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Transfer of the Cry1Ab Toxin in Tritrophic Bioassays Involving Transgenic Maize MON810, the Herbivore *Tetranychusurticae*Koch and the Predatory Ladybird Beetle *Adalia bipunctata* L. (Coleoptera: Coccinelidae)

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ABSTRACT

Laboratory bioassays of tritrophic were conducted to determine whether *Adalia bipunctata* L. (Coleoptera: Coccinelidae) larvae might accumulate and/ or degrade Cry1Ab toxin in relation to different exposure time with *Bt*-loaded prey. The spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae) was used as herbivore. The concentration of Cry1Ab toxin through the trophic chain decreased from *Bt* maize plants (60.49 µg/g^{-1} FW) to spider mites (0.12 µg/g^{-1} FW). The third trophic level, predatory larvae of *A. bipunctata*, showed different mean concentration of *Bt* toxin between feeding periods. In first repetition significant difference of *Bt* content (0.128 µg/g^{-1} FW) was obtained only during 72 hours feeding. The second repetition of bioassay showed higher mean contents of Cry1Ab toxin than the first. The highest concentration of *Bt* toxin content as was indicated in the first repetition during the 72 hours feeding time. The analysis in both bioassays showed predominantly similar pattern of Cry1Ab degradation through the tritrophic system (*Bt* maize plants-herbivorous prey-predatory ladybeetle larvae).

Key words: *Adalia bipunctata*; *Tetranychus urticae,* Cry1Ab; toxin; tritrophic, transfer, bioassay.

INTRODUCTION

Transgenic maize expressing insecticidal Cry toxins from *Bacillus thuringiensis* Berliner (*Bt*) has been cultivated in the USA since 1996 (James, 2011). *Bt* maize cultivars producing Cry1Ab provide an effective control of the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae), the most serious lepidopterous pest in Europe and North America. Other commercialized cultivars of *Bt* maize are those expressing the Cry1Ac or Cry1F proteins, providing control of lepidopterous pests and Cry34Ab1/Cry35Ab1 or Cry3Bb1 proteins to control *Diabriotica* spp. (Coleoptera: Chrysomelidae) (Archer *et al.* 2001, Bravo and Soberon 2008 and Hari *et al.* 2008).

Prior to the commercial release, *Bt* crops are subjected to an environmental risk assessment to identify potentially adverse effects on non-target arthropods, executing important ecological functions. Predators are one group of non-target arthropods which have been of special interest because of their high abundance in agro-ecosystems and since they are useful in the biological control of different agricultural systems. Some of the species could constitute appropriate indicators of potential ecological impacts of GM crops, but only when ingesting the toxin in an indirect way, *i.e.* through feeding on herbivorous prey. One prime example of a tested and contested group of non-target organisms (NTOs) are coccinellid beetle larvae that often execute two ecological functions in nature, biological control and pollination. Some laboratory studies did not find adverse effects on tested ladybird beetle species, like *Coleomegilla maculata*, *Hippodamia convergens* or *Stethorus punctillum* when exposed either directly to Cry proteins (US EPA 2001, 2003 and Duan *et al.* 2002) or when fed Cry1Ab or Cry3Bb1 expressing maize pollen or *Bt* maize fed herbivores (Pilcher *et al.* 1997; Lundgren and Wiedenmann, 2002; Ahmad *et al.* 2006, Alvarez-Alfageme *et al.* 2008 and Li and Romeis, 2010). However, other studies did reveal adverse effects or reported mixed results of different Cry toxins. For example, Dhillon and Sharma (2009) found direct toxic effects of Cry1Ab on larvae of *Cheilomenes sexmaculatus* (Linnaeus) in some bioassays. For larvae of the coccinellid *Adalia bipunctata*, adverse effects of Cry1 and Cry3 toxins were reported using continuous exposure protocol throughout the entire larval stage (Schmidt *et al.* 2009 and Hilbeck *et al.* 2012). Other authors (Porcar *et al.* 2010 and Alvarez-Alfgamene *et al.* 2011) did not observe statistically significant adverse effects of Cry1Ab or Cry3Bb1 on larvae of this species when using a different recovery/ exposure protocol or a short-term exposure assay protocol, although mortality in the Cry1 treatments at least in one of the studies was also about 10 and 13% higher than in the control treatments (Alvarez-Alfageme *et al.* 2011).

