Tri-Trophic Studies Using Cry1Ac-Resistant *Plutella xylostella* Demonstrate No Adverse Effects of Cry1Ac on the Entomopathogenic Nematode, *Heterorhabditis bacteriophora*

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J. Econ. Entomol. 107(1): 115–120 (2014); DOI: http://dx.doi.org/10.1603/EC13310

ABSTRACT The potential impacts on natural enemies of crops that produce insecticidal Cry proteins from *Bacillus thuringiensis* (Bt) are an important part of an environmental risk assessment. Entomopathogenic nematodes are important natural enemies of lepidopteran pests, and the effects of Bt crops on these nontarget organisms should be investigated to avoid disruption of their biological control function. The objective of this study was to investigate the effects of Cry1Ac-expressing transgenic Bt broccoli on the entomopathogenic nematode, *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae), under tri-trophic conditions. Using Cry1Ac-resistant *Plutella xylostella* L. (Lepidoptera: Plutellidae) larvae as hosts, we evaluated the potential impact of Cry1Ac-expressing Bt broccoli on several fitness parameters of *H. bacteriophora.* Virulence, reproductive potential, time of emergence, and preference of *H. bacteriophora* for the host (*P. xylostella*) were not significantly affected when Cry1Ac-resistant *P. xylostella* larvae were reared on leaves of Cry1Ac or non-Bt broccoli. Also the aforementioned parameters of the subsequent generation of *H. bacteriophora* did not differ between nematodes obtained from *P. xylostella* reared on Cry1Ac broccoli compared with those obtained from *P. xylostella* reared on non-Bt broccoli. To the best of our knowledge, the current study provides the first clear evidence that Cry1Ac does not affect important fitness parameters of *H. bacteriophora.*

KEY WORDS Cry1Ac, biosafety, nontarget effect, risk assessment

The development and commercialization of insect-resistant genetically modified crops producing insecticidal proteins (Cry proteins) from the bacterium *Bacillus thuringiensis* (Bt) has revolutionized insect management (Shelton et al. 2002) and greatly contributed to integrated pest management (IPM) programs (Romeis et al. 2008). Currently, Bt corn and cotton are the only two commercially available insect-resistant genetically modified crops. In 2012, these crops were grown on >69 million hectares worldwide (James 2012). Bt eggplant, cauliflower, cabbage, and rice are other Bt crops awaiting commercialization (Shelton et al. 2008, Chen et al. 2011, Shelton 2012). However, the safety of Bt crops to nontarget beneficial organisms warrants investigation as part of an environmental risk assessment (Romeis et al. 2006).

Diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae), is the major insect pest of brassica crops (Talekar and Shelton 1993) and occurs in every part of the world where brassica crops are grown. *P. xylostella* has a long history of becoming resistant to insecticides, beginning with DDT in 1950 (Ankersmit 1953). Since then, no new product has remained effective for more than a few years when applied intensively (Grzywacz et al. 2010). Indiscriminate and intensive use of insecticides has reduced populations of natural enemies important for control of *P. xylostella,* and has contributed to outbreaks of this devastating pest (Talekar and Shelton 1993). As a result, there is an urgent need for development and implementation of cost-effective and environmentally safe alternatives to keep *P. xylostella* populations below economically damaging levels. Introduction of Bt cotton and Bt maize have resulted in considerable reduction in insecticide use, decreases in environmental impact and increases in profit to growers (Brookes and Barfoot 2012), and less harm to important natural enemies (Wolfenbarger et al. 2008, Naranjo 2009). Our earlier studies (Metz et al. 1995a; Cao et al. 1999, 2002, 2005; Tang et al. 1999, 2001; Shelton et al. 2000; Zhao et al. 2000, 2003, 2005) have shown that *cry1* genes from Bt, when introduced in brassica crops, confer resistance to *P. xylostella*, although none have yet been commercialized (Shelton 2012). Further-

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more, modeling studies have shown that the introduction of Bt crucifers, in conjunction with biological control agents, can be a long-term solution to managing the pest density of *P. xylostella* and delaying its evolution to Bt plants (Onstad et al. 2013).

