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Leaf Tissue Assay for Lepidopteran Pests of Bt Cotton

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Abstract. Laboratory measurements of susceptibility to Bt toxins can be a poor indicator of the ability of an insect to survive on transgenic crops. We investigated the potential of using leaf tissue for evaluating heliothine susceptibilities to two dual-gene Bt cottons, *Gossypium hirsutum* L. A preliminary study with different cotton leaf disk combinations was performed to determine the best procedure for using cotton tissue to assay bollworm, *Helicoverpa zea* Boddie, and tobacco budworm, *Heliothis virescens* F. Neonate larvae from a laboratory colony were assayed simultaneously on leaf disks of non-Bt and two dual-gene transgenic cottons in addition to meridic diet overlaid with discriminating doses of a commercially-formulated Bt product. The bollworm colony in the laboratory was more tolerant than tobacco budworm to the commercially-formulated Bt product. When corrected for the non-Bt check, percentage of bollworm larval mortality was 42.1 and 71.9 on leaf disks of the two dual-gene Bt cottons. No tobacco budworm survived on dual-gene Bt cotton leaf disks in the study. Assays using transgenic cotton leaf disks may compliment current meridic diet-based methods by providing a linkage to insect survival on Bt cotton plants.

Introduction

Results from bioassays that estimate susceptibility of lepidopteran insects to transgenic insecticides are inherently variable. The relationship between laboratory measurements and survival on transgenic crops is difficult to determine. Unlike exposure to synthetic insecticides with one active ingredient, results from assays using a single protein from the soil bacterium, *Bacillus thuringiensis* Berliner (Bt), may not be indicative of survival on plant tissue expressing multiple proteins. Evaluating insect survival on living plant tissue expressing various Bt toxin combinations may offer a more realistic approach to creating a linkage between laboratory measurements of susceptibility and survival on transgenic cotton in the field. Currently, all commercially-available transgenic cottons express two or more Bt proteins for control of bollworm, *Helicoverpa zea* Boddie, and tobacco budworm, *Heliothis virescens* F. (heliothines). Therefore, bioassays with excised plant tissue may be a complimentary method to traditional meridic diet-based assays for determining heliothine susceptibilities to Bt crops expressing multiple toxins.

There are fundamental differences in bioassays to determine contact versus oral activities of an insecticide. Unlike contact exposure tests for many synthetic insecticides, insect assays measuring susceptibility to Bt toxins rely on food intake for delivery of a specific dose. This can create challenges when evaluating insect

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developmental parameters linked to the amount of the toxin ingested (Luttrell and Jackson 2012). The protein:carbohydrate ratio of meridic diet alone is reported to alter bollworm survival in dose-response assays with Bt proteins (Deans et al. 2016). Additionally, resistance ratios comparing offspring from feral populations to a susceptible colony in a laboratory that are adapted to a particular artificial diet may have limited interpretive meaning. Ultimately, individuals truly resistant to a transgenic crop need to survive on plant tissue to transfer resistant alleles to the next generation.

Lyophilized plant tissue or recombinant acrystalliferous strains of Bt subsp. kurstaki (HD73 cry-) that produce a single protein have historically been used in meridic diet-based assays to measure heliothine susceptibility to transgenic insecticides. Although selections of insect populations on individual Bt proteins have yielded measurements of high levels of tolerance in the laboratory (Gould et al. 1995, Luttrell et al. 1999), they are often a poor indicator of the capacity of an insect to survive on transgenic crops. For example, Gould et al. (1995) reported a resistance ratio of ~10,000 for a strain of tobacco budworm (YHD2) selected on Cry1Ac-amended meridic diet, but none survived when reared on non-Bt and Bt cotton plants (Tabashnik et al. 2003). Furthermore, laboratory bioassays have been adopted as an industry-wide standard for tracking heliothine susceptibility to Bt toxins. However, findings from the assays are limited to a single protein, which can be a poor indicator of insect survival on Bt crops expressing two or more toxins. Although toxin quantification of some Bt proteins may be a problem in tissue-based assays, they may compliment current meridic diet-based assays with purified proteins. This study assessed the potential of leaf tissue for evaluating heliothine susceptibility to transgenic cotton lines containing two Bt proteins.