In field trials, researchers reported recently that significantly fewer coccinellids of the species

Harmonia axyridis were found in *Bt* maize than in non-*Bt* maize and that the lifespan of the adult *H. axyridis* was significantly reduced by 38% feeding on *Bt*-containing diet versus those fed a *Bt*-free diet in laboratory trials. In laboratory feeding studies with third instar larvae and adults of *H. axyridis*, Dutra *et al.* (2012) found low concentration of Cry1Ab in predator bodies. Overall, the huge number of published papers provides no indication that the current *Bt* maize varieties cause direct impact on arthropods that are not closely taxonomically related to the target pest (Romeis *et al.* 2006; Wolfenbarger *et al.* 2008; Meissle and Romeis 2008; Naranjo 2009; Ricroch *et al.* 2010 and Duan *et al.* 2010).

The predatory ladybird species, *Adalia bipunctata* L., is a native coccinellid in many ecosystems in Europe, feeding predominantly on aphids, consuming also other soft-bodied arthropods and pollen (Hodek and Honek 1996). Thus, contact with Cry toxins might occur via multiple pathways, *e.g.* occur simultaneously, when feeding on plant material (pollen, nectar or leaf exudates) or on prey which consumed transgenic plant tissue and contains the Cry toxins. There is a lack of knowledge about transfer in time of Cry1Ab toxin through trophic chains.

Therefore, tritrophic bioassays to determine whether *A. bipunctata* larvae might accumulate and/ or degrade Cry1Ab toxin due of different time exposure with *Bt*-loaded prey were conducted. The spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae), was selected as the carrier because among arthropod preys of *A. bipunctata*, this species is known to contain the highest concentrations of Cry proteins, when fed on *Bt* maize (Dutton *et al.* 2002; Obrist *et al.* 2006; Meissle and Romeis 2009 and Li and Romeis 2010).

MATERIALS AND METHODS

Plant material

The plant material composed of transgenic maize (*Zea mays* L.) plants DKC 3421 Yield Gard (event MON810) from the Monsanto Company (St. Louis, MO, USA), expressing a gene encoding a truncated form of the Cry1Ab protein derived from *Bacillus thuringiensis* Berliner and the corresponding near-isogenic hybrid DKC 3420. In the DKC 3421 maize, the toxin is produced continuously by the plant because the CryIA(b) gene has been linked under the control of the strong constitutive-enhanced CaMV 35S promoter. The plants were grown individually in plastic pots (12 cm in diameter), using garden soil (Kronen) as substrate, and kept in a greenhouse at 25±0.3^{0}C, 60±5% RH and 16:8 (L: D) photoperiod. The maize plants were used for the spider mite rearing once they had reached the 4-5 leaf stage and were removed when they reached anthesis.

Arthropod material

A mass culture of *T. urticae* was established from a laboratory colony raised at the Department of Applied Entomology of Warsaw University of Life Sciences, Poland. The spider mite was reared on *Bt* maize or control plants (isogenic line) in growth chambers where the plants were grown. The second instar larvae (L_2) of *A. bipunctata* larvae were purchased from Biopartner (Poznań, Poland). Upon arrival, they were individually placed in a plastic Petri dish arena (diameter 90 mm) containing two filter paper into biological chamber (Sanyo MLR-351 H, Sanyo, Japan) at 24±0.3^{0}C, 70±5% RH, L:D 16:8 h photoperiod. Only larvae in good fitness were used in experiments. Each predatory larva was offered a piece of leaf with 20 spider mite adults.