Laboratory and field studies have demonstrated the potential use of entomopathogenic nematodes (EPNs) for the control of *P. xylostella* (Shinde and Singh 2000, Somvanshi and Ganguly 2007, Nyasani et al. 2008). Studies have also reported synergism between EPNs and Bt crops (Gassmann et al. 2006, 2008). The potential impacts of Bt Cry proteins in tri-trophic interactions on many arthropod parasitoids and predators have been studied, but little is known about Bt host plant-mediated interactions between herbivores and EPNs, an important source of natural mortality for many insect pests (Kaya and Gaugler 1993).

EPNs have a unique parasitic relationship with their hosts. Third-stage infective juveniles (IJs) enter the host hemocoel and release symbiotic bacteria; following this, host mortality occurs within 48 h (Boemare 2002, Dowds and Peters 2002). Symbiotic bacteria produce antibiotics that protect the host cadaver from microorganisms and supply nutrients essential for nematode growth and reproduction (Richardson et al. 1988, Ciche et al. 2001). IJs develop into adults and complete two to three generations within the host cadaver, feeding on digested host tissues and bacteria, and eventually produce a new generation of IJs in ≈10 d, depending on temperature and initial infestation density (Adams and Nguyen 2002).

If the host of the EPN feeds on Bt plants, it is possible that the EPN, in turn, might also be exposed to the Cry protein and it is important to determine if this will harm the EPN. The virulence and number of the IJs produced might be constrained by the quality and quantity of the host tissues. Using a Bt-resistant insect and a plant producing the same Bt protein is a method that allows investigators to effectively determine any direct and indirect effect (i.e., mediated by poor host quality) of the Bt protein on a natural enemy (Romeis et al. 2011).

The purpose of this study was to investigate the direct effects of the Cry1Ac protein on *Heterorhabditis bacteriophora.* Previous reports have shown that *H. bacteriophora* is very effective against *P. xylostella* on the basis of LD_{50} (9.16 IJs/larva), LT_{50} (43.26 h), the median lethal exposure time Lex T_{50} (3.24 h), and the propagation potential (271.42 IJs/mg) (Shinde and Singh 2000). In the future, if Bt brassica crops are commercialized for the control of *P. xylostella* (Shelton 2012), *H. bacteriophora* would likely be exposed to *P. xylostella* that have fed on Bt plants. Therefore, the following objectives were addressed in this study: 1) compare the virulence of *H. bacteriophora* against *P. xylostella* larvae fed Cry1Ac-expressing broccoli versus those fed non-Bt broccoli, 2) evaluate the effect of Cry1Ac-fed *P. xylostella* on the reproduction ability of *H. bacteriophora,* 3) test whether *H. bacteriophora* can discriminate between *P. xylostella* larvae that developed on Cry1Ac or non-Bt broccoli, and 4) evaluate if Cry1Ac broccoli plants negatively affect the ability of the progeny of *H. bacteriophora,* developed in the host fed on Cry1Ac broccoli, to use a subsequently provided host.

Materials and Methods

Insects. Two strains of *P. xylostella* were used in this study: 1) a laboratory-reared Cry1Ac-resistant strain that can survive on Cry1Ac broccoli plants and 2) a Cry1Ac-susceptible laboratory strain (Geneva 88) that cannot survive on Cry1Ac broccoli plants (Zhao et al. 2005). To confirm the expression of Cry1Ac in Bt plants, the susceptible strain was used, whereas in the tri-trophic bioassays, the resistant strain was used. Important life table parameters of Cry1Ac-resistant *P. xylostella* have been reported not to be significantly different when its larvae fed on Cry1Ac broccoli or non-Bt broccoli (Liu et al. 2011).

