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Degradation of transgenic *Bacillus thuringiensis* proteins in corn tissue in response to post-harvest management practices

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Abstract Knowledge of the persistence of Cry proteins in transgenic corn residue after harvest is necessary to assess the ecological risk to nontarget organisms. The amount of protein remaining in crop residue declines over time by a combination of microbial decomposition and leaching, both influenced by temperature, precipitation, and the amount of residue-soil contact. Here, we investigated how long biologically active Cry proteins persist in SmartStax corn residue expressing Cry1A.105, Cry1F, Cry2Ab2, Cry3Bb1, and Cry34/35Ab1, when subjected to four post-harvest practices (chisel plow tillage, flail mowing, cover crop planting, and undisturbed residue). Protein activity in residue samples collected up to 25 weeks after harvest was measured by *Ostrinia nubilalis* feeding bioassays and cross validated with detection frequencies determined by ELISA. All corn residue remained above ground in the flail-mowed and undisturbed treatments, while the cover crop and chisel plow treatments left 88.3 and 39.6% of the residue remaining above ground, respectively. Cry proteins retained biological activity for as long as 24 weeks after harvest when residue was left above ground with less soil contact, typical of no-till corn systems. ELISA detections were positively correlated with results of the feeding bioassays, which revealed the presence of active proteins beyond the point of ELISA detection.

Keywords Agriculture · *Ostrinia nubilalis* · Post-harvest practices · ELISA · Bioassay · No-till

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Introduction

Transgenic corn (*Zea mays*) expressing insecticidal proteins sourced from *Bacillus thuringiensis* (Bt) is a major pest management tool used in more than 80% of the US corn acreage (USDA-ERS 2020). This technology reduces yield loss and insecticide use, conserves biocontrol agents, suppresses regional pest populations, and provides economic benefits to farmers (Shelton et al. 2002; Carrière et al. 2003; Cattaneo et al. 2006; Wu et al. 2008; Hutchison et al. 2010; Edgerton et al. 2012; Lu et al. 2012; Kathage and Qaim 2012; NASEM 2016; Perry et al. 2016; Dively et al. 2018; Romeis et al. 2019). However, the extensive use of Bt corn has raised concerns regarding environmental risks, especially the potential negative

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impacts of Bt proteins to non-target organisms. This has inspired numerous investigations on Bt protein effects on non-target taxa and changes in the community composition, abundance and diversity of various organisms during the Bt crop cycle. Results of these studies have been summarized in several reviews and meta-analyses (Marvier et al. 2007; Wolfenbarger et al. 2008; Yu et al. 2011; Comas et al. 2014; Romeis et al. 2014), which together indicate that adverse effects from Bt crops are few compared to insecticide-treated controls and comparable to non-expressing isogenic controls. Additional studies have found no conclusive evidence that Cry proteins that remain in Bt corn residue after harvest have an adverse effect on non-target organisms (Saxena and Stotzky 2001; Clark and Coats 2006; Hönemann et al. 2008; Icoz and Stotzky 2008; Bai et al. 2012). Furthermore, information on the potential post-harvest exposure of transgenic Bt proteins to non-target organisms is not a major focus of regulatory risk assessments (Rose 2007).

There are several factors that may influence the post-harvest fate of Bt proteins in transgenic crops. Most Bt corn hybrids currently contain stacked and pyramided genes expressing multiple proteins to reduce the risk of pest resistance and provide broader control of pest populations (Carrière et al. 2016). These multiple proteins may persist longer in senescing crop tissue, resulting in a prolonged period of exposure to non-target organisms. No-till and reduced tillage agricultural systems, currently used on more than 70% of the crop acreage in the US (USDA-NASS 2017), allow significant amounts of corn residue to remain on the soil surface after harvest (Hickman and Schoenberger 1989). This residue represents the primary source of Bt proteins entering agricultural fields and neighboring habitats (Zwahlen et al. 2007; Jensen et al. 2010; Pisanie et al. 2019), and potentially exposes non-target organisms by contact or direct feeding on senescing tissue and proteins released into the soil environment. The amount of crop residue remaining in a field is reduced over time by microbial decomposition. Studies examining the decomposition rates of Bt and non-Bt post-harvest corn residue have reported conflicting results, with some studies reporting slower decomposition in Bt compared to non-Bt tissue (Saxena and Stotzky 2001; Castaldini et al. 2005; Flores et al. 2005; Poerschmann et al. 2005; Fang et al. 2007); while others report similar rates of decomposition (Zwahlen et al. 2007; Tarkalson et al. 2008; Lehman et al. 2008; Daudu et al. 2009; Zurbrügg et al. 2010; Londoño-R et al. 2013; Al-Kaisi 2019). The majority of studies indicate that the presence of Bt proteins does not influence the decomposition dynamics of corn residue. However, studies have shown significant effects of temperature, precipitation, and the amount of residue-soil contact on decomposition of corn residue. Burgess et al. (2002) reported that buried mesh litterbags containing post-harvest corn tissue decomposed significantly faster than those that remained on the soil surface. Al-Kaisi and Guzman (2013) also found that buried residue of simulated strip- and deep-tillage treatments tended to have greater decomposition than corn residue on the soil surface. Moreover, residues remaining on the soil surface experienced greater fluctuations in temperature and moisture content, subsequently reducing the amount of time that conditions are optimal for tissue decomposition (Parr and Papendick 1978; Summerell and Burgess 1989; Zwahlen et al. 2003). This suggests that the breakdown of Bt proteins may differ according to post-harvest management practices that can influence the amount of crop residue remaining on the soil surface.

Common post-harvest practices used in the mid-Atlantic US include tillage, cover crop planting, mowing, or leaving the corn residue undisturbed on the soil surface. Chisel plowing and disking, as the primary tillage methods, incorporate varying amounts of residue into the soil and usually leave less than 30% on the surface. Establishment of a cover crop using a no-till drill planter incorporates roughly 10–20% of the residue tissue into the soil (NRCS 2000) and flattens the standing stalks closer to the ground surface. Mowing the standing corn stalks chops the residue into smaller pieces and redistributes them on the soil surface, allowing closer residue-soil contact and presumably quicker breakdown of the residue.

