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Impact of *Bt* cotton expressing single (Cry1Ac) and dual toxins (Cry1Ac and Cry2Ab) on the fitness of the predator *Chrysoperla zastrowi sillemi* (Esben-Petersen): prey-mediated tri-trophic analysis

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Abstract

Transgenic *Bt* cotton with genes from soil inhabiting spore forming bacterium *Bacillus thuringiensis* Berliner produces δ-endotoxin for the control of lepidopteran insects. The prey-mediated effects of Cry protein on the third trophic level is the most realistic exposure pathway that needs to be addressed as an important component of environment risk assessment. The green lacewing, *Chrysoperla zastrowi sillemi* (Esben-Petersen) (Neuroptera: Chrysopidae) is the most important generalist predator in the cotton ecosystem in India. The tri-trophic interactions involving *Bt* cotton expressing single (Cry1Ac) and dual toxins (Cry1Ac and Cry2Ab) fed herbivores, i.e. mealybug, *Phenacoccus solenopsis* Tinsley, whitefly *Bemisia tabaci* (Gennadius) and leafhopper, *Amrasca biguttula biguttulla* (Ishida) on the fitness of *C. zastrowi sillemi*, were studied. The development, survival and body weight of *C. zastrowi sillemi* had no deleterious effect as there were insignificant differences in any of the studied fitness parameters regardless of having consumed prey fed on Bollgard, Bollgard II and non-*Bt* cotton plants. The feeding potential of *C. zastrowi sillemi* on mealybug was also not different on *Bt* or non-*Bt* cotton plants. ELISA studies confirmed the presence of Cry proteins in *Bt* cotton leaves; however, no Cry1Ac or Cry2Ab protein was detected in prey herbivores (*P. solenopsis*, *B. tabaci* and *A. biguttula biguttula*) or in the predator *C. zastrowi sillemi*. It could be concluded that transgenic cotton that expresses single (Cry1Ac) or dual (Cry1Ac and Cry2Ab) toxins had no apparent effect on the fitness of the predator through its preys *P. solenopsis*, *B. tabaci* and *A. biguttula biguttulla*.

Keywords: Transgenic *Bt* cotton, Cry1Ac, Cry2Ab, *Chrysoperla zastrowi sillemi*, Risk assessment, Non-target effects

Background

Genetically modified cotton with genes from soil inhabiting spore-forming bacterium *Bacillus thuringiensis* Berliner (*Bt*) produces δ-endotoxin, which is lethal to many lepidopteran pests. The area planted under biotech upland cotton globally in 2017 was 24.1 million hectares, India, having the largest area. In India, the area under *Bt* cotton has increased from 50,000 ha in 2002 to 11.4 million hectares in 2017, representing an unprecedented 227-fold jump in 16 years (James 2017). Initially, the cotton scenario was dominated by *Bt* cotton varieties/hybrids producing a single Cry protein (Cry1Ac), but these have now been replaced by a second generation of cotton producing two Cry proteins (Cry1Ac and Cry2Ab) (Kumar et al. 2014). The adoption of transgenic *Bt* cotton in countries such as India has changed the entire pest scenario in the cotton ecosystem. The pest status of bollworms and leaf-feeding insects has declined, but sap feeders, including whitefly, *Bemisia tabaci* (Gennadiaus); leafhopper, *Amrasca biguttula biguttula* (Ishida); mealybug, *Phenacoccus solenopsis* Tinsley; thrips, *Thrips tabaci* (Lindemann); aphid, *Aphis gossypii* (Glover); and mirid, *Creontiades biseratense* (Distant), have emerged as serious pests (Kumar et al. 2015).

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Studies conducted in Australia, USA, China and India have indicated that the Cry proteins in transgenic *Bt* cotton are available throughout the cropping season with temporal and spatial variations (Kranthi et al. 2005 and Shera and Arora 2016). So the target and non-target arthropods are continuously exposed directly or indirectly to Cry proteins, expressed in *Bt* cotton plants. At the third trophic level, predators and parasitoids may get exposed to Cry proteins, when they feed on herbivores that have consumed plant tissues having *Bt* protein (Torres et al. 2006; Mota et al. 2012; and Kumar et al. 2014). Exposure on the third trophic level through prey is the most realistic exposure pathway that needs to be addressed as an important component of environment risk assessment.