Nematodes. IJs of *H. bacteriophora* were obtained from a commercial supplier (The Green Spot Ltd, Nottingham, NH) and stored at 4°C until use. Before each bioassay, IJs were harvested by placing the sponge formulation in distilled water in a petri dish at room temperature.

Plants. Two types of broccoli (*Brassica oleracea* L.) were used in this study. The first produced high levels of Cry1Ac (Metz et al. 1995b) and expression was verified by screening 4–5-wk-old plants with susceptible *P. xylostella* neonates (Tang et al. 2001). The plants on which neonates showed 0% survival were used in the tests. A near-isoline non-Bt variety, *Brassica oleracea* variety 'Packman', was used as the control.

Virulence Bioassays. Median lethal dose (LD_{50}) and median lethal time (LT_{50}) were used to compare the virulence of *H. bacteriophora* against Cry1Ac on non-Bt broccoli-fed mid-fourth-instar *P. xylostella.* Tests were conducted in 5-cm petri dishes lined with filter paper. To determine the LD_{50}, the following concentrations were used: 1, 4, 8, 16, 32, and 40 IJs per *P. xylostella* larva. IJs were suspended in distilled water and the desired concentrations were obtained using serial dilutions. Each concentration was added to five petri dishes (replications) in 0.5 ml of distilled water. Five additional dishes of 0.5 ml distilled water without nematodes served as the untreated control. After 30 min, 10 mid-fourth instars of *P. xylostella* that had developed on Cry1Ac or non-Bt broccoli were placed in each petri dish. Non-Bt broccoli leaves were washed with distilled water and cut into 5-cm leaf discs and allowed to air-dry for 45 min before being used as the nutrient medium in the petri dishes. After inoculation, petri dishes were sealed and placed in a growth chamber maintained at 25°C, 50% RH, and a photoperiod of 16:8 (L:D) h. Mortality of *P. xylostella* was recorded 48 h after introduction of the larvae into the petri dishes. LT_{50} values were determined using the Glazer (1992) method, in which a single nematode concentration of 30 IJs/larva is applied. Ten mid-fourth instars of *P. xylostella* that developed on Cry1Ac or non-Bt broccoli were kept in contact with IJs for 0.5, 1, 2, 4, 8, 12, and 16 h, as described earlier. After each

exposure period, larvae were transferred to another petri dish and kept in the growth chamber under the conditions described earlier. Mortality was recorded after 48 h. Each treatment had five replications (50 larvae per treatment). Insect mortality data were corrected by Abbott's formula (Abbott 1925) and LD_{50} and LT_{50} values were calculated by probit analysis.

Choice Bioassays. For choice bioassays, IJs were presented with a choice of mid-fourth instars of *P. xylostella* that had developed on Cry1Ac or non-Bt broccoli. Tests were conducted in a cylindrical plastic tube (5 cm in height by 2 cm in diameter). Initially, each tube was filled with 2 cm of moist, autoclaved sand. Then ≈1,000 IJs of *H. bacteriophora* in 1 ml of distilled water were pipetted over the surface of the sand. After 30 min, additional moist, autoclaved sand was added to the tube so that the final level was 4.5 cm. By placing a plastic sheet (2 cm in length by 0.5 cm in width) vertically, the open end of the tube was divided into two equal semicircular chambers. Five mid-fourth-instar *P. xylostella* larvae that had developed on Cry1Ac or non-Bt broccoli were placed on either side of the vertical divider. Subsequently, the tube was sealed with Parafilm and transferred to a growth chamber maintained as described earlier. After 24 h of exposure to IJs, *P. xylostella* larvae were removed from the tube and placed in petri dishes provided with a non-Bt broccoli leaf as a source of nutrition until dissection. After 2 d, each *P. xylostella* larva was placed in distilled water and dissected and the number of nematodes was counted using a dissecting microscope at 40× magnification. The experiment was replicated 15 times using new tubes each time.