Knowledge of the persistence of active Cry proteins in corn residue after harvest is essential for assessing the ecological risk of transgenic Bt toxins to nontarget organisms. Root exudation and incorporation of senesced plant litter after harvest provide major sources of carbon for agricultural soils (Icoz and Stotzky 2008) and represent two pathways by which active Cry proteins are introduced into the soil rhizosphere (Saxena and Stotzky 2000). Protein degradation rates in the soil are influenced largely by

temperature and protein type (Sanvido et al. 2007; Hung et al. 2016). Under certain environmental conditions, Cry proteins have been detected in Bt corn residue and the soil rhizosphere of fields up to several months after the crop was harvested (Arias-Martín et al. 2016, Stotzky 2005, Palm et al. 1996). Consequently, the potential for long-term Cry protein persistence in agricultural soils has raised concern regarding their potential impact on non-target organisms (Andow and Zwahlen 2016; Arias-Martín et al. 2016). To date, studies investigating the effects of Cry proteins from Bt crops on post-harvest invertebrate communities have found no consistent or lasting adverse effects on a range of soil organisms including woodlice, collembolans, mites, earthworms, nematodes and protozoa (Saxena and Stotzky 2001; Icoz and Stotzky 2008; Bai et al. 2012; Clark and Coates 2006; Hönemann et al. 2008). The potential exposure and impact of crop residue containing Bt proteins entering aquatic systems has also been addressed (Swan et al. 2009; Chambers et al. 2010), and some aquatic invertebrates have been shown to be affected (Jensen et al. 2010; Axelsson et al. 2011, Venter et al. 2016). Although studies show no convincing evidence of adverse effects from expressed Bt proteins in crop residue on non-target communities, further research is needed to fully understand the movement and fate of Bt proteins for the regulatory risk assessment process.

To our knowledge, no studies have been conducted to determine the effects of post-harvest practices on the persistence of Bt proteins in corn residue. Here we determined how long biologically active Cry proteins persist in corn residues, when subjected to different post-harvest management practices. We hypothesized that Cry proteins would lose biological activity due to degradation and leaching at a faster rate if the corn residue is subjected to post-harvest practices that increase residue-soil contact. It is conceivable that certain practices may shorten the period of biological activity, thus reducing the potential risk to non-target organisms. Non-target studies that require confirmation of Bt protein exposure to sensitive non-target organisms often use an enzyme-linked immunosorbent assay (ELISA) to determine if Bt proteins are present in corn tissue (Zwahlen et al. 2003; Clark et al. 2005; Daudu et al. 2009). However, these assays can produce false-positives indicating the presence of structurally altered proteins but not biologically active ones (Marchetti et al. 2007; Gruber et al. 2011; Albright et al. 2016; Deng et al. 2019). In this study, all residue samples collected after harvest were analyzed using feeding bioassays and cross validated with detection frequencies determined by ELISA. We hypothesized that the ELISA method would detect the presence of Cry proteins beyond their period of biological activity.

Materials and methods

Study site and hybrids

The study was repeated twice in the same field during the fall and winter seasons of 2014–2015 (year 1) and 2016–2017 (year 2) at the Central Maryland Research and Education Center in Beltsville, MD. The field site (latitude: 39.012420°, longitude: − 76.825712°) is located in the Atlantic Coastal Plain eco-region. Total precipitation was approximately 612 mm during sampling months in 2014/2015 and 506 mm in 2016/2017. Soils at the field site are Elsinboro series fine-loamy, mixed, semiactive, mesic Typic Hapludults. The field was historically managed under a no-till corn-soybean rotation system. During both years, Dekalb® SmartStax (DKC62-08) was used as the Bt hybrid and compared to a near isoline non-Bt hybrid (DKC62-05) as a control. The SmartStax hybrid expressed Cry3Bb1 (event 88,017), Cry34Ab1 (event DAS-59122-7) and Cry35Ab1 (event DAS 59,122-7), all active against corn rootworms (*Diabrotica* spp.); Cry1A.105 (event MON 89,034), Cry2Ab2 (event MON 89,034), and Cry1F (event TCI507), active against a complex of lepidopteran pests [primarily European corn borer, *Ostrinia nubilalis* (Hubner); corn earworm/bollworm, *Helicoverpa zea* (Boddie); and fall armyworm, *Spodoptera frugiperda* (J.E.Smith)] and two traits providing tolerance to glufosinate and glyphosate herbicides. All seeds were treated with a standard fungicide combination and 0.25 mg a.i. per kernel of clothianidin (Poncho 250, Bayer CropScience) to control soil insects and diseases.

In both years, each hybrid was planted into a winter cover crop that was killed using conventional herbicides two weeks before planting. Corn was planted on May 20 (2014) and April 26 (2016) in 76 cm row spacings with a six-row no-till planter set at a seeding rate to achieve 65,000 corn plants per ha. Weed and

nutrient management was applied according to recommended production practices. Plots were harvested by combine (John Deere model s660) in late September after kernel moisture was less than 20%. The combine was equipped with a rotary spreader that evenly distributed the corn residue over a swath that was roughly equal to the width of the combine head (4.6 m).

