Cry1Ab protein treatments relative to the control ($P = 0.106$) (Fig. 1). However, significant reductions of survivorship ($P < 0.001$) were evident on C3 diet after 6 d of exposure, and on C2 and C3 diets after 10 d of exposure (Fig. 1). Weights of *S. frugiperda* larvae were significantly lower relative to the control on C1, C2, and C3 diets after 2, 6, and 10 d of exposure ($P < 0.001$) (Fig. 2). Larvae exposed to C2 and C3 diets gained little weight during the observation period (~15- and 4-fold, respectively), relative to the control (~139-fold) or C1 diet (~68-fold). Head capsule widths were significantly smaller ($P < 0.001$) on C2

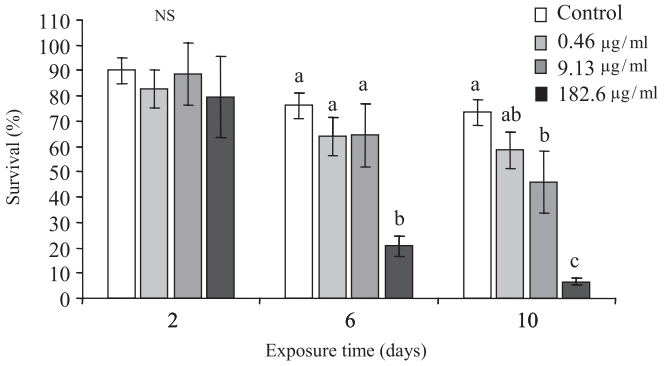

Fig. 1. Survival (%) of *S. frugiperda* larvae on artificial diet containing 0.46, 9.13, or 182.6 µg Cry1Ab protein/ml diet, or control diet (without Cry1Ab protein), after 2, 6, and 10 d of exposure. Within exposure times, columns sharing lower-case letters are not significantly different ($P < 0.05$) (2 d exposure, $\chi^2 = 6.13$, 3 df, $P = 0.106$; 6 d, $\chi^2 = 78.90$, 3 df, $P < 0.001$; 10 d, $\chi^2 = 107.75$, 3 df, $P < 0.001$).

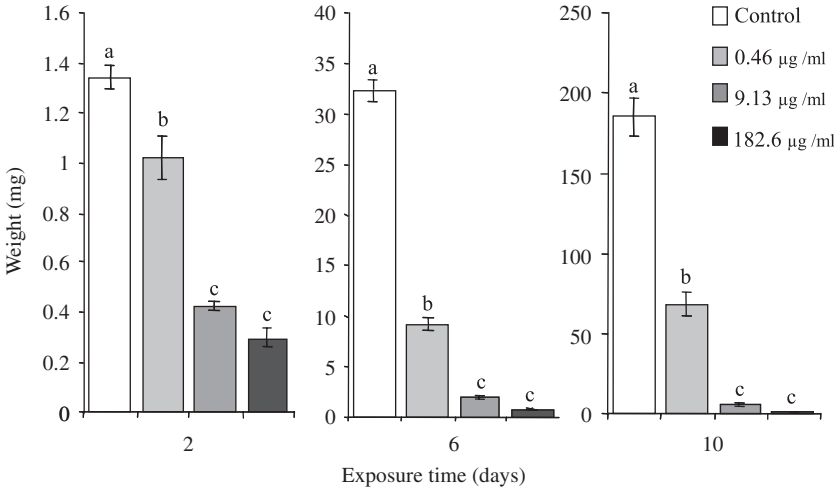

Fig. 2. Weight (mg) of *S. frugiperda* larvae developing on artificial diet containing 0.46, 9.13, or 182.6 µg Cry1Ab protein/ml diet, or control diet (without Cry1Ab protein), after 2, 6 and 10 d of exposure. Within exposure times, columns sharing lower-case letters are not significantly different (2 d exposure, $F_{3,\ 126} = 86.21$, $P<0.001$; 6 d, $F_{3,\ 115} = 503.12$, $P<0.001$; 10 d, $F_{3,\ 100} = 85.04$, $P<0.001$).

