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Differential Cry toxin detection and effect on *Brevicoryne brassicae* and *Myzus persicae* feeding on artificial diet

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Abstract

The literature about effects of genetically modified plants expressing Cry toxins has several contrasting results regarding detection and effects of Cry toxins on aphids. To test the hypothesis that this variation could be due to a lack of controlled exposure, and not just a lack of toxicity, we used an artificial aphid rearing system to detect acquisition and examine potential effects of Cry1Ac and Cry1F on mortality and population growth rate of cabbage aphid, *Brevicoryne brassicae* (L.), and green peach aphid, *Myzus persicae* (Sulzer) (both Hemiptera: Aphididae). Using enzyme-linked immunosorbent assay (ELISA), Cry1Ac and Cry1F were not detected in *B. brassicae* feeding on any of the Cry diets, whereas in *M. persicae*, Cry1Ac was detected in 55% of samples exposed alone or in combination with Cry1F, and Cry1F was detected in 67% of samples exposed alone or in combination with Cry1Ac. The toxins reduced the net population growth rate of *M. persicae*, but not of *B. brassicae*. For *M. persicae*, the reduction in growth rate was similar for Cry1Ac and Cry1F and there was no synergism from co-feeding the aphids with both Cry toxins. The toxins had no detectable effect on survival of either aphid species. Variability in detection and effects of Cry toxins on aphids may not depend only on the presence and level of a Cry toxin in the phloem, and on differential experimental conditions as previously suggested, but may also depend on some characteristic of the aphid that remains to be determined.

Introduction

Aphids are non-target pests of genetically modified, insect-resistant (GM-IR) plant technologies, and have the potential to mediate the transfer of entomotoxins, usually Cry toxins from *Bacillus thuringiensis* Berliner (Bt), to natural enemies through tritrophic exposure. Adverse effects on aphids reduce their quality or quantity as prey or host, potentially causing indirect detrimental effects on beneficial insects like natural enemies, and so might induce structural and functional changes in established food webs (e.g., Zhang et al., 2006; Stephens et al., 2012). However, the literature contains several contrasting results regarding detection and effects of Cry toxins on aphids.

Since Raps et al. (2001) demonstrated that Cry toxins have little or no presence in the phloem of Event 176 maize, aphids have been discounted by several researchers as a relevant exposure route to natural enemies. In some studies, Cry proteins were not detected in aphids (e.g., Head et al., 2001; Dutton et al., 2002, 2004; Schuler et al., 2005; Ramirez-Romero et al., 2008; Romeis & Meissle, 2011), and most researchers have not found any ecological effects on aphids in controlled environmental conditions (Lozzia et al., 1998; Schuler et al., 2001, 2005; Ramirez-Romero et al., 2008; Lawo et al., 2009; Zhang et al., 2012, 2013). Consequently, some of these researchers suggested that aphids generally do not acquire Cry proteins, and/or are not affected by the tested Cry proteins because Bt toxicity is order specific. In contrast, other researchers found aphicidal effects of some Bt toxins (Walters & English,

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1995; Porcar et al., 2009). Other studies demonstrated that detrimental effects were neutralized across generations (Lumbierres et al., 2004), and some effects might be beneficial for the aphid population (Liu et al., 2005). These works, together with the ones that demonstrated Cry protein acquisition by aphids (Zhang et al., 2006, 2012; Burgio et al., 2007, 2011), indicate that aphids can be a relevant exposure route for Cry toxins to natural enemies.

The common denominator in nearly all these studies is that they reared the aphids on Bt plants, in which the Cry toxin level can vary according to biotic and abiotic factors (Dong & Li, 2007). Only Porcar et al. (2009) tested aphids on an artificial diet with known constant exposure. Consequently, it is possible that the diversity of results regarding detection and effects on aphids is due to a lack of controlled exposure, and not just a lack of toxicity. To test this hypothesis, we used an artificial aphid rearing system to detect ingestion and examine potential effects of Cry1Ac and Cry1F on mortality and population growth rate of cabbage aphid, *Brevicoryne brassicae* (L.), and green peach aphid, *Myzus persicae* (Sulzer) (both Hemiptera: Aphididae). As Cry1Ac and Cry1F are present separately or together in several Bt crops, they were exposed to the aphids separately or in combination. *Myzus persicae* occurs on several of the commercially available Bt crops in Brazil and elsewhere in the world, and *B. brassicae* was used to examine the consistency of responses across another aphid species.