Treatments and experimental design

Post-harvest treatments were arranged in a randomized block split-plot design with hybrid type (SmartStax or non-Bt isoline) as the whole plot factor and post-harvest management practice as the subplot factor. Subplots were randomly assigned to four management practices: 1) tillage produced by a single pass of a chisel plow with straight points penetrating the soil to a depth of 38 cm; 2) mowing with a flail mower that chopped the standing stalks and evenly distributed the residue over the soil surface; 3) flail mowing followed by planting of a mixed cover crop of rye (*Secale cereale*), crimson clover (*Trifolium incarnatum*) and tillage radish (*Raphanus sativus*), seeded with a no-till drill (Great Plains model 1510) in 19 cm rows; and 4) the undisturbed corn residue following harvest. Each subplot contained 12 harvested rows of corn measuring 9.14 m by 15.24 m, and each management practice was randomized within whole plots replicated three times. In 2014, all plots were directly adjacent to each other within each whole plot, whereas subplots in 2016 were separated by a 9.14 m wide non-crop buffer to allow for equipment movement. Post-harvest management practices were applied within one week following corn harvest.

Corn residue tissue sampling

In year 1, leaf tissue samples were collected from both hybrids at growth stages R1 (silking) and R6 (harvest maturity) for bioassays to serve as the baseline level of toxin activity compared to the post-harvest activity. Immediately after establishment of each post-harvest treatment, a 0.42 m^2 circular frame was randomly placed over the soil surface and all plant material within the frame was collected. One sample was taken from each subplot, except for the undisturbed treatments that were assumed to contain the same amount of surface residue recorded in the flail mowed subplots. Samples were weighed in the laboratory and the wet biomass recorded.

Following application of the post-harvest treatments, residue tissue samples were collected every two weeks up to eight weeks, and then every four weeks thereafter until mid-April. For the flail mowed, cover crop and undisturbed treatments, approximately 100 g of plant residue was collected from the soil surface within each subplot per hybrid. Samples in year 1 consisted primarily of leaf tissue, except the flail mowed samples included residue from all tissue types which could not be easily differentiated. We standardized the sampling protocol in year 2 by collecting all available surface residue (leaf, stalk and husk tissue) in all subplot treatments. Because the chisel plow treatment buried the majority of the plant residue in the soil, it was not practical to collect individual pieces of the soil-incorporated tissue following tillage, especially in frozen soil. Alternatively, mesh litterbags were filled with approximately 100 g of plant residue collected from each subplot prior to the application of the chisel plow treatment. Bags used in 2014 measured 10 cm by 25 cm with 5 mm mesh openings but were changed to larger bags (38 cm by 63.5 cm) with wider mesh openings in 2016 (Fig. 1). After chisel plowing, eight litterbags per subplot were buried 0.6 m apart in the center of each subplot to a depth of 15–20 cm to mimic the depth of the chisel plow. One litterbag was removed from each subplot at each sampling period.

Bioactivity measurements

The leaf tissue samples prior to harvest and the post-treatment samples collected from each subplot were cut into small pieces and homogenized in the laboratory. A random subsample was then removed, frozen at − 80 °C and then freeze-dried (Labconco freeze drier model 195) at 0.2 Pa vacuum pressure to lyophilize the tissue to prevent denaturing of Bt proteins. Samples of the lyophilized Bt and non-Bt plant tissue from each subplot were then ground to a fine powder (IKA Works, Inc., model A11, Wilmington, DE), passed through a 500 μm sieve, and stored at − 80 °C.

To determine the biological activity of the lepidopteran-specific Cry1A.105, Cry2Ab2 and Cry1F proteins, laboratory feeding bioassays were performed on *O. nubilalis* obtained from a commercial insectary

Fig. 1 Litterbag method used during year 1 (left), compared to the litterbags used during year 2 (right)

(Benzon Research, Carlisle, PA) as eggs, which were placed in plastic cups containing artificial diet (Southland Products Inc., Lake Village, AR) and incubated in a growth chamber at 25 °C until early second instar larvae were available for bioassays. Three replicate samples of all treatment by hybrid combinations (n = 24) were assayed on the same day for each sampling date. For each bioassay, 1200 mL of artificial diet (adjusted with approximately 100 mL more water to offset the added tissue) was prepared and cooled in a water bath at 55 °C. For each sample, 25 mL of molten diet was drawn into a 60 mL plastic syringe and 600 mg of ground, lyophilized tissue was added to the diet and mixed thoroughly using a vortex shaker. The resulting mixture contained 24 mg of lyophilized tissue per mL of diet. Approximately 1.5 mL aliquots of the mixture were dispensed into each of 16 wells of a 128-well bioassay tray (C-D International). After the diet mixture cooled and solidified, one second-instar *O. nubilalis* larva was transferred to each well. Each 16-well section (consisting of 4 rows and 4 columns) of the bioassay tray was sealed with a perforated adhesive cover, and trays were held in a growth chamber at 25 °C, 14 h light/10 h dark cycle, and 40–60% RH. After 7 d, all live larvae within each row of four wells were recovered and weighed together, and the mean weight per larva was calculated for each sample. For each bioassay, three replicate groups of 10 larvae were weighed together at the beginning of the assay to calculate the average initial weight per larva. This average was used to adjust the final weight gain following 7 d of feeding.

ELISA strips (Quad Trait ImmunoStrip, Agdia Inc., Elkhart, IN) were used to determine the presence or absence of Cry1F, Cry2A, Cry34Ab1 and Cry3Bb1 proteins in samples of the three replicate subplots. Test strips for Cry35Ab1 and Cry1A.105 were not commercially available, so it was not possible to specifically detect these proteins, even though they were introduced into the corn plant as a single cassette with Cry34Ab1 and Cry2Ab2, respectively. A 20 mg sample of ground, lyophilized tissue was placed into a microcentrifuge tube along with 1 mL of 10% buffer solution. Each tissue mixture was vortexed for 15 s and then centrifuged for 2 min at 8000 rpm. The supernatant of each sample was transferred into individual vials, and the ELISA strips inserted for 10 min. At test completion, a darker control line indicated a valid test, and light but clearly visible test lines were recorded as a positive test for the presence of each Cry protein. All ELISA strips were preserved for documentation.

