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ORIGINAL PAPER

Impact of single-gene and dual-gene Bt broccoli on the herbivore *Pieris rapae* (Lepidoptera: Pieridae) and its pupal endoparasitoid *Pteromalus puparum* (Hymenoptera: Pteromalidae)

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Abstract Transgenic brassica crops producing insecticidal proteins from *Bacillus thuringiensis* (Bt) are being investigated as candidates for field release to control lepidopteran pests. Information on the potential impact of Bt brassica crops on pests and non-target natural enemies is needed as part of an environmental risk assessment prior to the commercial release. This first tier study provides insight into the tritrophic interactions among Bt broccoli plants, the herbivore *Pieris rapae* and its parasitoid *Pteromalus puparum*. We first evaluated the efficacy of three types of Bt broccoli plants, *cry1Ac*, *cry1C* and *cry1Ac* + *cry1C*, on different instars of *P. rapae*. Bt broccoli effectively controlled *P. rapae* larvae, although later instars were more tolerant. The efficacy of different Bt broccoli plants on *P. rapae* larvae was consistently *cry1Ac* > *cry1Ac* + *cry1C* > *cry1C*. When the parasitoid *P. puparum* developed in a *P. rapae* pupa (host) that had developed from Bt plant-fed older larvae, developmental time, total number and longevity of the *P. puparum* generated from the Bt plant-fed host were significantly affected compared with those generated from the non-Bt control plant-fed host. Simultaneously, negative effects on *P. rapae* pupae were found, i.e. pupal length, width and weight were significantly reduced after older *P. rapae* larvae fed on different Bt plants for 1 or 2 days. Cry1C toxin was detected using ELISA in *P. rapae* pupae after older larvae fed on *cry1C* broccoli. However, no Cry1C toxin was detected in newly emerged *P. puparum* adults developing in Bt-fed hosts. Only a trace amount of toxin was detected from entire *P. puparum* pupae dissected from the Bt plant-fed host. Moreover, no negative effect was found on the progeny of *P. puparum* developing from the Bt plant-fed host when subsequently supplied with a healthy host, *P. rapae* pupae. The reduced quality of the host appears to be the only reason for the observed deleterious effects on *P. puparum*. Our data suggest that the effects on *P. puparum* developing in Bt plant-fed *P. rapae* are mediated by host quality rather than by direct toxicity.

Keywords *Bacillus thuringiensis* · *Pieris rapae* · *Pteromalus puparum* · Transgenic broccoli · Tritrophic interactions

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Introduction

Lepidopteran pests of brassica vegetables, such as diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae) and imported cabbageworm, *Pieris rapae* L. (Lepidoptera: Pieridae), are severe economic threats to production. Moreover, the lack of a

diversity of strategies for managing lepidopteran pests has resulted in an almost total dependence on traditional synthetic insecticides that can cause negative impacts on non-target parasitoids and predators, and the rapid development of insecticide resistance (Shelton et al. 1993; Vásquez et al. 1997; Wold-Burkness et al. 2005). The development of plants expressing *Bacillus thuringiensis* (Bt) insecticidal crystal proteins (Cry toxins) offers an alternative strategy to control insect pests (Shelton et al. 2002b). Among the first commercialized transgenic crops containing Bt insecticidal proteins were Bt maize and Bt cotton, which were protected from attack by lepidopteran pests (i.e. corn borers in maize and the budworm-bollworm complex in cotton) (Shelton et al. 2002b). Bt plants were grown on 32.1 million ha worldwide in 2006 (James 2006). These crops have readily been adopted by farmers in a number of countries and have provided economic benefits to growers and reduced the use of insecticides (Shelton et al. 2002b; Brookes and Barfoot 2006; James 2006). Advances in insect-resistant transgenic crops in recent years have also offered a promising alternative to traditional synthetic insecticides for controlling lepidopteran pests in brassica vegetables. Previous studies in our and other laboratories have shown that *P. xylostella* can be controlled well using *Brassica oleracea* L. plants carrying a synthetic or fully modified *Bt* gene (Metz et al. 1995; Cao et al. 1999; Jin et al. 2000; Bhattacharya et al. 2002). Additional studies with a *cry1C* gene expressed in broccoli demonstrated that these transgenic plants are able to control Cry1Ac-resistant *P. xylostella* (Cao et al. 1999), and studies with pyramided *cry1Ac* and *cry1C* broccoli plants demonstrated that these plants have excellent control of both Cry1C-resistant and Cry1Ac-resistant *P. xylostella* (Cao et al. 2002). Similarly, transgenic collards with *cry1Ac* or *cry1C* genes showed complete control of susceptible *P. xylostella* larvae (Cao et al. 2005). It is noteworthy that both *cry1C* broccoli and Chinese cabbage have been reported to provide promising control of *P. rapae* and *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae) neonate larvae (Cao et al. 1999; Cho et al. 2001).

