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## Biocontrol Science and Technology

Publication details, including instructions for authors and subscription information:

<http://www.informaworld.com/smpp/title-content=t713409232>

### Effects of *Bacillus thuringiensis* -endotoxins Cry1Ab and Cry1Ac on the coccinellid beetle, *Cheilomenes sexmaculatus* (Coleoptera, Coccinellidae) under direct and indirect exposure conditions

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Online Publication Date: 01 April 2009

**To cite this Article** Dhillon, M. K. and Sharma, H. C. (2009) 'Effects of *Bacillus thuringiensis* -endotoxins Cry1Ab and Cry1Ac on the coccinellid beetle, *Cheilomenes sexmaculatus* (Coleoptera, Coccinellidae) under direct and indirect exposure conditions', *Biocontrol Science and Technology*, 19:4,407 — 420

**To link to this Article:** DOI: 10.1080/09583150902783801

**URL:** <http://dx.doi.org/10.1080/09583150902783801>

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## RESEARCH ARTICLE

### Effects of *Bacillus thuringiensis* $\delta$ -endotoxins Cry1Ab and Cry1Ac on the coccinellid beetle, *Cheilomenes sexmaculatus* (Coleoptera, Coccinellidae) under direct and indirect exposure conditions

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(Received 6 November 2008; returned 27 January 2009; accepted 28 January 2009)

Genes encoding *cry1Ab* and *cry1Ac*  $\delta$ -endotoxins from the bacterium, *Bacillus thuringiensis* (*Bt*) that have been incorporated in several crops to enhance their resistance to insect pests may possibly influence the activity and abundance of natural enemies of insect pests. The ladybird beetle, *Cheilomenes sexmaculatus* (L.) might ingest *Bt* toxins expressed by genetically modified plants by feeding on aphids, early instar larvae of lepidopterans, and other soft bodied insects feeding on transgenic plants. Therefore, we studied the effects of Cry1Ab and Cry1Ac *Bt* toxins on *C. sexmaculatus* under direct and indirect exposure conditions. For direct exposure, the neonate *C. sexmaculatus* larvae were fed either pure 2M sucrose (control) or sucrose solution containing Cry1Ab or Cry1Ac (0.1%), and on alternate days with aphids till pupation. Direct exposure of *C. sexmaculatus* larvae to *Bt* toxins resulted in reduced larval survival and adult emergence as compared to the controls, which might be due to long-term direct exposure. However, there were no adverse effects of the *Bt* toxins on *C. sexmaculatus* when the larvae were reared on *Aphis craccivora* Koch fed on different concentrations of Cry1Ab or Cry1Ac in the artificial diet. A significant and positive correlation was observed between the presence of *Bt* toxins in aphids, and coccinellid larvae and adults ( $r = 0.53^{**}$  to  $0.86^{**}$ ). The results suggested that a direct exposure to *Bt* toxins expressed in transgenic plants or predation on *H. armigera* on *Bt*-transgenic plants will have little effect on the activity and abundance of the ladybird, *C. sexmaculatus*.

**Keywords:** coccinellid predator; *Cheilomenes sexmaculatus*; *Bacillus thuringiensis*; nontarget effects; transgenics

#### Introduction

Over the past two decades, there has been a considerable progress in handling and introducing novel genes into crop plants to increase crop yields, improve nutrition, and impart resistance to biotic and abiotic stresses (Jouanin, Bonadé-Bottino, Girard, Morrot, and Giband 1998; Sharma, Sharma, Seetharama, and Crouch 2004). Area under transgenic crops is increasing at a fast rate, and has reached 114.3 million ha, of which transgenic crops with resistance to insect pests constituted more than 40 million ha during 2007 (James 2008). Pod borer, *Helicoverpa armigera* (Hubner) and the aphid, *Aphis craccivora* Koch are the two most important insect

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pests on chickpea and pigeonpea. The parasitoid, *Camponotus chlorideae* Uchida and the coccinellid predator, *Cheilomenes sexmaculatus* (L.) are important natural enemies of these pests in grain legumes. Transgenic chickpea and pigeonpea with *Bacillus thuringiensis* (Berliner) (*Bt*) genes for resistance to *H. armigera* are currently under development (Sharma, Ananda Kumar, Singh, and Sharma 2005), and therefore, it is important to assess the nontarget effects of *Bt* toxins to the natural enemies of crop pests in these crops.

Several studies have reported the direct and indirect effects of transgene products/transgenic plants on the beneficial insects (Dutton, Romeis, and Bigler 2003; Lövei and Arpaia 2005; Romeis, Meissle, and Bigler 2006; Sharma, Arora, and Pampapathy 2007; Dhillon, Lawo, Sharma, and Romeis 2008; Sharma, Dhillon, and Arora 2008). The *Bt* toxins are not transported to the phloem in some crops, and therefore, insect pests such as corn leaf aphid, *Rhopalosiphum maidis* (Fitch.) and the natural enemies feeding on it are not directly affected by the *Bt* toxins (Head, Brown, Groth, and Duan 2001; Raps et al. 2001; Dutton, Klein, Romeis, and Bigler 2002). The cotton aphid, *Aphis gossypii* Glover, is insensitive to *Bt* toxins, but trace amounts of *Bt* toxins were detected in the aphids when fed on *Bt* cotton (Zhang, Wan, Lövei, Wan, and Guo 2006a). Presence of Cry1Ac toxin in phloem sap from *Bt*-oilseed rape and in *Myzus persicae* Sulzer has indicated the importance of having an estimate of the effects of expected amounts of *Bt* toxins in the diets of non-target organisms predated on aphids fed on the transgenic crops (Burgio et al. 2007). Moreover, some *Bt* isolates such as INS 2.13, HFZ 24.8 and GU 9.1 exhibit different levels of toxicity (LC<sub>50</sub> values of 62, 328 and 114 ng/mL, respectively) to the cotton aphid, *A. gossypii* (Malik and Seikh 2006).

