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ORIGINAL PAPER

Effect of Bt broccoli and resistant genotype of *Plutella xylostella* (Lepidoptera: Plutellidae) on development and host acceptance of the parasitoid *Diadegma insulare* (Hymenoptera: Ichneumonidae)

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Abstract The ecological implications on biological control of insecticidal transgenic plants, which produce crystal (Cry) proteins from the soil bacterium *Bacillus thuringiensis* (Bt), remain a contentious issue and affect risk assessment decisions. In this study, we used a unique system of resistant insects, Bt plants and a parasitoid to critically evaluate this issue. The effects of broccoli type (normal or expressing Cry1Ac protein) and insect genotype (susceptible or Cry1Ac-resistant) of *Plutella xylostella* L. (Lepidoptera: Plutellidae) were examined for their effects on the development and host foraging behavior of the parasitoid, *Diadegma insulare* (Cresson) (Hymenoptera: Ichneumonidae) over two generations. Parasitism rate and development of *D. insulare* were not significantly different when different genotypes (Bt-resistant or susceptible) of insect host larvae fed on non-Bt broccoli plants. *D. insulare* could not discriminate between resistant and susceptible genotypes of *P. xylostella*, nor between Bt and normal broccoli plants with different genotypes of *P. xylostella* feeding on them. No *D. insulare* could emerge from Bt broccoli-fed susceptible and heterozygous *P. xylostella* larvae because these larvae were unable to survive on Bt broccoli. The parasitism rate, developmental period, pupal and adult weights of *D. insulare* that had developed on Bt broccoli-fed Cry1Ac-resistant *P. xylostella* larvae were not significantly different from those that developed on non-Bt broccoli-fed larvae. Female *D. insulare* emerged from Cry1Ac-resistant *P. xylostella* that fed on Bt plants could successfully parasitize *P. xylostella* larvae. The life parameters of the subsequent generation of *D. insulare* from *P. xylostella* reared on Bt broccoli were not significantly different from those from non-Bt broccoli. The Cry1Ac protein was detected in *P. xylostella* and in *D. insulare* when hosts fed on Bt broccoli. These results are the first to indicate that Cry1Ac did not harm the development or host acceptance of an important endoparasitoid after two generations of exposure. We suggest that using other Bt crops and resistant insect species would likely lead to similar conclusions about the safety of the presently used Bt proteins on parasitoids.

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Keywords *Bacillus thuringiensis* · Biosafety · Natural enemies · Transgenic crop · Host genotype

Abbreviations

RR	Cry1Ac-resistant strain or homozygous resistance genotype
RS	Heterozygous resistance genotype
SS	Susceptible strain or genotype
Bt	*Bacillus thuringiensis*

Introduction

Development and commercialization of insect-resistant genetically modified (IRGM) crops expressing insecticidal proteins (Cry toxins) from bacterium *Bacillus thuringiensis* (Bt) have offered an alternative to traditional synthetic insecticides for control of important agricultural insect pests. The only currently available IRGM crops for commercial planting are Bt corn and Bt cotton. In 2009 these crops were grown on 50.4 million hectares in 25 countries (James 2009). Bt rice received regulatory approve in China in 2009 and will likely be commercialized in the near future (http://www.stee.agri.gov.cn/biosafety/spxx/t20091022_819217.htm). Other IRGM crops are waiting in the wings, including Bt eggplant, cabbage and cauliflower (Shelton et al. 2008; Grzywacz et al. 2010).

The diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae), is the most destructive insect pest of brassica crops worldwide. The estimated annual cost for controlling this insect two decades ago was US $1 billion (Talekar and Shelton 1993). Like other transgenic crops, a range of brassica species expressing insecticidal proteins from Bt have been engineered to provide resistance to *P. xylostella* and other lepidopteran pests (Shelton et al. 2008). Our previous studies have demonstrated that brassica crops expressing Bt insecticidal proteins can effectively control *P. xylostella* (Metz et al. 1995; Tang et al. 1999, 2001; Cao et al. 1999, 2002, 2005; Shelton et al. 2000, 2008; Zhao et al. 2000, 2003, 2005).

