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Effects of *Bacillus thuringiensis* Cry1Ab and Cry3Aa endotoxins on predatory Coleoptera tested through artificial diet-incorporation bioassays

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Abstract

Traditional approaches to studying the effects of genetically modified (GM) crops on beneficial insects involve either field assays, comparing insect population levels between control and GM crops or tritrophic bioassays with contaminated insects – usually larvae or eggs of Lepidoptera – as preys. Here, we report the results of a bioassay using an artificial diet, suitable for predatory Coleoptera, to supply *Bacillus thuringiensis* (Bt) solubilized Cry1Ab and Cry3Aa as well as trypsin-activated Cry1Ab to *Atheta coriaria* and *Cryptolaemus montrouzieri* adults and young larvae of *Adalia bipunctata*. Water, solubilization buffer and trypsin-treated solubilization buffer were used as controls. In total, 1600 insects were assayed. Assays showed a relatively low mortality rate in the controls, ranging from as low as 7% after 15 days (*C. montrouzieri*) to about 15–20% after five days (*A. bipunctata*) or 15 days (*A. coriaria*). For all three predators, there were no statistical differences between the mortality recorded in any of the treatment groups and the corresponding controls. These results indicate a lack of short- (*A. bipunctata*) and long-term (*A. coriaria* and *C. montrouzieri*) mortality associated with oral ingestion of Cry1Ab and Cry3Aa at the high dose tested (50 $\mu g\ ml^{-1}$). We discuss the relevance of these findings for the ecology of beneficial Coleoptera and compatibility with Bt and GM Bt crops.

Keywords: *Bacillus thuringiensis*, biological control, non-target insects, *Adalia bipunctata*, *Atheta coriaria*, *Cryptolaemus montrouzieri*

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Introduction

Genetically modified (GM) crops expressing *Bacillus thuringiensis* (Bt) toxins raise concerns about their safety and compatibility with biological control agents. Many non-target insects, including parasitoids and predators, have been bioassayed in order to determine their sensitivity to Cry proteins from Bt (reviewed by Groot & Dicke, 2002; Lövei & Arpaia, 2005; Hilbeck & Schmidt, 2006); and, although most of the studies could not find significant deleterious effects, a few did report adverse effects on non-target organisms. In the case of predators, sensitivity to Bt can be studied with (i) bitrophic assays, which involve supplying them with plant matter (usually honeydew, nectar or pollen) or by feeding insects with sucrose solutions or artificial diets or (ii) by tritrophic assays, using other insects, previously fed with Bt, as preys. Methodological differences have been proposed to

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explain variations in sensitivity (Romeis *et al.*, 2004). Therefore, results obtained from a variety of methods to feed predators with Cry toxins should be considered. The aim of this work was to study predator mortality associated with oral ingestion of two Bt Cry toxins, Cry1Ab and Cry3Aa, which are two of the most widely used toxins in GM crop-based insect control strategies. We developed an artificial diet to supply the toxins to the commercial instars of three predatory Coleoptera species: the two-spot ladybird, *Adalia bipunctata*; the rove beetle, *Atheta coriaria*; and the mealybug destroyer, *Cryptolaemus montrouzieri*.

These species were chosen on the basis of their importance as biocontrol agents and due to the fact that, in two cases (*A. coriaria* and *C. montrouzieri*), no previous bioassays on Bt sensitivity have been reported.

A. bipunctata is a polyphagous predator that has been sold for aphid control in Europe since 1999. The compatibility of this native coccinellid with GM crops has received much attention, and several reports have been published on the effects transgenics have on this species (Birch *et al.*, 1999; Down *et al.*, 2000, 2003) although only one studied the effects of Cry toxins (Schmidt *et al.*, 2004).

A. coriaria is a common European soil-dwelling polyphagous predator. Both larvae and adults prey on several phytophagous Diptera larvae, mainly fungus gnats *Bradysia* spp. (Diptera: Sciaridae). *A. coriaria* adults have only recently been commercially supplied as biological control agents for fungus gnats and other soil insects such as shore flies and thrips.