Materials and methods

Preparation of the bioassays

The aphids were reared on collard plants in a greenhouse at L16:D8 photoperiod, 25 ± 2 °C, and 60 ± 10% r.h., or collected from collard leaves from organic farms. Purified, trypsinized Cry1Ac and Cry1F (94–96% purity) were purchased from M. Pusztai-Carey (Case Western Reserve University, Cleveland, OH, USA). Trypsin was removed using two high-performance liquid chromatography (HPLC) steps: ion exchange followed by gel filtration. The biological activity of the Cry proteins was confirmed against *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae) as described in Nakasu et al. (2013). Cry proteins or bovine serum albumin (BSA) were solubilized in 50 mM Na_2CO_3 buffer solution containing 100 mM NaCl pH 10 with 10 mM dithiothreitol added before use (details in Paula et al., 2014). They were then added to a liquid holidic diet that had been developed for several aphid species, including *M. persicae* (Dadd & Mittler, 1966). Control diets had the same buffer solution added without any Cry protein. Degradation of the Cry proteins in the diets was checked by measuring their concentrations by enzyme-linked immunosorbent assay (ELISA) after 24 and 72 h in the diet. Cages are described in detail by Douglas & van Emden (2007). Briefly, they were 25-mm plastic tubes with 300 µl of diet sandwiched in a sachet of two pieces of stretched Parafilm attached to one end of the tube. The degradation experiments were conducted concurrently with the Cry bioassay described below, using the same diets and tubes. To confirm aphid feeding on the holidic diet, we added food dyes to the diet, confined single apterous adults to the diet (11 *M. persicae* and eight *B. brassicae*), and recorded the presence of dyes in the gut and honeydew.

Bioassays

Five reproductive apterous adults of each aphid species were transferred to cages containing diet. As the apterae in each replicate experiment were not the same age, they were first collected from the plant source into a large tray, and then they were randomly allocated to cages and treatments. Thus, within a replicate experiment, aphid age was controlled to be similar in all cages. We then conducted a bioassay to explore the effects of two Cry toxins separately or together on two aphid species in one of four treatments: (1) control (no toxin, only diet and buffer); (2) Cry1Ac, 20 µg ml^{-1} (= 20 parts per million) (C20); (3) Cry1F, 20 µg ml^{-1} (F20); and (4) Cry1Ac, 20 µg ml^{-1} + Cry1F, 20 µg ml^{-1} (C20:F20). These concentrations were similar to that in leaves of WideStrike cotton (Siebert et al., 2009). Each treatment for each aphid species was replicated in 10 cages within one experiment, and this experiment was repeated twice for *B. brassicae* (n = 2) and 3× for *M. persicae* (n = 3). Twice a day the number of adults alive and the number of offspring were recorded to be able to estimate adult mortality and net population replacement rate [λ (per day)]. In addition, the number of apterae on the diet was recorded as an indication of the relative use of the diets. The evaluations stopped on the 3rd day based on preliminary experiments that showed that the high population density that occurred in some cages after 3 days depressed estimates of λ through density-dependent factors. The experiments were conducted in a controlled environment chamber (25 ± 2 °C and L16:D8 photoperiod).

Concurrent with the previous bioassay, we tested whether *M. persicae* was uptaking Cry protein into its body or just passing it through its digestive system without uptake. Uptake is defined as the absorption and incorporation of a substance into a living organism (USEPA, 2013). We measured protein using ELISA in five replicates of 100 aphids exposed for 24 h to Cry1F (F20) diet and then transferred to control diets for an additional 24 h. According to Wright et al. (1985), *M. persicae* excreted ingested

diet within 23 h. Therefore, if the *M. persicae* in our experiment were uptaking Cry protein, it would still be detectable after 24 h on the control diet, but if it was simply excreted in honeydew, there would be no detection. As no Cry proteins were detected in *B. brassicae*, it was not used in this test, and as Cry1F was more frequently detected in *M. persicae* than Cry1Ac, only Cry1F was used.

Finally, we conducted a bioassay to determine whether a Cry protein or neutral protein, such as BSA, could affect aphids in a similar way. There were three treatments in each experiment: (1) control (no protein added, only diet and buffer); (2) BSA, 20 μg ml^{-1} (BSA); and (3) Cry1F, 20 μg ml^{-1} (F20). Each treatment consisted of about 15 replicate cages. This experiment was done twice with *M. persicae*. Sampling and all other methods were done as described in the Cry bioassay.