Bt plants have provided economic benefits to growers and reduced the use of synthetic insecticides (Shelton et al. 2002; Qaim et al. 2008; Brookes and Barfoot 2010), but there has been considerable discussion about whether they are compatible with natural enemies that help suppress pest population. Numerous studies have investigated the effects of Bt plants and Cry proteins on parasitoids and predators. Predators are usually generalists and feed on several different prey species and the effects of Bt plants on them have been found to be negligible (Romeis et al. 2006; Naranjo 2009), although others have disagreed (Lövei et al. 2009, but also see Shelton et al. 2009a, b). Lawo et al. (2010) reported that larvae of the green lacewing, *Chrysoperla carnea* (Neuroptera: Chrysopidae), were adversely affected when fed Bt (Cry1Ac) cotton-fed caterpillars. In contrast, the predators remained unaffected when feeding on Bt cotton-fed caterpillars from a Cry1Ac-resistant strain. This demonstrated that the adverse effects seen with the susceptible caterpillar strain were prey-quality mediated and supports the concept of using Bt toxin resistant strains as a way to test the sensitivity of beneficial arthropods to Cry toxins (Romeis et al. 2010).

Host-parasitoid relationships are more intimate because parasitoids usually complete their larval development in a single insect host. Negative impacts of Bt toxins on non-target parasitoids have been reported when susceptible insect hosts were used in some studies (Baur and Boethel 2003; Liu et al. 2005a, b, c; Sanders et al. 2007), although the negative impacts most likely were host-quality mediated. When resistant insect hosts were used in some studies, no negative effects of transgenic plants on parasitoids were found (Johnson 1997; Atwood et al. 1998; Schuler et al. 1999, 2004; Chen et al. 2008). However, the impact of transgenic plants on the subsequent performance of adult parasitoids when resistant hosts were used has not been explored.

Diadegma insulare (Cresson) (Hymenoptera: Ichneumonidae) is a solitary, host-specific larval endoparasitoid and is an important biological control agent of *P. xylostella* in North America (Xu et al. 2001a, b; Sarfraz et al. 2005). Although our previous studies (Chen et al. 2008) indicated that Cry1C, which is effective against about 35–40 species and differs in its insecticidal host range from the Cry1A toxins (Avisar et al. 2004), did not have any direct toxicity on *D. insulare* during the first generation when it fed on *P. xylostella* feeding on Cry1C broccoli, neither our study nor any other study has investigated the possible chronic or cumulative

effects of Bt plants on subsequent generations of a parasitoid. Such longer term effects could be evident not only from direct toxicity, but from changes in the parasitoid's behavior. This is an important and yet uninvestigated question that has implications for both biological control and risk assessment of Bt crops.

Our resistant *P. xylostella* and Bt broccoli system allows us to investigate this question. For example, if Bt brassicas are commercialized for control of *P. xylostella*, *D. insulare* would likely be exposed in the field to different plant types (Bt and non-Bt plants) in the neighboring fields because a common strategy to delay the evolution of resistance to Bt plants is to use non-Bt plants as a refuge to conserve susceptible alleles (Bates et al. 2005). Likewise, as resistance to the Bt proteins evolves, parasitoids would also be exposed to different genotypes of insect hosts (resistant (RR), susceptible (SS) or heterozygous (RS) individuals). Thus, the interaction of Bt plants, genotypes of insect hosts and parasitoids might have complex effects, especially when viewed over multiple generations, including effects on the rate of resistance evolution.

The present study goes beyond the results of Chen et al. (2008) and other studies to explore whether host genotypes and plant types could affect the development and host acceptance behavior of *D. insulare* and its progeny over multiple generations. Specifically, the following objectives were addressed in this study: (1) determine if *D. insulare* could discriminate between resistant and susceptible genotypes of *P. xylostella*; (2) determine if *D. insulare* could discriminate plant types (Bt plants or non-Bt plants) hosting different resistance genotypes of *P. xylostella*, and; (3) quantify the effects of Cry1Ac broccoli plants on selected life history parameters of *D. insulare* when the plants are infested by RR, RS, and SS genotypes of *P. xylostella* for two generations.

Materials and methods

Insects

Three strains of *P. xylostella* were used: (1) a Cry1Ac-resistant strain (RR), which can survive on Cry1Ac Bt broccoli plants (Zhao et al. 2005); (2) a Cry1Ac-susceptible strain (SS, Geneva 88), which cannot survive on Cry1Ac Bt broccoli plants (Zhao et al. 2005); (3) a heterozygous strain (RS), which was developed by crossing RR with G88. The hymenopteran endoparasitoid, *D. insulare*, was originally field collected in Florida in 1999 and subsequently reared in our greenhouse according to the procedures of Xu et al. (2001a, b). Insects were kept in a climatic chamber at 27 ± 1°C, 50 ± 10% RH, and 16:8 h photoperiod.