The effects of *Bt* toxins on entomophagous insects could be due to direct exposure to the toxin, indirect exposure via reduction in prey quantity and quality, or through unintended changes in physico-chemical characteristics of the plant caused by the insertion of the transgene. Several coccinellid species are known to feed on maize pollen, which represent a substantial food source for *Coleomegilla maculata* DeGeer, an important predator in North America, and pollen feeding is addressed in many risk assessment studies of *Bt*-transgenic maize (Pilcher, Obrycki, Rice, and Lewis 1997; Duan et al. 2002; Lundgren and Wiedenmann 2002; Lundgren, Razzak, and Wiedenmann 2004). Therefore, it is likely that Cry1Ab or Cry1Ac *Bt* toxins expressed in transgenic chickpea and pigeonpea may be taken up directly by the ladybird, *C. sexmaculatus* from the plant sap, honeydew from aphids, and through predating on the aphid, *A. craccivora*, and the small *H. armigera* larvae. Such an exposure to *Bt* toxins may result in some direct or indirect effects on ladybird beetle, which is an important predator of aphids and other soft-bodied insects in grain legumes. The present studies were therefore conducted to assess the direct (through sucrose solution) and indirect (through *Bt*-intoxicated aphid, *A. craccivora*) effects of Cry1Ab or Cry1Ac *Bt* toxins to the larvae of the coccinellid beetle, *C. sexmaculatus*.

## Materials and methods

### *Insect culture*

Cultures of the aphid, *A. craccivora*; and the predatory coccinellid, *C. sexmaculatus* were maintained on cowpea, *Vigna unguiculata* (L.) Walp. plants in a nylon

net-house under ambient conditions. The aphids and coccinellids were obtained from *Glaricidia maculata* (Kunth.) Walp. growing at the ICRISAT farm. The *C. sexmaculatus* eggs were obtained from greenhouse-reared coccinellids as and when needed. The coccinellid eggs were removed from the oviposition substrate (to avoid fungus development and resultant larval mortality), and transferred on to a carbon paper in a plastic cup. The neonate *C. sexmaculatus* larvae from these plastic cups were used in the experiments.

To measure the indirect effects of *Bt* toxins on *C. sexmaculatus*, the aphids, *A. craccivora* were reared on an artificial diet described by Febvay, Delobel, and Rahbé (1988), with a sucrose content of 20% (w/v) as suggested by Hogervorst, Wackers, and Romeis (2007). The aphids (mixed stages) were fed on the artificial diets (control or *Bt* intoxicated) through a parafilm sachet using plexiglass cylindrical cages (diameter 3.4 cm, height 3.0 cm). The first layer of parafilm was stretched over the plexiglass cylinder and loaded with 125  $\mu$ L artificial diet, and then by a second layer of the parafilm was stretched over it. The bottom of the plexiglass cylinder was kept open on a plexiglass base for releasing the aphids and coccinellids for feeding.

#### **Feeding *C. sexmaculatus* larvae on sucrose solution**

The neonate *C. sexmaculatus* larvae were fed on one of the following food sources: (i) pure 2M sucrose solution, (ii) 2M sucrose solution containing Cry1Ab or Cry1Ac (0.1%), (iii) water, and (iv) no food. The survival of the *C. sexmaculatus* larvae was recorded daily to assess whether the predator larvae had actually fed on sucrose solution or sucrose solution containing the *Bt* toxins. The experiment was conducted twice with 15 replications each, thus forming a total of 30 replications for each treatment in a completely randomised design. Ingestion of *Bt* toxins by the coccinellid larvae was confirmed by ELISA. ELISA (Agdia®) test was carried out to detect the *Bt* toxins in 2M sucrose mixed Cry1Ab or Cry1Ac (0.1%), aphids, *C. sexmaculatus* larvae fed on different foods, and the coccinellid adults emerging from the larvae fed on these foods as per the method described by Sharma et al. (2008) using 0.3125, 1.25, 5, 10, and 20 ppb concentrations of Cry1Ac as standards. The test insects were thoroughly washed with PBS buffer before use in the ELISA test.