Detection of Cry toxins

Cry proteins were detected and quantified using ELISA in the aphids and diets using additional samples that were not a part of the experiments described above. These samples were conducted at the same time as the Cry bioassay. Replicates of 100 aphids from each species exposed for 24 h on each of the three Cry treatment diets and the control diet were weighed (five replicates per diet for *B. brassicae* and six replicates per diet for *M. persicae*), macerated, and homogenized in phosphate buffered saline with Tween 20 (Agdia, Elkhart, IN, USA) (PBST) in a volume (in μl) corresponding to 70× the fresh weight (FW) (in mg) to normalize the amount of total protein across samples. Each individual sample (aphids and diets of each treatment) was applied (100 μl per well), at least in duplicate technical replicates, in a double-sandwich ELISA PathoScreen plate (Agdia) for separate detection of Cry1Ac and Cry1F, according to manufacturer's instructions. The tested Cry1Ac and Cry1F proteins were also used as calibration standards at doses recommended by the manufacturer. The slope of the Cry1Ac calibration curve was much less than the slope of Cry1F, resulting in lower estimated protein concentrations in the Cry1Ac samples than the Cry1F samples. The absorbance was measured at 630 nm by a microtiter plate reader (TP Reader NM; ThermoPlate, São Paolo, Brasil). For each plate, the average absorbance of the buffer wells was subtracted from the absorbance of the other wells to correct for plate effects. The corrected control absorbance was subtracted from corrected treatment absorbance to estimate the Cry protein absorbance, and technical replicates were averaged. The limits of detection (LODs) for Cry1Ac and Cry1F were calculated using the standard deviation (SD) and slope method (IUPAC, 2014). For Cry1Ac, the LOD was 0.022 ng per well based on 19 samples tested in duplicate, and for Cry1F, the LOD was 0.006 ng per well based on 29 samples tested in duplicate. These values are similar to those reported in the literature.

Statistical analysis

Positive detection was determined using Welch's t-test (assuming unequal sample size and unequal variance) using a standard error calculated from the variances associated with the buffers, controls, and technical replicates. The amount of toxins in each sample (diets and aphids) was estimated from the measured absorbances using the average slope from the linear regressions obtained from the standard curves estimated for each plate. Adult mortality was compared among the treatments with logistic regression (Proc GENMOD, SAS 2014; SAS Institute, Cary, NC, USA). The percent of adults on the diet was calculated by averaging all observations on a cage during the experiment and the data were analyzed by ANOVA (Proc GLM). Net population replacement rate (λ) was calculated (Carey, 2001) and analyzed with ANOVA (Proc GLM). For the Cry bioassay experiment, a priori contrasts were used to test the hypotheses (1) the effect of Cry1Ac and Cry1F was the same, and (2) the two toxins did not interact (i.e., the effect of Cry1Ac and Cry1F together was the same as the sum of the two separate effects). In addition, means of significant effects were separated with Tukey's honestly significant difference (HSD) test. The Cry concentrations in the diets after 24 and 72 h were compared using a paired t-test.

Results

Cry toxins did not degrade during the bioassays

All Cry diet samples saturated the ELISA reader so we report minimum, underestimated diet concentrations based on a linear extrapolation of the standard curves. After 24 h, Cry1Ac concentration was greater than 8.3 μg ml^{-1} and Cry1F concentration was greater than 13.5 μg ml^{-1}. Thus, the diets likely contained the planned concentrations of Cry protein. Despite saturation of the reader, we could still determine from the paired diet samples that the amount of Cry proteins in the diets did not decline significantly during the exposure bioassays ($t = 0.63$, d.f. $= 11$, $P = 0.54$).

Confirmation of aphid diet consumption

Food dyes were detected in 10/11 *M. persicae* and 7/8 *B. brassicae* after 20 h on the artificial diet, indicating that nearly all aphids readily consumed the diet. In *M. persicae*, the food dye could be readily observed in a consolidated bolus inside the gut, whereas in *B. brassicae*, the food dye was observed as streaks of color inside the gut. Thus, it is

possible that *M. persicae* may have consumed more diet or retained the diet longer than *B. brassicae*.