A major concern regarding the utilization of Bt plants is their potential impact on non-target organisms (Romeis et al. 2006). As part of an ecological risk assessment before commercial release, it is crucial to evaluate the efficacy of Bt crops on target lepidopteran pests as well as their potential impact on non-target natural enemies. In the case of brassica vegetables, all of the work carried out to date has been conducted against lepidopteran pests, as cited above, with some additional studies conducted against one of their parasitoids (Schuler et al. 2004). However, there is as yet no report on the efficacy of Bt broccoli on different instars of *P. rapae*, except for neonates (Cao et al. 1999; Cho et al. 2001), nor on its impact on the non-target natural enemies of *P. rapae*. Negative impacts of Bt toxins on non-target parasitoids have been reported in tritrophic studies with Bt transgenic plants and susceptible herbivores that were fed with Bt plant tissue (Bernal et al. 2002; Baur and Boethel 2003; Meissle et al. 2004; Prütz and Dettner 2004). In general, parasitoids are very sensitive to changes in their hosts due to toxin ingestion, since the parasitoids usually complete their development in a single host. When Bt-susceptible hosts are treated with Bt toxins, their parasitoids are likely to be affected more than predators, which are often generalists and feed on several different prey species (Salama and Zaki 1983).

Pteromalus puparum L. (Hymenoptera: Pteromalidae) is a pupal endoparasitoid of *P. rapae*. *P. puparum* may lay up to 700 eggs in her lifespan and >200 offspring can be produced from a single pupa of *P. rapae* (Mahr 1996). A long-term survey in New York indicated that 44.4% of pupae of *P. rapae* were parasitized by *P. puparum* (Shelton et al. 2002a), while a study in Minnesota documented that *P. puparum* was the dominant parasitoid species of *P. rapae* with a maximum parasitism rate of 31% that effectively reduced insect pest pressure under field conditions (Wold-Burkness et al. 2005).

In order to evaluate the potential use of Bt vegetables, we investigated the efficacy of Bt broccoli plants with *cry1Ac* or *cry1C* single genes, or with the combination of the two genes (pyramided genes), on *P. rapae* larvae at different stages and the potential impact on the parasitoid, *P. puparum*, via the *P. rapae* host. The experiments were designed to address the following questions:

(1) Does the efficacy of single- and dual-gene Bt broccoli vary according to the different life stages of *P. rapae* larvae?
(2) Will *P. puparum* adults be adversely affected when developing in single-gene and dual-gene Bt broccoli-fed *P. rapae*?

(3) Does the Bt toxin transfer through different trophic levels in the *P. rapae–P. puparum* system? If so, can it be quantified?

Materials and methods

Bt broccoli plants

Three types of transgenic broccoli (*Brassica oleracea* L., var. *italica'* Green Comet) plants producing high levels of Cry1Ac (0.63 μg/g fresh leaf tissue), Cry1C (1.12 μg/g fresh leaf tissue) or both (Cry1Ac: 0.69–0.72 μg/g fresh leaf tissue; Cry1C: 0.27–1.23 μg/g fresh leaf tissue) and non-transgenic broccoli (control) were used in this study (Metz et al. 1995; Cao et al. 1999, 2002). The progenies of *cry1Ac*- and *cry1C*-expressing plants were verified by screening them with Cry1C-resistant (Cry1C-R) and Cry1Ac-resistant (Cry1Ac-R) *Plutella xylostella* neonates, respectively, when the plants were 4–5 weeks old (Tang et al. 2001). Both *cry1Ac* and *cry1C* plants also killed 100% of the neonates of the F_1 heterozygotes (susceptible strain of *P. xylostella* (S) × Cry1Ac-R) or 100% of all instars of the F_1 heterozygotes (S × Cry1C-R), respectively (Zhao et al. 2002), indicating that the plants expressed a high dose for the purpose of resistance management (US EPA 2001). Broccoli plants that expressed both the *cry1Ac* and *cry1C* genes were produced by sexual crosses between the two types of Bt broccoli and were characterized for Bt protein production and the control of S, Cry1Ac-R and Cry1C-R *P. xylostella* strains (Cao et al. 2002). Enzyme-linked immunosorbant assay (ELISA) analysis showed that Cry1Ac and Cry1C toxins were produced in the hybrids and in their F_1 progeny (Cry1Ac: 0.62–0.68 μg/g fresh leaf tissue; Cry1C: 0.94–1.38 μg/g fresh leaf tissue) at levels comparable to those in the original single-gene parental lines (Cao et al. 2002).

Insects

Pieris rapae adults and pupae were collected from the Fruit and Vegetable Research Farm of Cornell University's New York State Agricultural Experiment Station (Geneva, NY) in July, 2005 and continuously raised in a rearing chamber under the conditions of 27°C, a relative humidity (RH) of 75–80% and a photoperiod of 16:8 h (light:dark) based on the methods of Webb and Shelton (1988).

A starting population of the pupal endoparasitoid *Pteromalus puparum* was obtained from *Pieris rapae* pupae collected from the farm in September 2005 that had been naturally parasitized under field conditions. The field-collected *P. rapae* pupae were returned to the laboratory and placed in a wood-framed cage with netting sides (50 × 50 × 50 cm). After emergence, 20 pairs of *P. puparum* adults were moved from the wood-framed cage by mouth aspiration to a Plexiglas cylinder cage supplied with 10% sugar water, where random mating occurred for 2 h. The mated *P. puparum* adults were released to another wood-framed cage containing 40 *P. rapae* pupae (1–2 days old) collected from our insect culture. After the *P. rapae* pupae were inoculated with *P. puparum* adults for 48 h, they were transferred to a new wood-framed cage in a rearing chamber until emergence. The whole adult stage of *P. puparum* was supplied with 10% sugar water. The rearing work and experiments were carried out in the rearing chamber, except as noted below.