Table 1
Mean (±SE) head capsule width and frass production in *S. frugiperda* developing on artificial diet containing one of three Cry1Ab protein concentrations (C1 = 0.46, C2 = 9.13, C3 = 182.6 µg Cry1Ab/ml diet) or control diet (without Cry1Ab protein)

	Mean head capsule width (mm) Days after exposure			Mean frass production per larvae (mg) Days after exposure		
	2 d ($F_{3,\ 126} = 7.624$, $P<0.001$)	6 d ($F_{3,\ 115} = 114.25$, $P<0.001$)	10 d ($F_{3,\ 100} = 114.25$, $P<0.001$)	2 d ($H = 9.020$, 3df, $P>0.05$)	6 d ($H = 20.009$, 3df, $P<0.001$)	10 d ($H = 19.080$, 3df, $P<0.001$)
Control	0.43±0.02 a ($n = 32$)	1.30±0.05 a ($n = 32$)	2.30±0.05 a ($n = 32$)	0.12±0.02 a ($n = 99$)	27.7 4±6.93 a ($n = 84$)	207.84±14.80 a ($n = 81$)
C1 (0.46 µg Cry1Ab/ml)	0.41±0.01 ab ($n = 32$)	0.86±0.03 b ($n = 32$)	1.45±0.06 b ($n = 32$)	0.09±0.01 a ($n = 92$)	4.43±1.45 ab ($n = 71$)	59.93±12.92 ab ($n = 65$)
C2 (9.13 µg Cry1Ab/ml)	0.35±0.01 bc ($n = 33$)	0.51±0.02 c ($n = 29$)	0.68±0.03 c ($n = 29$)	0.004±0.0 a ($n = 85$)	0.35±0.12 b ($n = 62$)	3.38±1.38 b ($n = 44$)
C3 (182.6 µg Cry1Ab/ml)	0.34±0.01 c ($n = 30$)	0.41±0.01 c ($n = 22$)	0.46±0.07 c ($n = 7$)	0.003±0.0 a ($n = 85$)	0.05±0.01 b ($n = 22$)	0.146±0.07 b ($n = 7$)

Different lower case letters following the means within a column indicate significant differences ($P<0.05$).

and C3 diets after 2 d of exposure, relative to control diet, and on C1, C2, and C3 diets after 6 and 10 d of exposure (Table 1). Mean frass production after 2 d of exposure was not significantly different among treatments ($P>0.05$) (Table 1). However, significant differences were evident after 6 and 10 d of exposure in *S. frugiperda* larvae exposed to C2 and C3 diets relative to control diet ($P<0.001$) (Table 1). After 6 d, frass production on control diet was ~79- and 554-fold higher than on C2 diet, and these differences increased after 10 d (Table 1). Duration of *S. frugiperda* larval development increased significantly with Cry1Ab concentration ($P<0.001$), and was significantly longer for C1 (19.64±0.6 d) and for C2 (33±1.3 d) relative to the control diet (15.5±0.3 d). The percentage of pupation was not significantly affected by C1 diet (36.03±4.6%), but significantly lower on C2 diet (11.4±3.3%), relative to control diet (54.5±4.8%) ($P<0.001$). All larvae on C3 diet died prior to pupation. Pupal weight was not affected on C1 diet (169.4±7.5 mg), but was significantly lower on C2 diet (129.5±29.4 mg), relative to control diet (191.3±5.0 mg) ($F = 8.017$; 2 df; $P = 0.002$).

3.1.2. Host-mediated effects on *Cotesia marginiventris*

Observations were not made on parasitoids on C3 diet due to high host mortality (Fig. 1). Significant differences in proportions of successful parasitism, and egg-to-cocoon and cocoon-to-adult survival were not found among C1, C2, and control diet treatments ($P\geqslant0.475$) (Table 2). Differences were not found in egg to cocoon developmental periods among C1, C2, and control artificial diet, within sexes ($P\geqslant0.098$) (Table 3). The egg-to-adult developmental

Table 2
Rate of successful parasitism (%) and survivorship (%) in *C. marginiventris* developing on *S. frugiperda* exposed to artificial diet containing one of two Cry1Ab protein concentrations (C1 = 0.46, C2 = 9.13 μg Cry1Ab protein/ml diet) or control diet (without Cry1Ab protein), and on conventional or Bt maize tissue

	Control ($n = 43$)	C1 (0.46 μg Cry1Ab/ml) ($n = 78$)	C2 (9.13 μg Cry1Ab/ml) ($n = 114$)	Conventional maize ($n = 217$)	Transgenic maize ($n = 523$)
Success of parasitism	55.8% a	50.0% a	53.5% a	71.8% a	43.4% b
		($G = 1.086$; 2 df; $P = 0.581$)			($t = 4.502$; 92 df; $P < 0.001$)
Egg-to-cocoon survivorship	90.7% a	94.9% a	93.0% a	76.0% a	44.4% b
		($G = 1.505$; 2 df; $P = 0.475$)			($G = 65.269$; 1 df; $P < 0.001$)
Cocoon to adult survivorship	61.5% a	52.8% a	57.5% a	94.5% a	97.8% a
		($G = 1.045$; 2 df; $P = 0.593$)			($G = 3.076$; 1 df; $P = 0.079$)

Different lower case letters following the percentages within a row for each experiment (i.e. with artificial diet or maize) indicate significant differences.