The chrysopids (Neuroptera) commonly known as lacewings or aphid lions are among the most beneficial predators of agricultural ecosystems and a potent arsenal of biological control. *Chrysoperla zastrowi sillemi* (Esben-Petersen), previously known as *Chrysoperla carnea* (Stephen), is the most important generalist predator in the cotton ecosystem in India. Adults are free living while larvae feed on soft-bodied insects like aphids, whitefly, leafhoppers, thrips, mites, and eggs and larvae of many lepidopteran pests (Takalloozadeh 2015). Thus, this predator can be exposed to Cry toxins by feeding on preys, which may affect its fitness parameters and thus hamper its performance as a biocontrol agent.

Most studies on the ecological impact of transgenic *Bt* cotton (Cry1Ac) or *Bt* corn (Cry1Ab) on the chrysopids (*C. carnea*, *C. rufilabris*, *C. externa* and *Chrysopa pallens*), using non-target preys, have focused only on aphids or mites (Torres et al. 2006 and Mota et al. 2012). Studies on the interactions involving *Bt* cotton, non-target herbivores (*P. solenopsis*, *B. tabaci* and *A. biguttula biguttula*) and their predator *C. zastrowi sillemi* have not been previously reported.

The objectives of the study were (1) to quantify the impact of cotton cultivars expressing single toxin (Bollgard; Cry1Ac), dual toxins (Bollgard II; Cry1Ac + Cry2Ab) and non-toxin on the survival, development, and body weight of the predator *C. zastrowi sillemi* through non-target sucking insect pests (mealybug, whitefly and leafhopper); (2) to study the predatory potential of the predator when fed on cotton mealybug *P. solenopsis* reared on *Bt* cotton; and (3) to study whether Cry1Ac and Cry2Ab proteins can pass via the food chain to the third trophic level.

Materials and methods

Plant material

Two transgenic *Bt* cotton hybrids, MRC 6301 *Bt* (event Mon 831) and Ankur 3028 BG-II (event 15895), approved by Genetic Engineering Approval Committee (GEAC) in India and one non-*Bt* variety, LH 2108, recommended by Punjab Agricultural University, Ludhiana, were used for the experiments. MRC 6301 *Bt* plants express *Cry1Ac* gene from *B. thuringiensis* targeting bollworms, while Ankur 3028 BG-II plants express *Cry1Ac* and *Cry2Ab* genes targeting bollworms and leaf-feeding insects (Navarro and Hautea 2014). Seeds were individually sown in earthen pots (12 l), filled with humus rich soil and plants were raised for rearing of herbivores and further experimentation.

Insect materials (preys)

Plants raised in pots were used for rearing of sucking insect pests *P. solenopsis*, *B. tabaci* and *A. biguttula biguttula* in separate insect proof screen cages (1.5 × 1.5 × 1.5 m) under field conditions. The insects were initially collected from field-grown eggplant, *Solanum melongena* L., and okra, *Abelmoschus esculentus* (L.) Moench, crops. Different screen cages were also used for Bollgard, Bollgard II and non-*Bt* cultivars to prevent insects from moving between cultivars. The plants of the respective cotton cultivar/variety were changed from time to time for continuous supply of fresh food to the insects and availability of their culture throughout the study period. The cultures of all insects used as preys in bioassays were maintained for multiple generations.

