Environmental Pollution 209 (2016) 164–168

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Uptake and bioaccumulation of Cry toxins by an aphidophagous predator

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ARTICLE INFO

Article history:
Received 29 September 2015
Received in revised form
8 November 2015
Accepted 20 November 2015
Available online 10 December 2015

Keywords:
Bt toxin
Coccinellid
Persistence
Tritrophic exposure

ABSTRACT

Uptake of Cry toxins by insect natural enemies has rarely been considered and bioaccumulation has not yet been demonstrated. Uptake can be demonstrated by the continued presence of Cry toxin after exposure has stopped and gut contents eliminated. Bioaccumulation can be demonstrated by showing uptake and that the concentration of Cry toxin in the natural enemy exceeds that in its food. We exposed larvae of the aphidophagous predator, *Harmonia axyridis*, to Cry1Ac and Cry1F through uniform and constant tritrophic exposure via an aphid, *Myzus persicae*, and looked for toxin presence in the pupae. We repeated the experiment using only Cry1F and tested newly emerged adults. Both Cry toxins were detected in pupae, and Cry1F was detected in recently emerged, unfed adults. Cry1Ac was present 2.05 times and Cry1F 3.09 times higher in predator pupae than in the aphid prey. Uptake and bioaccumulation in the third trophic level might increase the persistence of Cry toxins in the food web and mediate new exposure routes to natural enemies.

1. Introduction

Many field and laboratory studies have been conducted to evaluate the potential effects of Cry toxins expressed by genetically modified (GM) plants on beneficial insects. They have focused on demonstrating presence or absence of adverse ecological effects on natural enemies, disregarding other ecological issues, such as toxin uptake and possible bioaccumulation. For example if Cry toxins can be uptaken by natural enemies and, if so, if they can bioaccumulate. The longer persistence of the Cry toxins in the food web and their availability to higher trophic levels would open up previously unconsidered exposure routes and ecological effects (e.g., Zhou et al., 2014).

Although many studies have documented the presence of Cry toxins in natural enemies, few of them have determined if the toxins were uptaken by the third trophic level (but see Zhang et al., 2006a). It is commonly believed that the Cry toxin presence in a natural enemy is ephemeral, being excreted and/or degraded after ingestion (Meissle and Romeis, 2009). Uptake is defined as the absorption and incorporation of a substance into a living organism (USEPA, 2013), and can be demonstrated by the continued presence of a substance, in this case a Cry toxin, after exposure has stopped and gut contents eliminated.

With respect to Cry toxins, the term “uptake” has been frequently misused and confused with “presence” in non-target organisms (e.g., Dutton et al., 2002; Harwood et al., 2005; Burgio et al., 2007, 2011; Svobodova et al., 2013). Such studies clearly demonstrated the presence of Cry toxin in non-target organisms, but they did not demonstrate that the Cry toxin was absorbed and incorporated into the organism.

Bioaccumulation is related to uptake, and refers to the accumulation of substances in an organism (Bryan, 1979). It occurs when an organism uptakes a substance at a rate greater than that at which the substance is lost. Thus, bioaccumulation can be demonstrated by first demonstrating uptake and then showing that the concentration of the substance in the organism exceeds that in its food or another environmental source. Bioaccumulation of a Cry toxin in a natural enemy has not yet been demonstrated. Instead, the common finding has been decreasing concentration of Cry toxin from plant to herbivore to natural enemy (e.g., Peterson et al., 2011).

To evaluate the possibility of uptake and bioaccumulation of Cry proteins at the third trophic level, we exposed larvae of a aphidophagous predator, the coccinellid *Harmonia axyridis*, to Cry1Ac and Cry1F toxins using a uniform and constant concentration of the toxins in diets for the aphid *Myzus persicae*. After exposing the

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http://dx.doi.org/10.1016/j.envpol.2015.11.036
0269-7491/

entire larval stage to Cry toxins, we tested the pupae and newly emerged, unfed adults for the presence of the toxins. If Cry toxin is found, this is proof of uptake and possibly, bioaccumulation, because all larval gut contents and cuticle are eliminated during pupation.

2. Materials and methods

2.1. Insect rearing

The prey aphid, *M. persicae* (Hemiptera: Aphididae), was reared on collard plants (*Brassica oleracea*) in a greenhouse at 13 h photophase at 25 ± 4 °C at 60 ± 10% RH. The generalist predator *H. axyridis* (Coleoptera: Coccinellidae) is common in Brazil, and adults were collected from an experimental field in Embrapa Genetic Resources and Biotechnology, Brasília, Brazil. The predator was reared in plastic cages, 10 × 15 cm, containing a daily supply of water in wet cotton balls and leaves containing aphids (*Aphis gossypii* and *Uroleucon ambrosiae*) collected from the field. Egg masses were transferred to separate cages and inspected daily to collect the neonate larvae (less than 24 h old) for use in the bioassays.