The third species, *C. montrouzieri*, is a voracious feeder of mealybug (Pseudococcidae) in both the larval and adult stages. Mealybug females feed on plant sap of citrus and other crops by attaching themselves to the plant while they suck the plant juices. Since a single *C. montrouzieri* larva may consume up to 250 young preys, this species, which is native to Australia, has been introduced into many countries to control mealybug populations.

The present work aimed to study the sensitivity of these predators to Cry proteins administered through artificial diet. The results presented here, together with future works using different methodologies (prey- mediated or field assay tests) will help understand the impact of Cry toxins on these beneficial Coleoptera and forecast their suitability as biological control agents in Bt crops.

Materials and methods

Purification of Cry proteins

A Cry1Ab-producing *Escherichia coli* recombinant strain was kindly provided by Dr Ruud de Maagd. The strain was cultured and inclusion bodies extracted and solubilized as previously described (Herrero *et al.*, 2004). TB medium, containing 100 μg ml^{-1} of ampicillin, was inoculated and grown for 48 h at 28°C. The culture was harvested by centrifugation and pellet resuspended in 3 ml g^{-1} of pellet of lysis buffer (50 mM Tris/HCl, pH 8.0, 5 mM EDTA, 100 mM NaCl). Lysozyme (800 μg per g of pellet) was added and the mixture was incubated at room temperature for 20 min. Deoxycholic acid was then added to a final concentration of 1 mg ml^{-1} and the mixture incubated at 37°C for 30 min. To remove DNA, DNase I was added to a final concentration of 50 μg ml^{-1} and incubation was continued at 37°C for 30 min. Pellet, containing the inclusion bodies, was harvested by centrifugation at 40,000 × g for 20 min and washed three times with washing buffer (20 mM Tris/HCl, pH 8.0, 5 mM EDTA, 100 mM NaCl) and then three times with a phosphate-buffered saline (PBS) solution. Cry1Ab was solubilized with carbonate buffer (50 mM Na2CO3, 100 mM NaCl, adjusted to pH 10.5) with DTT (di-thio-threitol, 10 mM) added just before use. The mixture was incubated for 2 h at 37°C in an orbital shaker (180 rpm). Supernatant, containing solubilized Cry proteins, was recovered by centrifugation, and the total soluble protein content was determined with the protein-dye method of Bradford (1976). Half of the volume of the solution was stored frozen (−20°C), and the other half was adjusted to pH 9.0 with 1 M TrisCl prior to the addition of a 10% (w:w) trypsin. Proteolytic digestion was performed for 2 h at 37°C and the supernatant was kept.

Cry3Aa crystals were produced in *Bacillus thuringiensis* strain BTS1. Crystal inclusions were purified from spores and cell debris by centrifugation in discontinuous 67%, 72%, 79%, 84% and 90% (w/v) sucrose gradients in 50 mM Tris–HCl, pH 7.5, as described by Thomas & Ellar (1983). The crystal band was removed and washed three times in 50 mM Tris–HCl, pH 7.5. Purity of the crystal preparation was monitored by phase contrast microscopy and analysed by 10% SDS-PAGE as described by Laemmli (1970). Crystal proteins were solubilized in extraction buffer (50 mM Na_2CO_3, pH 11.2) at 37°C for 2 h. Supernatant, containing solubilized Cry protein, was recovered by centrifugation, and the total soluble protein content was determined with the protein-dye method of Bradford (1976) using bovine serum albumin (BSA) (New England Bio-Labs, Beverly, MA) as a standard.

Aliquots of both proteins were analyzed by Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (10% polyacrylamide gel, 100:1 acrylamide:bis-acrylamide ratio). The gels were run at 35 mA for 2 h in a mini-Protean III apparatus (Bio-Rad, Hercules, CA) as previously described (Laemmli, 1970). Gels were stained with a solution containing 50% (v:v) ethanol, 10% (v:v) acetic acid and 0.1% (wt:v) Coomassie brilliant blue R250 for 30 min and then destained overnight with a solution containing 25% (v:v) ethanol and 10% (v:v) glacial acetic acid. Protein sizes were determined by comparison with broad range protein markers (Precision Plus Protein Standard, Bio-Rad, CA, USA and PageRuler^{TM} Prestained Protein Ladder).

