DEVELOPING A COTTON-BASED METHOD FOR BT RESITANCE MONITORING

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Abstract

Plant-based methods for detecting resistance in Bt crops can provide researchers a way to evaluate resistance without using diet-based bioassays that require protein or lyophilized tissue. For cotton, leaf tissues are generally used for this purpose. We wanted to design a bioassay more reflective of *Helicoverpa zea*'s feeding behavior by focusing on reproductive tissue. Preliminary experiments indicated that larvae could be confined on bract tissue. While larval weight was not a good metric to assess feeding, mass of bract tissue before and after feeding, converted to area that was consumed, was a good metric to assess feeding. Finally, we found that *H. zea* colony health is very important to the success of the assay.

Introduction

Plant-based methods for detecting resistance in Bt crops can provide researchers a way to evaluate resistance without using diet-based bioassays that require protein or lyophilized tissue. There are numerous examples from the field of using measurements of feeding on the plant to make comments about Bt resistance. For example, Fleming et al. (2018) noted an increase in cotton damage from heliothines over time and concluded that this was largely due to resistance. In his study, it is not clear if plant damage refers to leaf, square, flower or boll damage. Dively et al. (2016) noted that the kernel area consumed by *Helicoverpa zea* (Boddie), increased over time in both Cry1Ab and Cry1A.105 + Cry2Ab2 sweet corn. This observation, paired with diet-based bioassays, confirmed practical resistance to these Cry toxins. Both examples required additional studies beyond field observations to confirm resistance.

Gassmann et al. (2011) collected *Diabrotica virgifera virgifera* from both problem fields and control fields (defined as fields without unexpected amounts of feeding). Populations from both field types were then compared using a plant-based bioassay where non-Bt and Bt corn plants were challenged with different populations (each population was a replicate) and larval survival was assessed. There was three times higher survival in Cry3Bb1 plants challenged with populations from problem fields compared to control fields, but larval survival was similar for populations collected from problem fields and control fields on non-Bt plants or Cry34/35Ab1 plants. Therefore, they concluded that *D. virgifera virgifera* had evolved practical resistance to Cry3Bb1.

Plant based bioassays for *H. zea* in Bt cotton face a potential challenge. When *H. zea* larvae feed on squares, flowers or bolls, penetration into the carpel or boll wall triggers ethylene production, leading to abscission. Therefore, quantifying relative feeding amounts can be difficult. The Reisig program has three years of information measuring *H. zea* feeding on abscised tissue and four years of information measuring feeding on tissue that was fed but remained on the plant without abscission from non-Bt, Cry1Ac + Cry1F, Cry1Ac + Cry2Ab2, and Cry1Ab + Cry2Ae cotton. From these experiments, they determined that bract feeding on bolls does not cause tissue abscission. Although this is one type of feeding among several other types that does not cause abscission, it is easily quantifiable both in the field and in the lab.

Using this information, they challenged non-Bt and Bt cotton bolls removed from the plant with both Cry1Ac resistant and Cry1Ac susceptible *H. zea* (both were susceptible to Cry2). While the area of bract tissue fed on was similar among Bt cotton pyramids and reduced compared to non-Bt cotton for Cry1Ac susceptible larvae, for Cry1Ac resistant larvae, the amount of feeding was similar between non-Bt cotton and Cry1Ac + Cry1F cotton (Cry1F only has sublethal effects on *H. zea*) and reduced in both Cry1Ac + Cry2Ab2 and Cry1Ab + Cry2Ae cotton (Fig. 1). Therefore, measuring bract injury of bolls removed from cotton plants may serve as a plant-based bioassay to measure Bt resistance in *H. zea*.