Table 3
Developmental times (d) and adult longevity (d) and size (length of left hind tibia in mm) of *C. marginiventris* developing on *S. frugiperda* reared on artificial diet containing one of two Cry1Ab protein concentrations (C1 = 0.46, C2 = 9.13 μg Cry1Ab protein/ml diet) or control diet (without Cry1Ab protein), and on conventional or Bt maize tissue

	Control ♂: $n = 20$ ♀: $n = 4$	C1 (0.46 μg Cry1Ab/ml) ♂: $n = 29$ ♀: $n = 10$	C2 (9.13 μg Cry1Ab/ml) ♂: $n = 40$ ♀: $n = 21$	Conventional maize ♂: $n = 110$ ♀: $n = 46$	Transgenic maize ♂: $n = 169$ ♀: $n = 58$
Egg-to-cocoon development					
♂	6.35 (±0.2) a	6.62 (±0.2) a	6.6 (±0.2) a	8.6 (±0.1) a	9.5 (±0.1) b
		($H = 0.525$; 2 df; $P = 0.769$)			($t = 4.716$; 1 df; $P < 0.001$)
♀	6.5 (±0.5) a	6.3 (±0.2) a	7.19 (±0.3) a	8.2 (±0.2) a	9.1 (±0.2) b
		($H = 4.649$; 2 df; $P = 0.098$)			($t = 3.220$; 1 df; $P = 0.002$)
Egg-to-adult development					
♂	11.3 (±0.2) a	11.34 (±0.2) a	11.42 (±0.2) a	11.1 (±0.1) a	11.9 (±.0.1) b
♀		($H = 0.245$; 2 df; $P = 0.885$)			($t = 4.194$;1 df; $P < 0.001$)
	12.0 (±0.0) a	11.6 (±0.6) a	12.66 (±0.3) a	11.0 (±0.2) a	11.7 (±0.2) b
		($H = 2.735$; 2 df; $P = 0.255$)			($t = 2.420$;1 df; $P = 0.017$)
Longevity					
♂	14.55 (±1.3) a	13.27 (±1.4) a	12.16 (±0.9) a	5.0 (±0.3) a	4.5 (±0.2) a
		($H = 1.280$; 2 df; $P = 0.527$)			($t = 1.386$; 1 df; $P = 0.167$)
♀	20.0 (±1.2) a	16.75(±1.6) a	17.31 (±1.2) a	7.7 (±0.7) a	6.6 (±0.5) a
		($H = 1.825$; 2 df; $P = 0.401$)			($t = 1.627$; 1 df; $P = 0.107$)
Size					
♀	0.85 (±0.01) a	0.87 (±0.01) a	0.84 (±0.01) a	0.79 (±0.0) a	0.72 (±0.01) b
		($F = 2.454$; 2 df; $P = 0.104$)			($t = 7.368$; 1 df; $P < 0.001$)

Different lower case letters following the means within a row for each experiment (i.e. with artificial diet or maize) indicate significant differences ($P < 0.05$).

period of *C. marginiventris* did not vary significantly among treatments within sexes ($P \geqslant 0.255$) (Table 3). Adult longevity of both genders ($P \geqslant 0.401$) and female size ($P = 0.104$) of adult parasitoids did not differ among C1, C2, and control artificial diet treatments (Table 3). The sex ratios (% males) of *C. marginiventris* emerging from C1, C2 and control artificial diet treatments were male biased ($\chi^2 = 12.0$, 10.5, 7.0; 1 df; $P \leqslant 0.01$); 83.3%, 74.4% and 65.6%, for control, C1, and C2, respectively. Differences among sex ratios were not significant ($\chi^2 = 2.869$; 2 df; $P = 0.238$).

3.2. Host-mediated effects of Cry1Ab delivered via Bt-maize on Cotesia marginiventris

Rates of successful parasitism by *C. marginiventris* were ~1.7 × higher in the conventional versus Bt-maize treatment ($P < 0.001$) (Table 2). Egg-to-cocoon survival was significantly higher in the conventional maize treatment ($P < 0.001$), whereas cocoon to adult survival did not differ between maize treatments ($P = 0.079$) (Table 2). Egg-to-cocoon and egg-to-adult development periods were significantly longer by ~1 d in the Bt-maize versus

conventional maize treatment in both *C. marginiventris* females and males ($P \leqslant 0.017$) (Table 3). *C. marginiventris* females and males had similar longevities in both Bt- and conventional maize ($P = 0.107$, 0.167), though adult parasitoid size was significantly smaller in the Bt-maize treatment ($P < 0.001$) (Table 3). It was noteworthy that egg to cocoon period was ~2 d shorter in control artificial diet relative to conventional maize (Table 3). Increasingly larger parasitoids had increasingly larger egg loads in both the Bt- and conventional maize treatments, but the rate of gain in egg load was greater in the conventional relative to the Bt-maize ($P < 0.0001$) (Fig. 3). Moreover, *C. marginiventris* mean egg loads were significantly larger in the conventional versus Bt-maize ($P < 0.0001$), though *C. marginiventris* adult size was significantly greater in the former versus the latter treatment ($P < 0.0001$). The sex ratio (% males)