Predator

The culture of *C. zastrowi sillemi* was maintained on eggs of *Corcyra cephalonica* (Stainton) (Lepidoptera: Pyralidae), as a factitious host, in the Biocontrol Laboratory at Entomological Research Farm, Punjab Agricultural University, Ludhiana. Field-collected *C. zastrowi sillemi* adults were released in specially designed wooden oviposition cage (50 × 30 × 20 cm; Amar Chand & Company, India) with a sliding roof plank, covered with black muslin cloth. Standard adult diet (honey 1 g, sucrose 5 mg, protein 5 g, yeast 1 g and distilled water 40 ml) was provided twice daily as droplets on Perspex sheet strips by the help of a fine camel hair brush (Sattar and Abro 2011). The stalked eggs laid by the females on the roof plank were destalked after 24 h by the help of a sterilised razor blade. These eggs were transferred to individual plastic vials (4 × 3 cm) by the help of a soft camel hair brush. After hatching, fresh eggs of *C. cephalonica* were provided daily in each individual vial to the *Chrysoperla* larva till pupation. The adults emerging from the cocoons were collected individually and transferred again to the oviposition cages. The newly emerged adults were provided by a nutritional diet as described earlier. The culture of *C. zastrowi sillemi* was used for further experimentations.

Tri-trophic bioassay with *C. zastrowi sillemi*

Development, survival and body weight

All bioassays were conducted in an environmental chamber at 27 ± 2 °C and 65 ± 5% RH (Macro Scientific Works Ltd., India). Leaves from the upper third portion of 70-day-old Bollgard or Bollgard II or non-*Bt* plants were detached and placed in separate plastic jars (20 × 10 cm) lined with a muslin cloth for aeration. Similar sized mealybug nymphs ($n = 50$), whitefly adults ($n = 50$) and leafhopper nymphs ($n = 50$), from the stock cultures reared on Bollgard or Bollgard II or non-*Bt* leaves were collected and released in the respective jars. A soft camel hair brush was used for collection and release of mealybug nymphs, while an aspirator (Rescholar Equipment, India) was used to collect whitefly adults and leafhopper nymphs. The collected insects were released in separate plastic containers, with a hole in the screw cap. The cotton leaf was placed in each container before release of insects. The petiole of detached leaves was wrapped with water-soaked cotton swab so as to keep the leaves fresh and turgid for a longer period of time. A newly hatched single *Chrysoperla* larva was introduced in each plastic container for feeding. The hole was then plugged by a cotton wool. Each prey treatment (*P. solenopsis*, *B. tabaci* and *A. biguttula biguttula*), reared on Bollgard, Bollgard II or non-*Bt* leaves, was conducted simultaneously. The experiment was initiated with 30 *Chrysoperla* larvae for each treatment (one larva/replication). *Chrysoperla* larvae were inspected twice daily, and life-table parameters (development and mortality) were recorded. Last (3rd) instar *Chrysoperla* larvae, in each prey-predator combination, were weighed at the end of bioassay, using an electronic balance. The cocoons were collected, weighed and kept in separate glass vials individually to record the pupal period and percentages of adults' emergence. After emergence from cocoons, adults were provided by an adult diet as described earlier and their longevity was recorded (Additional file 1).

Predatory potential

An experiment on predatory potential was conducted in an environmental chamber set at 27 ± 2 °C and 65 ± 5% RH (Macro Scientific Works Ltd., India). In no-choice tests, 2nd instar mealybug nymphs ($n = 30$) reared on Bollgard or Bollgard II or non-*Bt* leaves were released by the help of a soft camel hair brush on respective leaves in separate plastic jars (20 × 10 cm) lined with muslin cloth for aeration (10 replications for each set). A newly hatched single *Chrysoperla* larva was introduced in each jar for feeding. The number of mealybugs consumed were counted daily and replaced with new sets till pupation. The mean consumption of mealybugs per day was worked out for each larval instar.

Cry toxin expression in tri-trophic pathway (cotton leaves, prey and predator)

To confirm Cry1Ac and Cry2Ab expression in bioassays, leaf samples were collected from the upper third portion of 70-day-old Bollgard (Cry1Ac) or Bollgard II (Cry 1Ac and Cry2Ab) or non-*Bt* plants. Leaf discs measuring 20 mm per replication were placed in separate 1.5 ml centrifuge tubes, and Cry protein measurements, using enzyme-linked immunosorbent assays (ELISA; see below), were made. To quantify *Bt* toxin in *P. solenopsis* nymphs, *B. tabaci* adults and *A. biguttula biguttula* nymphs, protein was extracted from each prey herbivore, reared either on Bollgard or Bollgard II or non-*Bt* leaves. Similarly, to assess the potential transfer of Cry1Ac and Cry2Ab proteins via food chain, *C. zastrowi sillemi* larvae fed on prey herbivores reared on Bollgard or Bollgard II or non-*Bt* leaves were collected and assayed, using ELISA. For all ELISA samples, five replications were maintained.