Effects of single-gene and dual-gene Bt broccoli on the herbivore *Pieris rapae*

Survival of first instar and later instars

To evaluate the efficacy of different Bt broccoli plants on first instar *P. rapae*, 50 first instars (2–2.5 days after hatching) were individually kept in 30-ml plastic cups (Wincup, Phoenix, AZ) with a punctured lid to allow ventilation. With 5 first instars as one replication, ten replications were set up for each type of Bt broccoli plants, i.e. *cry1Ac*, *cry1C* and *cry1Ac* + *cry1C*, and the non-Bt broccoli plants (control). Larvae were fed with Bt or non-Bt broccoli leaves (5–6 weeks old) and checked daily until pupation or death; the number of dead larvae in each group was recorded and the broccoli leaves were changed if needed. The efficacy of different Bt broccoli plants on second instar (4–6 days after hatching), third instar (6–8 days after hatching), fourth instar (8–10 days after hatching) and fifth instar (10–12 days after hatching) *P. rapae* was also

evaluated through the entire larval stage. The experimental design and conditions were the same as described above.

Sublethal effects on Pieris rapae *pupae developing from the fourth to fifth instars fed with Bt broccoli*

Based on the effects of different Bt broccoli plants on fourth and fifth instar *P. rapae* after 2 days of feeding, fourth to fifth instar *P. rapae* (9–11 days after hatching) were collected from the insect culture and fed *cry1Ac* broccoli plants leaves for 1 or 2 days, then transferred to non-Bt control broccoli plants and reared until pupation. A total of 50 pupae (1–2 days old) were randomly collected from the 1- to 2-day feeding treatments and divided into ten groups (replications) of five pupae in each. The length, width and weight of the pupae in each group from different treatments were measured and recorded. The same treatments were used for *cry1C* and *cry1Ac* + *cry1C* broccoli plants. Pupae used as controls were developed from non-Bt broccoli-fed fourth to fifth instar *P. rapae* larvae, and their characteristics were compared with those of pupae developed from larvae fed with *cry1Ac*, *cry1C* or *cry1Ac* + *cry1C* broccoli for 1 and 2 days.

Effects of single-gene and dual-gene Bt broccoli on the parasitoid *Pteromalus puparum*

Effects on parasitism, developmental time, proportion of females and longevity of P. puparum

After *P. rapae* pupae (1–2 days old) were evaluated for sublethal effects as described above, the pupae were submitted to *P. puparum* adults collected from our insect culture to assess the impact of single-gene and dual-gene Bt broccoli on the parasitoid. A total of 25 pupae from each type of broccoli plant (Bt and non-Bt) were individually placed into 30-ml plastic cups and divided into five groups (replications) with five pupae in each. Newly emerged *P. puparum* adults (<1.5 days) were transferred into a wood-framed cage using an aspirator (female: male 1:1) and allowed to mate randomly for 2 h. Each *Pieris rapae* pupa was inoculated with one pair of *P. puparum* adults for 48 h in a cylinder cage. The parasitized pupa was kept in a Plexiglas cylinder. The developmental time of *P. puparum* (from oviposition to adult emergence) in the *P. rapae* pupa was recorded. Once *P. puparum* adults had emerged from the pupa, they were supplied with 10% sugar water. The longevity of *P. puparum* and the proportion of females in each group were recorded and compared with the control group developing from non-Bt broccoli-fed *P. rapae*.

Effects on the progeny of Pteromalus puparum *developing from Bt broccoli-fed* Pieris rapae

In order to evaluate whether Bt broccoli plants would negatively affect the ability of the progeny of *P. puparum* to utilize a subsequently provided host, the parasitoid adults from *P. rapae* pupae that had developed from larvae fed on different Bt broccoli plants for 1–2 days were allowed to parasitize *P. rapae* pupae that had not been exposed to Bt plants (conventional *P. rapae* pupae). *P. puparum* adults collected from our insect culture were used as the control. A total of 25 pairs each of *P. puparum* adults that had developed from *cry1Ac*, *cry1C* and *cry1Ac* + *cry1C* broccoli plant-fed *P. rapae*, respectively, were allowed to parasitize 25 conventional *P. rapae* pupae after randomly mating for 2 h in a wood-framed cage. The experimental design and conditions were the same as above. Parasitism rate, developmental time, proportion of females and longevity of *P. puparum* adults emerging from the treatment groups were recorded and compared with those from the control.

Transfer of Cry1C toxin through different trophic levels

The food chain comprising *cry1C* broccoli, *Pieris rapae* and *Pteromalus puparum* was selected to examine whether Cry1C toxin can be transferred through the different trophic levels and accumulate in *P. rapae*, because *P. rapae* later instars are relatively more tolerant to *cry1C* broccoli (while the impact of different Bt broccoli plants on *P. puparum* was similar).

