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Effect of Bt broccoli and resistant genotype of *Plutella xylostella* (Lepidoptera: Plutellidae) on life history and prey acceptance of the predator *Coleomegilla maculata* (Coleoptera: Coccinellidae)

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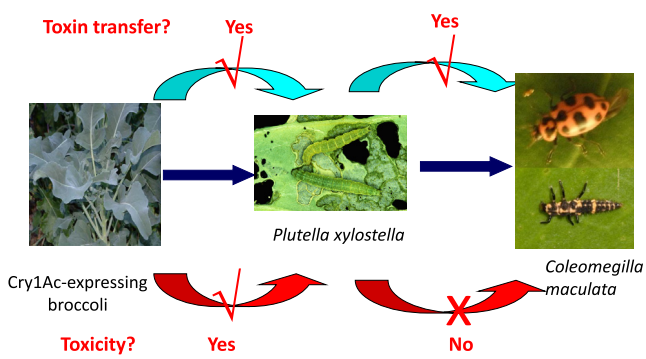
HIGHLIGHTS

- Plants expressing Cry1Ac, a Bt, control the caterpillar pest, *Plutella xylostella*.
- *Coleomegilla maculata* are predators of *Plutella xylostella* larvae.
- *C. maculata* could not discriminate between Cry1Ac and non-Bt plants.
- *C. maculata* could not discriminate between Cry1Ac-susceptibility of *P. xylostella*.
- Development and survival of *C. maculata* were not affected by Cry1Ac-fed prey.

GRAPHICAL ABSTRACT



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ABSTRACT

The ecological implications on biological control of insecticidal transgenic plants, which produce crystal (Cry) proteins derived from the soil bacterium *Bacillus thuringiensis* (Bt), remains a contentious issue and affects risk assessment decisions. In this study, we used a unique system of resistant insects, Bt plants and a predator 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 life history of the predator, *Coleomegilla maculata* DeGeer (Coleoptera: Coccinellidae) over two generations. Additional behavioral studies were conducted on prey choice. *C. maculata* could not discriminate between Bt-resistant and susceptible genotypes of *P. xylostella*, nor between Bt and normal broccoli plants with resistant genotypes of *P. xylostella* feeding on them. The larval and pupal period, adult weight and fecundity of each female were not significantly different when *C. maculata* larvae fed on different genotypes (Bt-resistant or susceptible) of insect prey larvae reared on Bt or non-Bt broccoli plants. The life-history parameters of the subsequent generation

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of *C. maculata* fed on Bt broccoli-reared resistant *P. xylostella* were also not significantly different from those on non-Bt broccoli. These results indicated that Cry1Ac did not harm the life history or prey acceptance of an important predator after two generations of exposure. Plants expressing Cry1Ac are unlikely to affect this important predator in the field.

1. Introduction

Genetically engineered (GE) plants expressing insecticidal proteins derived from the soil bacterium *Bacillus thuringiensis* (Bt) have heralded a new era in crop protection (Shelton et al., 2002; Meissle et al., 2005; Klümper and Qaim, 2014). Since their introduction in 1996, Bt crops have been widely adopted and in 2014 were planted on 78.8 million ha in 28 countries (James, 2014). Several major pests have been successfully controlled, and insecticides use for Lepidoptera and Coleoptera has been substantially reduced throughout most adopting countries (Shelton et al., 2002; Carrière et al., 2003; Wu et al., 2008; Hutchison et al., 2010; Brookes and Barfoot, 2012; Kathage and Qaim, 2012; Klümper and Qaim, 2014; Keweshan et al., 2015; Wangila et al., 2015). However, potential detrimental effects on non-target organisms, in particular natural enemies (i.e., predators and parasitoids) in the agricultural ecosystem, became a major topic in risk assessment to Bt crops. Generally, natural enemies could consume Bt protein in two ways: direct exposure to Bt proteins when they consume plants material (Cividanes et al., 2011) and indirectly when their prey fed on Bt plants. For endoporasitoids that are more intimate with their host because they complete their larval development in a single insect host, most studies have concluded that any adverse effects seen with a Bt-susceptible host were prey-quality mediated, meaning the prey was being harmed by the Cry protein and this in turn had a detrimental effect on the parasitoid (Baur and Boethel, 2003; Liu et al., 2005a,b,c; Sanders et al., 2007; Ding et al., 2009). When Bt-resistant pest hosts were used, no direct toxic effects were found (Johnson, 1997; Atwood et al., 1998; Schuler et al., 1999, 2004; Chen et al., 2008; Liu et al., 2011; Tian et al., 2014a).