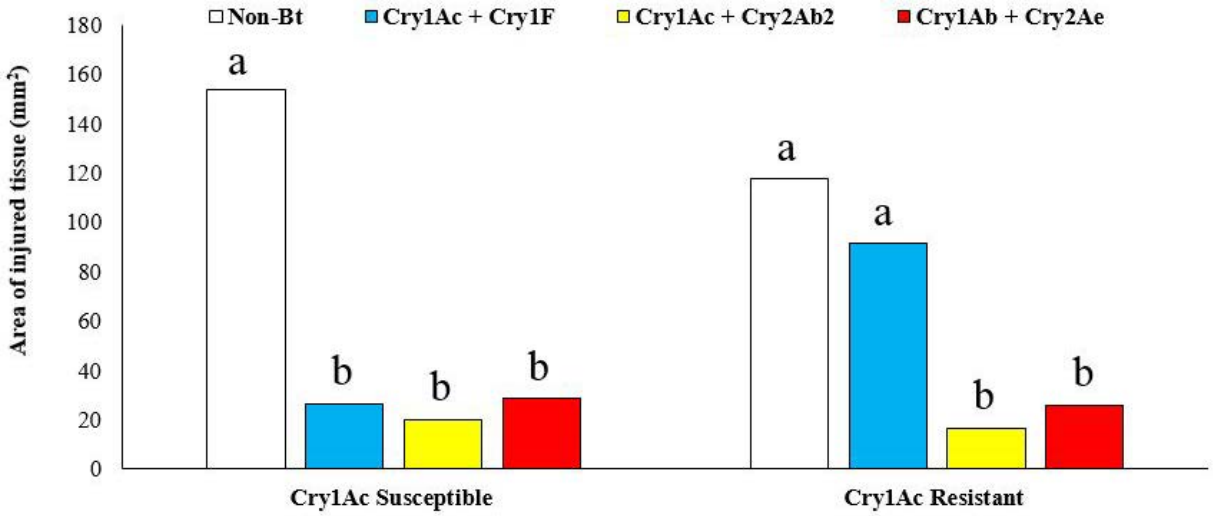

Figure 1. Differences in feeding on bracts of cotton bolls between Cry1Ac susceptible and Cry1Ac resistant *H. zea* larvae.

Materials and Methods

We intended to collect populations of *H. zea* from the crop fields across the southeastern US. We then wanted to screen populations for resistance to Cry1Ac, Cry2Ab2, and Vip3A using protein or lyophilized leaf tissue diet-based bioassays. Then we wished to subject these populations to an additional plant-based bioassay using cotton bolls from non-Bt, Cry1Ac + Cry1F, Cry1Ac + Cry2Ab2, and Cry1Ac + Cry1F + Vip3Aa19 cotton and bract feeding will be measured. Our hypothesis was that relative survival in the diet-based bioassays should reflect feeding in the boll bioassay. However, by the time these populations were screened on the protein and leaf tissue bioassays, the colonies were no longer healthy enough to screen. Therefore, we used a colony that had been selected for resistance to Vip3Aa39 in the lab, as well as a Bt-susceptible colony. Two cotton varieties (PHY499WRF expressing Cry1Ac +

Cry1F and a non-Bt variety, PHY425RF) were planted in the greenhouse. Plants were grown in four-inch pots containing standard potting mixture (Cornell Mix) supplemented with slow release fertilizer (14-14-14 N-P-K) under a 16:8 L:D cycle. Pots were checked daily for moisture and irrigated as needed. Potential confounding pests, like spider mites and aphids were controlled by releasing mite predators and aphid-parasitoids during the initial detection of the pest infestation.

When the plants started flowering, white and pink flowers were tagged with colored ribbon (#17021 Red std. Flag, C.H. Hanson Company, Naperville, IL.) once every six to eight days to obtain bolls of a known age. From each variety, eight day-old bolls were collected and transported to lab in a cooler with ice pack. Bolls were then bathed in a 5% bleach in deionized water for 10 minutes to clean the tissue surfaces and triple washed with deionized water to remove bleach from the surface. Excess water from the tissues was then removed by gently shaking the cotton bolls.