A total of five new *P. rapae* pupae (from the fourth to fifth instars fed with *cry1C* plants for 1, 2 and 3 days, respectively) were sampled and kept in Eppendorf vials

(1.5 ml) to detect the transfer and accumulation (if any) of Cry1C toxin in *P. rapae*. Each vial contained one pupa as one replication, and there were five replicates per treatment and 15 insects for the study.

Pieris rapae pupae developing from cry1C broccoli-fed fourth and fifth instars for 1–2 days were inoculated by *P. puparum* adults from the culture and kept in the chamber. *P. puparum* pupae were dissected from the parasitized *P. rapae* pupa 7–9 days after inoculation (without damaging the pupae) and washed five times in Wash Buffer (supplied in the Cry1C ELISA kit; EnviroLogix, Catalog no. AP 007, Portland, Me.). The pupae were then dried on a paper towel for 2 min and put into an Eppendorf vial (all pupae from one host) to detect the presence of Cry1C toxin. In addition, 20 *P. puparum* adults were collected following their emergence from the parasitized pupae, chilled at –20°C for 20 min and placed into a vial. Toxins from *cry1C* broccoli and non-Bt broccoli plants (6–7 weeks old) were extracted from 8-mm leaf discs to serve as controls. All samples were weighed and sealed in tubes, with five replications, and then stored at –20°C until the ELISA assays were conducted.

Detection and quantification of the amount of Cry1C toxins in all the samples were monitored by ELISA using the Cry1C kit from EnviroLogix. To extract soluble proteins, each sample was ground and homogenized in 0.5 ml Extraction/Dilution buffer (EnviroLogix). ELISA was conducted according to the manufacturer's instructions. Based on preliminary tests, sample extracts were used at a dilution of 1:51 for *cry1C* broccoli and 1:4 for *P. rapae* pupae. The optical density (OD) value of samples was measured using a microplate reader set at 450 nm, with 630 nm as the reference wavelength. Corresponding tissue blanks (control samples) were subtracted from Bt samples to eliminate any unspecific binding effects of proteins to ELISA test wells. The amount of Cry1C toxins in samples was calculated from standard concentrations obtained with Cry1C (Envirologix) and expressed as micrograms Bt protein/g fresh weight.

Statistical analysis

Survival analyses of different instar *Pieris rapae* on Bt broccoli and the non-Bt control were conducted using the Kaplan–Meier procedure and Logrank Test (Norusis 2005). Survival data were recorded until all *P. rapae* larvae fed with Bt plants were either dead or had pupated. All pupated individuals were recorded as survivors. Data on *P. rapae* pupae (length, width and weight) and *Pteromalus puparum* (parasitism rate, developmental time, total number of *P. puparum*, proportion of females and longevity) between plant types (Bt and non-Bt) and feeding time treatments were analyzed by two-way ANOVA and Fisher's protected LSD means separation test. Differences in the impact of Bt plants on the progeny of *P. puparum* developing from Bt plant-fed *P. rapae* pupae were analyzed by one-way ANOVA. All percentage data were transformed using arcsine square root before statistical analyses. All statistical analyses were conducted using SPSS FOR WINDOWS ver. 11.5 (SPSS, Chicago, IL).

Results

Effects of Bt broccoli plants on *Pieris rapae*

Survival of P. rapae *larvae*

The survival probability of *P. rapae* larvae was significantly affected by being exposed to Bt broccoli plants from the first instar compared with those exposed to non-Bt control plants (χ^2 =171.17, df = 3, $P < 0.001$) (Fig. 1). All larvae died within 3 days of

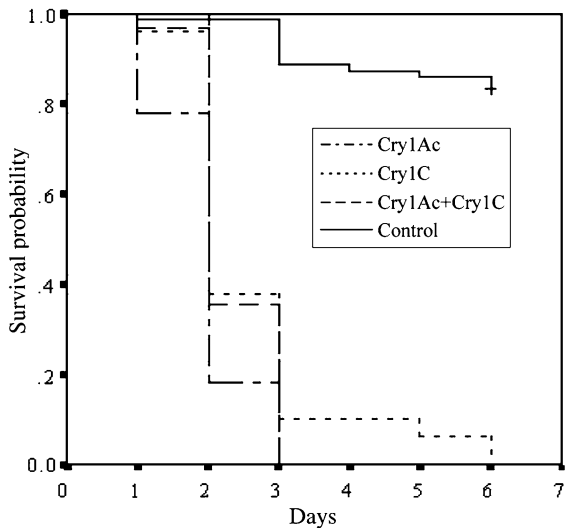

Fig. 1 Survival of *Pieris rapae* larvae after being fed *Bacillus thuringiensis* (Bt) broccoli plants and non-Bt control plants from first instar until pupation

feeding on *cry1Ac* or *cry1Ac* + *cry1C* broccoli plants and within 6 days of feeding on *cry1C* plants. Survival probabilities of *P. rapae* larvae were also significantly reduced when they were fed on Bt broccoli plants from the second, third, fourth and fifth instars compared with those fed on the non-Bt control (respectively, $\chi^2 = 210.4$, df = 3, $P < 0.001$; $\chi^2 = 172.63$, df = 3, $P < 0.001$; $\chi^2 = 142.02$, df = 3, $P < 0.001$ and $\chi^2 = 144.18$, df = 3, $P < 0.001$) (Fig. 2). The efficacies of Bt broccoli plants on different *P. rapae* instars were consistently *cry1Ac* > *cry1Ac* + *cry1C* > *cry1C* with significant differences between them except between *cry1C* and *cry1Ac* + *cry1C* plants for the first ($\chi^2 = 0.22$, df = 1, $P = 0.637$) and fourth instars ($\chi^2 = 0.22$, df = 1, $P = 0.638$), and between *cry1Ac* and *cry1Ac* + *cry1C* plants for fifth instars ($\chi^2 = 0.33$, df = 1, $P = 0.5634$).