In contrast to parasitoids, predators are usually generalists that feed on several different prey species and therefore have increased chances of coming into contact with prey that have consumed Bt proteins. However, when non-susceptible or resistant prey have been used as hosts, studies have shown that Bt proteins did not cause any detrimental effects to predators even though the ingested Bt protein by the predator was bioactive to the targeted pest (Romeis et al., 2006; Naranjo, 2009; García et al., 2010; Lawo et al., 2010; Li et al., 2011; Wang et al., 2012; Tian et al., 2012, 2013, 2014b; Zhao et al., 2013; Kumar et al., 2014; Su et al., 2015). However, a few studies have reported Bt crops harm natural enemies (Hilbeck et al., 1999; Meier and Hilbeck, 2001; Schmidt et al., 2009; Lövei et al., 2009) but these studies have been criticized for their methodology, including using susceptible prey (see Shelton et al., 2009a,b; Romeis et al., 2013).

The diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae), is considered the most important insect pest of cruciferous crops globally because of its high fecundity and capacity to disperse long distances (Talekar and Shelton, 1993; Zalucki et al., 2012). In order to control *P. xylostella* and other lepidopteran pests, several Bt crucifers express Cry1A protein have been developed (Shelton et al., 2008). Our previous studies have demonstrated that Bt crucifers 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). Studies have also been undertaken to assess the potential risk of Bt crucifers to natural enemies of *P. xylostella* and revealed no adverse effects on the tested species when resistant hosts were used (Chen et al., 2008; Liu et al., 2011; Tian et al., 2013, 2014b).

Coleomegilla maculata DeGeer (Coleoptera: Coccinellidae) is a very common and abundant predator in many cropping systems throughout the US. Both larvae and adults are important predators of many pest species. Several studies have evaluated the safety of Bt plants on *C. maculata* (e.g. Lundgren et al., 2005; Li et al., 2011; Tian et al., 2012), utilizing susceptible (SS) or resistant (RR) prey. While such studies are useful, they do not reflect the full range of insect genotypes found in the field, i.e., heterozygotes (RS). It is possible that predators that consume the RS-genotype larvae from the Bt crops could have their development affected indirectly. Furthermore, predators will not only encounter different insect genotypes, but also different plant types. The use of Bt crops in many countries requires that non-Bt crops be planted in the same area (i.e. for refuges) as part of a resistance management strategy (Bates et al., 2005). Thus, it is important to consider the genotype of the prey as well as the plant genotype on which it is found when studying their effects on the development and prey acceptance of predators such as *C. maculata.* Our resistant *P. xylostella* and Bt broccoli system allows us to investigate this question on a practical basis.

The present study explores whether prey genotype and plant type can affect the life history of *C. maculata* and its progeny over two generations. Specifically, the following objectives were addressed in this study: (1) determine if *C. maculata* can discriminate between resistant (RR) and susceptible (SS and RS) genotypes of *P. xylostella*; (2) determine if *C. maculata* can discriminate plant types (Bt plants or non-Bt plants) hosting resistant genotypes of *P. xylostella*, and; (3) assess the effects of Cry1Ac broccoli plants on selected life history parameters of *C. maculata* when the plants are infested by RR, RS, and SS genotypes of *P. xylostella* for two generations; (4) estimate the level of Cry1Ac protein after *C. maculata* have fed on Bt broccoli-fed RR *P. xylostella* larvae.