Bt broccoli plants

We used *Brassica oleracea* L., var. italica' Green Comet as the cultivar for our broccoli plants. The transgenic broccoli produces high levels of Cry1Ac (Metz et al. 1995). To ensure the activity of the Bt broccoli, the plants were screened with the susceptible *P. xylostella* neonates when plants were 4–5-week-old. In all the studies reported in this paper, broccoli plants with 8 true leaves were used and analysis by ELISA indicated that the Cry1Ac protein level was 12.33 ± 1.62 μg/g ($n = 7$) fresh leaf tissue.

Survival and development of different genotypes of *P. xylostella* fed on Bt or non-Bt broccoli plants

To evaluate the effect of Bt broccoli plants on different genotypes of *P. xylostella*, 20 neonates of RR, RS and SS genotypes were fed leaves from Cry1Ac broccoli or non-Bt broccoli in a Plexiglas cylinder cage (10 cm × 10 cm × 20 cm). In order to keep a leaf fresh, the leaf petiole was placed in a 100 ml flask with water. The old leaves were replaced with new ones every 2 days. All six treatments were replicated 4 times. The survival rate at 3 days and the duration of the egg and larval period, the length of the pupal period and the percent pupation and the pupal weight, and the adult emergence rate were recorded. The experiment was conducted in a climatic chamber at 27 ± 1°C, 50 ± 10% RH, and 16:8 h photoperiod.

Can *D. insulare* discriminate different genotypes of *P. xylostella*?

Choice tests were conducted in 1 m × 1 m × 1 m netted cages. Fifty *P. xylostella* second instars (either RR, RS, or SS) of a single genotype were placed on a

non-Bt broccoli leaf with its petiole inserted in a 100 ml flask filled with water for 1 day before being presented to the natural enemy. One flask hosting a different genotype of DBM (RR, RS or SS) was placed in a triangle with similar distance between the flasks. Three pairs of 3-day-old adult *D. insulare* were released in the center of the triangle in each cage. A flask of 10% sugar solution with a wick was placed into each cage as a food source for *D. insulare*. After 48 h, the *P. xylostella* larvae were retrieved and transferred into diet cups (Shelton et al. 1991) and allowed to develop into *P. xylostella* adults or *D. insulare* adults. All 50 caterpillars from one leaf were placed in one diet cup. All three treatments were replicated six times. Parasitism rates (% parasitism = (number of *D. insulare*/(number of *D. insulare* + number of *P. xylostella*) *100) caused by *D. insulare* on each genotype of *P. xylsotella* were recorded.

Can *D. insulare* discriminate plant types hosting different genotypes of *P. xylostella*?

In order to evaluate whether the parasitoids could discriminate plant types hosting different genotypes of *P. xylostella*, a 2 × 3 design (plant types: Bt or non-Bt) × (*P. xylostella* genotypes: RR, RS and SS) was utilized. Each cage (1 m × 1 m × 1 m) had six treatments: RR on Cry1Ac plant, RS on Cry1Ac plant, SS on Cry1Ac plant, RR on non-Bt plant, RS on non-Bt plant, and SS on non-Bt plant. Each treatment had 50 *P. xylostella* second instars (RR, RS, or RS) on each leaf and each leaf was placed in the center of the cage in a flask and evenly separated. *P. xylostella* larvae were allowed to feed on the corresponding leaves for 1 day before being exposed to *D. insulare*. Four pairs of new emerged *D. insulare* adults were put into a Plexiglas cylinder cage (10 cm × 10 cm × 20 cm) with sugar water to mate for 3 days. Then the four mated females were released into the center of each cage. After 48 h, the larvae were retrieved and transferred to diet cups and allowed to develop into *P. xylostella* adults or *D. insulare* adults. Parasitism rates (percentages) caused by *D. insulare* on each genotype of *P. xylsotella* were recorded. The six treatments were replicated six times.