#### **Direct effects of *Bt* toxins on survival and development of *C. sexmaculatus***

Trypsin activated (95% pure) Cry1Ab and Cry1Ac *Bt* toxins were dissolved in a 2M sucrose solution at a concentration of 0.1% (330 and 500 times greater than the estimated LC<sub>50</sub> against the neonate *H. armigera* larvae (Sharma et al. 2008) to assess the direct effects, if any, of the *Bt* toxins to the ladybird, *C. sexmaculatus*. Neonate *C. sexmaculatus* larvae were fed on: (i) pure 2M sucrose solution (sucrose + aphids), (ii) 2M sucrose solution containing Cry1Ab (Cry1Ab + aphids) or Cry1Ac (Cry1Ac + aphids) at 0.1% on alternate days. The *C. sexmaculatus* larvae were provided *ad libitum* *A. craccivora* (mixed stages) after every 24 h of feeding on one of the above foods till pupation. One set of *C. sexmaculatus* larvae were fed on *A. craccivora* only. The neonate *C. sexmaculatus* larvae were kept individually in bioassay cups (3.3 cm in diameter, 3.5 cm in depth), and fed on above mentioned foods in the insectary at  $26 \pm 1^\circ\text{C}$ ,  $80 \pm 5\%$  RH, and a 12-h photoperiod. The experiment was conducted twice with 15 replications each, thus, forming a total of 30 replications for

each treatment in a completely randomised design. Observations were recorded on larval and pupal periods, larval survival, weights of male and female larvae, adult emergence, and weights of male and female adults of *C. sexmaculatus*.

### ***Indirect effects of Bt toxins on survival and development of C. sexmaculatus***

Indirect effects of *Bt* toxins on *C. sexmaculatus* were measured through *A. craccivora* fed on 0.5x, 1x, 5x, and 10x concentrations of Cry1Ab and Cry1Ac in the artificial diet [where 'x' represents the LC<sub>50</sub> of the *Bt* toxins against *H. armigera*] (Sharma et al. 2008). The range of *Bt* concentrations were selected to address the effects of *Bt* toxins on the *C. sexmaculatus* through the aphid, *A. craccivora* under different *Bt*-toxin expression levels in the transgenic plants. The aphids were reared on artificial diets amended with different amounts of Cry1Ab (1.5, 3, 15, and 30 ppm) and Cry1Ac (0.9, 1.8, 9, and 18 ppm) for 3 days, and then offered to the *C. sexmaculatus* larvae in the insectary as described above. One set of aphids was also reared on control artificial diet. The *C. sexmaculatus* larvae were provided *ad libitum* *A. craccivora* (mixed stages) reared on different concentrations of *Bt* toxins, until pupation. The experiment was conducted twice with 30 replications each, thus forming a total of 60 replications for each treatment in a completely randomised design. Observations were recorded on larval and pupal periods, larval survival, weights of male and female larvae, adult emergence, and weights of male and female adults of *C. sexmaculatus*.

### ***Detection of Bt toxins in artificial diet, A. craccivora, and C. sexmaculatus***

The artificial diet with different *Bt* concentrations (30 µL), *A. craccivora* reared on different *Bt* concentrations (30–40 mg), and the *C. sexmaculatus* larvae and adults (30–40 mg) fed on *Bt*-exposed aphids along with their controls were collected in Eppendorf tubes in four replications (two from each repeat of experiments) for each treatment, and crushed in PBS buffer in the ratio of 1:10 (diet/insect sample: buffer) with a plastic pestel. The semi-quantitative ELISA (Agdia®) was performed using the procedure given by Sharma et al. (2008), but with *Bt* standards as 0.3125, 1.25, 5, 10, and 20 ppb of Cry1Ac.

### ***Statistical analysis***

The data were analysed using the SAS 9.1.3 version statistical program (SAS Institute 2008). All the experiments were conducted twice. There was no change in trends or their significance differences between the treatments, and hence, the results from both the sets were pooled for analysis. The pooled data were subjected to homogeneity test before final analysis. Longevities of *C. sexmaculatus* larvae on different foods were analysed using analysis of variance (ANOVA) in a completely randomised design (CRD) and the significance of differences were judged by *F*-test, however, the pairwise comparisons for longevities on different foods were done through Wilcoxon *U*-test with an adjusted  $\alpha = 0.025$ . Larval and pupal periods, larval survival, weights of male and female larvae and adults, and adult emergence of *C. sexmaculatus* on different concentrations of Cry1Ab or Cry1Ac were analysed using one-way ANOVA in a CRD, while the interaction effects of *Bt* toxins (Cry1Ab

and Cry1Ac)  $\times$  concentrations were analysed using two-way ANOVA in a randomised block design (RBD), and the significance of differences were judged by *F*-test. The pairwise comparisons between different foods (direct exposure) and different concentrations of Cry1Ab or Cry1Ac with the controls (indirect exposure) for larval and pupal periods were done by Wilcoxon *U*-test with an adjusted  $\alpha = 0.025$ , larval survival and adult emergence by contingency table analysis ( $\chi^2$ -test) with Fisher's exact test, and the weights of *C. sexmaculatus* male and female larvae and adults were done by Student's *t*-test.

## Results

### *Consumption of sucrose solution and Bt toxins by C. sexmaculatus larvae*

There were significant differences in longevity of *C. sexmaculatus* larvae fed on 2M sucrose, water, and the starved insects ( $F_{2,58} = 48.36$ ,  $P < 0.0001$ ). However, there were no significant differences in the longevity of *C. sexmaculatus* larvae fed on 2M sucrose mixed with Cry1Ab or Cry1Ac, or 2M sucrose alone ( $F_{2,58} = 0.58$ ,  $P = 0.56$ ). Longevity of *C. sexmaculatus* larvae on water ( $1.5 \pm 0.09$  days) or without food ( $1.1 \pm 0.05$  days) was significantly shorter than those fed on 2M sucrose mixed with Cry1Ab ( $3.2 \pm 0.22$  days) or Cry1Ac ( $3.0 \pm 0.17$  days), or 2M sucrose alone ( $2.9 \pm 0.21$  days) (Figure 1). The prolonged survival of *C. sexmaculatus* larvae on sucrose as compared to those fed on water or kept without food suggested that the coccinellid larvae actually ingested the sucrose solution and the *Bt* toxins dissolved in sucrose.