Tritrophic feeding study

The tritrophic bioassay assessed the transfer of *Bt* protein-expressing maize through food chain. The second larvae instar (L_2) was fed *ad libitum* with *T. urticae* reared on *Bt*-transgenic plants or on the respective non-transformed near isolines for several generations as described above. The bioassay was conducted in two subsequent runs with 10 replications each, resulting in a total of 60 *A. bipunctata* larvae per treatment. For every run, the *A. bipunctata* larvae were obtained from a different shipment and were fed by *T. urticae* reared on different plants. Experimental arenas consisted of small plastic Petri dishes (diameter 55 mm x height 30 mm) containing two filter paper discs. The predatory ladybird larvae were placed individually into dishes. The exposure treatments used in a trial were 24, 48 and 72 hours feeding periods, prey-*Bt* or non-*Bt* maize, respectively. All experiments were conducted in a climate chamber (Sanyo MLR-351 H, Sanyo, Japan) at 24±0.3^{0}C, 70±5% RH, L:D 16:8 h photoperiod. At the end of the assay, *A. bipunctata* larvae were transferred into 2.0 ml eppendorf tubes and frozen at -20ºC until Cry1Ab determination.

Samples of maize leaves and *T. urticae* were collected from *Bt* plants. Individuals of the spider mites were collected directly from maize leaves by using a brush. Maize leaf material and *T. urticae*, obtained from the 7th leaf from one plant, were separately placed into 2.0-ml micro-reaction tubes. For each of the experiments, 10 samples of leaves and *T. urticae* were collected from different maize plants, resulting in a total of 20 samples. Leaf material and *T. urticae* was similarly obtained from isogenic plants. Each sample contained 20 adults of spider mites. Larvae of *A. bipunctata* were pooled into a 1.5-ml micro-reaction tube as one sample. Twenty samples were used for each treatment (24, 48 and

72 h feeding periods) with a total of 60 samples.

Cry1Ab detection in maize leaves and insects

The level of the Cry1Ab toxin in maize leaves, herbivores and predatory larvae was determined by a sandwich ELISA adapted for quantitative purposes, using the EnviroLogix QualiPlate kit for Cry1Ab/Cry1Ac (EnviroLogix Inc. Portland, Maine, USA.). A *Bt*-Cry1Ab protein molecular weight 130 kDa, isolated from *Bt* spores, 10 µg/ml stock solution from Fitzgerald Industries International was used as a standard for the Cry1Ab standards with a concentration ranged from 0 to 10 ng/ml were used as calibrators. Samples were ground in an eppendorf tube with the help of liquid nitrogen, by rotating the pestle against the sides of the tube. One ml of extraction buffer was added to the crushed material and all was incubated for 90 minutes with slight shaking (200 rpm). After 5 min of centrifugation at 16 000 x g and 15ºC, 50 µl of the supernatant was directly used in the ELISA assay. Samples were analyzed in duplicates and the mean absorbance was calculated. The absorbance was measured at 450 nm using a microplate reader (Fluostar Optima, BMG LABTECH). Cry1Ab concentrations were determined by calculating the mean optical density reading against the standard curve. Cry1Ab expression was quantified as µg per fresh weight after multiplication with the dilution factors.

Data analysis

Because of outlier observations, linear models did not work well, as was clear from graphical methods for model diagnosis (Pinheiro and Bates, 2000, and Quinn and Keough, 2002). Thus, instead Kruskal test was employed (Quinn and Keough, 2002). Since the results were different for the two repetitions of the experiment, the repetitions were analyzed separately. After detecting significant differences among the treatments, pair-wise comparisons were verified by means of the Wilcoxon test (Quinn and Keough, 2002), but without adjustment for multiple testing (Webster 2007 and Kozak 2009).