Effect of plant type and host genotype on the development of the F_1 parasitoid

Because the first experiment of survival of different genotypes of *P. xylostella* fed on Bt and our earlier studies (Zhao et al. 2000, 2005) showed that the RS and SS strains could not survive on Cry1Ac broccoli plants, here were only four treatments in this study: RR on Bt broccoli, RR on non-Bt broccoli, RS on non-Bt broccoli and SS on non-Bt broccoli. For the RR treatments, 50 *P. xylostella* larvae were placed on a Cry1Ac leaf in a 100 ml flask filled with water and then placed in a Plexiglas cylinder cage (10 cm × 10 cm × 20 cm) for 1 day. Then two pairs *D. insulare* were released into the cage and, after 24 h, the parasitoids were removed and the *P. xylostella* RR larvae were transferred onto a new Cry1Ac broccoli leaf. The *P. xylostella* larvae were kept in the cage until *D. insulare* pupa or *P. xylostella* adult emergence. Old Cry1Ac leaves were replaced with fresh ones as needed. Each *D. insulare* pupa was weighed and placed individually into a 30 ml cup. Moreover, the developmental time of *D. insulare* (from oviposition to adult emergence), adult longevity (without food) and dry weight were recorded at the end. RR, RS and SS larvae fed on non-Bt leaf in each cage were also set up as described above and exposed to *D. insulare* with the same data endpoints being recorded. The four different treatments were replicated 4 times.

Development of F_2 parasitoids reared from different genotypes of *P. xylostella* exposed to Bt or non-Bt broccoli

In order to evaluate whether Bt broccoli plants and *P. xylostella* genotypes would affect the ability of *D. insulare*'s progeny to utilize a susceptible host, we studied the development of the second generation *D. insulare* whose parents emerged from the different genotypes of *P. xylostella* larvae on Bt or non-Bt broccoli. Two pairs of *D. insulare* that developed from RR on Bt, RR on non-Bt broccoli, RS on non-Bt broccoli, and SS on non-Bt broccoli, separately, were introduced to 50 SS larvae fed on non-Bt broccoli plants for 1 day. After 24 h, the parasitoids were removed and the *P. xylostella* larvae were transferred onto a new non-Bt broccoli leaf. All four treatments

were replicated 4 times. Parasitism rate, the developmental time, pupal weight, adult longevity (without food) and dry weight of F_2 *D. insulare* were recorded.

Quantification of Cry1Ac in *P. xylostella* and in *D. insulare*

The amounts of Cry1Ac in larvae, pupae and adults of the RR genotype, which were fed on Bt broccoli from neonates, and the Cry1Ac in larvae, pupae and adults of *D. insulare* were monitored by ELISA using the EnviroLogix Cry1Ac/Cry1Ab kit. RR neonates were fed on Bt broccoli until the second instar, then the larvae were provided to *D. insulare* for 48 h. The parasitized larvae were fed on Bt broccoli to develop into *P. xylostella* adults or *D. insulare* pupae. The mature larvae, pupae, adults and pupal coccons were sampled and kept in Eppendorf vials to detect the transfer and accumulation of Cry1Ac protein in *D. insulare*.

Each sample included 20 larvae, pupae or adults, separately, and was ground and homogenized in 0.3 ml Extraction/dilution buffer (EnviroLogix). In order to keep samples from contamination, each sample were washed three times with dilution buffer prior to the analysis. ELISA was conducted according to the manufacturer's instructions. Based on preliminary tests, sample extractions were diluted by 1:50 for *P. xylostella* larvae, by 1:20 for the pupae and adults of *P. xylostella* and the larvae and pupae of *D. insulare*, and by undiluted extraction for *D. insulare* adults. Each treatment was replicated 5–7 times. The optical density value of sample was measured using a microplate reader set at 450 nm. The larvae fed on non-Bt broccoli were used as the controls.