### *Direct effects of Bt toxins on development and survival of C. sexmaculatus*

The larval period was prolonged by 1.6 days when the larvae were fed on sucrose + aphids than on aphids alone. However, there were no differences in the pupal periods of the insects reared on sucrose + aphids or aphids alone. Larval period of *C. sexmaculatus* was prolonged by 1.2 and 1.5 days when fed on Cry1Ab + aphids and Cry1Ac + aphids than those fed on sucrose + aphids (Table 1). The pupal period was also prolonged in insects fed on Cry1Ab + aphids and Cry1Ac + aphids than those fed on sucrose + aphids. Pupal period was comparatively longer in insects fed on

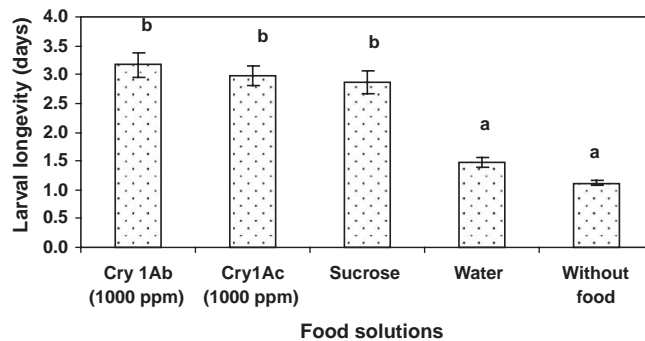


Figure 1. Longevity (mean  $\pm$  SE) of predatory coccinellid, *Cheilomenes sexmaculatus* on different food solutions. The bars with different letters are significantly different at  $P \leq 0.05$ .

Table 1. Direct effects of *Bt* toxins on different biological parameters of the coccinellid, *Cheilomenes sexmaculatus*.

Treatments	Larval weight (mg ± SE)#		Larval period (days ± SE)£	Larval survival (% ± SE)¶	Pupal period (days ± SE)£	Adult emergence (% ± SE)¶	Adult weight (mg ± SE)#	
	Male	Female					Male	Female
(i) Cry1Ab <sup>1</sup> + aphids	3.99 ± 0.76 <sup>a</sup>	4.83 ± 0.78 <sup>a</sup>	9.1 ± 0.22 <sup>a</sup>	60.0 ± 9.10 <sup>a</sup>	3.3 ± 0.14 <sup>a</sup>	56.7 ± 9.36 <sup>a</sup>	6.06 ± 0.54 <sup>a</sup>	7.70 ± 0.23 <sup>a</sup>
(ii) Cry1Ac <sup>1</sup> + aphids	3.33 ± 0.38 <sup>a</sup>	3.66 ± 0.20 <sup>a</sup>	9.4 ± 0.17 <sup>a</sup>	90.0 ± 5.57 <sup>b</sup>	3.0 ± 0.04 <sup>a</sup>	90.0 ± 0.00 <sup>c</sup>	6.35 ± 0.25 <sup>a</sup>	8.44 ± 0.23 <sup>a</sup>
(iii) Sucrose + aphids	7.44 ± 0.73 <sup>b</sup>	8.7 ± 1.27 <sup>b</sup>	7.9 ± 0.14 <sup>c</sup>	90.0 ± 5.57 <sup>b</sup>	3.6 ± 0.10 <sup>b</sup>	86.7 ± 3.70 <sup>b</sup>	7.00 ± 0.29 <sup>b</sup>	9.76 ± 0.61 <sup>b</sup>
(iv) Aphids alone	10.60 ± 0.61 <sup>c</sup>	14.64 ± 0.57 <sup>c</sup>	6.2 ± 0.08 <sup>b</sup>	96.7 ± 3.33 <sup>c</sup>	3.8 ± 0.08 <sup>c</sup>	96.7 ± 0.00 <sup>d</sup>	9.43 ± 0.24 <sup>c</sup>	11.53 ± 0.34 <sup>c</sup>
(i) vs. (ii) <sup>2</sup>	<i>P</i> = 0.4070	<i>P</i> = 0.1851	<i>P</i> = 0.4566	<i>P</i> = 0.0146	<i>P</i> = 0.0440	<i>P</i> = 0.0073	<i>P</i> = 0.5751	<i>P</i> = 0.3710
(i) vs. (iii) <sup>2</sup>	<i>P</i> = 0.0100	<i>P</i> = 0.0181	<i>P</i> < 0.0001	<i>P</i> = 0.0048	<i>P</i> = 0.0305	<i>P</i> = 0.0048	<i>P</i> = 0.1070	<i>P</i> = 0.0057
(i) vs. (iv) <sup>2</sup>	<i>P</i> < 0.0001	<i>P</i> < 0.0001	<i>P</i> < 0.0001	<i>P</i> = 0.0012	<i>P</i> = 0.0020	<i>P</i> = 0.0006	<i>P</i> < 0.0001	<i>P</i> < 0.0001
(ii) vs. (iii) <sup>2</sup>	<i>P</i> < 0.0001	<i>P</i> = 0.0016	<i>P</i> < 0.0001	<i>P</i> = 0.6400	<i>P</i> < 0.0001	<i>P</i> = 1.0000	<i>P</i> = 0.0900	<i>P</i> = 0.0700
(ii) vs. (iv) <sup>2</sup>	<i>P</i> < 0.0001	<i>P</i> < 0.0001	<i>P</i> < 0.0001	<i>P</i> = 0.3000	<i>P</i> < 0.0001	<i>P</i> = 0.3000	<i>P</i> < 0.0001	<i>P</i> < 0.0001
(iii) vs. (iv) <sup>2</sup>	<i>P</i> = 0.0142	<i>P</i> = 0.0021	<i>P</i> < 0.0001	<i>P</i> = 0.5500	<i>P</i> = 0.2555	<i>P</i> = 0.3000	<i>P</i> < 0.0001	<i>P</i> = 0.0155