Materials and Methods

Laboratory assays in 2016 were used to determine the suitability of cotton leaf tissue as a substrate for measuring susceptibility of heliothines to Bt toxins. Laboratory-reared insect colonies of the two heliothine species have been maintained since 1971 at the United States Department of Agriculture (USDA), Agricultural Research Service (ARS), Southern Insect Management Research Unit (SIMRU) at Stoneville, MS, and were used in all laboratory assays. The colonies are routinely reared following details outlined in Blanco et al. (2009) for tobacco budworm to provide large numbers of insects to assay, and are all considered to be susceptible to insecticides and Bt toxins. Non-Bt cottons [DP1441RF®, Delta and Pine Land Company™, Scott, MS], Bollgard II® [DP1321B2RF®, Delta and Pine Land Company™, Scott, MS], and WideStrike® [PHY499WRF®, Dow AgroSciences™, Indianapolis, IN] were grown with no insecticide in a greenhouse to provide fresh tissue for laboratory assays.

In a preliminary study, non-Bt cotton leaves 6 to 8 cm in diameter were removed from the top five nodes and taken to the laboratory. Cork borers were used to cut 1.15- and 2.54-cm disks from leaves. Forceps were used to transfer leaf disks into one of the treatments: 1) two 1.15-cm leaf disks per well in a 128-well bioassay tray (Frontier Agricultural Sciences, Newark, DE), 2) three 1.15-cm leaf disks per well in a 128-well bioassay tray, or 3) one 2.54-cm disk in a 36.9-ml clear container (Dart Container, Mason, MI). A 5-mm layer of agar was added to the bottom of all wells and cups before leaf disks were transferred. A single neonate larvae was placed into each well or cup and covered with a lid. All

containers were placed in an incubation chamber for 7 days at 27°C, 70% relative humidity, and a photoperiod of 16:8 light:dark hours. Four replications of 16 insects each were used for each species and treatment. Each individual treatment was paired with the same leaf disk combination, but without a larva to correct for leaf weight gain from absorption of moisture. Leaves and neonate larvae were weighed individually at the beginning and immediately following the 7-day test. The weight gain from the check leaf disk(s) (no larva) was subtracted from the weight of the leaf disk(s) fed to larvae to determine the amount of leaf tissue consumed. Leaf weight loss and larval weight gain and instar were recorded at the conclusion of the test. Differences in leaf weight loss, larval weight gain, and larval instars were analyzed with a general linear mixed model as a randomized complete block design (SAS Institute 2013). Least squares means were estimated and compared using Fisher's least significant difference at $\alpha = 0.05$.

Upon determining the most suitable and efficient method for using cotton tissue in bioassays for both species, an additional experiment was used to evaluate larval mortality when exposed to different transgenic cotton leaf disks and a commercially-formulated Bt product, Dipel DF® (Valent BioSciences™, Libertyville, IL). Four replications of neonate larvae of both species were simultaneously assayed on 2.54-cm non-Bt, Bollgard II, and WideStrike leaf disks in 36.9-ml clear containers with a 5-mm layer of agar and meridic diet overlaid with discriminating doses of Dipel DF. Each replication consisted of 16 individual larvae. Repeater pipets were used to dispense 1.5 ml of Nutri-Soy Wheat Germ diet (Blanco et al. 2009) into 128-well bioassay trays (C-D International, Pitman, NJ). After the diet cooled at room temperature, 50 µl of a given concentration of Dipel DF suspended in water was pipetted into each well. Concentrations of Dipel DF in diet overlay assays were 0, 0.08, 0.25, 0.74, 2.22, 6.67, 20, and 33.33 µg/cm^2 for bollworm, and 0, 0.001, 0.003, 0.01, 0.03, 0.08, 0.25, and 0.74 µg/cm^2 for tobacco budworm. Percentage of larval mortality for both assay procedures, insect weight gain, and leaf disk weights for tissue-based assays were recorded at 7 days. Percentage of mortality on Bollgard II and WideStrike leaf disks were corrected for check (non-Bt) mortality using Abbott's (1925) formula. Confidence intervals were calculated using Rosenheim and Hoy's (1989) iteration of Elston's (1969) method of correcting assay data for check responses. Probit analyses were used to develop regressions for estimating the lethal concentration of Dipel DF needed to kill 50% (LC_{50}) of neonate larvae of each species (SAS Institute 2013).

Results

There were no species, treatment, or species-by-treatment interactions with regard to the amount of non-Bt leaf tissue consumed or larval instars at the conclusion of the 7-day preliminary study. However, bollworms weighed significantly less than tobacco budworm larvae at the end of the 7-day preliminary test ($F = 10.6$; df = 1; $P = 0.0053$), regardless of treatment (Table 1). Because no differences were detected between leaf disk size and using 2.54-cm disks in a 36.9-ml clear container was more efficient for laboratory personnel, the method was selected for further testing. Percentage of mortality, corrected percentage of mortality, and 95% confidence intervals of bollworm and tobacco budworm larvae assayed on 2.54-cm non-Bt, WideStrike, and Bollgard II cotton leaf disks are presented in Table 2. Mean mortality of bollworm ranged from 10% on non-Bt cotton leaf disks to 73.3% on Bollgard II. The percentage of tobacco budworms that