Biological activity of Cry1Ab and Cry3Aa protoxins was confirmed by bioassaying two control susceptible species, *Ostrinia nubilalis* and *Leptinotarsa decemlineata*, respectively. The former species was fed with an artificial diet for Lepidoptera, containing dilutions of Cry1Ab, whereas the latter was bioassayed with disks of potato leaves dipped into Cry3Aa solutions.

Artificial diet

An artificial diet for predatory Coleoptera was modified from a recipe previously reported for ladybugs (Majerus *et al.*, 1989). The main differences were that beef extract and honey were used instead of desiccated liver and maple syrup, respectively, and that the mold-inhibitor Nipagin (Methylparaben) was added. In addition, low EEO agarose (Laboratorios Conda, Madrid, Spain) was used instead of agar to enable Cry proteins to be added to the liquid diet at

low temperature. Diet was prepared as follows: beef extract (60 g), yeast extract (40 g), sucrose (100 g) and agarose (13 g) were solubilized in 800 ml of sterile water by heating in a microwave oven. The mixture was cooled at room temperature before adding 65 g of honey, 9 g of Ain Vitamin Mixture 76 (MP Biomedicals, Solon, OH, USA) and 1.1 g of Nipagin. Water was added to a final volume of one litre and the diet was kept at 40–50°C in a water bath. One-ml artificial food doses were prepared by pouring liquid diet into 4 × 6 Corning® Costar® flat-bottom cell-culture plates (Sigma-Aldrich, St Louis, Missouri, USA). Seven stocks of doses, containing the seven different solutions to be tested, were prepared. Three independent control treatments were prepared by adding 70 µl of water, solubilization buffer (pH 10.5) or trypsin-treated solubilization buffer (pH 9) to 1 ml of liquid diet. Three additional treatments containing solubilized Cry1Ab and Cry3Aa as well as trypsin-activated Cry1Ab were produced by adding 70 µl of a concentrated solution to 1 ml of diet, to yield a final protein concentration of 50 µg ml^{-1}. Positive controls containing 5% Boric acid or 0.5% of the pyrethrin- and piperonyl butoxide-based insecticide ZZ Cooper (Zelnova) were also included for the assays against *A. coriaria* and *C. montrouzieri* (boric acid), and *A. bipunctata* (ZZ Cooper).

As a control of the bioactivity of the toxins in the diet, large (third instar) larvae of target *L. decemlineata* were also supplied with Cry3Aa-containing doses prepared exactly as for the three non-target species. A control group of *L. decemlineata* feeding on doses without toxin was set.

Bioassays

The three insect species were purchased from Biobest Biological Systems (Westerlo, Belgium) and immediately used in bioassays upon reception. All bioassays were performed in small (5 cm in diameter) Petri dishes containing a 1-ml dose of artificial diet. Preliminary experiments showed that high relative humidity led to rapid bacterial development in assays with *A. bipunctata* larvae. Therefore, a round (1 cm in diameter) ventilation grid was used to assure aeration in these bioassays. *A. coriaria* and *C. montrouzieri* were bioassayed in the original Petri dish without additional aeration. *A. bipunctata* young (first- and second-instars) larvae were individually placed in the dishes, and 30 dishes per treatment were used. *A. coriaria* and *C. montrouzieri* adults were placed in groups of four and five adults per dish, respectively, and five (*A. coriaria*) and two (*C. montrouzieri*) dishes per treatment were used. The whole bioassay was repeated three (*A. bipunctata* and *C. montrouzieri*) to four times (*A. coriaria*). The total number of assayed insects was thus 1600 (240 *C. montrouzieri*, 640 *A. coriaria* and 720 *A. bipunctata*).