The mean values within a column following different letters for male and female larval and adult weights (Student's *t*-test, *P* < 0.05)#, larval and pupal periods (Mann-Whitney *U*-test, *P* < 0.05)£, and larval survival and adult emergence ( $\chi^2$ -test, *P* < 0.05)¶ are significantly different from each other.

<sup>1</sup>Cry1Ab or Cry1Ac (0.1%, w/v) mixed in 2M sucrose solution.

<sup>2</sup>The *P*-values are based on  $\alpha$ -levels adjusted for two pairwise comparisons ( $\alpha = 0.025$ ).

Cry1Ab+aphids than on Cry1Ac+aphids, while no significant differences were observed in the larval period.

Larval survival and adult emergence of *C. sexmaculatus* was significantly lower when the coccinellid larvae were fed on Cry1Ab+aphids than on Cry1Ac+aphids, sucrose+aphids, or aphids alone. Larval survival and adult emergence were 30–37% and 30–40% lower in the coccinellid larvae fed on Cry1Ab+aphids as compared to those on Cry1Ac+aphids, sucrose+aphids, or aphids alone (Table 1). However, no significant differences were observed for larval survival and adult emergence in the insects fed on Cry1Ac+aphids vs. sucrose+aphids, Cry1Ac+aphids vs. aphids alone, and sucrose+aphids vs. aphids alone.

Weights of male and female *C. sexmaculatus* larvae and the adults were significantly greater in the coccinellid larvae fed on sucrose+aphids and aphids alone than in insects fed on Cry1Ab+aphids or Cry1Ac+aphids, except the adult male weight on Cry1Ab+aphids, and male and female larval weights in case of Cry1Ac+aphids (Table 1). The larvae of *C. sexmaculatus* fed on Cry1Ab+aphids and Cry1Ac+aphids had almost half the weight of the insects fed on sucrose+aphids or aphids alone. However, no significant differences were observed in the weights of male and female larvae and adults when the larvae of *C. sexmaculatus* were fed on Cry1Ab+aphids or Cry1Ac+aphids.

The ELISA test detected high amounts of *Bt* toxins in the 2M sucrose mixed with Cry1Ab ( $12.6 \pm 0.44$  ppb) and Cry1Ac ( $17.3 \pm 0.34$  ppb), and the coccinellid larvae fed on Cry1Ab ( $10.2 \pm 2.34$  ppb) and Cry1Ac ( $10.20 \pm 4.50$  ppb) in sucrose solution, while only traces of the *Bt* toxins were detected in the coccinellid adults ( $1.12 \pm 0.54$  to  $1.7 \pm 0.64$  ppb). In general, lower amounts of *Bt* toxins were detected in insects fed on Cry1Ab or Cry1Ac mixed in 2M sucrose as compared to the *Bt* concentration added, indicating that some amount of *Bt* toxins might have been metabolized in the insect gut.

#### **Indirect effects of *Bt* toxins on development and survival of *C. sexmaculatus***

One-way ANOVA for the comparison of different concentrations of Cry1Ab and Cry1Ac, and two-way ANOVA for interaction of *Bt* toxins (Cry1Ab and Cry1Ac)  $\times$  concentrations revealed that there were no significant effects of *Bt* on the larval and pupal periods, weights of male and female larvae and adults, and larval survival and adult emergence of *C. sexmaculatus* when the larvae were fed on *A. craccivora* reared on *Bt*-intoxicated artificial diets (Table 2). Furthermore, Wilcoxon *U*-test did not indicate any significant differences in larval and pupal periods of *C. sexmaculatus* fed on *A. craccivora* reared on control diets or different concentrations of Cry1Ab and Cry1Ac (Table 3). Student's *t*-test indicated that weights of *C. sexmaculatus* male and female larvae and adults fed on *A. craccivora* reared on control diets were statistically similar to those reared on diets with different concentrations of Cry1Ab and Cry1Ac (Table 3).  $\chi^2$ -test indicated that there were no significant differences in larval survival and adult emergence of *C. sexmaculatus* whose larvae were fed on aphids reared on control diet or diets with different concentrations of Cry1Ab and Cry1Ac (Table 3).