Statistical analyses

A one-way analysis of variance (ANOVA; SAS Institute 1997) tested for differences in the amount of plant residue remaining on the soil surface following each post-harvest treatment. This analysis included data from both hybrids and treated replicate block and hybrid as random factors. For the bioassay

data, a three-way mixed model ANOVA tested for main and interaction effects of hybrid, post-harvest treatment, and sampling week on larval weights. Percent growth inhibition resulting from exposure to Cry proteins was calculated as the difference in mean weight gain of cohorts feeding on diets incorporated with Bt and non-Bt tissue from samples from the same replicate. This difference was divided by the larval weight gain of cohorts feeding on non-Bt tissue diet and then multiplied by 100. A two-way mixed model ANOVA tested for main and interaction effects of post-harvest treatment and sampling week on percent growth inhibition. Before each analysis, data were tested for normality and homogenous variance using the Shapiro–Wilk *W* test, Spearman's rank correlation, and by examining residual plots. Data transformations and grouping of variance were performed as necessary. For each analysis, sampling week was modeled as a repeated measure, and replicate blocks were treated as a random factor. Significant effects among means were separated by using Tukey's adjustment for pairwise comparisons ($P \leq 0.05$). Back transformed means ($\pm$ SE) are presented in the results.

The ELISA data, expressed for each sample as 1 = positive detection or 0 = negative detection, were analyzed by ANOVA using SAS PROC GLIMMIX to test for differences in the frequency of positive detections among post-harvest treatments over sampling weeks. A separate analysis was conducted for each protein (Cry1F, Cry2Ab2, Cry34Ab1 and Cry3Bb1), assuming a binary distribution of the response variable. In each analysis, the r-side modeling approach and MMPL estimation method were used, post-harvest treatment, sampling week and their interaction were treated as fixed effects, and a RANDOM statement modeled the repeated measures of binary outcomes. Means were estimated using the LSMEANS statement and tested for differences at $\alpha = 0.05$ by the pdiff option.

Results

Plant residue on soil surface

The crushing and shredding of corn plants by the combine head removed ears and the upper portion of stalks and leaves, leaving 25–30 cm of the basal portion of stalks and roots intact. The plant material that passed through the combine consisted of cobs, husks, intact pieces of cornstalks, and smaller chaff. The spinning disks of the chaff spreader distributed the discharged material uniformly behind the combine. Visual assessments estimated that 20–30% of the plant residue, particularly standing stalks and lower leaves, did not directly contact the soil surface. The flail mower chopped the standing cornstalks into smaller pieces, resulting in more direct contact with the soil without any incorporation. The total amount of plant residue on the soil surface in the flail-mowed and undisturbed treatments averaged 11.1 $\pm$ 1.0 Mg/ha. This residue amount was significantly greater than the other post-harvest treatments ($F_{(2, 1)} = 18$, $P < 0.0001$). The chisel plow treatment incorporated 60.7% of the cropped residue into the soil, with an average 4.4 $\pm$ 0.7 Mg/ha remaining on the soil surface. For the cover crop treatment, the action of the planting drill incorporated 11.7% of the residue into the soil via the seed furrows, with an average 9.8 $\pm$ 0.5 Mg/ha remaining above ground.

Feeding bioassays (year 1)

Weight gain of larvae feeding on non-Bt tissue incorporated in diet was not affected by the post-harvest treatments over time ($F_{(3, 72)} = 1.8$, $P = 0.155$). Percent growth inhibition of *O. nubilalis* larvae feeding on Bt leaf tissue was 99% at fresh silking (R1) and 98% at physiological maturity (R6), indicating very little degradation of the Cry proteins occurred prior to harvest. Bt toxin expression in the senesced plant residue also inhibited growth by 96% prior to the application of the post-harvest treatments (week 0).

Differences in growth inhibition were relatively consistent among post-harvest treatments from week 4 through week 22, as indicated by a non-significant interaction effect (Fig. 2A). Larvae feeding on diet incorporated with Bt residue from the chisel plow and undisturbed plots generally resulted in the highest growth inhibition. The week effect was significant ($F_{(8, 70)} = 9.15$, $P < 0.001$), showing a steady decline in overall percent inhibition from 97.2% at week 0–28.6% by week 25. Pooled over weeks, post-harvest treatments had a significant main effect on larval growth inhibition ($F_{(3, 70)} = 3.73$, $P = 0.015$; Fig. 2B). Overall inhibition was highest in the chisel plow (70.9% $\pm$ 5.59) and undisturbed

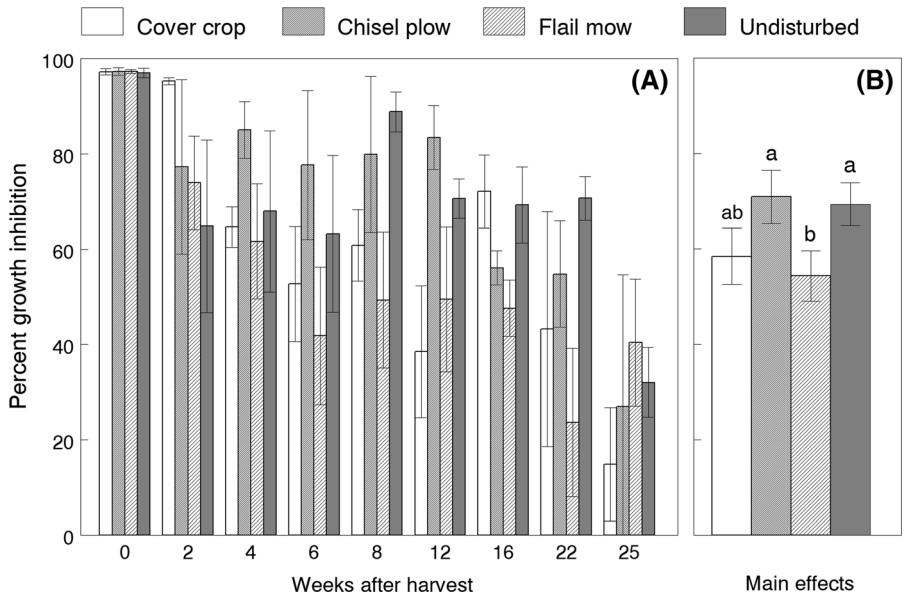

Fig. 2 Mean (± SEM) percent growth inhibition of early 2nd instar *O. nubilalis* after feeding for 7 days on diet incorporated with lyophilized Bt residue collected over 25 weeks post-harvest from plots managed under different post-harvest treatments. Percent inhibition was calculated as the difference in weight gain of larvae feeding on diets incorporated with Bt corn residue relative to those larvae exposed to non-Bt residue. Graph A shows the interaction means by week, whereas graph B shows the main effect treatment means pooled across sampling weeks. Mean bars bearing the same letter are not significantly different at the 5% probability level. Year 1