Reproductive Potential. Ten mid-fourth-instar *P. xylostella* that had developed on Bt or non-Bt broccoli were infested with *H. bacteriophora* in the same way as described in the virulence bioassay, using a single concentration of 30 IJs per larva. After 48 h, five infected cadavers, recognized by their red color, were removed from the petri dish, rinsed, weighed, transferred to a White trap (White 1927), and incubated in the growth chamber under the conditions described earlier. After 3 d, observations were made every 6 h and the time was recorded when IJs were observed in the White trap. Emerging IJs were collected from the White traps daily over 10 d and stored in 50-ml plastic tubes at 4°C. The content of each tube (nematode suspension from an individual White trap) was mixed thoroughly with a pipette, 10 samples of 10 μl from each suspension were examined under a dissecting microscope at 40× magnification, and the total numbers of IJs per White trap were calculated. To control for variations in larval weights of *P. xylostella* and its potential to influence production of IJs, the values for IJs produced were calculated per milligram of *P. xylostella.* The experiment was replicated 15 times.

Effect of Bt Proteins on Second-Generation IJs. To evaluate whether Cry1Ac plants would negatively affect the ability of the progeny of *H. bacteriophora* to use a subsequently provided host, the IJs obtained from mid-fourth-instar *P. xylostella* fed on Cry1Ac broccoli or non-Bt broccoli plants were tested against mid-fourth-instar *P. xylostella* that had developed on artificial diet (Shelton et al. 1991). To mitigate direct or indirect effects on fitness parameters of IJs produced due to initial inoculation density, IJs used for second-generation bioassays were obtained by infecting mid-fourth-instar *P. xylostella* that had developed on Cry1Ac or non-Bt broccoli with a single concentration of 30 IJs per larva. The experimental design and conditions were the same as described earlier for the LD_{50}, LT_{50}, and reproductive potential experiments.

Statistical Analyses. Data on LD_{50} and LT_{50} were analyzed by a generalized linear model with the Probit link function. Data on choice bioassays were analyzed with paired *t*-tests. Data on larval weight, time of emergence, and reproductive potential of *H. bacteriophora* were analyzed using the Student *t*-test. All statistical calculations were performed with the R version 2.15.1 package (R Development Core Team 2012). For all tests, $\alpha = 0.05$.

Table 1. Virulence of *Heterorhabditis bacteriophora* against mid-fourth-instar Cry1Ac-resistant *Plutella xylostella* fed on Cry1Ac (Bt) or non-Bt broccoli (N)

Plant type	95% Fiducial limits			95% Fiducial limits		
	LD_{50}	Lower	Upper	LT_{50}	Lower	Upper
Bt	9.3a	7.5	11.0	3.4a	2.6	4.2
N	8.8a	7.2	10.4	3.6a	2.8	4.4

LD_{50} values expressed in number of nematodes per larvae. LT_{50}: the time in hours at which 50% of larvae used in the treatment were killed.
LD_{50} and LT_{50} values followed by the same letter within the same column are not significantly different ($P < 0.05$).

Results

Virulence Bioassay. The LC_{50} and LT_{50} values for *H. bacteriophora* against *P. xylostella* larvae that had developed on Cry1Ac broccoli were 9.3 IJs per larva and 3.4 h, respectively, and there were no significant differences compared with the non-Bt broccoli values of 8.8 IJs per larva and 3.6 h, respectively (Table 1). Similarly, there were no significant differences found for the second generation (Table 2).

Choice Bioassays. *H. bacteriophora* exhibited no significant preference for *P. xylostella* larvae that had developed on Cry1Ac or non-Bt broccoli (paired *t*-test, $t = -0.06$, df = 14, $P = 0.95$). An average of 59 IJs

Table 2. Virulence of second-generation *Heterorhabditis bacteriophora* emerged from Cry1Ac-resistant *Plutella xylostella* fed on Cry1Ac (Bt) or non-Bt broccoli (N) against mid-fourth-instar Cry1Ac-resistant *P. xylostella* reared on artificial diet

Plant type	95% Fiducial limits			95% Fiducial limits		
	LD_{50}	Lower	Upper	LT_{50}	Lower	Upper
Bt	7.7a	6.1	9.3	3.2a	2.5	3.9
N	8.3a	6.7	9.9	3.0a	2.4	3.6

LD_{50} values expressed in number of nematodes per larvae. LT_{50}: the time in hours at which 50% of larvae used in the treatment were killed.
LD_{50} and LT_{50} values followed by the same letter within the same column are not significantly different ($P < 0.05$).