2. Material and methods

2.1. 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 has been maintained on a wheat germ-casein artificial diet for over 300 generations (Shelton et al., 1991); (3) a heterozygous strain (RS), which was developed by crossing RR with G88. SS and RS strain larvae cannot survive on Cry1Ac Bt broccoli plants (Liu et al., 2011).

Both larvae and adult *C. maculata* originated from Pioneer Hi-Bred International, Inc. (Johnston, IA) and were reared on decapsulated eggs of brine shrimp *Artemia franciscana* (Kellogg) (Decapoda, Penaeus) (Brine Shrimp Direct, Ogden UT) (Li et al., 2011) and a 1.5% agar solution provided separately for >20 generations. These insects were maintained in a climatic chamber at 27 ± 1 °C, 50 ± 10% RH and 16:8 L:D photoperiod. In this article we use the terms G1 and G2 to refer to the generations of the predator since exposure to Bt broccoli plants.

2.2. Bt broccoli plants

We used *Brassica oleracea* L., var. italica' Green Comet as the cultivar for our Bt broccoli plants. The transgenic broccoli produces high levels of Cry1Ac (Metz et al., 1995). To ensure the biological activity of the Bt broccoli, the plants were screened with the susceptible *P. xylostella* neonates when plants were 4 to 5 wk old. In all the studies reported in this paper, broccoli plants with 8 true leaves were used. Analysis by ELISA indicated that the Cry1Ac protein level was 12.33 ± 1.62 μg/g fresh leaf tissue (Liu et al., 2011). Packman, a near-isoline, was used as the non-Bt broccoli.

2.3. Can C. maculata discriminate between different genotypes of P. xylostella?

Sixty *P. xylostella* 2nd instars of a single genotype (Cry1Ac-RR, RS, or SS) were placed on a non-Bt broccoli leaf, with its petiole inserted in a 100 ml flask filled with water. Larvae from the three genotypes *P. xylostella* that fed on three non-Bt broccoli leaves were placed into the cage (42 cm × 42 cm × 42 cm) for 1 d, then two newly emerged *C. maculata* adults or two 3rd instars were released in the center of each cage. The cages with 60 Cry1Ac-RR, RS, or SS *P. xylostella* 2nd instars on each leaf, but without *C. maculata*, served as controls in order to determine the numbers of lost or dead larvae. After 48 h, the numbers of remaining larvae on each leaf were counted in the cages. Predation rates (% predation = (initial number of *P. xylostella* – number of remaining larvae – number of lost or dead larvae)/initial number of *P. xylostella*) were recorded. The larval treatment was repeated six times and the adult treatment four times.

2.4. Can C. maculata discriminate between plant types hosting resistant genotypes of P. xylostella?

Sixty 2nd instar *P. xylostella* Cry1Ac-RR were placed on a non-Bt broccoli leaf and another 60 2nd instar *P. xylostella* Cry1Ac-RR were placed on a Bt leaf. Each leaf was inserted with its petiole in a 100 ml flask filled with water for 1 d before being presented to the natural enemy. The Bt broccoli and non-Bt broccoli leaf with Cry1Ac-RR larvae were placed into the cages (42 cm × 42 cm × 42 cm), then two new emerged *C. maculata* adults or two 3rd instars were released in the center of each cage. After 48 h, the numbers of remaining larvae were counted in the cages. The cages with 60 Cry1Ac-RR *P. xylostella* 2nd instars on a Bt broccoli leaf and 60 Cry1Ac-RR *P. xylostella* 2nd instars on non-Bt broccoli leaf, but without *C. maculata*, served as controls. The predation rate was calculated in a similar way as in the previous experiment. Predation rates (% predation = (initial number of *P. xylostella* – number of remaining larvae – number of lost or dead larvae)/initial number of *P. xylostella*). The experiments with both *C. maculata* larvae and adults were replicated 6 times.