Bioactivity of Cry1Ac after ingestion by *P. xylostella* larvae

To confirm that *D. insulare* was exposed to active Cry1Ac toxin when it developed inside Cry1Ac-resistant *P. xylostella* larvae, the biological activity of the Cry1Ac toxin being consumed by Cry1Ac-resistant *P. xylostella* was checked according to the methods of Chen et al. (2008). *P. xylostella* second instars from the Cry1Ac-resistant (RR) strain were fed Cry1Ac plants. Non-Bt broccoli plants were used as control. After RR larvae fed on the Cry1Ac plants for 2 days, they were collected into separate 1.5 ml Eppendorf vials. The RR larvae were washed with distilled water 4 times then ground with a pestle in 500 µl Cry1Ac toxin extraction buffer (supplied in Cry1Ac ELISA kit, EnviroLogix Inc., Portland, ME). The solution was diluted to 5,000 µl and was applied to cabbage leaf disks that were fed to Cry1Ac-susceptible (SS) larvae. Ten second instars from the SS strain were placed on each of the leaf disks inside 30-ml plastic cups with 5 replications. Mortality was determined after 3 days at 27 ± 1°C.

Statistical analyses

Data were analyzed using one-way ANOVA and differences between treatment means were tested with the Tukey test at a 5% level of significance. All statistical analyses were conducted using SPSS 17.0 Windows (1998) (SPSS, Chicago, IL).

Results

Survival and development of different genotypes of *P. xylostella* fed Bt or non-Bt broccoli plants

The results confirmed that RS and SS larvae could not survive on Bt plants, while Cry1Ac-resistant neonates could (Table 1). The egg to larval period and pupal period of RR fed Bt plants was significantly longer in comparison with the RS and SS strains fed on non-Bt plants. No significant differences were found between RR fed Cry1Ac plants and RR fed non-Bt plants. The *P. xylostella* pupation rates, pupal weights, and adult emergence rates were not significantly different between the treatments of RR on Bt broccoli, RR on non-Bt broccoli, RS on non-Bt broccoli and SS on non-Bt broccoli.

Can *D. insulare* discriminate different genotypes of *P. xylostella*?

The results show that *D. insulare* did not discriminate between different genotypes of *P. xylostella*. The parasitism rates caused by *D. insulare* on RR, RS and SS genotypes hosted on non-Bt broccoli plants were 30.14 ± 6.51%, 21.45 ± 3.33% and 28.44 ± 6.98%

Table 1 Development of different genotypes of *Plutella xylostella* on Bt or non-Bt broccoli plants

Genotype	Plant type	Survival rate (%)	Egg to larval period (days)	Pupal rate (%)	Pupal weight (mg)	Pupal period	Adult emergence (%)
RR	Bt	93.8 ± 2.40a	10.6 ± 0.11a	85.0 ± 2.89a	7.3 ± 0.26a	3.1 ± 0.07a	92.4 ± 3.03a
	Non-Bt	95.0 ± 3.54a	10.1 ± 0.04ab	76.3 ± 4.73a	7.3 ± 0.13a	3.0 ± 0.09ab	90.3 ± 1.53a
RS*	Non-Bt	92.5 ± 3.23a	9.9 ± 0.22b	80.0 ± 4.56a	7.2 ± 0.18a	2.8 ± 0.09b	93.8 ± 2.74a
SS*	Non-Bt	95.0 ± 2.04a	9.8 ± 0.21b	87.5 ± 3.23a	7.5 ± 0.08a	2.6 ± 0.11b	95.9 ± 2.55a
df		3,12	3, 12	3, 12	3, 12	3, 12	3, 12
F		0.175	5.592	1.639	0.613	5.529	0.868
P		0.912	0.014	0.233	0.619	0.015	0.484

Means (±SEM) within the same column followed by different letters are significantly different ($P < 0.05$, Tukey test)

* No survivor of RS and SS fed on Bt broccoli

(mean ± SE), respectively ($F = 0.618$, $df = 2,12$, $P = 0.556$).

Can *D. insulare* discriminate plant types hosting different genotypes of *P. xylostella*?

Because RS and SS larvae could not survive on Bt broccoli plants, no parasitoids emerged from these hosts. Parasitism rates caused by *D. insulare* were similar when RR larvae were on Bt broccoli (31.3 ± 4.76%) and non-Bt broccoli (34.9 ± 8.52%). The parasitism rates on RS and SS larvae were 29.6 ± 6.97% and 32.1 ± 8.32%, respectively, when these larvae were on non-Bt broccoli plants. There were no significant differences between the four treatments ($F = 0.092$, $df = 3, 20$, $P = 0.964$).