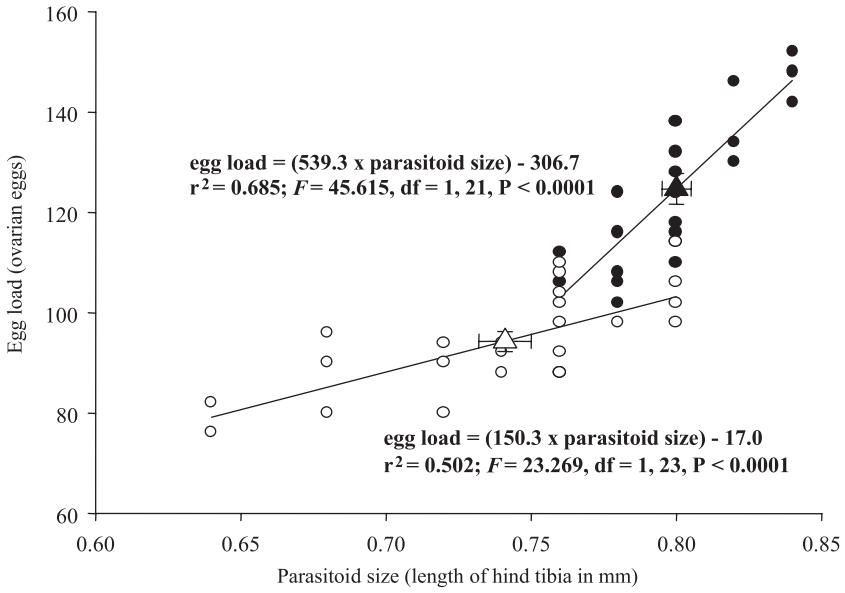

Fig. 3. Relationships between adult size (length in mm of left hind tibia) and egg load (ovarian eggs in newly emerged females) in *C. marginiventris* parasitizing *S. frugiperda* feeding on either transgenic (empty circles) or conventional maize (filled circles); the corresponding mean (±SE) adult sizes and egg loads are indicated by triangles. Differences are significant between the slopes of the relationships ($t = 4.81$, df = 44, $P < 0.0001$), mean parasitoid sizes ($t = 5.55$, df = 46, $P < 0.0001$), and mean egg loads ($t = 8.48$, df = 46, $P < 0.0001$).

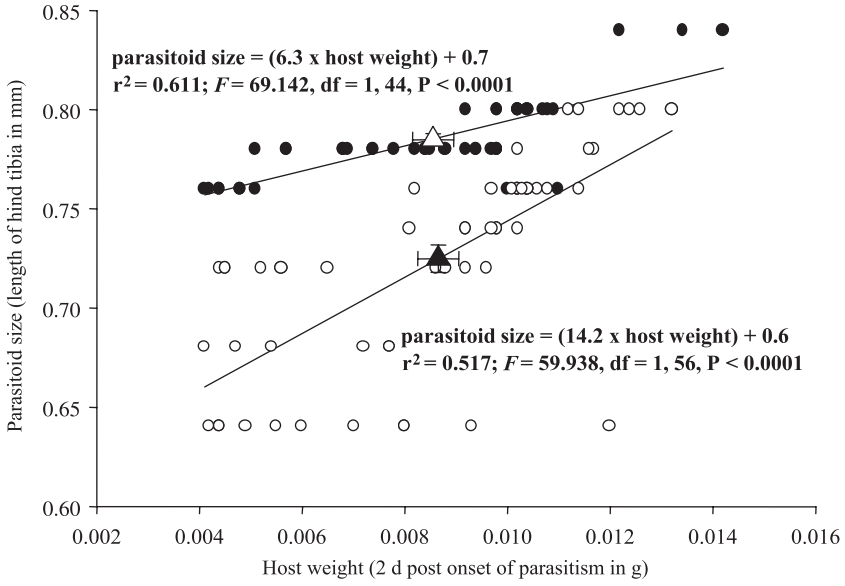

Fig. 4. Relationships between host weight (weight in g 2 d after onset of parasitism) and parasitoid adult size (length in mm of left hind tibia) in *C. marginiventris* parasitizing *S. frugiperda* feeding on either transgenic (empty circles) or conventional maize (filled circles); the corresponding mean (±SE) host weights and parasitoid adult sizes are indicated by triangles. Differences are significant between the slopes of the relationships ($t = 3.49$, df = 100, $P = 0.001$) and mean parasitoid adult sizes ($t = 7.37$, df = 102, $P < 0.0001$), but not between mean host sizes ($t = 0.20$, df = 102, $P = 0.844$).