2.2. Bioassay preparation

Trypsinized and purified Cry1Ac and Cry1F toxins (both ca 65 kDa) were purchased from Dr. M. Pusztai-Carey (Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio) and their biological activities were confirmed as described in Nakasu et al. (2013). The tritrophic system for the predator bioassays was prepared based on (Douglas and van Emden, 2007), and summarized as follow. The artificial system consisted of a sachet made with two layers of parafilm M, inside of which was 300 μl of liquid holidic diet for rearing aphid (Dadd and Mittler, 1996), attached to one end of a transparent acrylic tube (2.5 cm diameter × 2.5 cm height, wall thickness 0.35 cm). Before the system assembly, the tube and parafilm pieces were sterilized by UV radiation for 30 min in a laminar flow hood, and the artificial liquid diet was filtered using a sterilization filter (pore size of 0.22 μm). When the tubes were finished, aphids were carefully transferred into them using a paint-brush (#2) 24 h before being offered to the predator. All bioassays were conducted inside a controlled environment chamber (25 ± 2 °C and 13 h photophase).

2.3. Exposure of larval predators

Unfed neonate predator larvae were individually transferred to cages containing *M. persicae* aphids feeding on diet of one of the treatments: 1. Control (no Cry toxin added); 2. Cry1Ac 20 μg/ml (C20); 3. Cry1F 20 μg/ml (F20); and 4. Cry1Ac 20 μg/ml and Cry1F 20 μg/ml (C20:F20). These Cry1Ac and Cry1F concentrations were similar to that in leaves of WideStrike® cotton (Siebert et al., 2009). Water was supplied daily in each cage on wet filter paper (1 cm^2). The cages were inspected daily until the pupal stage to evaluate survival and developmental stage. Pupae (within 24 h of pupation) were weighed and stored at −20 °C for ELISA. An additional bioassay, using the same method as above, was performed to provide additional evidence of Cry1F uptake in *H. axyridis*. Neonate *H. axyridis* were individually exposed to aphids feeding on control or F20 diets and reared to adult eclosion. Adults were provided water but no food, and within 24 h of eclosion, they were sexed, weighed and stored at −20 °C for ELISA.

2.4. Cry1Ac and Cry1F detection

The Cry toxins were detected and quantified using double sandwich enzyme-linked immunosorbent assay (ELISA PathoScreen plate, Agdia, USA) according to the manufacturer's instructions. For each diet at least six replicates of 100 *M. persicae* were fed for 24 h, collected and weighed for Cry quantification. All predators from the two bioassays described above were analyzed individually. All insect samples were macerated using a glass pestle and homogenized in PBST in a volume (in μl) corresponding to 70× the fresh weight (FW) (in mg). The samples were centrifuged at 15,500× *g* for 15 min and the supernatant was used for the analysis. Each sample was applied (100 μl/well) in duplicate or triplicate. Each plate had duplicate calibration standards to estimate a linear calibration curve for each plate. The absorbance was measured at 630 nm with a microtiter plate reader (TP Reader NM Thermo Plate®, USA).

The LODs (Limit of Detection) for Cry1Ac and Cry1F detection in the predator samples were calculated using the standard deviation and slope method. The Cry1Ac LOD was 0.0016 ng/mg FW and the Cry1F LOD was 0.0004 ng/mg FW. Based on the dilutions and the technical specifications of the reader, the linear part of the standard curve indicated accurate estimations for the predator samples of Cry1Ac up to 5 ng/mg FW and for Cry1F up to 20 ng/mg FW.

2.5. Statistical analysis

Each ELISA plate was set up to contain multiple blanks, and controls that matched the aphid and predator samples of each Cry treatment. This allowed ELISA absorbances for each sample (aphids and predator pupae and adults) to be normalized for each plate and for each respective control. Technical replicates were then averaged and absorbances were converted to concentrations of Cry toxin (ng of Cry/mg FW) using the average slope of the standard curve and sample weight. The mean concentration of Cry toxin was calculated for each species by averaging values for all samples in a diet treatment. Cry concentrations were analyzed using ANOVA (Proc GLM, SAS 9.4). Sufficient statistics are provided in the Supplementary Material.

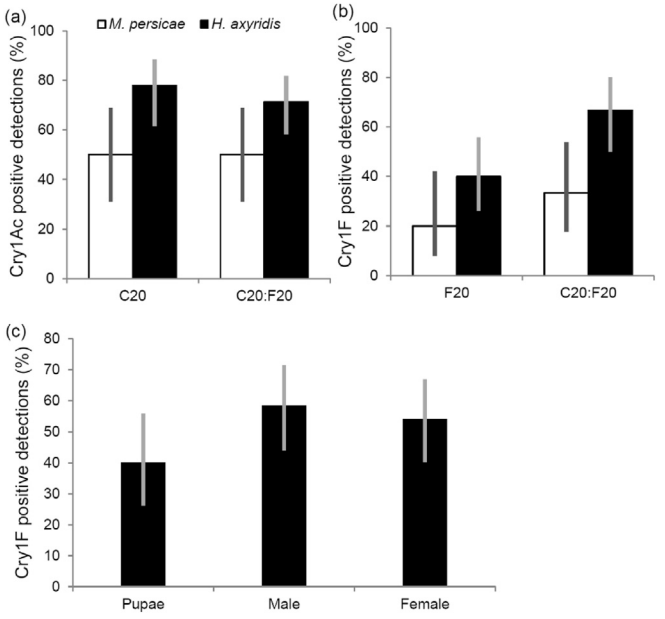

Fig. 1. Uptake of Cry toxins in *H. axyridis*. Detection of Cry toxins in the prey and predator pupae (mean ± SE): (a) Cry1Ac; (b) Cry1F; (c) Cry1F comparing pupae and adults of *H. axyridis*.