Effect of plants type and host genotype on the development of the F_1 parasitoid

When *D. insulare* parasitized RR, RS and SS genotype larvae fed Bt or non-Bt broccoli plants, the parasitoids developed normally to adults if the insect hosts were able to survive on Bt broccoli plants (Table 2). *D. insulare* parasitism rates, the developmental times from egg to adult and adult weights were not significantly different when *D. insulare* parasitized RR larvae on Bt broccoli, and RR, RS and SS on non-Bt broccoli. Pupal weights and adult longevity of *D. insulare* were similar when insects developed from the RR genotype on Cry1Ac broccoli and non-Bt broccoli plants, but there were some differences in pupal weights and adult longevity between RR on Cry1Ac broccoli and RS or SS on non-Bt broccoli.

Development of F_2 parasitoids reared from different genotypes of *P. xylostella* exposed to Bt or non-Bt broccoli

Diadegma insulare adults developed from RR larvae that fed on Bt or non-Bt broccoli plants successfully parasitized susceptible *P. xylostella* (Table 3). *D. insulare* parasitism rates, developmental time, pupal weight, adult weight, and adult longevity were not significantly affected by plant types (Bt or non-Bt) or host resistance genotype.

Quantification of Cry1Ac in *P. xylostella* and in *D. insulare*

Cry1Ac levels in *P. xylostella* and in *D. insulare* are presented as μg/g of fresh tissue and μg × 10^{-3}/insect (Table 4). For the tissue data, the highest concentrations were detected in the larvae of *P. xylostella*. The Cry1Ac concentration in the second instar larvae was 16 times higher than in the pupae and 48 times higher than in the adult. The Cry1Ac levels in larvae and pupae of *D. insulare* were much lower than that of *P. xylostella*. Adults of *D. insulare* had very low levels of Cry1Ac (0.01), while a much high level (0.92) was detected in the pupal cocoons, presumably because the Cry1Ac was excreted with other waste in the pupal meconium.

Bioactivity of Cry1Ac after ingestion by *P. xylostella* larvae

The survival rate was 93% ± 2.1 and 91% ± 2.3 when the SS larvae fed on the leaf with the solution of non-Bt broccoli plants and on the leaf with

Table 2 Development of F1 *Diadegma insulare* in different genotypes of *Plutella xylostella* fed on Cry1Ac or non-Bt broccoli

Genotype	Plant type	Parasitism rate (%)	Egg to adult period (days)	Pupae weight (mg)	Adult weight (mg)	Adult longevity (days)
RR	Bt	86.7 ± 2.92a	13.7 ± 0.11a	5.1 ± 0.13a	1.2 ± 0.03ab	2.6 ± 0.07a
	Non-Bt	72.3 ± 13.35a	13.8 ± 0.14a	5.3 ± 0.21ab	1.1 ± 0.03ab	2.4 ± 0.11ab
RS	Non-Bt	78.2 ± 7.49a	14.1 ± 0.12a	5.7 ± 0.13b	1.1 ± 0.03a	2.4 ± 0.09ab
SS	Non-Bt	87.8 ± 2.0a	14.0 ± 0.14a	5.6 ± 0.17ab	1.2 ± 0.03b	2.4 ± 0.05b
df		3, 12	3, 221	3, 107	3, 197	3, 194
F		1.361	1.655	3.258	2.717	3.778
P		0.305	0.178	0.024	0.046	0.012

Means (±SEM) within the same column followed by different letters are significantly different ($P < 0.05$, Tukey test)

Table 3 Development of F2 *D. insulare* whose parents emerged from different genotypes of *Plutella xylostella* fed on Cry1Ac or non-Bt broccoli

Parental genotype	Plant type	Parasitism rate (%)	Developmental time (days)	Pupae weight (mg)	Adult weight (mg)	Adult longevity (days)
RR	Bt	89.5 ± 1.89a	13.5 ± 0.12a	5.8 ± 0.20a	1.2 ± 0.06a	2.5 ± 0.17a
	Non-Bt	93.0 ± 1.32a	13.2 ± 0.16a	5.2 ± 0.15a	1.1 ± 0.05a	2.5 ± 0.11a
RS	Non-Bt	79.1 ± 6.31a	13.6 ± 0.10a	5.5 ± 0.13a	1.3 ± 0.05a	2.4 ± 0.10a
SS	Non-Bt	88.5 ± 2.06a	13.3 ± 0.19a	5.6 ± 0.21a	1.3 ± 0.07a	2.6 ± 0.17a
df		3, 12	3, 113	3, 117	3,43	3, 102
F		2.854	1.373	2.245	1.063	0.204
P		0.105	0.255	0.087	0.375	0.894