Bioassays were performed at 25 ± 1°C, under an 18:6 h (L:D) photoperiod. *A. coriaria* assay dishes were covered with an inverted opaque plastic box. Doses with artificial food containing the appropriate treatment were replaced every three days. Casualties were recorded daily for six days for *A. bipunctata* and for 15 days for the remaining two non-target species, as well as for *L. decemlineata*.

Results

Figure 1 shows the protein contents of solubilized Cry1Ab and Cry3Aa and trypsin activated Cry1Ab stock solutions prepared as described in 'Materials and methods'. The solution containing Cry1Ab was mainly composed of a 135 kDa band that was proteolitically cleaved to a 62 kDa protease resistant fragment, corresponding to the toxin core (fig. 1a). Solubilized Cry3Aa exhibited a major protein band of about 65 kDa (fig. 1b). As expected, Cry1Ab and Cry3Aa were highly active on susceptible target species (*Ostrinia nubilalis* and *Leptinotarsa decemlineata*, respectively), as confirmed through standard bioassays (data not shown). Additionally, the bioactivity of the artificial diet containing Cry3Aa was confirmed by feeding *L. decemlineata* with treated diets prepared in the same manner as those given to the test organisms. Indeed, 100% mortality was recorded after one week, whereas control insects feeding on the same artificial diet but without Cry3Aa showed virtually no mortality after 15 days. Preliminary assays with the artificial diet showed it was voraciously consumed by the three predatory species tested; and, also, it was suitable to keep insects alive long enough to carry out bioassays (data not shown). There was no evidence of fungal contamination during the assay, but bacterial colonies could be observed in two day-old diet doses. Adult *C. montrouzieri* were bioassayed for 15 days, and a very low (7% or less) mortality rate was recorded in the controls. Mortality values in *A. coriaria* controls were about 20% or lower after 15 days. *A. bipunctata* was bioassayed for six days, and larvae were observed to moult during bioassays and exhibited relatively low mortality (lower than 20%) on days 1–5. However, a rapid increase in mortality was observed from day five onwards (fig. 2), suggesting that the artificial diet was only suitable for *A. bipunctata* bioassays lasting less than six days.

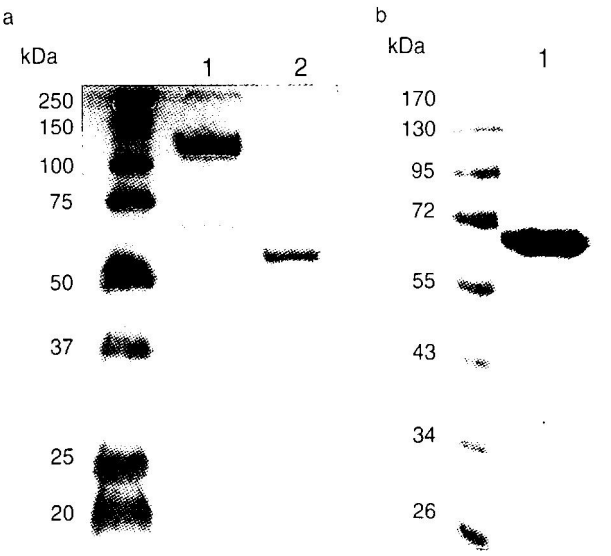

Fig. 1. SDS-PAGE showing protein contents of (a) Cry1Ab and (b) Cry3Aa. Solubilized and trypsin-digested Cry1Ab (a: lanes 1 and 2) and solubilized Cry3Aa (b: lane 1) are shown. Molecular masses are given to the left in kDa.

Insect mortalities associated with the seven treatments tested (water, solubilization buffer, trypsin-treated buffer, solubilized Cry1Ab, trypsin-treated Cry1Ab, solubilized Cry3Aa and positive control) on the three species are shown in fig. 2 (*A. bipunctata*), fig. 3a (*A. coriaria*) and fig. 3b