Differential Cry toxin detection in aphid species

No Cry1F or Cry1Ac were detected in *B. brassicae* feeding on any of the Cry diets (n = 15), whereas in *M. persicae* Cry1Ac was detected in 55% of samples exposed alone or in combination with Cry1F (both n = 11), and Cry1F was detected in 67% of samples exposed alone (n = 10) or in combination with Cry1Ac (n = 11). The measured quantities of Cry1Ac and Cry1F were bimodally distributed in *M. persicae* (Figure 1), indicating that some aphid samples acquired Cry protein after 24-h exposure while others did not. In general, samples with detectable Cry protein were above the LOD and those with undetectable protein were below the LOD. If this variation was due to normal variation in protein acquisition rates, the distributions would be unimodal. Detection of the two Cry proteins was highly correlated. *Myzus persicae* samples that acquired Cry protein acquired both Cry1Ac and Cry1F, and when one of the proteins was not detected, neither was the other. In the *M. persicae* samples that acquired Cry protein, the average amount (mean ± SE) in *M. persicae* was 0.54 ± 0.087 ng Cry1Ac mg^{-1} FW and 4.3 ± 0.552 ng Cry1F mg^{-1} FW, and these were less than the concentrations in the diets. When averaged to include the aphid samples that did not acquire any Cry protein, *M. persicae* would have 0.27 ng Cry1Ac mg^{-1} FW and 2.87 ng Cry1F mg^{-1} FW.

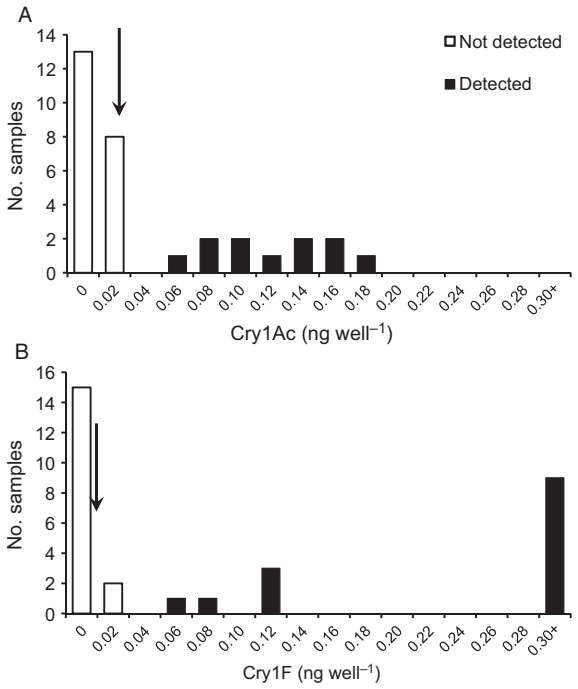

Figure 1 Frequency of amounts (ng per well) of (A) Cry1Ac and (B) Cry1F proteins in *Myzus persicae* samples showing samples with statistically detectable and not detectable values. Arrows indicate the calculated limits of detection (ng per well).

No Cry1F was detected in any of the *M. persicae* samples that were first exposed to Cry1F diet and then transferred to control diet for 24 h. This suggests that *M. persicae* excreted all of Cry1F in honeydew and did not uptake it from the diet. Acquisition of Cry protein was temporary, and possibly related to the quantity of diet consumed, and the degradation and retention of the protein in the digestive system of *M. persicae*.

Neutral protein has no detectable effects

Bovine serum albumin had similar effects as the control on *M. persicae* mortality, association with the holidic diet, and net population growth rate (Figure 2). F20 also had similar effects on *M. persicae* mortality and association with the diet, but net population growth rate was lower than in the control (Figure 2C), repeating the result of the Cry bioassay. That in all of the treatments a similar proportion of adult apterae was associated with the diet may indicate that the diets did not affect the feeding behavior of the aphids. These results indicate that the effect of F20 on the population growth rate was not due to the presence of protein in the diet, as BSA had no effect on the population growth rate. Because the concentration of the proteins was only 20 μg ml^{-1}, the proteins were unlikely to have greatly affected the physical properties of the diet.