(69.4% ± 4.49) treatments. Weight gain of larvae fed diet incorporated with cover crop and flail mowed residue experienced 58.5% ± 5.92 and 54.4% ± 5.27 inhibition, respectively.

ELISA tests (year 1)

ELISA data for each Cry protein were averaged and plotted as the probability of positive detection to show trends among post-harvest treatments over sampling weeks (Fig. 3). All samples collected prior to the post-harvest treatments (week 0) tested positive for each protein, whereas all samples collected at week 22 tested negative. It was not possible to test for treatment by week effects for each protein, because the GLIMMIX model could not converge. However, rates of positive detections varied considerably among the different Cry proteins. For overall comparison, the probability of detection for each protein was averaged over weeks and treatments, excluding weeks 0 and 22. Cry3Bb1 consistently showed the lowest detection rates, averaging 12.5% ± 0.05. In contrast, the probability of positive detections for Cry34Ab1, Cry2Ab2 and Cry1F proteins averaged 90.3% ± 0.08, 59.7% ± 0.16 and 80.6% ± 0.11%, respectively, with most negative detections occurring after week 6. Pooled over proteins and weeks, probabilities of positive detections for samples collected in the cover crop, chisel plow, flail mow and undisturbed treatments averaged 50.0% ± 0.07, 66.7% ± 0.06, 54.2% ± 0.07 and 72.2% ± 0.04, respectively. There were significant week effects for all proteins, as evident by the decline in positive detections particularly after week 6.

Significant main effects of the post-harvest treatments depended on the Cry protein. For Cry3Bb1, overall probabilities of detections ranged from 5.6 to 16.7% and did not differ significantly among treatments. Similarly, treatments had no significant effect on the presence of Cry34Ab1, which was detected in 77.8–100% of samples. However, the persistence of the Cry2Ab2 ($F_{(3,56)} = 231.1$, $P < 0.001$) and Cry1F ($F_{(3,12)} = 7.31$, $P = 0.005$) proteins was significantly affected by post-harvest treatments. Overall, positive detection probabilities for Cry2Ab2 averaged 44.4% for samples collected from cover crop and flail mow plots, whereas 72.2 and 66.7% of the cover crop and flail mow samples tested positive for Cry1F,

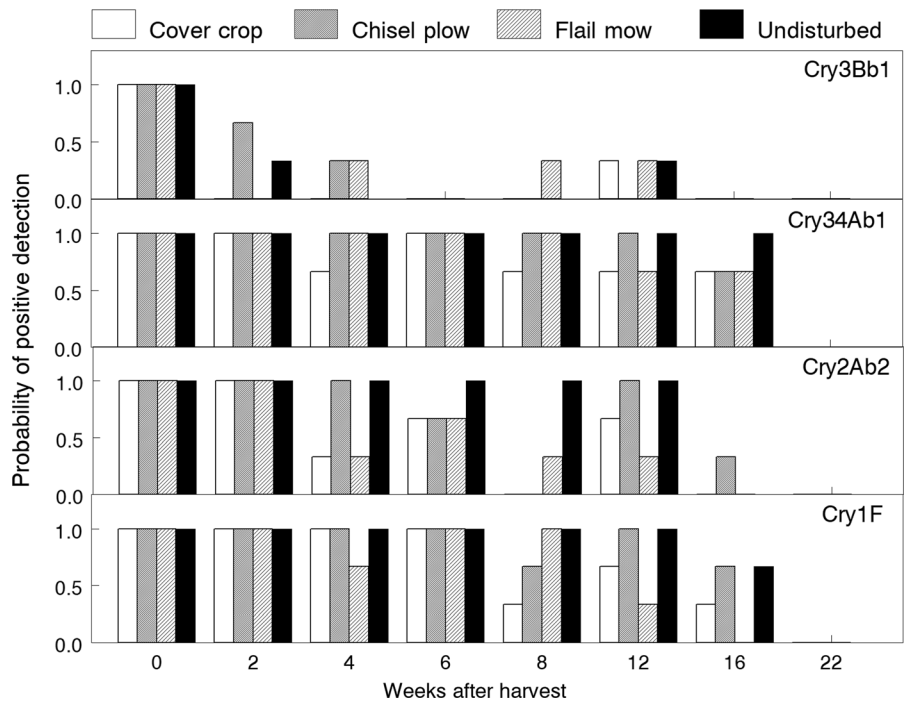

Fig. 3 Probability of positive detection of Cry3Bb1, Cry34Ab1, Cry2A and Cry1F proteins in SmartStax field corn based on an enzyme-linked immunosorbent assay (ELISA). Means of each protein are given for each post-harvest by week combination. Year 1

respectively. In comparison, detection levels were significantly higher for Cry2Ab2 and Cry1F in the chisel plow (66.7 and 88.9%, respectively) and the undisturbed plots (83.3 and 94.4%, respectively).