Means (±SEM) within the same column followed by different letters are significantly different ($P < 0.05$, Tukey test)

Table 4 Cry1Ac concentration in fresh Bt broccoli leaf and in *Plutella xylostella* and in *Diadegma insulare* (Means ± SEM) ($n = 5$)

Sample	Cry1Ac concentration per fresh g tissue (μg/g)	Cry1Ac concentration per insect (μg × 10^{-3}/insect)
Bt broccoli leaf	12.33 ± 1.618	–
2nd instar *P. xylostella*	4.86 ± 0.333	0.99 ± 0.061
3rd instar *P. xylostella*	1.70 ± 0.225	3.15 ± 0.413
4th instar *P. xylostella*	1.89 ± 0.189	13.29 ± 1.178
P. xylostella pupae	0.31 ± 0.025	2.17 ± 0.190
P. xylostella adult	0.10 ± 0.011	0.32 ± 0.038
D. insulare larvae	0.15 ± 0.027	1.05 ± 0.193
D. insulare pupae	0.22 ± 0.033	1.23 ± 0.202
D. insulare adult	0.01 ± 0.002	0.01 ± 0.004
D. insulare pupal coccon	0.92 ± 0.039	1.10 ± 0.046

extraction buffer, respectively. By comparison, the survival rate of SS insects was 51 ± 4.0% when they fed on the cabbage leaf with the solution of Cry1Ac toxin ingested by RR larvae ($F = 63.414$, $df = 2, 27$, $P < 0.0001$), thus indicating the Cry1Ac was still active against susceptible *P. xylostella*.

Discussion

Biological control, using predators and parasitoids of crop pests, is a key component in integrated pest management (IPM) systems and these beneficial organisms should be conserved (Croft 1990). Another key element in IPM is host plant resistance, but we have had little host plant resistance to key lepidopteran and coleopteran species in our major crops prior to the advent of Bt crops (Shelton et al. 2008). A key question is whether host plant resistance using Bt technology is compatible with biological control.

There has been considerable published work in the laboratory and field on the potential effects of Bt proteins on natural enemies. The effects of Cry toxins on predators were reviewed by Romeis et al. (2006)

and the authors suggested that predators were not susceptible to lepidopteran—active proteins. For example, Lawo et al. (2010) confirmed that Cry1Ac protein has no directly toxicity to the predator, *Chrysoperla carnea*, a predator others (e.g. Hilbeck et al. 1998) suggested was susceptible. For parasitoids the situation is more complex, whereas an insect predator is characterized by feeding on multiple and various hosts during its lifetime, a parasitoid usually completes its entire life in a single host and derives all its nutritional requirements from the host tissues. Sanders et al. (2007) reported that adult *Campoletis sonorensis* reared on Bt maize-fed *Spodoptera frugiperda* larvae were significantly smaller than those reared in hosts fed either of the conventional maize hybrids. Ramirez-Romero et al. (2007) assessed host-mediated effects of Cry1Ab on the parasitoid *Cotesia marginiventris* and showed that the exposure to Cry1Ab protein via Bt-maize tissue affected parasitoid developmental times, adult size, and fecundity. If hosts fed on Bt cotton or the diet with Cry1Ac toxin, the survival and development of the hymenopteran endoparasitoids were affected (Baur and Boethel 2003; Liu et al. 2005a, b, c; Ding et al. 2009). Although some (e.g. Romeis et al. 2006; Naranjo 2009) would argue that these negative impacts on parasitoids were likely due to poor host-quality, others may argue that the parasitoids were directly harmed by the Bt proteins. It is important to sort this out and the use of Bt-resistant hosts is the best way to do so. Demonstrating that the parasitoid is simply not susceptible to the Bt protein is best done using resistant hosts and showing that the protein that the parasitoid was exposed to was biologically active. The present study is the second example of such a critical test. However, unlike the first example by Chen et al. (2008), in this study we examined the effect of host genotypes, their interaction with plant type and any potential longer-term effect in subsequent generations.