Impact on P. rapae *pupae*

Pupal length, width and weight were measured on *P. rapae* developed from fourth to fifth instars fed with different Bt broccoli plants for 1 and 2 days, respectively (Table 1). Pupal length was significantly affected by the instar being fed Bt broccoli plants or non-Bt plants ($F = 8.808$, df = 3, $P < 0.001$) and by feeding time on Bt plants ($F = 8.766$, df = 1, $P = 0.006$). Similarly, *P. rapae* pupal width was significantly affected by plant type (Bt/non-Bt) ($F = 9.332$, df = 3, $P < 0.001$) and feeding time ($F = 13.521$, df = 1, $P = 0.001$). Pupal weight was also significantly affected by plant type ($F = 4.023$, df = 3, $P = 0.016$), but not by feeding time ($F = 1.481$, df = 1, $P = 0.233$). Important indicators of pupal quality, the length and the weight of *P. rapae* pupae, were both significantly reduced after the older larvae fed on either *cry1Ac*, *cry1C* or *cry1Ac* + *cry1C* broccoli plants for 2 days compared with their counterparts fed on non-Bt plants (Table 1).

Effects on parasitism, developmental time, proportion of females and longevity of *Pteromalus puparum*

Overall, the parasitism rate (%) of *P. puparum* from the culture on Bt-fed *Pieris rapae* pupae was lower

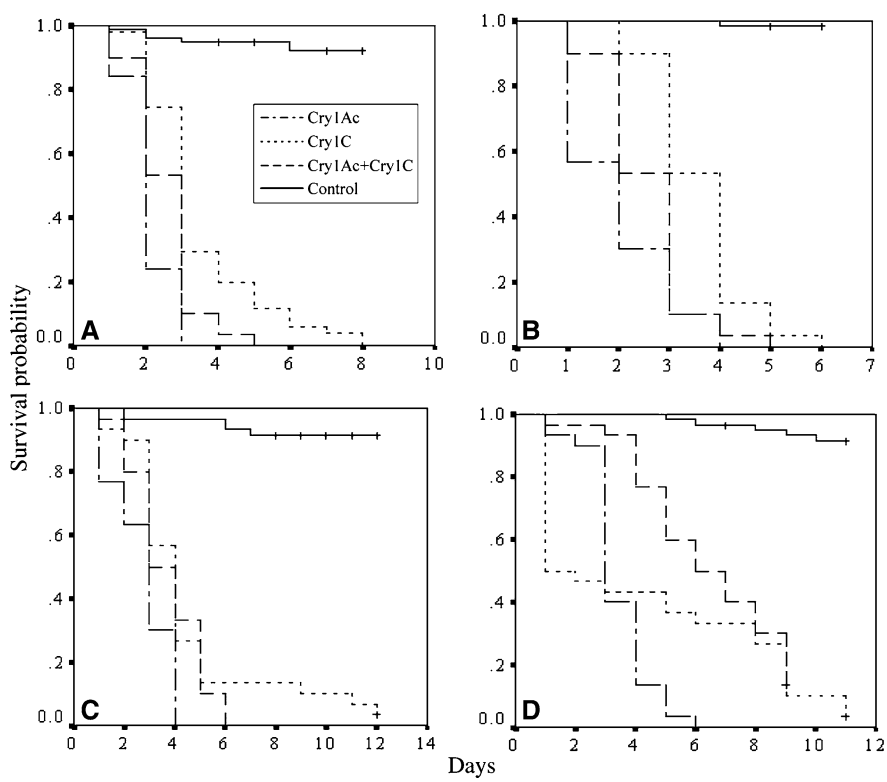

Fig. 2 Survival of *P. rapae* larvae after being fed with Bt broccoli plants and non-Bt control plants from later instars until pupation. (**A**) Second instars, (**B**) third instars, (**C**) fourth instars, (**D**) fifth instars

Table 1 Impact of different Bt broccoli plants on *Pieris rapae* pupal size after larvae had fed on *Bacillus thuringiensis* (Bt) and non-Bt plants

Plant type	Feeding time (days)	Length (mm)	Width (mm)	Weight (mg)
cry1Ac	1	17.1 ± 0.3 a	4.3 ± 0.2 a	106.8 ± 3.0 a
	2	16.4 ± 0.4* a	4.0 ± 0.2 a	99.2 ± 4.7* a
cry1C	1	17.1 ± 0.5 a	4.4 ± 0.2 a	99.6 ± 14.1* a
	2	16.3 ± 0.2* a	4.1 ± 0.1 a	89.0 ± 3.8* a
cry1Ac + cry1C	1	17.2 ± 0.2 a	4.6 ± 0.1 a	110.1 ± 5.0 a
	2	15.2 ± 0.2* b	3.6 ± 0.1* b	91.2 ± 3.3* a
Control		18.1 ± 0.2	4.8 ± 0.1	129.7 ± 18.5