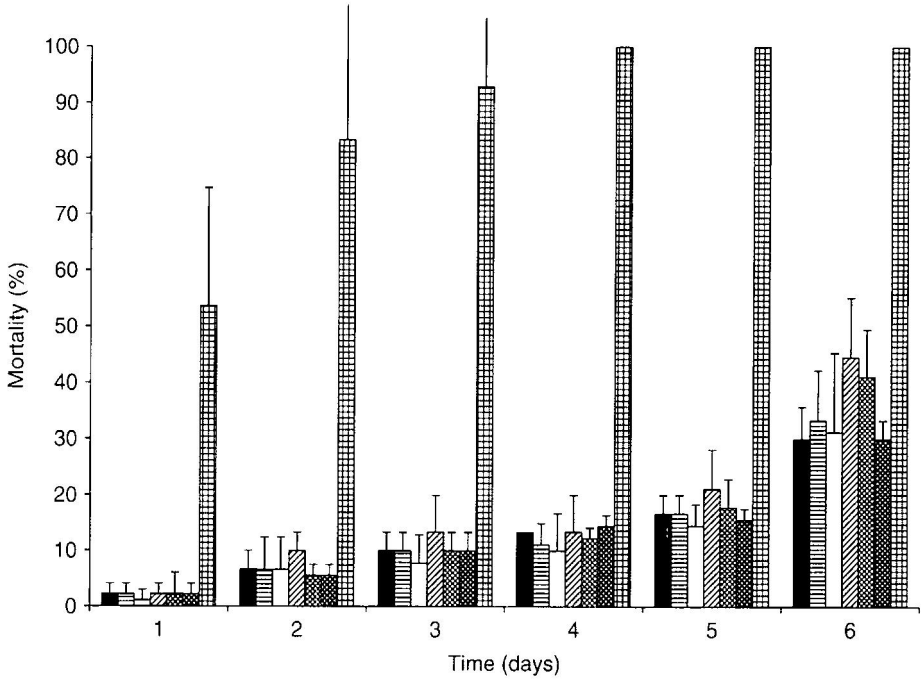

Fig. 2. Daily mortality (%) associated with seven treatments: water, solubilization buffer (50 mM Na2CO3, 100 mM NaCl, 10 mM DTT, pH 10.5), trypsin-treated buffer, solubilized Cry1Ab, trypsin-treated Cry1Ab, solubilized Cry3Aa and ZZ Cooper (positive control), orally administered to young *Adalia bipunctata* larvae through artificial diet. All treatments with Cry proteins were set at a protein concentration of 50 $\mu g\,\mu l^{-1}$. Standard deviations (SD) are shown (■, H_2O; ▤, buffer; □, trypsin; ▨, Cry1Ab; ▩, Cry1Ab-T; ▩, Cry3A; ▦, C+).

(*C. montrouzieri*). Oral ingestion, of both solubilized and trypsin-treated Cry1Ab, led to between 18% and 24% mortality in *A. bipunctata* after five days, and between 41% and 46% after six days. These values are slightly, but not significantly, higher than those of the solubilization buffer and trypsin-treated buffer controls at days five and six. Mortality in the controls after day six, including water, was ≥30%, which was considered too high to continue with the assay.

The seven treatments showed no effect on *A. coriaria* adults after 15 days (fig. 3a). Mortality associated with all the treatments exhibited a narrow distribution, ranging from 16% to 20%. No significant differences were found between the groups.

Finally, very low toxicity was recorded for all treatments in bioassays on *C. montrouzieri* adults after 15 days, ranging from no mortality at all (Cry3Aa) to a maximum of 7% (two dead insects out of 30) for Cry1Ab, trypsin-treated Cry1Ab, solubilization buffer and trypsin-treated buffer treatments (fig. 3b).