ELISA procedures

Bt protein concentrations in cotton leaves, insect prey and predator were measured, using sandwich ELISA, using quantiplate kits (Wu et al. 2014). The concentration of Cry1Ac protein was measured, using Cry 1Ab/Cry1Ac kit (AP 003 QT V50) and Cry2Ab using Cry2A kit (AP 005 QT BC V50) of ENVIROLOGIX 500 Riverside Industrial Parkway Portland, ME, USA. Before analysis, all insects (preys and predator) were washed in phosphate-buffered saline with Tween-20 buffer to remove any protein from their outer surface. Leaf samples weighing 20 mg were homogenised in 0.5 ml buffer solution and diluted (1: 10 and 1: 50) for Cry1Ac and Cry2Ab, respectively. Quantities (mg/replication) of material and buffer dilutions used from different prey herbivores and predator were ≈20 mg *P. solenopsis* (in batch) homogenised in 0.5 ml buffer (no dilution), ≈20 mg *B. tabaci* (in batch) homogenised in 0.5 ml buffer (no dilution), ≈20 mg *A. biguttula biguttula* (in batch) homogenised in 0.5 ml buffer (no dilution) and 15 ± 2 mg *C. zastrowi sillemi* homogenised in 0.5 ml buffer (no dilution). All the samples in buffer were ground by hands, using a plastic pestle. For every sample, a fresh pestle was used to avoid any possible cross contamination of the individual sample. After vortexing for 3 h on a vortex shaker (Spinix; Tarson Products Ltd., India), centrifugation for 1 min (at 10,000 rpm in microcentrifuge) (Eppendorf AG 5415D, Germany) and appropriate dilution of supernatants, ELISA was performed according to manufacturer's protocol. The absorbance of each well solution was recorded at 450 nm by using micro filter plate reader (Thermo Electron Corporation, China). The optical density (OD) value of each calibrator and corresponding concentrations of Cry1Ac and Cry2Ab

(standards provided in the kit) were used to prepare the standard curve. The proteins' concentration of each sample was determined by finding its OD value and the corresponding concentration level in the linear curve, using regression analysis. The results from standard curve were multiplied by the dilution factor incurred during extraction. To determine the dilution factor, the volume in milliliter of extraction solution was divided by weight of samples in grams. For leaf samples, protein concentrations in μg g^{-1} fresh weight were calculated by multiplying these results by (1:10 or 1:50) dilutions made for Cry1Ac and Cry2Ab, respectively.

Data analyses

The data on the life parameters (larval period, pupal period and adult longevity) and weight (larval and cocoon weight) were subjected to one-way ANOVA and Tukey's multiple range test. Data are presented as mean ± standard error. Data on *Chrysoperla* larval and pupal survivals were analysed and presented in the form of Wald Chi-square χ2 test and *P* values. Data on feeding performance were subjected to two-way ANOVA and Tukey's multiple range tests. All statistical tests were carried out using IBM SPSS 22.0 for Windows (IBM Corporation, Armonk, New York, USA).

Results and discussion

Development, survival and weight of *C. zastrowi sillemi* fed on *P. solenopsis* reared on *Bt* and non-*Bt* cotton leaves

Different life parameters, i.e. larval period ($F = 0.01$; df = 2.70; $P = 0.994$), pupal period $F = 0.08$; df = 2.60; $P = 0.927$) and adult longevity ($F = 0.01$; df = 2.60; $P = 0.987$) of *C. zastrowi sillemi* did not differ when fed on *P. solenopsis* reared on Bollgard or Bollgard II or non-*Bt* cotton leaves (Table 1). There was also insignificant difference in larval ($\chi^2 = 0.324$; df = 2; $P = 0.956$) and pupal survival ($\chi^2 = 0.333$; df = 2; $P = 0.954$) among the treatments. The prey mediated effects of Bollgard or Bollgard II on the body weight of last larval instar ($F = 0.09$; df = 2.57; $P = 0.912$), cocoon ($F = 0.04$; df = 2.57; $P = 0.958$) and adults ($F = 0.06$; df = 2.27; $P = 0.938$) of *Chrysoperla* also showed insignificant difference than the non-*Bt* cotton (Table 1).