Values are means ± standard error. Those followed by an asterisk (*) within a column are significantly different from the non-Bt control. Means from different feeding time treatments for the same plant type followed by different lowercase letters are significant different (two-way ANOVA and Fisher's protected LSD means separation test, $P < 0.05$)

than that on a non-Bt-fed host (Table 2). As the feeding time on Bt broccoli plants increased from 1 day to 2 day, the parasitism rate significantly decreased compared with the parasitism rate of non-Bt-fed *P. rapae* ($F = 6.825$, df = 1, $P = 0.014$), but no significant difference was found between plant types ($F = 1.649$, df = 3, $P = 0.198$). Developmental time and total number of *P. puparum* emerging from *P. rapae* pupae were significantly affected by plant type ($F = 5.747$, df = 3, $P = 0.003$ and $F = 6.179$, df = 3, $P = 0.002$, respectively), but not by feeding time ($F = 0.001$, df = 1, $P = 0.980$ and $F = 0.007$, df = 1, $P = 0.933$, respectively). The longevity of emerged *P. puparum* was significantly affected both by plant type ($F = 64.727$, df = 3, $P < 0.001$) and feeding time ($F = 17.675$, df = 1, $P < 0.001$). However, the proportion of females was not significantly affected by plant type ($F = 1.493$, df = 3, $P = 0.235$) except for feeding time ($F = 4.981$, df = 1, $P = 0.033$).

Effects on the progeny of *Pteromalus puparum* developing from Bt broccoli-fed *Pieris rapae*

Pteromalus puparum adults developing from Bt broccoli-fed *P. rapae* pupae were negatively affected, as seen by the results above (Table 1). In order to assess whether this impact could be carried over to the next generation and affect subsequent host utilization, *P. puparum* adults developing from Bt plant-fed *P. rapae* were inoculated to conventional hosts (Table 3). The parasitism rate of *P. puparum* from different Bt treatments was not significantly impacted compared with the non-Bt control ($F = 0.197$, df = 3, $P = 0.897$). Developmental time, total number and longevity of *P. puparum* were also not significantly affected by Bt plants ($F = 2.540$, df = 3, $P = 0.093$, $F = 0.105$, df = 3, $P = 0.956$ and $F = 1.484$, df = 3, $P = 0.257$, respectively). The proportion of females from *cry1Ac + cry1C* plants

Table 2 Impact of Bt broccoli plants on the parasitoid *Pteromalus puparum* developing from Bt plant-fed *Pieris rapae*

Plant type	Feeding time (days)	Parasitism (%)	Developmental time (days)	Total number of *P. puparum*	Proportion of females (%)	Longevity (days)
cry1Ac	1	80 ± 8.9 a	13.8 ± 0.3 a	51.3 ± 3.0 a	47.9 ± 2.2 b	27.8 ± 0.7* a
	2	60 ± 8.9 a	13.4 ± 0.2 a	60.3 ± 4.8 a	65.0 ± 6.4 a	21.3 ± 0.5* b
cry1C	1	76 ± 7.5 a	15.6 ± 0.5 a	53.1 ± 4.5 a	50.4 ± 2.1 a	38.1 ± 0.8 a
	2	64 ± 9.8 a	15.8 ± 1.0* a	54.1 ± 5.8 a	56.5 ± 5.3 a	32.6 ± 1.5* b
cry1Ac + cry1Ac	1	80 ± 6.3 a	14.7 ± 0.1a	48.4 ± 2.0 a	59.7 ± 2.1 a	25.8 ± 1.4* a
	2	48 ± 10.2* b	15.0 ± 0.8 a	37.3 ± 2.4* b	65.4 ± 7.8 a	20.1 ± 1.6* b
Control		84 ± 4.0	14.1 ± 0.3	60.1 ± 4.6	55.5 ± 3.3	40.6 ± 2.2

Values are means (± standard error. Those followed with an asterisk (*) within a column are significantly different from the non-Bt control. Means from different feeding time treatments for the same plant type followed by different lower-case letters are significantly different (two-way ANOVA and Fisher's protected LSD means separation test, $P < 0.05$)

Table 3 Impact of Bt broccoli plants on the progeny of *Pteromalus puparum* developing from Bt plant-fed *Pieris rapae*

Plant type	Parasitism (%)	Developmental time (days)	Total number of *P. puparum*	Proportion of females (%)	Longevity (days)
cry1Ac	80 ± 0 a	13.5 ± 0.3 b	56.4 ± 2.9 a	53.2 ± 1.7 a,b	38.2 ± 1.1 a
cry1C	76 ± 9.8 a	14.3 ± 0.1 a	57.7 ± 3.1 a	54.6 ± 2.3 a,b	37.0 ± 1.3 a
cry1Ac + *cry1C*	80 ± 8.9 a	13.7 ± 0.1 a,b	58.3 ± 3.9 a	36.6 ± 3.7 b	40.3 ± 1.7 a
Control	84 ± 4 a	14.1 ± 0.3 a,b	60.1 ± 4.6 a	55.5 ± 3.3 a	40.6 ± 2.2 a

Values are means ± standard deviation. Those followed by different lower-case letters within a column are significantly different (one-way ANOVA and Fisher's protected LSD means separation test, $P < 0.05$)

was significantly lower than those from the non-Bt control ($F = 10.012$, df = 3, $P = 0.001$).