2.5. Effect of plant type and prey genotype on the life history of the G1 C. maculata

Because the RS and SS strains could not survive on Cry1Ac broccoli plants (Zhao et al., 2005), there 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 treatment with the RR on Bt broccoli, RR neonates were fed on Bt broccoli until the 2nd instar. The 2nd instar *P. xylostella* larvae were then transferred to a Cry1Ac leaf in a 40 ml cup containing a 1st instar *C. maculata* larva. We added new 2nd instar *P. xylostella* to the *C. maculata* each day until the *C. maculata* larva began to pupate. For the other three treatments, the neonates of RR, RS and SS fed on non-Bt broccoli plants, but in all other ways the experiment was the same as that described for RR on Bt broccoli. Each treatment used 30–40 *C. maculata* larvae with each larva as a replicate. The experiments were conducted in a climatic chamber at 27 ± 1 °C, 50 ± 10% RH and 16:8 L:D photoperiod. After *C. maculat*a adults emerged, one female and one male were put together in a Petri dish of 10 cm diameter, which has been reared on decapsulated eggs of brine shrimp *A. franciscana* (Brine Shrimp Direct, Ogden UT) and not exposed to Cry1Ac. We recorded the following data: *C. maculata* larval weight at 7 d, the number of larval and pupal days, adult fresh weight (<24 h after emerge), the fecundity of each female over one month, and the hatching rate of the eggs.

2.6. Life history of G2 C. maculata 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 development of *C. maculata*'s progeny, we studied the life history during the 2nd generation of *C. maculata* whose parents had fed on the different genotypes of *P. xylostella* larvae reared on Bt or non-Bt broccoli. The methods were similar to those for the experiment described above. Each genotype of *P. xylostella* neonates were fed on Cry1Ac (RR on Bt treatment) or non-Bt (RR, RS and SS on non-Bt treatments) broccoli until the 2nd instar, then provided to the 1st instar *C. maculata* whose parents had fed on the different genotypes of *P. xylostella* larvae on Bt or non-Bt broccoli. We added new 2nd instars to the *C. maculata* each day until the *C. maculata* larva begun to pupate. Each treatment used 30–40 *C. maculata* larvae with each larva as a replication. We recorded the following data: *C. maculata* larval weight at 8 d, larval and pupal development time, adult's fresh weight (<24 h after emerge), the fecundity of each female over one month, and the hatching rate of the eggs.

2.7. Quantification of Cry1Ac in C. maculata and P. xylostella

RR neonates of *P. xylostella* were fed on Bt broccoli until the 2nd instar, and then the larvae were provided to *C. maculata*. The amount of Cry1Ac in the RR larvae of *P. xylostella*, and in 4th instar, pupae and adults of *C. maculata* were determined by ELISA using the EnviroLogix Cry1Ac/Cry1Ab kit (Portland, ME). Kits were identified as: QualiPlate™ Kit for Cry1Ab/Cry1Ac – AP 003 CRBS. Each sample included 20 *P. xylostella*, 5 larvae, pupae or adults of *C. maculata*, separately, and was ground and homogenized in 0.2 ml Extraction/dilution buffer (EnviroLogix). ELISA was conducted according to the manufacturer's instructions. Based on preliminary tests, each sample extraction was diluted by 1:20 and the optical density value of the sample was measured using a microplate reader set at 450 nm. The larvae fed on non-Bt broccoli were used as the controls. Each treatment was replicated 6–10 times.

2.8. Statistical analyses

Descriptive statistics are given as mean values and standard errors of the mean. Because the data fit the assumptions for parametric analysis, data were analyzed using one-way ANOVA and differences between treatments 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).

3. Results

3.1. Can C. maculata discriminate different genotypes of P. xylostella?

C. maculata did not discriminate between different genotypes of *P. xylostella* (Fig. 1). There were no significant differences in

percentages of RR, RS or SS genotype *P. xylostella* consumed by *C. maculata* larvae ($F = 3.860$; df = 2.9; $P = 0.062$) or adults ($F = 0.434$; df = 2.12; $P = 0.658$).

