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Use of *Bt*-resistant caterpillars to assess the effect of Cry proteins on beneficial natural enemies

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Abstract: A concern related to the use of insect-resistant *Bt*-transgenic plants is their potential to harm non-target organisms, especially natural enemies of important crop pests. A few studies purporting to show negative effects of *Bt* plants on non-target organisms had tremendous negative effects on the perception of *Bt* plants and on regulatory decisions. Focusing on the tri-trophic non-target studies it became evident that the design of these studies often did not account for the quality of the hosts being fed to the natural enemies. This occurred when *Bt*-susceptible hosts that had ingested *Bt* (Cry) proteins and became compromised were fed to natural enemies, causing indirect prey/host-quality mediated effects. The result was that the natural enemy often developed more slowly, had higher mortality, or decreased fecundity due to the poor host quality, not the Cry protein. Here we review studies that overcame this methodological problem in testing Cry proteins against natural enemies by feeding them strains of pest insects that had evolved resistance to Cry proteins expressed in the *Bt* plants. The studies utilized natural enemies from multiple orders and families of insect predators and parasitoids, and an entomopathogenic nematode. The study results provide unambiguous evidence on the lack of effects of these Cry proteins on important natural enemies and provide guidance for future non-target studies. These data confirm the large and sound body of literature demonstrating that the Cry proteins currently used in *Bt* crops for control of Lepidoptera are not harmful to natural enemies that are important for biological control of these and other pest species.

Key words: *Bacillus thuringiensis*, genetically modified crops, non-target risk assessment, study design

Background

The biological control function provided by natural enemies should not be harmed by the application of any new pest management practice. Plants producing insecticidal (Cry) proteins from the bacterium *Bacillus thuringiensis* (*Bt*), have become a major tactic for controlling pest Lepidoptera on cotton and maize, and pre-release risk assessment studies are conducted to ensure they do not harm important natural enemies (Romeis *et al.*, 2008).

Such risk assessment studies need to be carefully designed (Romeis *et al.*, 2011, 2013; De Schrijver *et al.*, 2016) to produce results that are reliable and robust. In particular, the test species needs to be exposed to biologically active Cry protein to avoid false negative results. To avoid false positive results, care has to be taken that observed effects can be related to the insecticidal protein and are not an artifact of a poor study design. This is particularly challenging in the case of tri-trophic studies deploying *Bt* plants, herbivores, and non-target natural enemies where potential effects of host/prey-quality have to be taken into account. Such indirect effects on predators or parasitoids usually result when they feed on susceptible hosts that have ingested Cry proteins and become less suitable as food for the natural enemy. The outcome is that as the host suffers, so does the natural enemy, leading some to suggest there is a direct effect of the Cry protein on the natural enemy. The need to separate indirect, host/prey-quality related effects from direct toxic effects of the Cry proteins has repeatedly been demonstrated (Romeis *et al.*, 2006; Naranjo, 2009; Shelton *et al.*, 2012).

One way of overcoming the effects of host/prey-quality is to feed non-susceptible herbivores the Cry protein and then allow the predator or parasitoid to feed on this uncompromised organism and study the life history traits (development time, survivorship, fecundity, etc.) of the natural enemy. One suggested method for removing host effects consists of using once-susceptible hosts that have developed resistance to the Cry protein and natural enemies that typically feed on the host in the field.

Non-target studies deploying Cry protein-resistant caterpillars as host or prey

A number of tri-trophic studies were conducted using lepidopteran species that had evolved resistance to Cry proteins expressed in plants as host or prey for natural enemies (Table 1). Studies were conducted to assess the non-target effects of *Bt* proteins expressed in cotton (Cry1Ac/Cry2Ab), corn (Cry1F), broccoli (Cry1Ac or Cry1C), and oilseed rape (Cry1Ac). The studied Cry proteins are also common in commercialized *Bt* crops (e.g., Bollgard® II cotton expressing Cry1Ac+Cry2Ab; Herculex® I corn expressing Cry1F). The resistant caterpillars were allowed to feed on the plants before they were subjected to the natural enemies. In all studies the respective non-*Bt* isolines (or near-isolines) were used as controls.

For parasitoids, in no case were there any differences in the percent parasitism, emergence rate of the parasitoids and fecundity of parasitoids that developed on hosts that had consumed any of the Cry proteins, compared to hosts that developed on the corresponding non-*Bt* plants. Similarly, the studies on predators did not reveal any differences in the development, survival or fecundity of predators that fed on a prey that consumed *Bt* foliage, compared to the prey that had fed on non-*Bt* plants. Likewise the entomopathogenic nematode was not affected in important fitness parameters such as virulence, reproductive potential, time of emergence, and host preference.

To avoid false negative results, the majority of the studies quantified the amount of Cry protein in the host or prey caterpillars. In addition, many studies confirmed with sensitive insect bioassays that the Cry proteins detected in the caterpillars were still biologically active. Thus, the results that the tested Cry proteins are not adversely affecting the tested natural enemies are very robust.

Discussion

Using non-susceptible, Cry protein resistant lepidopteran hosts or prey avoids the problems encountered by others (e.g., Ponsard *et al.*, 2002; Lövei *et al.*, 2009) who have claimed that lepidopteran-active *Bt* proteins harm important natural enemies (Shelton *et al.*, 2009; 2012). The studies listed in Table 1 provide assurance that the Cry proteins tested do not present a hazard to a diverse set of predators in five different families belonging to three insect orders (Neuroptera, Hemiptera, Coleoptera), to three species of parasitoids belonging to two families of Hymenoptera, and to the entomopathogenic nematode *H. bacteriophora*.