Transfer of Cry1C toxin through different trophic levels

The stable expression of the *cry1C* gene, with an average of 0.84 μg/g fresh weight was detected in *cry1C* broccoli plants using ELISA. After fourth to fifth instar *P. rapae* larvae had fed upon Cry1C broccoli plants for 1 day, Cry1C toxin (0.18 ± 0.02 μg/g fresh weight) was detected in *P. rapae* pupae at a significantly lower level than that found in *cry1C* broccoli plants ($F = 35.587$, df = 3, $P < 0.001$). After *P. rapae* had continuously fed on *cry1C* plants for 2 and 3 days, no further accumulation of Cry1C toxin was detected compared with the 1-day feeding (Fig. 3). No Cry1C toxin was detected from the parasitoid *P. puparum* adults developing from *cry1C* plant-fed *P. rapae* pupae; however, a trace amount (<1 ppb) of Cry1C protein was detected from *P. puparum* pupae.

Discussion

Bt transgenic plants provide excellent control of several important lepidopteran pests of field crops. However, although many field studies to date have shown that Bt transgenic plants have a negligible impact on non-target organisms, including beneficials (Naranjo et al. 2005), a number of laboratory studies have shown negative effects (Ferry et al. 2003). As yet there are no clear international guidelines for assessing the effects of Bt plants with insecticidal protein on selected non-target arthropods. A tiered system adopted from the ecotoxicological evaluation of plant protection products is being suggested for such evaluation, and this approach includes a first tier of "worst-case" study under laboratory conditions

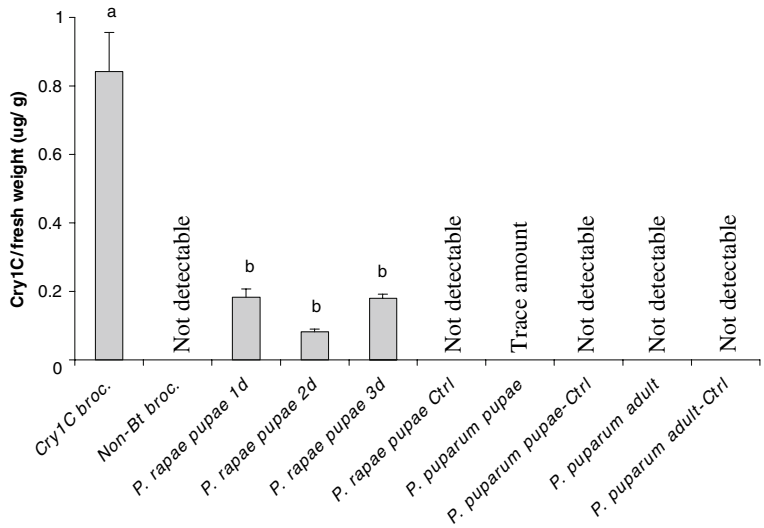

Fig. 3 Presence of Cry1C toxin in *cry1C* broccoli plants, in *Pieris rapae* pupae developing from larvae that had fed on *cry1C* broccoli plants for 1, 2 and 3 days and in its parasitoid *Pteromalus puparum*, as detected by ELISA. Means (±SE) denoted with same *lower-case letters* are not significantly different (one-way ANOVA, $P < 0.05$)

with additional studies if needed (Dutton et al. 2003; Poppy and Sutherland 2004; Rose 2006).

In the present "worst-case" study, we first evaluated the efficacy of the three types of Bt broccoli plants on different life-stage larvae of the herbivore *Pieris rapae*. The results indicated that single-gene Bt broccoli plants containing the *cry1Ac* or *cry1C* gene effectively controlled *P. rapae* larvae. All first to third instars were killed by either single-gene or dual-gene (*cry1Ac* + *cry1C*) Bt broccoli plants (Figs. 1, 2). No *P. rapae* larvae was able to survive on *cry1Ac* broccoli for more than 6 days regardless of larval stage. Similarly, dual-gene broccoli plants also provided full control of the first to fourth instars, and only 3.33% of the fifth instars were able to survive. Our results with *P. rapae* are in line with the excellent efficacy of Bt broccoli plants against *Plutella xylostella* (Cao et al. 1999, 2002). Furthermore, our previous studies have shown that dual-gene broccoli plants can delay the evolution of *P. xylostella* resistance (Zhao et al. 2003, 2005), indicating that dual Bt gene broccoli plants should be considered for effective and sustainable control of lepidopteran pests on brassica vegetable crops.