3.2. Can C. maculata discriminate plant types hosting resistant genotypes of Plutella xylostella?

Predation by *C. maculata* was independent of whether host plants expressed Bt toxins or not. The lady beetle larvae attacked 42.2 ± 7.0% and 39.4 ± 5.5% when RR *P. xylostella* larvae were feeding on a Cry1Ac leaf or non-Bt leaf, respectively. Similarly, adult beetles killed 50.6 ± 5.3% and 48.9 ± 10.7% of the RR *P. xylostella* larvae. There were no significant differences between the Bt and non-Bt treatments for larvae ($t = 0.585$, df = 5, $P = 0.584$) or adults ($t = 0.133$, df = 5, $P = 0.900$).

3.3. Effect of plant type and prey genotype on the life history of the G1 C. maculata

When *C. maculata* larvae fed on Bt or non-Bt broccoli-reared RR, RS and SS genotype larvae, they developed normally to adults (Table 1). Larval weight, the developmental time of larvae and pupae, adult weights, female fecundity and egg hatching rate were not significantly different in the four treatments.

3.4. Effect of plant type and prey genotype on the life history of the G2 C. maculata

When *C. maculata* were reared on Bt broccoli-reared RR, or non-Bt broccoli-reared RR, RS and SS genotype for the 2nd generation, there were no significant differences in larval weight, the developmental time of larvae and pupae, adult weights, fecundity of each female and egg hatching in the four treatments (Table 2).

3.5. Quantification of Cry1Ac in C. maculata and P. xylostella

Cry1Ac levels in *C. maculata* are presented as μg/g of fresh tissue and $\mu g \times 10^{-3}$/insect (Table 3). For the tissue data, the highest concentrations (2.35 μg/g) were detected in 3rd instar *C. maculata*. However, on a per-insect basis, the highest Cry1Ac level (17.20 $\mu g \times 10^{-3}$/insect) was found in the 4th instar. The pupae of *C. maculata* had very low levels of Cry1Ac (0.02 $\mu g \times 10^{-3}$/insect), while a much high level (1.36 $\mu g \times 10^{-3}$/insect) was detected in the pupal cocoon. No Cry1Ac was found in newly emerged adults without food, whereas Cry1Ac was found if the adults fed on Bt broccoli-reared RR *P. xylostella* larvae for 3, 4 and 6 d. Cry1Ac protein did not accumulate in *C. maculata* with increased feeding days (per tissue: $F = 1.021$; df = 2.16; $P = 0.382$; per insect: $F = 1.068$; df = 2.18; $P = 0.367$).

4. Discussion

In agroecosystems, using predators and parasitoids to control pests is a key component in integrated pest management (IPM) systems and these beneficial organisms should be conserved (Croft, 1990). *C. maculata* is one of the most important and widely distributed natural enemies from southern Canada to northern South America (Gordon, 1985). Adults and larvae prey on aphids, mites, lepidopteran eggs and larvae (Krafsur and Obrycki, 2000). The compatibility of transgenic Bt plants with this predator has been discussed (e.g. Lundgren et al., 2005; Li et al., 2011; Tian et al., 2012; Liu et al., 2014). Lundgren et al. (2005) reported that the fitness parameters of *C. maculata* were similar when its larvae were reared to pupation on aphids that had consumed Bt or non-Bt corn plants, but aphids contain very little if any Cry protein from Bt