Differential Cry toxin effects on aphid species

Neither of the two toxins affected the survival of either aphid species, whether tested in combination or separately (*B. brassicae*: $F_{3,55} = 0.509$, $P = 0.68$; *M. persicae*: $F_{3,88} = 1.56$, $P = 0.21$; Figure 3A). The toxins also did not affect the number of the adults on the diet (*B. brassicae*: $F_{3,55} = 0.25$, $P = 0.86$; *M. persicae*: $F_{3,88} = 1.53$, $P = 0.21$; Figure 3B), possibly indicating that the diets did not affect aphid feeding behavior. The toxins affected the net population growth rate (Figure 3B) of *M. persicae* ($F_{3,88} = 12.25$, $P<0.0001$), but not of *B. brassicae* ($F_{3,55} = 1.29$, $P = 0.29$). For *M. persicae*, the reduction in growth rate was similar for Cry1Ac and Cry1F and there was no synergism detected from co-feeding the aphids with both Cry toxins.

Discussion

It has been previously reported that when Cry toxins were detected in aphids, they were not observed in all aphid

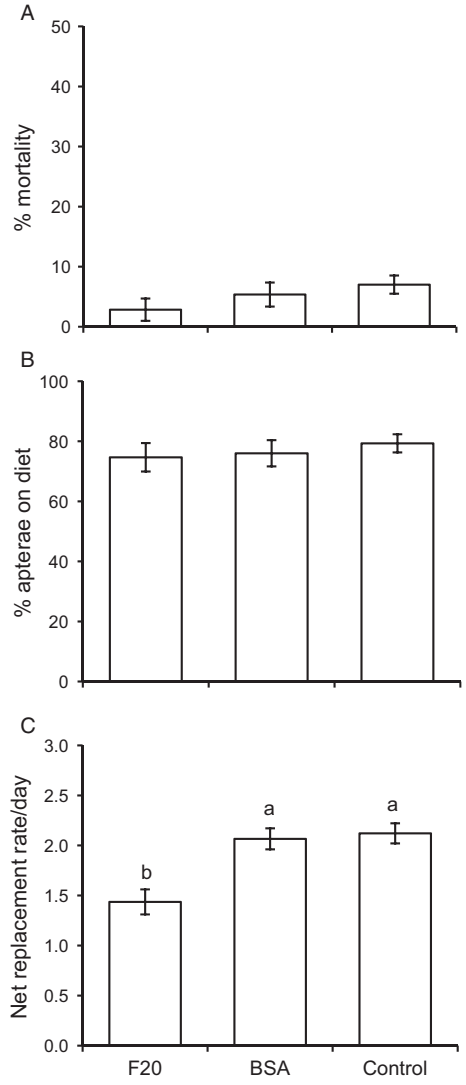

Figure 2 Effect of bovine serum albumin (BSA) and Cry1F at 20 μg ml^{-1} offered to *Myzus persicae* in an artificial diet on mean (± SE) (A) adult mortality (%; $F_{2,70} = 1.55$, $P = 0.22$), (B) fraction of adults on diet (%; $F_{2,70} = 0.55$, $P = 0.58$), and (C) net population growth rate ($F_{2,70} = 11.65$, $P = 0.00004$). Means capped with different letters are significantly different (Tukey's HSD: $P<0.05$).