Table 2. ANOVA for comparing indirect effects of Cry1Ab and Cry1Ac *Bt* toxins and their concentrations on different biological parameters of *Cheilomenes sexmaculatus* reared on *Bt*-intoxicated artificial diet fed *Aphis craccivora*.

Insect parameters	One-way ANOVA <sup>1</sup>						Two-way ANOVA <sup>2</sup>		
	Cry1Ab			Cry1Ac			<i>Bt</i> toxins × concentrations		
	df	F-value	P-value	df	F-value	P-value	df	F-value	P-value
Male larval weight	4,135	2.32	0.06	4,144	1.53	0.2	9,279	1.71	0.09
Female larval weights	4,109	1.27	0.29	4,106	1.04	0.39	9,215	1.02	0.42
Larval period	4,251	1.67	0.16	4,262	1.52	0.2	9,513	1.43	0.17
Pupal period	4,242	0.38	0.83	4,249	0.23	1.42	9,491	0.78	0.64
Larval survival	4,286	0.78	0.54	4,290	1.92	0.22	9,576	1.63	0.1
Adult emergence	4,285	0.77	0.55	4,290	1.57	0.38	9,575	1.46	0.16
Adult male weight	4,130	1.12	0.35	4,138	0.24	0.91	9,268	0.59	0.81
Adult female weight	4,107	1.16	0.33	4,106	1.3	0.27	9,213	1.1	0.36

<sup>1</sup>One-way ANOVA for comparing different concentrations of Cry1Ab and Cry1Ac.

<sup>2</sup>Two-way ANOVA for comparing *Bt* toxins and their concentrations.

### Detection of *Bt* toxins in artificial diets, *A. craccivora*, and *C. sexmaculatus*

The ELISA test detected high amounts of *Bt* toxins in artificial diets intoxicated with Cry1Ab and Cry1Ac (17.7–20.1 ppb) (Table 4), indicating that there was no degradation of *Bt* toxins in the artificial diet. However, lower amounts of *Bt* toxins were detected in artificial diets as compared to the actual amounts of *Bt* added to the diet. This may be because of limited efficacy of ELISA test to detect the *Bt* (calibration up to 20 ppb) or interaction of *Bt* toxins with the diet ingredients. The aphids reared on Cry1Ac-amended artificial diet at 18 ppm imbibed traces of *Bt* toxin (1.7 ppb), which magnified to 2.5 ppb in coccinellid larvae, traces of which were detected in the adults. The aphids reared on artificial diets amended with Cry1Ab at 15 ppm (5.0 ppb) and 30 ppm (9.9 ppb) imbibed comparatively more *Bt* toxin than Cry1Ac, which magnified to 11.0 to 12.3 ppb in coccinellid larvae, and the traces were detected in the adults of *C. sexmaculatus* (Table 4). A significant and positive association was observed between the presence of *Bt* toxins in aphids and coccinellid larvae ( $r=0.86^{**}$ ), aphids and coccinellid adults ( $r=0.53^{**}$ ), and coccinellid larvae and adults ( $r=0.72^{**}$ ).

Table 3. Indirect effects of *Bt* toxins on different biological parameters of the coccinellid, *Cheilomenes sexmaculatus* reared on *Bt*-intoxicated artificial diet fed *Aphis craccivora*.

Treatments	Larval weight (mg $\pm$ SE)#		Larval period (days $\pm$ SE)£	Larval survival (% $\pm$ SE)¶	Pupal period (days $\pm$ SE)£	Adult emergence (% $\pm$ SE)¶	Adult weight (mg $\pm$ SE)#	
	Male	Female					Male	Female
<i>Cry1Ab</i>								
1.5 ppm	7.0 $\pm$ 0.41	10.5 $\pm$ 0.56	5.9 $\pm$ 0.25	83.3 $\pm$ 8.82	3.4 $\pm$ 0.11	79.1 $\pm$ 8.08	7.0 $\pm$ 0.46	10.0 $\pm$ 0.59
3 ppm	7.5 $\pm$ 0.46	11.0 $\pm$ 0.62	5.7 $\pm$ 0.31	86.7 $\pm$ 9.89	3.5 $\pm$ 0.18	83.3 $\pm$ 9.89	6.9 $\pm$ 0.34	10.6 $\pm$ 0.60
15 ppm	8.2 $\pm$ 0.74	11.4 $\pm$ 0.86	5.8 $\pm$ 0.32	88.0 $\pm$ 8.42	3.4 $\pm$ 0.22	86.3 $\pm$ 8.10	7.3 $\pm$ 0.46	10.3 $\pm$ 0.88
30 ppm	8.2 $\pm$ 0.44	10.8 $\pm$ 0.51	5.9 $\pm$ 0.32	93.3 $\pm$ 3.33	3.3 $\pm$ 0.12	88.3 $\pm$ 4.01	7.6 $\pm$ 0.34	10.0 $\pm$ 0.76
Control	7.7 $\pm$ 0.40	10.6 $\pm$ 0.56	6.1 $\pm$ 0.45	89.6 $\pm$ 6.34	3.3 $\pm$ 0.15	89.6 $\pm$ 6.34	7.3 $\pm$ 0.52	11.1 $\pm$ 0.74
<i>Cry1Ac</i>								
0.9 ppm	8.1 $\pm$ 0.59	10.9 $\pm$ 1.01	5.9 $\pm$ 0.32	88.0 $\pm$ 4.18	3.2 $\pm$ 0.07	84.6 $\pm$ 3.54	7.2 $\pm$ 0.57	10.1 $\pm$ 1.17
1.8 ppm	8.1 $\pm$ 0.49	10.7 $\pm$ 0.67	5.8 $\pm$ 0.23	98.3 $\pm$ 1.67	3.3 $\pm$ 0.11	91.7 $\pm$ 4.01	7.2 $\pm$ 0.45	10.3 $\pm$ 0.69
9 ppm	7.7 $\pm$ 0.41	11.7 $\pm$ 1.06	5.7 $\pm$ 0.33	87.8 $\pm$ 5.21	3.3 $\pm$ 0.12	84.4 $\pm$ 5.94	7.0 $\pm$ 0.59	10.1 $\pm$ 0.81
18 ppm	6.8 $\pm$ 0.32	10.6 $\pm$ 0.68	6.2 $\pm$ 0.46	81.7 $\pm$ 10.46	3.5 $\pm$ 0.16	75.0 $\pm$ 10.57	7.1 $\pm$ 0.65	10.4 $\pm$ 1.07
Control	8.0 $\pm$ 0.45	10.8 $\pm$ 0.51	5.6 $\pm$ 0.23	94.6 $\pm$ 2.41	3.4 $\pm$ 0.14	93.0 $\pm$ 3.42	7.3 $\pm$ 0.45	10.2 $\pm$ 0.77