Development, survival and weight of *C. zastrowi sillemi* fed on *B. tabaci* reared on *Bt* and non-*Bt* cotton leaves

The prey (*B. tabaci*)-mediated effects of *Bt* cotton, expressing single (Cry1Ac) or dual (Cry1Ac Cry2Ab) toxin, on the larval ($F = 0.01$; df = 2.73; $P = 0.991$) and pupal periods ($F = 0.08$; df = 2.60; $P = 0.926$) of *C. zastrowi sillemi* were not different than the non-*Bt* cotton. Similarly, the larval survival ($\chi^2 = 0.317$; df = 2; $P = 0.957$) and pupal survival ($\chi^2 = 0.463$; df = 2; $P = 0.927$) survival also were not significantly affected by the treatments. The body weight of larvae ($F = 0.03$; df = 2.57; $P = 0.971$), and cocoons ($F = 0.07$; df = 2.57; $P = 0.929$) had insignificant difference when Bollgard or Bollgard II or non-*Bt* cotton leaves fed *B. tabaci* adults were offered to *Chrysoperla* as prey (Table 2).

Development, survival and weight of *C. zastrowi sillemi* fed on *A. biguttula biguttula* reared on *Bt* and non-*Bt* cotton leaves

The developmental period of *C. zastrowi sillemi* immature stages, i.e. larval ($F = 0.01$; df = 2.87; $P = 0.980$) and pupal periods ($F = 0.07$; df = 2.54; $P = 0.930$), had insignificant difference, when fed on the prey (*A. biguttula biguttula*), reared on cotton cultivars expressing single (Cry1Ac) or dual (Cry1Ac and Cry2Ab) or no toxin. Further, there were also insignificant differences in larval ($\chi^2 = 0.342$; df = 2; $P = 0.952$) and pupal survivals ($\chi^2 = 0.375$; df = 2; $P = 0.945$) of the predator among different treatments. Larval ($F = 0.03$; df = 2.57; $P = 0.957$) and cocoon weights ($F = 0.02$; df = 2.54; $P = 0.979$) of *Chrysoperla* were also not affected, regardless to the prey that had fed on cotton cultivars with or without toxin (Table 3).

Table 1 Development time, survival and weight of *Chrysoperla zastrowi sillemi* fed on *Phenacoccus solenopsis* reared on *Bt* and non-*Bt* cotton cultivars

Parameters	Bollgard (Cry1Ac)	Bollgard II (Cry1Ac and Cry2Ab)	Non-*Bt*	Statistical analysis
*Larval period (days)	11.13 ± 0.33 (24)	11.12 ± 0.29 (25)	11.08 ± 0.35 (24)	$F = 0.01$; df = 2.70; $P = 0.994$
#Larval survival (%)	80.0	83.3	80.0	$\chi^2 = 0.324$; df = 2; $P = 0.956$
*Pupal period (days)	7.19 ± 0.15 (21)	7.14 ± 0.16 (21)	7.24 ± 0.21 (21)	$F = 0.08$; df = 2.60; $P = 0.927$
#Pupal survival (%)	87.5	87.5	88.0	$\chi^2 = 0.333$; df = 2; $P = 0.954$
*Adult longevity (days)	29.19 ± 0.97 (21)	29.14 ± 1.05 (21)	29.38 ± 1.18 (21)	$F = 0.01$; df = 2.60; $P = 0.987$
*Larval weight (mg)	10.85 ± 0.29 (20)	10.90 ± 0.35 (20)	11.05 ± 0.37 (20)	$F = 0.09$; df = 2.57; $P = 0.912$
*Cocoon weight (mg)	13.10 ± 0.59 (20)	13.00 ± 0.54 (20)	13.25 ± 0.68 (20)	$F = 0.04$; df = 2.57; $P = 0.958$
*Adult weight (♀) (mg)	13.70 ± 0.37 (10)	13.60 ± 0.40 (10)	13.80 ± 0.42 (10)	$F = 0.06$; df = 2.27; $P = 0.938$