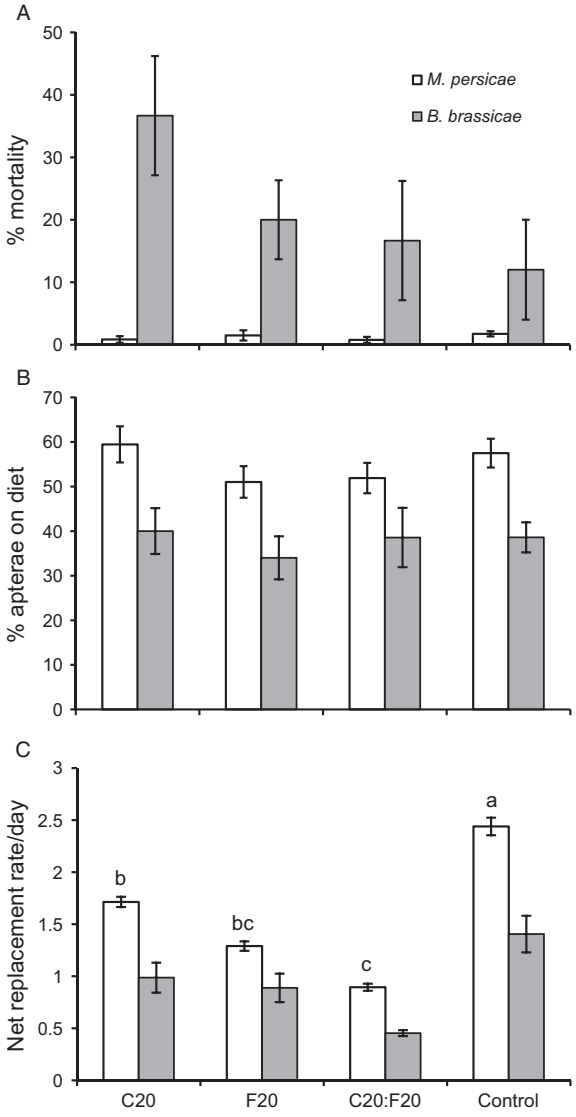

Figure 3 Effect of Cry1Ac and Cry1F at 20 μg ml^{-1} offered in combination (C20:F20) or separately (C20, F20) to *Myzus persicae* and *Brevicoryne brassicae* in an artificial diet on mean (± SE) (A) adult mortality (%), (B) fraction of adults on diet (%), and C) net population growth rate. Means capped with different letters are significantly different (Tukey's HSD: $P<0.05$).

samples (Zhang et al., 2006, 2012; Burgio et al., 2007, 2011). Romeis & Meissle (2011) asserted that such variation was the result of sample contamination, but this is not a likely explanation either for the previously published results (refuted by Burgio et al., 2011; Zhang et al., 2012) or for ours. The variation in detection reported in the literature could be attributed to variable Cry concentrations in the Bt plant. In our case, however, even using an artificial system with uniform and constant Cry toxin exposure, the histogram of Cry toxin concentrations was bimodal, indicating that some aphid samples acquired Cry toxins and some did not. Only *M. persicae* and not *B. brassicae* acquired Cry toxins, but not by all aphid samples as the toxins were not detected in about 33% of the samples. The reason for this variation is not clear, but it may be related to intra- and inter-specific variation in some characteristic of the aphid in relation to the artificial diet, possibly related to ingestion, protein degradation in the digestive

system (Li et al., 2011), and excretion rates. An implication of this study is that the variability in detection and effects of Cry toxins on aphids may not depend only on the presence and level of a Cry toxin in the phloem, and the differential experimental conditions as previously suggested, but may also depend on some characteristic of the aphid population that remains to be determined.

Acquisition of the toxins resulted in lower population growth of *M. persicae*, even though these toxins were expected to have toxicity only for lepidopterans (Höfte & Whiteley, 1989). The effects were not great enough to affect aphid survival, but a longer study may have revealed additional effects. Under the same experimental conditions, *B. brassicae* either did not acquire or acquired too little Cry toxin to be detected or to be affected. Our results corroborate the previously published contrasting results regarding detection and effects of Cry toxins on aphids. No synergistic effects of the Cry toxins were observed, similar to that observed by Porcar et al. (2009), who examined synergistic effects of Cry3A, Cry4Aa, Cry11Aa, and Cyt1Aa. Survival rates of *M. persicae* on the diet were uniform and high, whereas survival was lower in *B. brassicae*, possibly because the diet had not been optimized for this species. Despite this possibility, many of the remaining live *B. brassicae* had high reproduction and visible honeydew production, indicating significant ingestion of diet.

The effects of Cry toxins in Bt plants on aphids may be difficult to observe because the plants may not express detectable levels of Cry toxin in phloem tissue. Cry toxin concentrations in plant phloem have been reported only for Event 176 maize (Raps et al., 2001). In addition, physiological changes in the plant may have stronger effects on the aphids. For example, Lumbierres et al. (2004) found that on Bt maize plants aphids initially had a higher growth rate than on non-Bt maize, but this benefit disappeared as the maize matured during the growing season. There are many nutritional changes in maturing maize plants that could affect aphids. Faria et al. (2007) suggested that effects of Bt maize on aphids were related to amino acid concentrations in the phloem, but did not characterize the Cry toxin profile in the plants. Our result is not related to changes in a plant, but its relevance to the field is not certain. Whether there is exposure to Cry toxin on a Bt plant and whether physiological or ontogenetic changes in plants will obscure the effects of Cry toxins is unknown.

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