Discussion

Artificial diet incorporation bioassays are widely used to determine susceptibility of target (mainly Lepidoptera) pests to Bt preparations and they are also used to study the effects of Bt and Bt crops on predators (Sims, 1995, 1997; Romeis *et al.*, 2004; Duan *et al.*, 2006; Raybould *et al.*, 2007). Artificial diet assays are used for regulatory approvals of Bt crops to test for adverse effects of the particular Cry proteins on non-target organisms (a primary route for US EPA Biopesticides Registration Action Documents, BRADs, is available at http://www.epa.gov/oppbppd1/biopesticides/ingredients/index_ab.htm#b). However, diet-based bioassays are not always included in research works on non-target organisms. There are two main reasons for this: (i) artificial diets have only been developed for a few predator species, and (ii) prey-mediated bioassays are considered to more realistically mimic the toxin uptake through the food chain, which may take place in the field. However, when insects used as prey are reared on Bt food, their nutritive qualities may be reduced; thus, mortality might be prey-mediated rather than toxin-mediated (Hilbeck *et al.*, 1999; Romeis *et al.*, 2004). A bioassay method using artificial diet, such as that reported here, has the advantages of being able to supply a precise amounts of Cry toxin to predators and to eliminate the 'prey quality' factor. Additionally, and compared to bitrophic plant-predator assays, using artificial diet eliminates nutritional differences among plant hybrids, which have been reported to account for statistical differences in the growth of non-target phytofagous insects (Clark *et al.*, 2006). Antibiotics were deliberately excluded from the diet composition since bacteria occurring in the insect midgut naturally might be critical for sensitivity (Broderick *et al.*, 2006). Nipagin was the chosen preservative because it has been used as a fungicide for decades in artificial diets for insect rearing; moreover, it is known not to interfere with treatment toxicity.

The results of our bioassays suggest that Cry1Ab and Cry3Aa, two Bt toxins expressed in transgenic crops genetically protected against Lepidoptera and Coleoptera key pests, respectively, are innocuous to three important predatory Coleoptera. The concentration tested, 50 $\mu g\,ml^{-1}$, is about fivefold higher than the concentration of Cry1Ab in

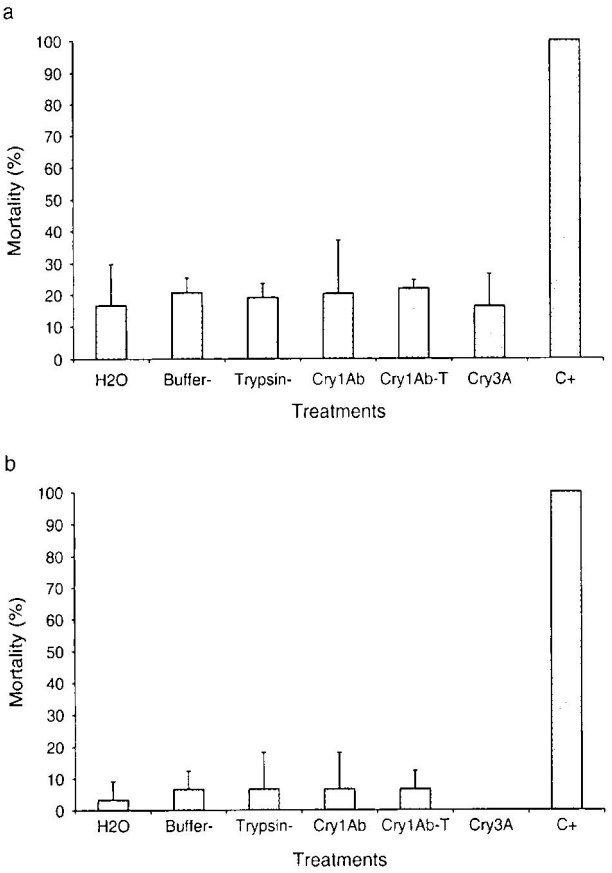

Fig. 3. Mortality values (%) after a 15-day bioassay on (a) *Atheta coriaria* and (b) *Cryptolaemus motrouzieri* adults fed with an artificial diet with one of the following treatments: water, solubilization buffer, trypsin-treated buffer, solubilized Cry1Ab, trypsin-treated Cry1Ab, solubilized Cry3Aa and 5% Boric Acid (positive control). Treatments with Cry proteins were set at a protein concentration of $50\,\mu g\,\mu l^{-1}$. Standard deviations (SD) are shown.

transgenic corn and 20- to 250-fold higher than the concentration of Cry1Ab detected in predatory Coleoptera sampled from Bt crops (Harwood *et al.*, 2005, 2007). Therefore, the amount of toxin supplied to the predators in the assays is probably much higher than that available to the predator through the food chain in the agricultural ecosystem.