Means (± SE), number of replicates given in parentheses, the experiment started with 30 larvae in each treatment; *one-way ANOVA ($p < 0.05$), #chi-square test ($p < 0.05$)

Table 2 Development time, survival and weight of *Chrysoperla zastrowi sillemi* fed on *Bemisia tabaci* reared on *Bt* and non-*Bt* cotton cultivars

Parameters	Bollgard (Cry1Ac)	Bollgard II (Cry1Ac and Cry2Ab)	Non-*Bt*	Statistical analysis
*Larval period (days)	10.85 ± 0.26 (26)	10.84 ± 0.29 (25)	10.80 ± 0.21 (25)	$F = 0.01$; df = 2.73; $P = 0.991$
#Larval survival (%)	86.7	83.3	83.3	$\chi^2 = 0.317$; df = 2; $P = 0.957$
*Pupal period (days)	7.14 ± 0.17 (22)	7.10 ± 0.14 (20)	7.05 ± 0.18 (21)	$F = 0.08$; df = 2.60; $P = 0.926$
#Pupal survival (%)	84.6	80.0	84.0	$\chi^2 = 0.463$; df = 2; $P = 0.927$
*Larval weight (mg)	11.00 ± 0.28 (20)	11.00 ± 0.36 (20)	11.15 ± 0.32 (20)	$F = 0.03$; df = 2.57; $P = 0.971$
*Cocoon weight (mg)	13.65 ± 0.55 (20)	13.55 ± 0.60 (20)	13.75 ± 0.61 (20)	$F = 0.07$; df = 2.57; $P = 0.929$

Means (± SE), number of replicates given in parentheses, the experiment started with 30 larvae in each treatment; *one-way ANOVA ($p < 0.05$), #chi-square test ($p < 0.05$)

Bt proteins in genetically modified crops may pose a risk to non-target beneficial arthropods providing biological control, pollination and decomposition services in the ecosystem. The possible lethal effects posed by insecticidal proteins in biological control agents can be determined under laboratory studies in different ways (Tian et al. 2013): (1) directly, the organism can be exposed to the protein incorporated in an artificial diet or mixed with non-genetically and genetically modified plant materials; and (2) indirectly, predatory species can be offered target or non-target prey herbivores fed on genetically modified plants. The latter way involving prey-mediated effects at higher trophic level provides a very realistic exposure pathway and has been experimented in numerous tri-trophic interaction studies with genetically modified *Bt* crops. Target prey herbivores susceptible to *Bt* proteins may result in prey quality-mediated effects at the third trophic level. One way to avoid this impact is to use non-target herbivores, which are not susceptible to proteins expressed in transgenic plants (Romeis et al. 2011).

Obtained results revealed that the larval period and survival, pupal period and survival, and adult longevity of *C. zastrowi sillemi* as well the body weight (larval, cocoon or adult) of the predator were not different when the predator was offered *P. solenopsis* or *B. tabaci* or *A. biguttula biguttula* as food that had consumed cotton leaves expressing single toxin (Cry1Ac) or two toxins (Cry1Ac and Cry2Ab) or no toxin. Non-significant differences might be due to the fact that Cry1Ac and Cry2Ab proteins were not detected in any of the herbivore (lack of exposure) and consequently not reflected in the third trophic level. The results agree with Guo et al. (2008). Similar findings have been reported in tri-trophic interactions between *Bt* cotton (Cry1Ac), *A. gossypii* and *C. carnea* (Magar et al. 2012) and *C. externa* (Mota et al. 2012). In a comprehensive review, Romeis et al. (2014) concluded that no direct impact on survival and developmental parameters has also been noticed in *C. carnea* feeding on *Bt* maize pollen containing Cry 1Ab or Cry 3Bb1 (Li et al. 2008) and Cry 1Ab protein mixed in sucrose diet (Romeis et al. 2014). Simon et al. (2006) reported that Cry1Ab or Cry2Ab toxins did not have detrimental effects on *C. carnea* when ingested either directly or through prey as the larval midgut lacks specific receptors for these proteins. No prey-mediated effects of Cry proteins have also been reported in other predatory species including the coccinellids (Wu et al. 2014), the rove beetles (Garcia et al. 2010) and the wolf spiders (Niu et al. 2017).