of *C. marginiventris* emerging from conventional and Bt-maize was male biased ($\chi^2 = 25.4$, 53.3; 1 df; $P < 0.001$), 70.5% and 74.5% for conventional and Bt-maize, respectively. The difference in sex ratio between cultivars was not significant ($\chi^2 = 0.539$; 1 df; $P = 0.469$). Increasing host weight led to increasing adult parasitoid size in the Bt- and conventional maize treatments, but the rate of gain in parasitoid size with host weight was greater on Bt-maize ($P = 0.001$) (Fig. 4). Importantly, while mean parasitoid size differed significantly between maize treatments ($P < 0.0001$), corresponding host weights did not differ significantly between treatments ($P = 0.844$). The concentration of Cry1Ab protein in *S. frugiperda* larvae in the Bt-maize treatment was 0.037 ± 0.005 μg/ml.

4. Discussion

Overall, the results of the present study showed that, as expected, purified Cry1Ab protein had dose-related deleterious effects on *S. frugiperda* survival, weight, and development times within 6 d of exposure. Its quality as a host for *C. marginiventris*, assessed as larval weight and size, was significantly reduced under both of the Cry1Ab protein levels evaluated in the present study, so a host-mediated effect on parasitoid development was expected. However, significant host-mediated effects on *C. marginiventris* were not detected under either of the Cry1Ab protein levels evaluated, although high host mortality at the highest Cry1Ab level (182.6 μg Cry1Ab/ml diet) (effects on *C. marginiventris* not evaluated at this Cry1Ab level) would likely have a significant effect on parasitoid populations in the field. Notably, however, host-mediated effects on *C. marginiventris* were evident when *S. frugiperda* were fed Bt-maize tissue. Parasitoid developmental periods and adult size and egg load were significantly affected despite the relatively low levels of Bt toxin present in hosts. Particularly noteworthy was the finding that adult sizes, and so egg loads, were smaller in *C. marginiventris* developing on hosts fed Bt- versus conventional maize tissue, independently of host quality.

While *S. frugiperda* survival was not significantly affected after 2 d of exposure to Cry1Ab protein, significant effects were evident after 10 d of exposure to 9.13 and 182.6 μg Cry1Ab/ml diets. These results suggested that Cry1Ab is not acutely lethal, and mortality depends on level (dosage) and length of exposure. A study aimed at determining the LD_{50} of Cry1Ab toxin among field populations of *S. frugiperda* also showed that mortality was dose-related (Lynch et al., 2003), though time-dependent mortality was not studied. The most likely explanation for the delayed effect on *S. frugiperda* survival evident in this study is a reduction of food intake when larvae were exposed to the Cry1Ab protein. This is in agreement with the known mode of action of Cry1Ab in *S. frugiperda*, in which activated toxin molecules bind weakly to the peritrophic membrane (Aranda et al., 1996), and likely lead to partial paralysis of the gut and mouth parts, as generally known in lepidopteran species intoxicated with Bt δ-endotoxins (Hoy and Hall, 1993; Regev et al., 1996). Sublethal effects of Cry1Ab protein on *S. frugiperda* were evident in the differences in size, i.e. larval weight and head capsule widths, among treatments. These differences likely resulted from a reduction in food consumption, and possibly cytological and physiological alterations (Monette et al., 1997; Certiaens et al., 2001). The reductions of *S. frugiperda* pupal formation rate and size due to Cry1Ab protein could affect *S. frugiperda* population growth and reproduction in the field, even at the lowest concentration tested. According to the results of Bokonon-Ganta et al. (2003) and Vojtech et al. (2005), the concentration of Cry1Ab in Bt-maize MON810 (0.7 and 1.6 μg/ml, resp.) varies in a range close to our C1 (0.46 μg/ml) concentration. Thus, similar effects are evident in this study at C1 and those of Bokonon-Ganta et al. (2003) and Vojtech et al. (2005).