Table 3. Weight, time of emergence, and reproductive potential of *Heterorhabditis bacteriophora* from mid-fourth-instar Cry1Ac-resistant *Plutella xylostella* fed on Cry1Ac (Bt) or non-Bt broccoli (N)

Observations	Plant type	(Mean ± SE)
Larval wt (mg)	Bt	24.4 ± 3.3a
	N	25.6 ± 3.1a
Mean time to emergence (h)	Bt	154.4 ± 10.0a
	N	153.2 ± 10.8a
Mean nematodes per White trap	Bt	2918.0 ± 2014.0a
	N	3430.0 ± 1924.0a
Mean nematodes per mg *P. xylostella*	Bt	119.0 ± 78.0a
	N	136.0 ± 78.0a

Within each observation category, means followed by the same letter are not significantly different (Student *t*-test, $P < 0.05$).

were found in each infected larvae from both treatments.

Reproductive Potential. The number of *H. bacteriophora* produced per milligram of *P. xylostella* larvae that had developed on Cry1Ac or non-Bt broccoli was compared and the difference between the numbers of IJs was not significant (Student *t*-test, $t = -0.71$, df = 28, $P = 0.482$) (Table 3). The emergence time of IJs from the host cadaver was also not significantly affected by Cry1Ac plants (Student *t*-test, $t = 0.315$, df = 28, $P = 0.76$) (Table 3). Similar results were found for the second-generation IJs (Table 4).

Discussion

Bt crops, when used as an element of IPM programs, provide opportunities to reduce the use of synthetic insecticides and increase environmental and economic benefits (Shelton et al. 2002, Qaim et al. 2008, Brookes and Barfoot 2009). However, target and nontarget organisms will be exposed to the toxins while feeding on Bt crops, and natural enemies may be indirectly exposed to Bt toxins from prey that have ingested Bt toxins. Therefore, it is important to assess the compatibility of Bt crops with biological control. The biosafety of Bt plants has been studied extensively (Romeis et al. 2006, Wolfenbarger et al. 2008, Naranjo 2009, Liu et al. 2011) and, with the exception of a few studies that mistook the effect of host quality for that of the Cry protein (see discussion by Shelton et al. 2009a,b), research has not shown any negative effect of Cry proteins on natural enemies. Analyzing the potential effects of Bt crops on natural enemies using resistant hosts is considered to be an effective and efficient method of overcoming any prey or host quality effects (Romeis et al. 2011). Studies to date have focused on the effects of Bt proteins on parasitoids and predators, but few studies have investigated the effects of Cry proteins on soil-dwelling arthropod natural enemies and EPNs. The relationship between EPNs and their host is intimate and complex, as they complete two to three generations within their host and derive nutrition from the host tissues and symbiotic bacteria. Therefore, the nutritional content of an insect host can affect the host susceptibility to EPNs and its fitness (Shapiro-Ilan et al. 2008). Toxicity of Cry1Ab and Cry3Bb to free soil-dwelling nematodes, *Caenorhabditis elegans*, has been reported in the laboratory; however, the observed effects could not be explained by the direct toxicity of the Cry proteins (Höss et al. 2008, 2011).