The mean values of male and female larval and adult weights (Student's *t*-test,  $P < 0.05$ )#, larval and pupal periods (Mann-Whitney *U*-test,  $P < 0.05$ )£, and larval survival and adult emergence ( $\chi^2$ -test,  $P < 0.05$ )¶ for *Cry1Ab*/*Cry1Ac* were statistically at par to the controls.

Table 4. Amounts of *Bt* toxin in artificial diet, aphids, and the larvae and adults of predatory coccinellid, *Cheilomenes sexmaculatus*.

Treatment	<i>Bt</i> toxin concentration (ppb) (mean <sup>1</sup> ± SE)			
	Artificial diet	Aphids	Coccinellid larvae	Coccinellid adults
<i>Cry1Ab</i>				
Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
1.5 ppm	19.2 ± 0.9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
3 ppm	17.8 ± 0.9	0.1 ± 0.1	0.2 ± 0.2	0.0 ± 0.0
15 ppm	17.7 ± 0.6	5.0 ± 0.7	11.0 ± 6.0	0.3 ± 0.2
30 ppm	19.9 ± 0.7	9.9 ± 5.6	12.3 ± 6.7	0.5 ± 0.3
<i>Cry1Ac</i>				
Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
0.9 ppm	18.8 ± 1.8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
1.8 ppm	20.1 ± 1.8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
9 ppm	19.0 ± 1.2	0.1 ± 0.1	0.3 ± 0.3	0.0 ± 0.0
18 ppm	18.8 ± 0.7	1.7 ± 1.0	2.5 ± 1.6	0.2 ± 0.2

<sup>1</sup>Mean values are based on four replications.

## Discussion

The risk that transgenic plants pose to the nontarget organisms is a function of feeding behaviour, expression of the transgene in the plant, mode of exposure to the insecticidal toxin, and the toxicity of the toxin towards the specific organism. Coccinellids have earlier been reported to feed on pollen, and Cry1Ab endotoxin has been found in foreguts at detectable levels (Lundgren et al. 2004; Lundgren, Huber, and Wiedenmann 2005). The ladybird, *C. sexmaculatus* might ingest Cry1Ab or Cry1Ac *Bt* toxins expressed in transgenic plants through leaf exudates, honeydew produced by aphids feeding on these plants, and indirectly through herbivores such as *A. craccivora*, *H. armigera*, *Spodoptera litura* (Fab.), *Spodoptera exigua* (Hub.), or some other lepidopteran larvae, and soft bodied insects feeding on transgenic plants. Therefore, we designed three bioassays to expose *C. sexmaculatus* larvae to Cry1Ab and Cry1Ac *Bt* toxins directly or indirectly at high concentrations for use in toxicological studies as a worst-case scenario. Direct exposure of neonate *C. sexmaculatus* larvae to Cry1Ab and Cry1Ac *Bt* toxins in 2M sucrose increased larval longevity by 2 to 3 times over those fed on water or kept without food, and the ELISA test did detect *Bt* toxins in the coccinellid larvae fed on *Bt* mixed sucrose, suggesting that the coccinellid larvae actually ingested the *Bt* toxins with the food. However, the exposure period of *C. sexmaculatus* larvae to the *Bt* toxins was quite short (3–4 days), to measure any detectable effects on the coccinellid larvae.