Although host-mediated effects on parasitoid development and fitness were expected under the two Cry1Ab protein levels that were evaluated, the results of this study did not show differences in *C. marginiventris* emergence rates, egg-to-cocoon and egg-to-adult development times, offspring production, nor adult longevity and size relative to the control. A plausible explanation for such lack of effects is that parasitoid larvae may have had access to sufficient resources, prior to host death or quality reduction, to ensure adequate development. In addition, the levels within the host of activated Cry1Ab protein, if it affects *C. marginiventris*, may not have been high enough to affect developing parasitoid larvae. This suggestion, however, implies a direct effect of Cry1Ab protein on *C. marginiventris* larvae. Although unprecedented, this possibility should not be dismissed and is discussed below.

Parasitism rates, larval survival, developmental times, and adult size and fecundity were significantly affected in *C. marginiventris* developing on *S. frugiperda* fed Bt-maize tissue, though other parameters such as cocoon-to-adult mortality and sex ratios were not affected. It is noteworthy that: (i) these effects occurred despite the relatively low levels of Bt toxin present in hosts (0.037 ± 0.005 μg/ml), viz. ~5% of the levels occurring in transgenic maize tissue (0.77 μg/ml, Bokonon-Ganta et al., 2003), and (ii) parasitoid adult size was smaller on hosts fed Bt- versus conventional maize tissue, though the corresponding hosts did not show differences in size, assessed as weight. In particular, the finding that *C. marginiventris* females were smaller, so less fecund, on Bt- compared to conventional maize, despite a lack of difference in the sizes of their hosts suggests a direct effect of the Cry1Ab protein on *C. marginiventris* larvae. Such an effect has not been documented previously for hymenopteran parasitoids, so merits further inquiry.

In part, the results of this study agreed with those presented by Vojtech et al. (2005) who reported longer development periods of *C. marginventris* developing on hosts fed Bt-maize. Similarly, previous research showed

significant effects on developmental times and longevity in the parasitoids *Parallorhogas pyralophagous* (Marsh) (Bernal et al., 2002) and *C. marginiventris* (Baur and Boethel, 2003), when hosts were exposed to Bt-maize (expressing Cry1Ab toxin) or Bt-cotton (expressing Cry1Ac toxin), respectively. However, some contrasting results were found in the present study compared with those of Vojtech et al. (2005). For example, significant changes in the sex ratio, and no changes in the rates of parasitism were reported in that study, while the sex ratio was not affected and rates of parasitism were reduced in this study. These discrepancies may be due to the difference in host species between the studies. *S. littoralis* seems to be more sensitive than *S. frugiperda* (higher mortality and longer larval developmental periods) to Bt-maize (Vojtech et al., 2005; Bokonon-Ganta et al., 2003), which could lead to differences in effects at the third trophic level. Alternatively, these discrepancies could be due to differences in methodological approaches between the studies. While in their study Vojtech et al. (2005) used Bt-plant tissue integrated in artificial diet, the present study relied on Bt-maize tissue delivered directly to host larvae. The contrasting results between the present study and that by Vojtech et al. (2005) highlight the importance of developing effective methodological approaches for risk assessment of transgenic crops.

Combined, the results of this study showed that although the use of purified, full-length protein using artificial diet uncovered some effects of Cry1Ab at the second trophic level, it failed to do so at the third trophic level, while the use of Bt-plant tissue uncovered effects of Cry1Ab at both trophic levels (e.g., Bokonon-Ganta et al., 2003). At least three explanations for the discrepancy at the third trophic level are plausible. (i) Faster larval development in *C. marginiventris* on hosts fed artificial diet containing Cry1Ab protein (~6–7 d) versus Bt-maize tissue (~8–10 d) facilitated coping with reductions of host quality over time. (ii) While the artificial diet contained full-length, inactive Cry1Ab protein, Bt-maize delivered the truncated, active form of the protein. And, (iii) the Bt transgene could have pleiotropic and/or epistatic effects in Bt-maize plants (e.g., Olesinski et al., 1995; Saxena and Stotzky, 2001), and subsequently on *S. frugiperda* and *C. marginiventris*.

On the basis of currently available information (Baur and Boethel, 2003; Vojtech et al., 2005; this study) it is reasonable to predict the occurrence of significant negative impacts of Bt-maize on *C. marginiventris* populations in the field, as extensions of individual-level impacts detected in the laboratory. Population- and field-level impacts of Bt crops are particularly likely in the case of specialist parasitoids, such as *C. marginiventris* (Sisterson and Tabashnik, 2005). So, future studies should be conducted in the field, and should test the hypothesis that Bt crops do not significantly affect the population dynamics of *C. marginiventris*.