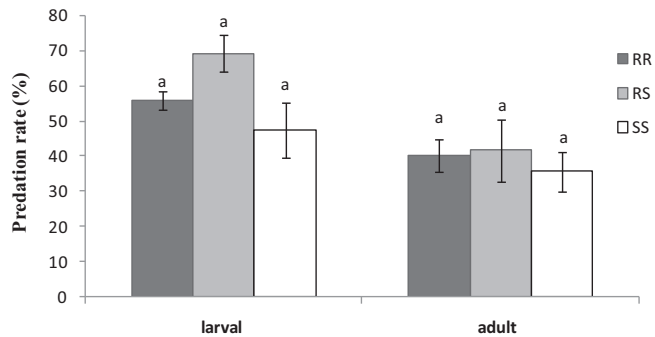

Fig. 1. Predation (%) of *Plutella xylostella* fed on Bt resistance genotypes by *Coleomegilla maculata* on normal (non-Bt) broccoli. The letters reflect the lack of significant differences at the 0.05 error level.

plants. Tian et al. (2012) reported that adult weight and female fecundity of *C. maculata* were not different when they were fed resistant *Spodoptera frugiperda* larvae reared on either Bt or control maize leaves during both generations. When *C. maculata* larvae were fed with resistant *Trichoplusia ni* larvae reared on either Bt or control cotton, the fitness parameters of *C. maculata* were also not affected (Li et al., 2011). Because the lady beetle also feeds on plant tissues, for example, pollen and nectar (Cividanes et al., 2011), the direct effects of Bt crop have been evaluated (Duan et al., 2002; Moser et al., 2008). Duan et al. (2002) reported that transgenic corn pollen expressing Cry3Bb1 protein had no measurable negative effects on the survival and life history of *C. maculata* larvae, nor any adverse effect on adult survival and reproductive capacity. However, Moser et al. (2008) found developmental time of 4th instar *C. maculata* increased when they fed on Bt corn seedlings daily but the results are questionable since the studies did not include a proper negative control. Generally, most published research supports the conclusion that Bt proteins are safe for this important predator.

In the present study, we evaluated more dimensions by examining the effect of prey genotypes, their interaction with plant type and any potential longer-term effect in subsequent generations. Our results showed that the life-history parameters of *C. maculata* fed on resistant (RR) or susceptible (RS and SS) larvae were similar when the larvae fed on Bt broccoli and non-Bt broccoli over two generations. Additionally, our research has shown that when Cry1Ac toxin is consumed by Cry1Ac-resistant *P. xylostella* larvae, it remains biologically active (Liu et al., 2011). In this study, Cry1Ac protein was detected in *C. maculata* that fed on Bt broccoli-reared resistant *P. xylostella* larvae. Moreover, we exposed *C. maculata* to higher concentrations of Cry1Ac than under field conditions. Our present results further confirm that this Cry protein is safe for this important predator even though *C. maculata* was exposed to biologically active Cry1Ac.

The effect of Cry toxins on important predators was reviewed by Romeis et al. (2006) who suggested that most predators are not susceptible to lepidopteran –active proteins. Recent research reported that several Cry proteins have no direct toxicity to some important predators, including *Chrysoperla carnea* (Lawo et al., 2010), *Stethorus punctillum* (Li and Romeis, 2010), *Chrysoperla sinica* (Wang et al., 2012), *Propylaea japonica* (Zhao et al., 2013), *Chrysoperla rufilabris* (Tian et al., 2013), *Geocoris punctipes* and *Orius insidiosus* (Tian et al., 2014b).

In the present study we added the potential interaction between predation and prey resistance to Bt proteins. Some strains of *P. xylostella* have evolved resistance to Bt sprays in the field (Shelton et al., 2008), and resistant strains can also survive on Bt broccoli in the lab (Zhao et al., 2000). If a field is sprayed with Bt

Table 1
Life history parameters of G1 *Coleomegilla maculata* fed on Bt or non-Bt broccoli plants hosting RR, RS or SS genotypes *Plutella xylostella* (Means ± SEM).