Table 1. Studies that have deployed Bt-resistant strains of lepidopteran herbivores to assess the Cry protein effects on parasitoids and predators.

Species	**Order: Family**	**Bt-resistant host/prey species**	**Family**	**Cry proteins tested**	**Test material used (variety, event)**	**Cry protein presence/ bioactivity confirmed**[a]	**References**
Parasitoids							
Cotesia plutellae	Hym.: Braconidae	*Spodoptera frugiperda*	Noctuidae	Cry1Ac	Bt oilseed rape (Oscar, line O52)	N / N	Schuler *et al.*, 2003, 2004
Diadegma insulare	Hym.: Ichneumonidae	*Plutella xylostella*	Plutellidae	Cry1C	Bt broccoli (Cornell H12, H14)	Y / Y	Chen *et al.*, 2008
				Cry1Ac	Bt broccoli (Cornell Q23)	Y / Y	Liu *et al.*, 2011
Cotesia marginiventris	Hym.: Braconidae	*Spodoptera frugiperda*	Noctuidae	Cry1F	Bt maize (Mycogen 2A496, TC1507)	Y / N*	Tian *et al.*, 2014a
Predators							
Chrysoperla carnea	Neu.: Chrysopidae	*Helicoverpa armigera*	Noctuidae	Cry1Ac	Bt cotton (MECH 12, BG-I)	Y / N	Lawo *et al.*, 2010
Chrysoperla rufilabris	Neu.: Chrysopidae	*Trichoplusia ni*	Noctuidae	Cry1Ac, Cry2Ab	Bt cotton (Bollgard II, event 15985)	Y / Y	Tian *et al.*, 2013
				Cry1Ac	Bt broccoli (Cornell Q23)	Y / Y	Tian *et al.*, 2013
		Spodoptera frugiperda	Noctuidae	Cry1F	Bt maize (Mycogen 2A517, TC1507)	Y / Y	Tian *et al.*, 2013
Coleomegilla maculata	Col.: Coccinellidae	*Trichoplusia ni*	Noctuidae	Cry1Ac, Cry2Ab	Bt cotton (Bollgard II, event 15985)	Y / Y	Li *et al.*, 2011
		Spodoptera frugiperda	Noctuidae	Cry1F	Bt maize (Mycogen 2A517, TC1507)	Y / Y	Tian *et al.*, 2012
		Plutella xylostella	Plutellidae	Cry1Ac	Bt broccoli (Cornell Q23)	Y / N*	Liu *et al.*, 2015
Geocoris punctipes	Hem.: Geocoridae	*Trichoplusia ni*	Noctuidae	Cry1Ac, Cry2Ab	Bt cotton (Bollgard II, event 15895)	Y / N*	Tian *et al.*, 2014b
		Spodoptera frugiperda	Noctuidae	Cry1F	Bt maize (Mycogen 2A517, TC1507)	Y / N*	Tian *et al.*, 2014b
Orius insidiosus	Hem.: Anthocoridae	*Trichoplusia ni*	Noctuidae	Cry1Ac, Cry2Ab	Bt cotton (Bollgard II, event 15895)	Y / N*	Tian *et al.*, 2014b
		Spodoptera frugiperda	Noctuidae	Cry1F	Bt maize (Mycogen 2A517, TC1507)	Y / N*	Tian *et al.*, 2014b
Zelus renardii	Hem.: Reduviidae	*Trichoplusia ni*	Noctuidae	Cry1Ac, Cry2Ab	Bt cotton (Bollgard II, event 15895)	Y / N*	Su *et al.*, 2015
		Spodoptera frugiperda	Noctuidae	Cry1F	Bt maize (Mycogen 2A517, TC1507)	Y / N*	Su *et al.*, 2015
Entomopathogenic nematode							
Heterorhabditis bacteriophora	Strongylida: Heterorhabditidae	*Plutella xylostella*	Plutellidae	Cry1Ac	Bt broccoli (Cornell Q23)	N / N*	Gautam *et al.*, 2014

[a] Cry protein presence was confirmed by ELISA, Cry protein bioactivity in sensitive-insect bioassays; Y – yes, N – no; n.a. – not analyzed; * bioactivity was confirmed in previous studies (Cry1Ac + Cry2Ab: Li *et al.*, 2011; Cry1F: Liu *et al.*, 2011, Tian *et al.*, 2012);

The physiology and feeding behaviors of the different predators represent the main feeding behaviors found in predatory arthropods. Likewise, the three hymenopteran parasitoids used represent a common life history in which the parasitoid's egg is laid inside the host and the parasitoid larva develops within the host by feeding on its tissues. A similar parasitic behavior is displayed by the entomopathogenic nematode except that juveniles infect the host. The fact that none of the natural enemies was harmed by any of the Cry proteins indicates that they, and other similar species, are not at risk.

The results from the studies listed in Table 1 are in accordance with the large body of literature that shows that the spectrum of activity of the lepidopteran-active *Bt* Cry proteins deployed in today's *Bt* crops is restricted to the target insects order (Romeis *et al.*, 2006; Naranjo, 2009). The safety of those Cry proteins to natural enemies has an added benefit for managing lepidopteran pests of *Bt* crops. Modeling (Onstad *et al.*, 2013) and empirical studies (Liu *et al.*, 2014) have shown that the conservation of natural enemies by the use of *Bt* plants can delay the evolution of resistance to *Bt* plants by the pest species.

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