In conclusion, the results of the present study showed the occurrence of host-mediated, sublethal effects of Cry1Ab protein as delivered by Bt-maize on the parasitoid *C. marginiventris*, and did not show similar effects of full-length Cry1Ab protein delivered via artificial diet. This finding highlights the need to evaluate non-target effects of transgenic Bt crops using transgenic plants rather than purified, full-length Cry proteins alone. Also, the results of this study suggested that Cry1Ab protein as expressed in Bt-maize may have a direct effect on *C. marginiventris*. The occurrence of direct effects of Cry1Ab protein on a hymenopteran parasitoid, such as *C. marginiventris*, merits further research because of the importance of these parasitoids as natural enemies in agroecosystems.

Acknowledgments

We thank F. Diaz-Fleischer (INECOL, A.C., Mexico), S.E. Naranjo and two anonymous reviewers for constructive comments on an earlier version of the manuscript, and M. Sétamou, A. Bokonon-Ganta, P. Gillogly (all formerly at TAMU), D. Fortini (INRA, Le Magneraud-Surgères, France), and S. Alvarez (Texas Agricultural Experiment Station, Weslaco, USA) for logistical support. This work was partially supported by the program of Agricultural French Ministry, 'Impact des OGM' (Coordination of M.H. Pham-Delègue), USDA Hatch Project H-8707 (J. Bernal), and a Ph.D. scholarship from the Mexican Council for Science and Technology (CONACyT) and the 'UDLA-P' (R. Ramirez-Romero).

References

Andow, D.A., Hilbeck, A., 2004. Science-based risk assessment for nontarget effects of transgenic crops. Bioscience 54, 637–649.

Aranda, E., Sanchez, J., Peferoen, M., Guereca, L., Bravo, A., 1996. Interactions of *Bacillus thuringiensis* crystal proteins with the midgut epithelial cells of *Spodoptera frugiperda* (Lepidoptera: Noctuidae). J. Invertebr. Pathol. 68, 203–212.

Ashley, T.R., 1979. Classification and distribution of fall armyworm parasites. Fla. Entomol. 62, 144–153.

Ashley, T.R., Waddill, V.H., Mitchell, E.R., Rye, J., 1982. Impact of native parasites on the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in South Florida and release of the exotic parasite, *Eiphosoma vitticole* (Hymenoptera: Ichneumonidae). Environ. Entomol. 4, 833–837.

Baur, M.E., Boethel, D.J., 2003. Effect of Bt-cotton expressing Cry1A(c) on the survival and fecundity of two hymenopteran parasitoids (Braconidae, Encyrtidae) in the laboratory. Biol. Control 26, 325–332.

Bernal, J., Griset, J., Gillogly, P., 2002. Impacts of developing on Bt maize-intoxicated hosts on fitness parameters of a steam borer parasitoid. J. Entomol. Sci. 37, 27–40.

Blumberg, D., Navon, A., Keren, S., Goldenberg, S., Ferkovich, S.M., 1997. Interactions among *Helicoverpa armigera* (Lepidoptera: Noctuidae), its larval endoparasitoid *Micropilis croceipes* (Hymenoptera: Braconidae), and *Bacillus thurigiensis*. J. Econ. Entomol. 90, 1181–1186.

Bokonon-Ganta, A.H., Bernal, J.S., Pietrantonio, P.V., Setamou, M., 2003. Survivorship and development of fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera : Noctuidae), on conventional and transgenic maize cultivars expressing *Bacillus thuringiensis* Cry9C and Cry1A(b) endotoxins. Int. J. Pest Manage. 49, 169–175.

Certiaens, A., Verleyen, P., Van Rie, J., Van Kerkhove, E., Schwartz, J.-L., Laprade, R., De Loof, A., Schoofs, L., 2001. Effect of *Bacillus*

thuringiensis Cry1 toxins in insect hemolymph and their neurotoxicity in brain cells of *Lymantria dispar*. Appl. Environ. Microb. 67, 3923–3927.

Chaufaux, J., Seguin, M., Swanson, J.J., Bourguet, D., Siegfried, B.D., 2001. Chronic exposure of the European Corn Borer (Lepidoptera: Crambidae) to Cry1Ab *Bacillus thuringiensis* toxin. J. Econ. Entomol. 94, 1564–1570.

Chilcutt, C.F., Tabashnik, B.E., 1999. Effects of *Bacillus thuringiensis* on adults of *Cotesia plutellae* (Hymenoptera: Braconidae), a parasitoid of the Diamondback Moth, *Plutella xylostella* (Lepidoptera: Plutellidae). Biocontrol Sci. Technol. 9, 435–440.

Hoy, C.W., Hall, F.R., 1993. Feeding behavior of *Plutella xylostella* and *Leptinotarsa decemlineata* on leaves treated with *Bacillus thuringiensis* and esfenvalerate. Pestic. Sci. 38, 335–340.