Treatments	Larval weight at 7th days (mg)	Larval period (d)	Pupal period (d)	Adult weight (mg)	Eggs per female in one month	Eggs hatching rate (%)
RR on Bt broccoli	4.1 ± 0.3a	13.9 ± 0.3a	4.3 ± 0.1a	9.1 ± 0.3a	28.0 ± 4.9a	82.8 ± 6.0a
RR on non-Bt broccoli	4.3 ± 0.7a	13.0 ± 0.4a	4.0 ± 0.2a	8.6 ± 0.7a	48.1 ± 10.9a	68.1 ± 4.9a
RS on non-Bt broccoli	5.2 ± 0.9a	13.0 ± 0.3a	3.8 ± 0.2a	9.2 ± 0.4a	26.3 ± 2.8a	75.5 ± 4.7a
SS on non-Bt broccoli	4.4 ± 0.3a	13.4 ± 0.4a	4.1 ± 0.1a	8.5 ± 0.4a	50.3 ± 22.5a	65.0 ± 6.5a
	$F = 0.682$, df = 3.70, $P = 0.566$	$F = 1.678$, df = 3.103, $P = 0.176$	$F = 1.686$, df = 3.72, $P = 0.178$	$F = 0.580$, df = 3.70, $P = 0.630$	$F = 0.805$, df = 3.24, $P = 0.503$	$F = 1.498$, df = 3.31, $P = 0.234$

One-way ANOVA followed by Tukey HSD when significant difference was detected. Means followed by the same letter in the same column are not significantly different at the 0.05 probability level.

Table 2
Life history parameters of G2 *Coleomegilla maculata* fed on Bt or non-Bt broccoli plants hosting RR, RS or SS genotypes *Plutella xylostella* (Means ± SEM).

Treatments	Larval weight at 8th days (mg)	Larval period (d)	Pupal period (d)	Adult weight (mg)	Eggs per female in one month	Eggs hatching rate (%)
RR on Bt broccoli	6.6 ± 0.6a	12.3 ± 0.4a	3.7 ± 0.1a	9.4 ± 0.5a	33.3 ± 10.5a	55.6 ± 6.8a
RR on non-Bt broccoli	6.5 ± 0.5a	11.9 ± 0.3a	3.875 ± 0.2a	9.0 ± 0.4a	61.0 ± 20.3a	67.0 ± 7.6a
RS on non-Bt broccoli	6.7 ± 0.8a	12.1 ± 0.a	3.3 ± 0.2a	9.0 ± 0.7a	23.4 ± 3.1a	61.2 ± 9.7a
SS on non-Bt broccoli	6.6 ± 0.6a	11.3 ± 0.3a	3.6 ± 0.1a	10.3 ± 0.4a	48.3 ± 12.0a	65.2 ± 8.3a
	$F = 0.028$, df = 3.85, $P = 0.9$	$F = 1.620$, df = 3.75, $P = 0.192$	$F = 1.188$, df = 3.64, $P = 0.322$	$F = 1.949$, df = 3.65, $P = 0.130$	$F = 1.577$, df = 3.24, $P = 0.221$	$F = 0.428$, df = 3.29, $P = 0.735$

One-way ANOVA followed by Tukey HSD when significant difference was detected. Means followed by the same letter in the same column are not significantly different at the 0.05 probability level.

Table 3
Cry1Ac concentration in *Plutella xylostella* and in *Coleomegilla maculata* (Means ± SEM).

	Cry1Ac concentration per fresh g tissue (μg/g)	Cry1Ac concentration per insect (μg × 10^{-3}/insect)
2nd *P. xylostella* larvae	2.3 ± 0.3	1.7 ± 0.4
2nd *C. maculata* larvae	2.2 ± 0.4	2.9 ± 0.5
3rd *C. maculata* larvae	2.4 ± 0.4	11.2 ± 1.2
4th *C. maculata* larvae	1.8 ± 0.3	17.2 ± 2.4
C. maculata pupae	0.0 ± 0.0	0.2 ± 0.0
C. maculata pupae cocoon	1.4 ± 0.4	0.8 ± 0.3
New emerged *C. maculata* adult	0	0
C. maculata adult feed for 3 d	0.7 ± 0.1	11.9 ± 2.4
C. maculata adult feed for 4 d	1.0 ± 0.1	16.0 ± 2.1
C. maculata adult feed for 6 d	0.8 ± 0.1	12.3 ± 1.2

or contains Bt plants, over time one would expect the field to contain resistant (RR), heterozygous (RS) and susceptible (SS) larvae. If a predator has a preference to remove RR individuals, then this would reduce the likelihood of the population evolving resistance to the Bt crop.