Table 4. Weight, time of emergence, and reproductive potential of second-generation *Heterorhabditis bacteriophora* obtained from Cry1Ac-resistant *Plutella xylostella* fed on Cry1Ac (Bt) or non-Bt broccoli (N), from mid-fourth-instar Cry1Ac-resistant *Plutella xylostella* reared on artificial diet

Observations	Plant type	(mean ± SE)
Larval wt (mg)	Bt	36.0 ± 2.4a
	N	35.6 ± 2.3a
Mean time to emergence (h)	Bt	154.0 ± 10.0a
	N	152.8 ± 11.0a
Mean nematodes per White trap	Bt	3,156.0 ± 1019.0a
	N	3,048.0 ± 1508.0a
Mean nematodes per mg *P. xylostella*	Bt	88.0 ± 29.0a
	N	85.0 ± 39.0a

Within each observation category, means followed by the same letter are not significantly different (Student *t*-test, $P < 0.05$).

Liu et al. (2011) confirmed the presence of bioactive Cry1Ac in resistant *P. xylostella* larvae that fed on Bt broccoli and the presence of Cry1Ac in the endoparasitoid *Diadegma insulare* (Hymenoptera: Ichneumonidae) that fed on *P. xylostella* larvae. Likewise, in this study, it appears that the EPN was also exposed to Cry1Ac since it fed internally on *P. xylostella* that had consumed Cry1Ac. On entering the host and reaching its hemocoel, IJs of *H. bacteriophora* release their symbiotic bacteria, ultimately killing the host within 48 h. However, host mortality does not ensure progeny reproduction. To become self-fertilized hermaphrodites with a female phenotype (Poinar 1975), IJs must feed within the host hemocoel on the bacteria and host tissues (Kaya and Gaugler 1993) and eventually they give rise to a second generation consisting of amphimictic males, females, and IJs (Strauch et al. 1994). If nutritive conditions are favorable, IJs will develop into hermaphrodite females; otherwise they emerge from the host. Therefore, it can be concluded that *H. bacteriophora* feeding on mid-fourth-instar *P. xylostella* that had developed on Bt broccoli are also exposed to bioactive Cry1Ac. However, despite being exposed, there were no significant differences in the LD_{50} or LT_{50} values, time of emergence from the host cadaver, or reproductive potential of *H. bacteriophora* when developing in *P. xylostella* that had fed on Cry1Ac or non-Bt broccoli. Furthermore, in the current study, our results from choice bioassays also indicate that *H. bacteriophora* could not discriminate between Cry1Ac or non-Bt broccoli-fed hosts using the methods we employed. While there may be some concern about using what might be considered a high number of IJs and saturating the soil and potentially masking any behavioral preference, further studies would have to be conducted to eliminate this speculation.

The components in the insect host diet can also affect the efficacy of IJs of subsequent generations

(Shapiro-Ilan et al. 2008). In our study, there were no significant differences in the aforementioned parameters for the second-generation *H. bacteriophora* obtained from Cry1Ac or non-Bt broccoli-fed *P. xylostella* against larvae that developed on artificial diet. Combinations of EPNs and Bt crops have been shown to be synergistic in insect suppression in the field (Gassmann et al. 2006). Our study provides strong evidence there is no effect from Cry1Ac on *H. bacteriophora,* and this has important implications for its role as a natural enemy in IPM.

In conclusion, based on the results from the current study and our previous studies (Cao et al. 1999, 2002; Zhao et al. 2003; Chen et al. 2008; Liu et al. 2011), we have shown that Cry1Ac brassica crops can effectively control *P. xylostella* without any demonstrated negative effects on important natural enemies due to the high specificity of the toxins. Furthermore, the current study provides valuable information to regulatory authorities about the safety of Cry1Ac to EPNs. We expect similar results will be obtained using other lepidopteran-active Bt proteins.

Acknowledgments

An assistantship for the senior author's (S. G.) MS program was provided by the Cornell Sathguru Foundation. Additional laboratory support was provided by the Biotechnology Risk Assessment Program Competitive Grant No. 2010-33522-21772 from the USDA, National Institute of Food and Agriculture.

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Received 4 July 2013; accepted 4 November 2013.