Feeding bioassays (year 2)

At week 0, Bt toxin expression in the senesced Bt plant tissue resulted in 90% inhibition of *O. nubilalis* larval growth (Fig. 4A). Because week 2–6 samples from the chisel plow subplots were lost, only data from weeks 8 through 24 were analyzed. Weight gain of larvae feeding on non-Bt tissue incorporated in diet was not affected by the post-harvest treatments over time ($F_{(3,\ 18.6)} = 1.82$, $P = 0.179$). The treatment by week interaction was not significant, indicating that differences in growth inhibition among post-harvest treatments were relatively consistent over the sampling period. Bioassays of residue from the chisel plot treatment consistently resulted in lower *O. nubilalis* growth inhibition, while inhibition was generally higher when fed residue from the flail mow and undisturbed subplots. There was an overall steady decline in growth inhibition from 89.7% at week 0 to 19.5% at week 24. Post-harvest treatment had a significant main effect on larval growth inhibition ($F_{(3,\ 38)} = 18.01$, $P < 0.001$; Fig. 4B), with the chisel plow residue showing significantly less protein activity (9.9% ± 4.32) than the other treatments. Larvae reared on diet incorporated with residue from the undisturbed, flail mow and cover crop treatments experienced similar levels of inhibition, averaging 55.7% ± 5.41, 53.1% ± 5.56 and 48.7% ± 4.80, respectively.

ELISA tests (year 2)

The probability of detecting Cry3Bb1, Cry24Ab1, Cry2Ab2 and Cry1F proteins in plant residues collected from post-harvest treatments varied over sampling weeks (Fig. 5). Note that data for the chisel plow treatment are missing for weeks 2–6. At week 2, all residue samples in the cover crop and flail mow treatments tested positive for each Cry protein, whereas all but one replicate of the undisturbed treatment tested positive for the Cry3Bb1 protein. Positive readings were detected in all treatments through week 24 for at least one of the four proteins tested. However, declines in positive detections varied over weeks depending on protein and post-harvest treatment. The persistence of Cry3Bb1 and Cry2Ab2 proteins declined at a faster rate in all treatments

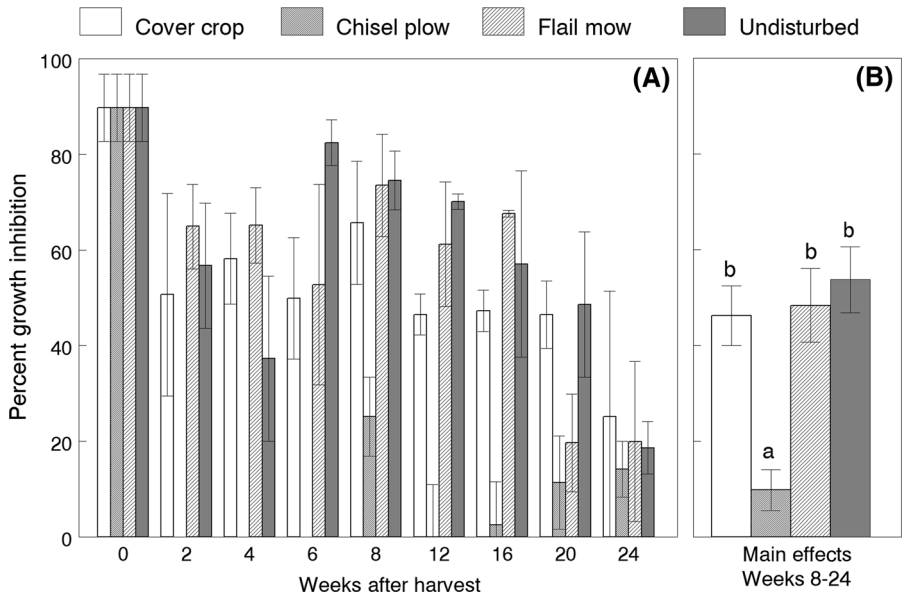

Fig. 4 Mean (± SEM) percent growth inhibition of early 2nd instar *O. nubilalis* after feeding for 7 days on diet incorporated with lyophilized Bt residue collected over 24 weeks post-harvest from plots managed under different post-harvest treatments. Percent inhibition was calculated as the difference in weight gain of larvae feeding on diets incorporated with Bt corn residue relative to those larvae exposed to non-Bt residue. Graph A shows the interaction means by week, whereas graph B shows the main effect treatment means pooled across sampling weeks. Mean bars bearing the same letter are not significantly different at the 5% probability level. Year 2

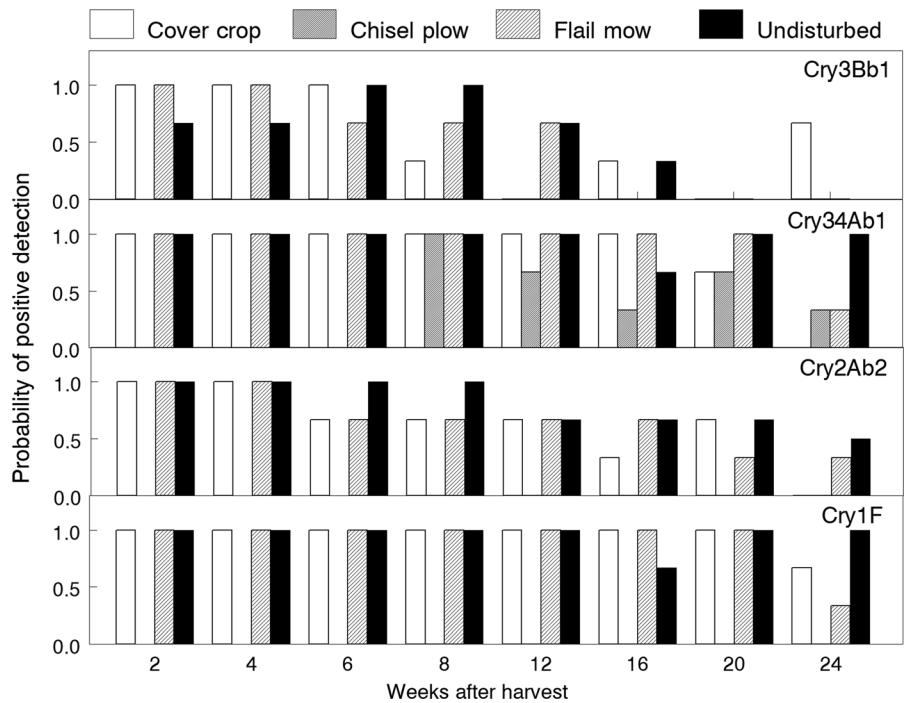

Fig. 5 Probability of positive detection of Cry3Bb1, Cry34Ab1, Cry2A and Cry1F proteins in SmartStax field corn based on an enzyme-linked immunosorbent assay (ELISA). Means of each protein are given for each post-harvest treatment by sampling week combination. Year 2