Therefore, we designed a life long direct exposure assay to measure the effects of *Bt* toxins on different life stages of *C. sexmaculatus*. There was a slight, but significant increase in developmental period (1.5–2 days) when the *C. sexmaculatus* larvae were fed on Cry1Ab or Cry1Ac mixed with sucrose as compared to the larvae fed on sucrose only, and on alternate days on aphids, which might be due to long-term ingestion of *Bt* toxins. Larval survival and adult emergence were lower in insects fed on Cry1Ab+aphids than on Cry1Ac+aphids, sucrose+aphids, and

aphids alone, suggesting some adverse effects of Cry1Ab on *C. sexmaculatus*. ELISA test detected *Bt* toxins in food offered to the coccinellids (2M sucrose mixed Cry1Ab and Cry1Ac), and the coccinellid larvae that fed on them. Traces of *Bt* toxins were also detected in the coccinellid adults, suggesting that the observed effects of *Bt* toxins on different life-stages of *C. sexmaculatus* were due to ingestion of *Bt* toxins during different larval instars. Schuler et al. (2005) also reported reduced weight and survival of *Chrysoperla carnea* Stephen on *Bt*-transgenic oilseed rape under low *M. persicae* density (lack of *ad libitum* food), which at high aphid density retained normal growth and survival.

Studies earlier have reported that aphids feeding on *Bt*-transgenic plants do not ingest *Bt* toxin (Head et al. 2001; Raps et al. 2001; Schuler et al. 2001, 2005). However, the Cry1Ab has been detected in the phloem sap of *Bt*-oilseed rape, and the aphids, *M. persicae* feeding on the *Bt*-oilseed rape plants (Burgio et al. 2007). The *Bt* toxins were also detected in the coccinellid, *Propylaea japonica* Thunberg larvae and the prey, *A. gossypii* when reared on *Bt* cottons (Zhang et al. 2006a). Expression of transgene product in the phloem sap might be transformation protocol/promoter/candidate gene/environment/plant species specific. Therefore, to mimic the *Bt* expression patterns in different plant parts, and assess the effects of *Bt* toxins on the predatory coccinellid at the level expressed in different plant parts indirectly through the herbivores ingesting the *Bt* toxins, we designed a bioassay for indirect exposure of *C. sexmaculatus* to *Bt* toxins through *A. craccivora* reared on *Bt*-intoxicated artificial diets. There were no significant differences in larval and pupal periods, larval survival, adult emergence, and the male and female larval and adult weights of *C. sexmaculatus* when fed on *A. craccivora* reared on Cry1Ab- or Cry1Ac-intoxicated artificial diets. A significant and positive correlation was observed between *Bt* detection in aphids, coccinellid larvae, and coccinellid adults. The amount of *Bt* toxins detected in coccinellid larvae was greater than that detected in the aphids, suggesting that coccinellid larvae accumulated the *Bt* toxins from the food source without much processing by the gut proteases. Processing of *Bt* toxins by the insect gut proteases is known to alter specificity of *Bt* toxins (Haider, Knowles, and Ellar 1986; Rukmini, Reddy, and Venkateswerlu 2000).

No adverse effects of *Bt*-transgenic crops have been reported on the development, survival, and reproduction of ladybeetles, *C. maculata*, *Hippodamia convergens* (Guérin-Méneville), and *P. japonica* through their aphid preys on *Bt*-transgenic crops (Dogan, Berry, Reed, and Rossignol 1996; Duan et al. 2002; Lundgren and Wiedenmann 2002; Zhu et al. 2006). *Chrysoperla carnea* larvae were also not affected when fed on *Bt*-maize reared aphids (Lozzia, Furlanis, Manachini, and Rigamonti 1998; Dutton et al. 2002), or through the *Bt*-maize reared spider mites, *Tetranychus urticae* (Koch), even though the spider mites had much more amounts of Cry1Ab toxin than the lepidopteran larvae (Dutton et al. 2002). However, poor prey quality and Cry1Ac toxin mediated negative effects have been observed on the predatory beetle, *P. japonica* when fed on young *S. litura* larvae fed on *Bt*-transgenic cotton (Zhang, Wan, Wan, and Guo 2006b). Such negative effects of *Bt* toxins on the coccinellid, *C. sexmaculatus* were also observed when fed on young *H. armigera* larvae reared only on *Bt*-amended artificial diet (HC Sharma, unpublished data), indicating that the adverse effects of *Bt* toxins to the coccinellids might depend on the processing of the *Bt* toxins and the quality of the insect host. Hilbeck, Moar, Pusztai-Carey, Filippini, and Bigler (1998) observed some adverse effects of the *Bt*-fed

lepidopteran, *Spodoptera littoralis* (Boisduval) larvae on the chrysopid, *C. carnea*. Under natural conditions in the field, no significant differences were observed in the abundance of coccinellid beetles between *Bt*-transgenic and non-transgenic cottons (Sharma et al. 2007). The present studies suggested that the Cry1Ab and Cry1Ac at levels expressed in transgenic grain legumes are unlikely to have any adverse effects towards the predatory coccinellid, *C. sexmaculatus* feeding on aphids colonizing *Bt*-transgenic plants. However, direct exposure to very high *Bt* concentrations or indirect exposure through predation on *H. armigera* and/or other lepidopteran larvae feeding on *Bt*-transgenic plants in absence of *ad libitum* aphids may have some adverse effects on the survival and development of *C. sexmaculatus*.

### Acknowledgements

The technical support of ICRISAT entomology staff during the studies, help in statistical analysis of the data by Mr Ravi Devulapalli, and funding by Swiss Agency for Development and Cooperation (SDC), Berne, Switzerland and the Department of Biotechnology (DBT), New Delhi, India, is gratefully acknowledged.

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