However, our results indicated that *C. maculata* could not discriminate between different genotypes of RR, RS and SS larvae fed on non-Bt broccoli (Fig. 1). Thus in theory, *C. maculata* could not directly alter prey resistance evolution. On the other hand, our research in the greenhouse demonstrated that *C. maculata*, combined with unsprayed, non-Bt refuge plants delayed resistance to Bt broccoli plants in the *P. xylostella* population (Liu et al., 2014). Arpaia et al. (1997) proposed a mathematical model to simulate the impact of natural enemies on the rate of *Leptinotarsa decemlineata* Say adaptation to Bt-toxin-expressing transgenic potato plants when the Bt-expressing plants are mixed at the plot-to-plot level with normal potato plants. Their modeling results suggested that *C. maculata* predatory behavior could decrease the rate at which *L. decemlineata* adapted to Bt-toxins if plot-to-plot mixed-planting were used. Prey resistance evolution is complex and whether the predator would accelerate or slow down resistance evolution needs further study in the field.

Predation by both *C. maculata* adult and larvae was not significantly different when resistant *P. xylostella* larvae fed on Bt broccoli or non-Bt broccoli. Therefore, we conclude that *C. maculata* could not distinguish Bt plants, which would likely have implications on the role of natural enemies regulating resistance evolution of targeted insect pests to Bt crops (Bates et al., 2005; Onstad and Knolhoff, 2014). We believe that this is the first study to report that a predator could not distinguish between Bt plants and non-Bt plants, although some studies have addressed foraging behavior of parasitoids (Schuler et al., 1999, 2003). In our earlier research, we studied the effects of different genotype *P. xylostella* larvae fed on Bt crops on *Diadegma insulare* (Liu et al., 2011) and the results showed that the parasitoid also did not discriminate host genotype, nor between Bt and normal broccoli plants with different genotype of *P. xylostella* feeding on them.

In the present study, Cry1Ac was found in *C. maculata* larvae that fed on Bt broccoli-reared resistant *P. xylostella*, but no Cry1Ac was found in new emerged adults (Table 3). It is worth noting that the Cry1Ac level was very high in pupal cocoon. A possible reason is that Cry1Ac was excreted with other waste in the pupal meconium. We also obtained similar results in our study with the pupal cocoon of the parasitoid *D. insulare* (Liu et al., 2011),

and noted that the adult parasitoid had very low levels of Cry1Ac. When *C. maculata* fed on Bt broccoli-reared resistant *P. xylostella* larvae for 3, 4 and 6 d, Cry1Ac was found in *C. maculata* adults, but there were no differences between the length of feeding. Therefore, we conclude that Cry1Ac can transfer from the plant to the prey to the host, but does not accumulate in the body of the predator.

In conclusion, transgenic Bt broccoli plants expressing Cry1Ac can effectively control *P. xylostella* but has no direct effects on *C. maculata.* Cry1Ac is one of the main Bt protein used in Bt cotton (Naranjo et al., 2008) and structurally very similar to the main protein used in Bt maize (Hellmich et al., 2008). Thus, based on our results and other studies (Duan et al., 2002; Lundgren et al., 2005; Li et al., 2011; Tian et al., 2012) we conclude that Cry1Ac is safe to *C. maculata.* For other predators, other studies have reached similar conclusions (Lawo et al., 2010; Li and Romeis, 2010; Wang et al., 2012; Zhao et al., 2013; Tian et al., 2013, 2014b). Therefore, the evidence suggests that Bt broccoli, and probably other plants, expressing Cry1Ac does not harm important natural enemies and would be compatible with biological control within an overall integrated pest management program.

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