Predatory potential of *C. zastrowi sillemi* fed on *P. solenopsis* reared on *Bt* and non-*Bt* cotton leaves

Daily consumption rate of *P. solenopsis* by *C. zastrowi sillemi* on non-*Bt* cotton was not different than the *Bt* cotton expressing single (Cry1Ac) or dual (Cry1Ac Cry2Ab) toxin ($F = 0.04$; df = 2.81; $P = 0.961$) (Table 4). However,

Table 3 Development time and survival of *Chrysoperla zastrowi sillemi* fed on *Amrasca biguttula biguttula* reared on *Bt* and non-*Bt* cotton cultivars

Parameters	Bollgard (Cry1Ac)	Bollgard II (Cry1Ac and Cry2Ab)	Non-*Bt*	Statistical analysis
*Larval period (days)	14.48 ± 0.21 (23)	14.45 ± 0.29 (22)	14.43 ± 0.26 (23)	$F = 0.01$; df = 2.65; $P = 0.993$
#Larval survival (%)	76.7	73.3	76.7	$\chi^2 = 0.342$; df = 2; $P = 0.952$
*Pupal period (days)	7.68 ± 0.20 (19)	7.63 ± 0.21 (19)	7.58 ± 0.18 (19)	$F = 0.07$; df = 2.54; $P = 0.930$
#Pupal survival (%)	82.6	86.4	82.6	$\chi^2 = 0.375$; df = 2; $P = 0.945$
*Larval weight (mg)	10.05 ± 0.35 (20)	10.00 ± 0.37 (20)	10.11 ± 0.32 (20)	$F = 0.02$; df = 2.57; $P = 0.979$
*Cocoon weight (mg)	12.85 ± 0.63 (19)	13.00 ± 0.54 (19)	13.05 ± 0.68 (19)	$F = 0.03$; df = 2.54; $P = 0.969$

Means (± SE), number of replicates given in parentheses, the experiment started with 30 larvae in each treatment; *one-way ANOVA ($p < 0.05$), #chi-square test ($p < 0.05$)

Table 4 Predatory potential of *Chrysoperla zastrowi sillemi* fed on *Phenacoccus solenopsis* reared on *Bt* and non-*Bt* cotton cultivars

Chrysoperla stages	Mean number (± SE) of *Phenacoccus solenopsis* nymphs consumed per day when reared on			
	Bollgard (Cry1Ac)	Bollgard II (Cry1Ac and Cry2Ab)	Non-*Bt*	Mean
1st instar	14.2 ± 0.36	14.0 ± 0.33	14.3 ± 0.45	14.17 ± 0.38c
2nd instar	18.4 ± 0.34	18.2 ± 0.47	18.1 ± 0.41	18.23 ± 0.40b
3rd instar	35.9 ± 0.53	36.0 ± 0.60	36.1 ± 0.46	36.00 ± 0.53a
Mean	22.83 ± 0.41	22.73 ± 0.47	22.83 ± 0.44	–
ANOVA				
Instars	$F = 1803.73$; df = 2.81; $P < 0.001$			
Cultivars	$F = 0.04$; df = 2.81; $P = 0.961$			
Interaction	$F = 0.15$; df = 4.81; $P = 0.861$			

Means (± SE) followed by the same letter are not significantly different (LSD, $P < 0.05$)

consumption by different larval instars were significantly different from each other ($F = 1803.73$; df = 2.81; $P < 0.001$). Third instar *Chrysoperla* larvae consumed more preys, followed by the 2nd and 1st instar larvae. The interaction between the factors, cotton type and *Chrysoperla* larval stages, was also insignificant and indicating that differential consumption rate by larval stages was not a function of the cotton type (*Bt* or non-*Bt* cotton) ($F = 0.15$; df = 4.81; $P = 0.861$).