In the present study, the life parameters of *P. xylostella* with resistance to Cry1Ac were not significantly different when the larvae fed on Bt broccoli and non-Bt broccoli. The heterozygous and susceptible larvae could not survive on the Bt broccoli plants (Table 1), which indicated that the Bt broccoli used in the present study was effective against susceptible *P. xylostella*. Our results also indicate that the Cry1Ac-resistant strain was highly resistant to Cry1Ac since most RR larvae could survive on the Cry1Ac broccoli plants, the larvae developed at a similar rate and the size of larvae parasitized by *D. insulare* was similar (Table 1). The equally high rates of parasitism by *D. insulare* when on RR hosts on Bt and non-Bt plants and when on RS and SS hosts on non-Bt plants suggest that Cry1Ac has no effect on *D. insulare* and there is also no indication of sublethal effects.

Diadegma insulare consumed most of its host's tissue before emergence and was thus exposed to toxins present in the gut of the host (Harvey and Strand 2002). In our study, Cry1Ac protein was detected in *D. insulare*, although the toxin was diluted when moving though the trophic levels. Furthermore, our result also confirmed the Cry1Ac protein the parasitoid was exposed to was still biologically active. Despite these findings, *D. insulare* parasitism rate, egg to adult period, pupal weight, adult weight and adult longevity were not affected when Bt broccoli-fed RR larvae were used as hosts. The fact that *D. insulare* did not complete its development when Bt broccoli-fed SS larvae were used as hosts was due to the earlier death of the susceptible *P. xylostella* on Bt broccoli plants rather than direct toxicity by Cry1Ac protein. Therefore, our present results further confirmed that this Cry protein is safe to this important parasitoid. Schuler et al. (1999, 2004) reported similar results in *P. xylostella* and another of its parasitoids, *Cotesia plutella*, that developed to maturity in Bt-resistant hosts fed on Bt oilseed rape leaves. However, in their study, they did not confirm the bioactivity of the ingested Cry protein nor make the assessment past the first generation.

Another facet of the present study is the potential interaction between parasitism and host resistance to Bt proteins. It has been reported that some strains of *P. xylostella* have developed resistance to microbial Bt sprays in the field (Shelton et al. 2007) and that resistant strains can also survive on Bt broccoli (Zhao et al. 2000). Thus, if Bt crucifers are deployed commercially (Shelton et al. 2008), it is possible there would be resistant, heterozygous and susceptible larvae in the fields. If there is a preference by a parasitoid to remove resistant individuals, then this would reduce the likelihood of the population becoming resistant to the Bt crop. In the present study, we addressed the question but our results indicated that *D. insulare* parasitism rates were not

significantly different when RR, RS and SS larvae fed on non-Bt broccoli; therefore, we conclude that at least *D. insulare* could not discriminate different genotype hosts.

Host location and acceptance by parasitoids relies on a number of cues, such as volatiles released by plants in response to feeding damage and by insect frass (Turlings et al. 2005) and changes in these signals in plants may prevent parasitoids from locating hosts effectively (Sanders et al. 2007). However, Schuler et al. (1999, 2003) reported that *C. plutella* females did not distinguish between Bt and wild type oilseed rape plants, and were more attracted to Bt plants damaged by Bt-resistant hosts than by susceptible hosts due to more extensive feeding damage.

In our study, we examined the foraging behavior and host acceptance of *D. insulare* under the interactions of plant and insect hosts. The parasitism rates were not significantly different when a resistant host fed on Cry1Ac broccoli plants or when resistant, heterozygous and susceptible hosts fed on non-Bt broccoli plants. Therefore, we conclude that *D. insulare* could not discriminate between plant types (Bt or non-Bt), insect host genotypes (RR, RS, or SS) and any interaction effects of plant types and insect genotypes, which may have implications on the role of natural enemies regulating resistance evolution of target insect pests to Bt crops (Bates et al. 2005; Onstad and Knolhoff 2008).

In conclusion, transgenic Bt brasscia plants can effectively control *P. xylostella* but have no direct effects on hymenopteran parasitoids based on the results herein and those from our previous studies (Cao et al. 1999, 2002; Zhao et al. 2003; Chen et al. 2008). These conclusions were derived from experiments using our unique system of Bt broccoli and resistant *P. xylostella*. However, we expect that similar conclusions could be reached using other Bt crops and resistant insect species if they were available. If this broader conclusion could be verified, then we suggest that Bt plants are fully compatible with biological control within an overall integrated pest management program.

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