In the field, *A. coriaria* might come in contact with Cry toxins through tritrophic interaction with its main prey. In fact, fungus gnat larvae feed on root tissues, so they might uptake Cry toxins either from roots, which may contain larvicidal Cry toxins (Saxena *et al.*, 2004) or after standard Bt insecticidal preparations have been sprayed. The present work is the first report on the sensitivity of *A. coriaria* to Bt. The lack of toxicity of Cry toxins on *A. coriaria* we found after 15 days, which represents 70% of the life-span of the adults, suggests that adults of this species are not sensitive to the tested Bt toxins and, therefore, are compatible with transgenic Cry1Ab and Cry3Aa crops.

Similarly, *C. montrouzieri* was not affected by the Cry toxins tested. To the best of our knowledge, the compatibility of the mealybug destroyer with Bt has not previously been reported, possibly because phloem-feeding pests are thought not to incorporate Cry toxins from Bt crops. However, a recent report (Burgio *et al.*, 2007) demonstrates that Cry toxins can be found in plant phloem and insects feeding on phloem. Therefore, they could pass through the trophic chain up to predators feeding on sucking insects. Alternatively, *C. montrouzieri* may be exposed to Cry toxins by consuming mealybugs after a Bt insecticidal suspension has been sprayed onto crops. Our results after a 15-day bioassay suggest that this species might not be affected by Cry1Ab- and Cry3Aa-based control methods, including transgenic crops producing these toxins.

It has to be noted that, in our bioassays, we chose adults rather than larvae of *A. coriaria* and *C. montrouzieri* because they are the preferred instars for commercialisation purposes of these species as biological control agents. However, since sensitivity of insect larvae to Bt is often higher than that of adults, it cannot be concluded from our results that larvae of *A. coriaria* and *C. montrouzieri* are not sensitive to Bt.

In the third predatory species tested, *A. bipunctata*, there was no increase in larval mortality associated to oral ingestion of either Cry1Ab or Cry3Aa. These results are in contrast with a previous work by Schmidt *et al.* (2004), who reported significantly higher mortality rates in *A. bipunctata* fed with *Ephestia* spp. eggs sprayed with Cry1Ab, as compared to buffer-sprayed controls. They tested the same concentration we used, $50\,\mu g\,ml^{-1}$, and also two lower ones, 5 and $25\,\mu g\,ml^{-1}$, with all three treatments giving a significant increase in mortality compared to the controls, although lacking a clear dose-response. Both studies cannot be fully compared because of important differences in the bioassay methodologies. In fact, it should be noted that our bioassays lasted up to six days, whereas the whole immature life stage was toxin-fed by Schmidt and co-workers. Therefore, *A. bipunctata* may not be significantly sensitive to Cry1Ab when exposed relatively short-term but could be sensitive after long-term exposure. The fact that we used an artificial diet instead of sprayed Lepidoptera eggs is of importance since interactions between compounds in the diet and the toxins are theoretically possible. However, such toxin-diet interactions that have previously been hypothesized to explain differences between prey-fed and diet-fed studies (Romeis *et al.*, 2004) have not been demonstrated, and it must be noted that artificial diet-based bioassays have been carried out for decades with no reported synergism between toxins and diet compounds. In concordance with this, we have confirmed the bioactivity of Cry3Aa in the artificial diet, which killed 100% of the *L. decemlineata* larvae tested.

Although our results on the three studied predatory Coleoptera indicate their lack of sensitivity under the particular conditions of the bioassay methodology used, they only contribute partially to characterizing the effects of Cry toxins on these species. Further studies should be performed to confirm the lack of sensitivity of the tested predators to Cry1Ab and Cry3Aa. Particularly, assays with new diets allowing long-term tests on *A. bipunctata* as well as studies on the sensitivity of larval instars of *A. coriaria* and *C. montrouzieri* to Bt toxins should be undertaken.

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