compared to the other two proteins. Cry3Bb1 and Cry2Ab2 showed the lowest detection rates, averaging an overall 23.3% ± 0.15 and 43.3% ± 0.27, respectively. Cry34Ab1 and Cry1F proteins were generally

detected over time in most residue samples, except for the chisel plow treatment that showed positive detections only for Cry34Ab1. The overall probability of positive detections for the Cry34Ab1 and Cry1F proteins averaged 78.3% ± 0.13 and 68.3% ± 0.40, respectively.

GLIMMIX analysis was able to statistically test for post-harvest treatment effects on the probability of positive detections for weeks 8–24. Main treatment effect was significant for all proteins but differed among treatments. For Cry3Bb1, detection probabilities ranged from 0 to 26.7% and were significantly lower in the chisel plow treatment ($F_{(3,\ 56)} = 367.9$, $P < 0.001$) compared to the other treatments. Similarly, detection probabilities for the Cry2Ab2 and Cry1F proteins ranged from 0 to 73.3% and 0 to 93.3%, respectively, with significantly fewer detections in the chisel plow treatment than the other treatments (Cry2Ab2: $F_{(3,\ 56)} = 452.2$, $P < 0.001$; Cry1F: $F_{(3,\ 12)} = 283.5$, $P < 0.001$). For Cry34Ab1, positive detections in undisturbed (93.3%) and flail mow (86.7%) residue were significantly higher ($F_{(3,\ 12)} = 14.1$, $P < 0.001$), compared to the cover crop (73.3%) and chisel plow (60%) residue.

Discussion

This study investigated the persistence of Cry proteins (Cry1F, Cry2Ab2, Cry34Ab1 and Cry3Bb1) expressed in Bt SmartStax corn residue subjected to different post-harvest management practices. Feeding bioassays confirmed high levels of Cry2Ab2 and Cry1F expression in plant tissue at crop senescence. Growth of *O. nubilalis* larvae was inhibited by 90–96% when fed residue collected immediately after harvest before post-harvest treatments were applied. ELISA tests also revealed 100% positive detections of all Cry proteins at that time.

In both years, levels of Cry protein detection and larval growth inhibition significantly declined with increasing weeks after harvest. Across sampling weeks, larval weight gain was not affected by feeding on diet with non-Bt corn residue collected from the different post-harvest treatments. We assumed that differences in growth inhibition were the result of the Bt concentration and any nutritional changes in the residue samples since harvest. Overall growth inhibition averaged 48% at week 22 (year 1) and 31% at week 20 (year 2), based on data pooled over the post-harvest treatments. Additionally, ELISA detected the presence of Cry proteins up to 16 weeks post-harvest in year 1 and through the last collection date at 24 weeks post-harvest in year 2. Taken together, these results provide strong evidence that biologically active Bt proteins remained in corn residue for as long as 24 weeks after harvest. Given the low concentration of lyophilized residue added to the bioassay diet (approx. 2.4%) and the high sensitivity of *O. nubilalis* larvae to Cry proteins (Huang et al. 2006, Priesnitz et al. 2016; Hilbeck et al. 2018), it is plausible that biologically active proteins of Cry2Ab2 and Cry1F in corn residue could be present even longer than 24 weeks. This prolonged persistence of biological activity is particularly noteworthy because reported levels of Cry2Ab2 and Cry1F expression in corn residue at harvest are 52 to 98% less compared to peak levels of each protein during the R4 growth stage (EPA 2010a, b).

Published studies addressing the environmental fate of Cry proteins in the soil produced conflicting results (Clark et al. 2005). Some studies report half-lives less than 7 days for purified proteins in soil (Hung et al. 2016), while other studies using plant tissue buried or left on the soil surface found detectable levels of Bt proteins after 2–6 months (Zhang et al. 2015). Soil dissipation studies submitted by registrants for the current registrations of Bt corn events report similar rates of protein loss in a soil environment. For example, a Monsanto study used three types of field-collected soil spiked with 500-fold more Cry2Ab2 protein than the maximum amount found in the field and then quantified degradation with a corn earworm (*Helicoverpa zea*) feeding bioassay. Results indicated that the protein concentration decreased by 50% in 1–6 days, and by 90% in 3–14 days (EPA 2010b). It should be noted that most environmental fate studies in support of registration were designed to quantify Cry protein dissipation (leaching and degradation) when corn residue is ploughed into the soil, typical of the post-harvest tillage practices of corn production in the Midwest Corn Belt. Although these studies show rapid loss of purified proteins spiked in soils, our results provide clear evidence that the loss of biological activity of Cry proteins expressed in decomposing corn residue on the soil surface behaves differently.