James, C., 2004. Preview: Global Status of Commercialized Transgenic Crops: 2004. ISAAA, Briefs No. 30.

Knowles, B.H., Dow, J.A.T., 1993. The crystal δ-endotoxins of *Bacillus thuringiensis*: models for their mechanism of action on the insect gut. BioEssays 15, 469–476.

Kumar, H., Mihm, J.A., 1996. Damage by fall armyworm, *Spodoptera frugiperda* (J.E. Smith), southwestern corn borer *Diatraea grandiosella* Dyar and surcane borer *Diatraea saccharalis* Fabricius on maize in relation to seed treatment with selected insecticides in the fields. Maydica 41, 235–239.

Lynch, R.E., Wiseman, B.R., Plaisted, D., Warnick, D., 1999. Evaluation of transgenic sweet corn hybrids expressing CryIA (b) toxin for resistance to corn earworm and fall armyworm (Lepidoptera: Noctuidae). J. Econ. Entomol. 92, 246–252.

Lynch, R.E., Hamm, J.J., Myers, R.E., Guyer, D., Stein, J., 2003. Baseline susceptibility of the fall armyworm (Lepidoptera : Noctuidae) to Cry1Ab toxin: 1998–2000. J. Entomol. Sci. 38, 377–385.

Martinez, A.J., Bard, J., Holler, T.A., 1988. Mass rearing sugarcane borer and Mexican rice borer for production of parasites *Allorhogas pyralophagous* and *Rhaconotus roselinsis* USDA-APHIS-PPQ, APHIS.

Miranda, R., Zamudio, F.Z., Bravo, A., 2001. Processing of Cry1Ab δ-endotoxin from *Bacillus thuringiensis* by *Manduca sexta* and *Spodoptera frugiperda* midgut proteases: role in protoxin activation and toxin inactivation. Insect Biochem. Mol. Biol. 31, 1155–1163.

Monette, R., Potvin, L., Baines, D., Laprade, R., Schwartz, J.L., 1997. Interaction between calcium ions and *Bacillus thuringiensis* toxin activity against Sf9 cells (*Spodoptera frugiperda*, Lepidoptera). Appl. Environ. Microb. 63, 440–447.

Obrycki, J., Ruberson, J., Losey, J., 2004. Interactions between natural enemies and transgenic insecticidal crops. In: Ehler, L.E., Sforza, R., Mateille, T. (Eds.), Genetics, Evolution and Biological Control. CAB International, Wallingford, UK, pp. 183–206.

Olesinski, A.A., Lucas, W.J., Galun, E., Wolf, S., 1995. Pleiotropic effects of tobacco-mosaic-virus movement protein on carbon metabolism in transgenic tobacco plants. Planta 197, 118–126.

Poitout, S., Bues, R., 1970. Elevage de plusieurs espèces de lepidoptères Noctunidae sur milieu artificiel riche et sur milieu artificiel simplifié. Ann. Zool. Ecol. Anim. 2, 79–91.

Regev, A., Keller, M., Strizhov, N., Sneh, B., Prudovsky, E., Chet, I., Ginzberg, I., Koncz-Kalman, Z., Koncz, C., Schell, J., Zilberstein, A., 1996. Synergistic activity of a *Bacillus thuringiensis* d-endotoxin and a bacterial endochitinase against *Spodoptera littoralis* larvae. Appl. Environ. Microb. 62, 3581–3586.

Romeis, J., Meissle, M., Bigler, F., 2006. Transgenic crops expressing *Bacillus thuringiensis* toxins and biological control. Nat. Biotechnol. 24, 63–71.

Saxena, D., Stotzky, G., 2001. Bt corn has a higher lignin content than non-Bt corn. Am. J. Bot. 88, 1704–1706.

Sisterson, M.S., Tabashnik, B.E., 2005. Simulated effects of transgenic Bt crops on specialist parasitoids of target pests. Environ. Entomol. 34, 733–742.

SPSS, 2000. Systat 10 Statistics I & II. Chicago, USA.

Vojtech, E., Meissle, M., Poppy, G.M., 2005. Effects of Bt maize on the herbivore *Spodoptera littoralis* (Lepidoptera: Noctuidae) and the parasitoid *Cotesia marginiventris* (Hymenoptera: Braconidae). Transgenic Res. 14, 133–144.

Wabiko, H., Raymond, K.C., Bulla Jr., L.A., 1986. *Bacillus thuringiensis* entomocidal protoxin gene sequence and gene product analysis. DNA 5, 305–314.

Zar, J.H., 1998. Biostatical Analysis, fourth ed. Pearson Prentice Hall, New Jersey.