RESULTS AND DISCUSSION

The time treatment affected the level of the Cry1Ab toxin in maize leaves in both repetitions of the experiment ($P < 0.001$ for both of them). However, pair-wise differences among treatments differed between the repetitions. In the first repetition, no significant difference was noticed in the level of the Cry1Ab toxin in *A. bipunctata* larvae between the 24 h (median of 0.019) and 48 h (median of 0.011) feeding treatments ($P = 0.060$). The 72 h feeding treatment (median of 0.128) significantly differed from both of them (in both cases $P < 0.001$). In the second repetition, all the three treatment times differed from each other in the level of the Cry1Ab toxin in predatory larvae. The smallest level was noticed at the 24 h treatment (median of 0.026) and then at the 48 h treatment (median of 0.085). The difference was significant at $P < 0.001$. The 72 h treatment (median of 0.133) was significantly greater than both of them (with $P < 0.001$ for the difference from the 24 h treatment and $P = 0.034$ for the difference from the 48 h treatment).

Cry1Ab toxin transfer through the trophic chain

A mean concentration of 60.49 µg Cry1Ab toxin per g of fresh weight was detected in *Bt* maize leaves. At the second trophic level, herbivore *T. urticae* contained an average of 0.12 µg per g of fresh weight. The third level, predatory larvae showed different concentrations of *Bt* toxin, depending on feeding period and number of repetition (first and second) (Table 1). As expected, the *Bt* toxin was not detected in non-*Bt* maize leaves, *T. urticae* raised on non-*Bt* maize plants or predatory larvae fed on herbivore.

The current study provides data about transfer of Cry1Ab toxin among trophic levels. Tritrophic bioassays were conducted using second instar larvae of predatory *A. bipunctata*, which were fed on *Bt* maize-fed spider mites. Bioassays results provided information about the fate of toxin along the tritrophic food chain. The experiments showed that predatory larvae ingested this toxin via *Bt*-prey in different volumes due to feeding time. The transfer of Cry1Ab from genetically modified maize through higher trophic levels has been reported for other coleopteran predators: the ground beetle *Poecilus cupreus* L. (Coleoptera: Carabidae) (Meissle *et al.* 2005, Alvarez-Alfgamene *et al.* 2009, Priesnitz 2010 and Grabowski *et al.* 2013, in press), the ladybird *Stethorus punctillum* Weise (Alvarez-Alfgamene *et al.* 2008), *Adalia bipunctata* L. (Coleoptera: Coccinellidae) (Schmidt *et al.* 2009 and Alvarez-Alfgamene *et al.* 2011) or the rove beetle *Atheta coriaria* Kraatz (Coleoptera: Staphylinidae) (Garcia *et al.* 2010). Moreover, Cry toxins might also reach

Table (1): Concentrations of *Bt* toxin in *A. bipunctata* larvae between different feeding periods

	Treatment	Median level of the Cry1Ab toxin in predatory larvae µg/g FW
Repetition 1	24 h	0.019 a[1]
	48 h	0.011 a
	72 h	0.128 b
Repetition 2	24 h	0.026 a
	48 h	0.085 b
	72 h	0.133 c

[1] Different letters in a column for a particular repetition represent significantly different median level of the Cry1Ab toxin in maize leaves, according to pair-wise Wilcoxon test, at $P \leq 0.05$.