We hypothesized that Cry proteins will lose biological activity at a faster rate if the post-harvest

management practice increases the residue-soil contact. Surface residue amounts measured in year 1 at week 0 were significantly different among treatments. All standing corn stalks and plant residue on the soil surface remained above ground in the flail-mowed and undisturbed treatments, while the cover crop and chisel plow treatments left 88.3 and 39.6% of the residue remaining above ground, respectively. In general, our results show that the Cry proteins in corn residues left above ground with less soil contact were more persistent and retained biological activity much longer than what has been reported in the literature. Nonetheless, differences in biological activity among treatments were not consistent between years. In year 1, growth inhibition was greater when *O. nubilalis* larvae fed on diet incorporated with residue from the chisel plow and undisturbed treatments, indicating that lepidopteran active proteins degraded slower; whereas in year 2, significantly faster degradation was observed in residue from the litterbags of the chisel plow treatment, as hypothesized. We suspect that the slow degradation rate in the chisel plow treatment in year 1 was due to the type of litterbag used and the condition of the residue packed in the bags. These bags had much smaller openings and were tightly packed with residue compared to bags used in year 2 (Fig. 1), which likely decreased soil-residue contact, resulting in slower decomposition and protein degradation. The loosely packed bags used in year 2 more closely mimicked the soil incorporation of corn residue resulting from chisel plow tillage. Litter bags provide a convenient tool to assess the decomposition of residue over time; however, future studies should consider a litter bag design that mimics the distribution and soil contact of crop residue when incorporated by different tillage practices.

Planting a winter cover crop after corn harvest is a common practice in the mid-Atlantic US and its adoption in the Midwest is increasing (Zulauf et al. 2019). We predicted that this practice would increase residue-soil contact resulting in faster degradation of Cry proteins compared to the flail mow and undisturbed treatments. However, no significant differences in growth inhibition or ELISA detection frequencies were observed among these treatments in either year. The no-till seeding action of the planter drill incorporated 11.7% of the plant residue into the seed slots. However, we collected only surface residue and not the soil-incorporated tissue; therefore, the residue used for bioassay and ELISA testing was the same as that used in the flail mow treatment. Although the drill pressed residue closer to the soil surface, shading of the cover crop canopy probably reduced the surface temperatures, which could have negated any increase in microbial degradation brought about by increased soil contact. Studies show that Cry proteins degrade more slowly under cooler temperature conditions (Feng et al. 2011; Li et al. 2007; Zhang et al. 2015). In general, cooler surface temperatures during the winter will likely reduce the rate of Cry protein degradation in corn residue on the soil surface compared to residues tilled into the soil.

ELISA testing revealed consistent differences in the detection probabilities of the four Cry proteins. Of the rootworm-active proteins, Cry3Bb1 degraded much faster than Cry34Ab1, which is coupled with Cry35Ab1. Although these proteins target rootworms, they are expressed in leaf tissues at equal or higher concentrations than in root tissues. Depending on the corn growth stage, the concentration of Cry3Bb1 and Cry34Ab1 ranges from 189 to 240 µg/g dry weight (dwt) and 50–220 µg/g dwt, respectively. However, the Cry3Bb1 concentration in the post-harvest residue is considerably lower, ranging from 20 to 35 µg/g dwt (EPA 2010c, 2010d). Thus, there is less of this protein present after harvest compared to the Cry34Ab1 protein. Moreover, Cry34Ab1 may take longer to degrade because it is coupled with Cry35Ab1; however, it is unclear whether a negative ELISA test for Cry34Ab1 means that Cry35Ab1 is not present.

ELISA detection probabilities of the lepidopteran active proteins in all residue samples were positively correlated with the level of *O. nubilalis* growth inhibition from the bioassays (Cry2Ab2, $r = 0.75$, $P = 0.033$; Cry1F, $r = 0.69$, $P = 0.056$). We could not separate individual effects of the two proteins on larval growth; however, Cry2Ab2 protein showed an overall lower probability of detection during the early sampling weeks compared to the Cry1F protein, suggesting that the growth inhibition during the later weeks was mainly due to the Cry1F protein. Moreover, the Cry1A.105 protein likely contributed to *O. nubilalis* growth inhibition but we could not make any specific conclusions about its persistence compared to the other two lepidopteran active proteins. We hypothesized that the ELISA tests would indicate protein persistence in corn residue beyond the period of biological activity measured by the feeding bioassays.

Contrary to our hypothesis, ELISA testing in both years showed similar patterns of detection frequencies that corresponded with results of the feeding bioassays during most sampling weeks. There was no evidence that ELISA tests generated false positive Cry protein detections for samples collected during the later weeks following harvest. On the contrary, growth inhibition of *O. nubilalis* larvae was observed through the final sampling weeks, indicating the presence of active Cry proteins beyond the point when these proteins were no longer detected by ELISA testing. These findings corroborate the study by Albright et al. (2016) that showed bioactivity retained in detectable fragments of Cry proteins using ELISA.

In summary, this study clearly demonstrated that Cry proteins retain biological activity significantly longer in no-till corn systems than was previously reported in conventionally tilled systems. Although few adverse effects on non-target organisms have been documented to date, the potential for non-target effects following harvest exists for future genetically engineered crops, particularly if the majority of crop residue remains on the soil surface. Post-harvest practices that increase soil-residue contact and promote faster protein degradation, may help to mitigate these concerns by reducing the period of protein exposure to soil and epigeal organisms. Altogether, our findings suggest that future environmental fate studies in support of registrations of Bt crop events should not only quantify Cry protein degradation in crop residue incorporated into the soil but also the degradation rates of crop residue following conservation tillage practices. Furthermore, the tiered risk assessment framework for testing non-target effects should also focus on sensitive taxa exposed to biologically active proteins following harvest. As more and more farmers adapt minimum and no-tillage cover cropping practices, there will be less Bt crop residue being incorporated into the soil. As such, environmental fate studies will need to adapt to changing post-harvest practices.

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Author contributions VY, GPD, WOL and CRRH designed the experiments. VY and GPD performed the experiments, and GPD analyzed the data. VY, GPD and AL wrote the manuscript, and WOL and CRRH edited the manuscript.

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Declaration

Conflict of interest We do not have any conflicting financial, personal, or professional interests that are related to this manuscript.

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