Quantification of Cry proteins in tri-trophic pathway

The concentration of Cry1Ac protein in MRC 6301 *Bt* cotton leaves averaged (2.52 ± 0.02 μg/g fresh weight). The expressed levels of Cry1Ac and Cry2Ab proteins in Ankur 3028 BG-II leaves averaged (1.95 ± 0.02 μg/g) and (21.64 ± 0.32 μg/g) fresh weight, respectively. ELISA studies revealed that prey herbivores (*P. solenopsis*, *B. tabaci* and *A. biguttula biguttula*) reared on Bollgard and Bollgard II leaves did not contain detectable amounts of the Cry1Ac or Cry2Ab protein. Similarly no Cry1Ac or Cry2Ab protein was detected in *C. zastrowi sillemi* larvae fed on any of the three prey herbivore. None of non-*Bt* cotton leaves or prey herbivores or *C. zastrowi sillemi* samples was found to contain any Cry1Ac or Cry2Ab protein in respective tri-trophic pathway.

The expression of Cry proteins in Bollgard (Kranthi et al. 2005) and Bollgard II (Kranthi et al. 2009) cotton have been known to be high in leaves as compared to other plants parts. In the present study, high concentrations of both Cry1Ac and Cry2Ab proteins in plant leaves were recorded. Moreover, Cry2Ab protein was expressed at higher level in Bollgard II than Cry1Ac which is consistent with earlier studies (Kranthi et al. 2009 and Shera and Arora 2016).

The majority of earlier studies that aimed to quantify the Cry protein concentration in non-target herbivores that have fed on *Bt* transgenic plants have shown Cry proteins, either absent or detected at very low levels. Highly varying concentrations of Cry proteins in herbivores depend on their feeding mode and feeding location on the plant (Eisenring et al. 2017). Phloem feeders (mealybugs, whiteflies, aphids) do not ingest the Cry proteins, but cell content feeders (spider mites), herbivores that do not target the phloem (thrips, plant bugs), or tissue feeders (beetles, caterpillars) ingest relatively high concentrations of Cry proteins (Eisenring et al. 2017; Meissle and Romeis 2018). Raps et al. (2001) reported that *Bt* toxin is not present in the phloem sap, so aphids do not ingest the toxin feeding on *Bt* maize plants, thereby excluding its direct effects on aphids or on other phloem feeding arthropods. Similarly, the toxin content (Cry1Ab) from *Bt* maize was found to be negligible in leafhoppers (Obrist et al. 2006). In contrast, the leafhoppers *Empoasca pteridis* (Dahlbom), *Eupteryx atropunctata* (Goeze) and *Zyginidia scutellaris* (Herrich-Schäffer) also feed on mesophyll cells and thus ingest certain amount of Cry protein. This has been shown previously for *Z. scutellaris*, which was found to acquire certain amount of Cry protein (Cry1Ab) from *Bt* maize (Dutton et al. 2004).

Conclusions

It could be concluded that phloem feeders like *P. solenopsis*, *B. tabaci* and *A. biguttula biguttula* contained no detectable Cry proteins (Cry1Ac or Cry2Ab) despite their high expression levels in Bollgard or Bollgard II cotton leaves. Considering that the green lacewing *C. zastrowi sillemi* is mostly a generalist predator of phloem feeders in the cotton crop, it is unlikely that these non-target herbivores feeding on *Bt* cotton pose any risk to this beneficial predator. Thus, this important generalist predator will continue to render its biological control services in the cotton ecosystem dominated by transgenic *Bt* cotton cultivars.

Additional file

Additional file 1: Development time, survival and weight of *Chrysoperla zastrowi sillemi* fed on *Phenacoccus solenopsis* reared on *Bt* and non-*Bt* cotton cultivars. (DOCX 31 kb)

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