natural enemies when they feed on plant tissues (Obrist *et al.* 2006). In the present bioassays, we hypothesized that feeding time of predatory larvae will increase the amount of Cry1Ab toxin ingested via Bt-loaded-prey. We proved that toxin content declined through the trophic chain, however, on short exposure periods (24, 48 h). Descent of its movement was: *Bt* maize (60.49 µg/ g F.W.) - *T.urticae* (0.12 µg/ g F.W.) – *A. bipunctata* (0.12 µg/ g F.W.). Accordingly, highest accumulation of toxin (0.12 µg/ g F.W.) was observed during 72 h feeding time. Alvarez-Alfgamene *et al.* (2011) detected that a drastic decrease (90%) of the *Bt* concentration through the food chain occurred for both toxins (Cry1Ab and Cry3Bb1). However, other authors reported that young stages of tested arthropods accumulated higher amount of *Bt* toxins than their adult stages. For example, Garcia *et al.* (2010) proved that the acquisition of the toxin was higher in the second and the third larval instars of *A. coriaria*, when they were fed on *Bt*-fed spider mites. The same model of Cry transfer was observed by Obrist *et al.* (2006) both, laboratory and field collected insects, in case of the omnivorous predator *Orius majusculus* (Reuter) (Heteroptera: Anthocoridae) after they were fed on *Bt*-loaded spider mites and *Bt* pollen. Nevertheless, the toxin is not always detected in all chains of tritrophic bioassays. Harwood and Obrycki (2006) did not find Cry1Ab toxin in *Scarites subterraneus* Fabricius (Coleoptera: Carabidae) when they were fed on *Deroceras leave* (Müller) (Mollusca: Agriolimicidae) reared on *Bt* maize. Even field evidence of Cry1Ab toxin uptake could not show any content in this species (Peterson *et al.* 2009). However, some results showed a lower content of Cry1Ab in ladybird beetle larvae collected in Cry1Ab- or Cry3Bb1-*Bt* maize fields (Harwood *et al.* 2007, Obrist *et al.* 2006 and Meissle and Romeis, 2009), but other laboratory studies showed higher amount of this toxin (Alvarez-Alfgamene *et al.* 2011). Dutra *et al.* (2012) reported mean concentrations of Cry1Ab in *Bt* maize leaves ranged 23-33 µg/g dry weight. The herbivore, *Spodoptera frugiperda* (Lepidoptera: Noctuidae), feeding on *Bt* maize leaves from 24 to 72 h was found on one order of magnitude less Cry1Ab than first trophic level (maize leaves) (2.1-2.2 µg/g dry weight).

Obtained results showed relatively high amount of Cry1Ab levels in *Bt* maize plants (60.49 µg/g fresh weight) growing in the greenhouse. Alvarez-Alfgamene *et al.* (2011) reported similar the Cry1Ab and Cry3Bb1 leaf-expression levels of climate chamber grown *Bt* maize plants to those showed from the field (Monsanto, 2002, Nguyen and Jehle, 2007 and 2009). It is very often that Cry toxin concentrations in GM plants have high variability among laboratories, cultivation sites and also sometimes within one cultivar in the same location (Then and Lorch, 2008). Moreover, Cry toxins are known to be produced in GM plants in a tissue- and time-specific manner (Nguyen and Jehle 2007, Szekacs *et al.* 2010a, b and Szekacs *et al.* 2012). Grabczyńska *et al.* (2011) established (in cooperation with RWTH Aachen University) the performance of two qualitative ELISA kits in 96 well plate format: Envirologix-QualiPlate™ Kit for Cry1Ab/Cry1Ac and Agdia-Bt-Cry1Ab/1Ac ELISA Kit with available Cry1Ab standards: the first from the Fitzgerald Industries, the second from Agdia Inc. Authors showed two graphs - one for Fitzgerald and Agdia Cry1Ab standards used with Agdia kit and one for Fitzgerald and Agdia Cry1Ab standards used with Envirologix kit. The Cry1Ab levels in MON810 leaves varied from 12.08 µg/g f.w. on Agdia kit with Agdia standard to 78.94 µg/g f.w. on Envirologix kit with Fitzgerald standard. In the present analysis, qualitative ELISA kit Envirologix QualiPlate and Cry1Ab standard from Fitzgerald Industries International were used.

Since we do not know all potential *Bt* toxin sources in coccinellids via multi-trophic food webs in agro-ecosystems, an accurate environmental risk assessment of genetically modified plants could not be made. One of the solutions is to combine laboratory bioassays and field surveys as a quantitative approach for such predatory arthropods.

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