Our studies on the impact of Bt broccoli plants on *Pteromalus puparum*, an endoparasitoid of *P. rapae*, indicated that *P. puparum* was significantly affected by *P. rapae* hosts that had fed upon Bt plants (Table 2). Parasitism rate, developmental time, total number and longevity of *P. puparum* were either significantly affected by plant type (Bt/non-Bt) or by feeding time on Bt plants. In one previous "worst-case" study, Vojtech et al. (2005) reported that the cocoons of the parasitoid *Cotesia marginiventris* (Hymenoptera: Braconidae) were smaller when developing in *Spodoptera littoralis* (Lepidoptera: Noctuidae) larvae that had fed on transgenic Bt maize. Also, the developmental times of *C. marginiventris* were longer than those developing in a non-Bt-fed host. However, the negative impact of Bt maize on *C. marginiventris* when developing in susceptible *S. littoralis* turned out to be indirect, i.e. host-mediated effects (Vojtech et al. 2005). Longer developmental time and reduced fecundity and longevity of parasitoids developing in or on Bt plant-fed hosts have also been reported in other studies (Bernal et al. 2002; Baur and Boethel 2003; Meissle et al. 2004). To date, however, we suggest that the mechanism of the negative impact of Bt plants on parasitoids is likely to be mediated by poor host quality. Our results indicate that the size (length and width) and weight of the host, *P. rapae* pupae developing from Bt broccoli-fed, were significantly affected compared with non-Bt plant-fed larvae. In addition, Bt toxin is known to change the amino acid and ion composition in the hemolymph of herbivores such as *S. littoralis* (Salama et al. 1983). This may suggest that less biomass and nutrition were available for *P. puparum* to complete development in Bt-treated hosts. In a recent study, Walker et al. (2007) evaluated the impact of Bt toxins on two larval parasitoids, *Cotesia kazak* Telenga (Hymenoptera: Braconidae) and *Meteorus pulchricornis* Wesmael (Hymenoptera: Braconidae), by feeding their herbivore hosts with diets amended with commercial preparations of Bt sprays and Cry1Ac toxin preparations. These researchers found that the larval development of both parasitoids was delayed only when there was a significant impact on the host. Reduced nutrition of hosts had a comparatively greater effect on parasitoid survival than Bt toxins (Walker et al. 2007). A number of earlier studies revealed that Bt-maize expressing the *cry1Ab* gene caused negative effects on the predator *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae), i.e. higher mortality, prolonged development and a decrease in weight (Hilbeck et al. 1998a, b, 1999; Dutton et al. 2002). However, Romeis et al. (2004) fed high concentrations (10,000 times higher than that ingested when feeding on Bt-reared lepidopteran larvae) of Cry1Ab toxin directly to *C. carnea* and found no direct toxic effect of the toxin on *C. carnea* larvae. Moreover, feeding first instar *C. carnea* with the Cry1Ab toxin did not affect the utilization of subsequently provided prey. Such direct studies are very appropriate and help to explain some spurious results with predators, but they are very difficult to perform with parasitoids whose survival relies on their host's survival.

Cry1C toxin was detected in the herbivore, *P. rapae* pupae, which meant that *P. puparum* were exposed to Bt toxin when they were parasitizing *P. rapae* pupae. Thus, direct effects of Bt toxin on the parasitoid cannot be ruled out, although it is very unlikely there would be a direct effect since Bt proteins need specific receptors in the target insect gut epithelium (Gill et al. 1992; Knowles 1994). To date, no direct effects on hymenopteran species have been demonstrated (Vojtech et al. 2005). Moreover, no Bt toxin was detected in newly emerged *P. puparum* adults, and only a trace amount of Bt toxin was

detected in *P. puparum* pupae (Fig. 3). Foremost, these results suggest that *P. puparum* larvae did not take up Bt toxin. The trace amount of Bt toxin on *P. puparum* pupae is likely to be the result of tissues of *P. rapae* pupae clinging to the surfaces of the pupae because a trace amount of Bt toxin was also detected in the Wash Buffer that was used for washing the pupae before the ELISA tests (data not shown). Secondly, the Bt toxin, if ingested, may have been excreted or metabolized before the emergence of *P. puparum* adults from *P. rapae* pupae. Similar phenomena (toxin excretion and degradation) were reported in the predator *Pirata subpiraticus* Böesenberg et Strand (Araneae: Lycosidae) (Chen et al. 2005). In addition, our results indicate that parasitism rate, developmental time and longevity of the progeny of *P. puparum* developing from Bt broccoli-fed *P. rapae* were not significantly affected when inoculated with subsequently provided hosts (Table 3). Taken together, we conclude that Bt broccoli did not have an clear-cut direct effect on *P. puparum.*

Transgenic Bt vegetable brassicas are being considered as candidates for future release to farmers to control damage from lepidopteran pests (Srinivasan et al. 2005; Christey et al. 2006). Based on the results herein and those from our previous studies (Cao et al. 1999, 2002; Zhao et al. 2003), we conclude that such transgenic brassicas can be effective against a number of the more important Lepidoptera. We also conclude that, in the case of *P. rapae*, the negative impact of Bt broccoli on *P. puparum* resulted from poor quality of the host rather than direct effects of the Bt toxin and that this host quality-mediated impact did not adversely affect the progeny of *P. puparum* to utilize subsequently provided hosts. These results provide additional evidence that Bt plants can contribute to biological control within an overall integrated pest management program.

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