The experimental arena base was 4.8 cm x 42.5 cm x 28 cm Styrofoam boards. The boards were covered with thin plastic so that *H. zea* larvae would not chew through the Styrofoam when confined. Each board was subdivided into 12 different arenas and surface sterilized with ethanol. The Styrofoam at each arena was penetrated with a floral water tube that was filled with water. 2.5 cm bolls were removed from greenhouse plants and immediately placed in the water tubes. Bolls were then covered with a clear 37 ml plastic diet cup (# 9091 1.25 oz plastic cups, Frontier Agriculture Sciences, Newark, DE) that was secured to the board using pins, but the bracts were splayed out so that they overlapped underneath the diet cup.

The experimental design was unbalanced, due to constraints from the *H. zea* colonies. The experiment was a completely randomized design, with three treatments: 1) Bt-susceptible larvae on non-Bt cotton; 2) Vip3Aa39-selected larvae on non-Bt cotton; and 3) Bt-susceptible larvae on Cry1Ac + Cry1F cotton. Before placing them on the arena, larvae were weighed and the surface area of the bracts was determined using tracings over a 10 x 10 cm translucent 1000H vellum paper marked with 2 x 2 mm grid (# 1020-6510, DEW Drafting supplies, Gilbert, AZ). Eight day-old *H. zea* larvae were then placed on the bracts and confined with a 240 ml clear plastic cup (Rensow plastic cups, Royal Disposable, Maspeth, NY), also secured with pins. In this way, larvae were prevented from accessing the boll and, if they chose to feed, they fed only on the bracts. Arenas were placed in lighted growth chambers at 27 degrees C, 60 RH for six hours. Larvae were then removed and weighed and the surface area of the bracts was again determined using tracings. The translucent graph paper was placed over the bract area outline traced before the feeding assay and enlarged to 2x. The area that was fed on was extracted using a razor blade and weighed. Weights were converted back to surface area by counting and weighing known areas on the graph paper. Larval weight and bract area remaining after 6 hours were compared using a mixed models ANOVA.

Results

Larval weight was not different among treatments prior to placement on the assay ($F = 2.6$, d.f. = 2, 23.2, $P = 0.0959$), nor was it different after the assay ($F = 2.41$, d.f. = 2, 22.1, $P = 0.1134$). Moreover, percentage weight gain was not different after the assay ($F = 2.91$, d.f. = 2, 22.5, $P = 0.075$). Finally, bract area fed on after six hours was different $F = 13.8$, d.f. = 2, 21.6, $P = 0.0001$). Bt-susceptible larvae fed on more non-Bt cotton area (581 mm^2 per individual), but Vip3Aa39-selected larvae on non-Bt cotton (93 mm^2 per individual) and Bt-susceptible larvae on Cry1Ac + Cry1F cotton (14 mm^2 per individual) fed on less.

Discussion

Our hypothesis was that relative survival in the diet-based bioassays should reflect feeding in the boll bioassay. Although we were not able to test this hypothesis, we expected that Bt-susceptible larvae would feed on more non-Bt cotton area and feed on less Bt cotton area. Furthermore, we expected that the Vip3Aa39-selected larvae would feed on comparable cotton area when placed on both non-Bt and Bt cotton. We were not able to test the Vip3Aa39-selected larvae on Bt cotton, however, since the colony health declined. Hence, this assay seems to be both a reflection of colony health and Bt-susceptibility. Bt-susceptible larvae fed on much more non-Bt cotton compared to Vip3Aa39-selected larvae. Vip3Aa39-selected larval feeding on non-Bt cotton was so suppressed, that we were not able to statistically separate feeding from Bt-susceptible larvae on Bt tissue.

Because bracts are irregularly shaped, determining the area of tissue that larvae feed on is very time-intensive. Larval weight is much quicker to determine, but cannot be used as a proxy for the amount of tissue fed on in this assay since

it did not separate out in any analysis. Future work will be conducted on this assay to link it to diet-based bioassays. Once the assay is developed and confirmed it may be possible to simplify how bract area is determined.

Acknowledgements

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