Table 1. Estimates of Leaf Weight Loss and Weight Gain and Instar of Bollworm and Tobacco Budworm Larvae on Non-Bt Cotton Leaf Disks at 7 Days

	Bollworm			Tobacco budworm		
Treatment	Leaf loss[d] (g)	Larval gain[d] (g)	Mean instar[d]	Leaf loss[d] (g)	Larval gain[d] (g)	Mean instar[d]
1[a]	0.0152	0.0029	2.7	0.0204	0.0044	2.8
2[b]	0.0156	0.003	2.6	0.0198	0.0044	2.8
3[c]	0.0243	0.0029	2.5	0.0168	0.0037	2.7

[a]Two 1.15-cm leaf disks placed in 128-well bioassay tray.
[b]Three 1.15-cm leaf disks placed in 128-well bioassay tray.
[c]One 2.54-cm leaf disk placed in 36.9-ml clear container.
[d]Means were determined from four replications of 16 individuals.

Table 2. Percentage of Mortality of Bollworm and Tobacco Budworm Larvae Assayed on 2.54-cm WideStrike and Bollgard II Cotton Leaf Disks and LC_{50}s for Dipel DF

	Bollworm			Tobacco budworm		
Treatment	% Mortality[a]	Corr. % Mort.[ab]	LC_{50}[acd]	% Mortality[a]	Corr. % Mort.[ab]	LC_{50}[acd]
Non-Bt	10.0			8.3		
WideStrike	45.0	42.1 (20.5)		100	100 (0)	
Bollgard II	73.3	71.9 (30.5)		100	100 (0)	
Dipel DF			3.518 (3.241-3.829)			0.016 (0.014-0.019)

[a]Means were determined from four replications of 16 individuals.
[b]Corrected percentage of mortality. Means were corrected using Abbott's (1925) formula. Values in parentheses are 95% confidence intervals calculated using Rosenheim and Hoy's (1989) iteration of Elston's (1969) method of correcting assay data for check responses.
[c]Units are displayed as $\mu g/cm^2$.
[d]Values in parentheses are 95% confidence intervals.

died ranged from 8.3% on non-Bt to 100% on WideStrike and Bollgard II cottons. When corrected for non-Bt cotton, the percentage of bollworm mortality on WideStrike and Bollgard II leaf disks ranged from 42.1 to 71.9, respectively. Mortality of tobacco budworm that is very susceptible to Bt toxins, was 100% on WideStrike and Bollgard II cottons. Significant regressions were obtained for bollworm ($p < 0.0001$) and tobacco budworm ($p < 0.0001$) larvae exposed to diet overlaid with various doses of Dipel DF. LC_{50}s for bollworm and tobacco budworm were 3.518 and 0.016 $\mu g/cm^2$, respectively.

Discussion

The development of insect resistance to transgenic insecticides is a major challenge to sustainable agricultural production in the United States (Ferre and Van Rie 2002, Gahan et al. 2005). Pyramiding, which is the stacking of multiple genes for simultaneous expression of two or more toxins, is one strategy for combatting the development of resistance. Assay procedures are needed that can link

laboratory measurements of insect susceptibility to an ever-growing pyramid of Bt toxins to survival on transgenic crops. Because cotton terminal leaves are known to express higher levels of Cry1Ac than other parts of the plant (Greenplate 1999, Adamczyk et al. 2001, Kranthi et al. 2005, Sivasupramaniam et al. 2008, Willrich Siebert et al. 2009), we determined the most practical and efficient procedure for using this tissue in assays. We used our new procedure to determine the mortality caused by larval feeding on dual-gene cottons to compare with meridic diet overlaid with Dipel DF, a commercial formulation of Bt.

The study demonstrated that at the higher levels of tolerance observed for bollworm versus tobacco budworm on meridic diet overlaid with Dipel DF, there were differences in survival of larvae on WideStrike and Bollgard II leaf disks. No tobacco budworm larvae survived on either of the dual-gene transgenic cottons, and none was expected considering the high susceptibility of tobacco budworm to Bt toxins. Although bollworms prefer feeding on cotton fruiting structures, neonate larvae are known to feed on terminals and leaves (Schmidt et al. 1988). Bioassays are typically invalidated when check mortality is high (Miller et al. 2010). Mortality on check leaf disks (non-Bt) for any replication of either species did not reach 15% in our study. An increase in the number of replications might be needed to better estimate survival on leaf disks containing pyramided Bt toxins. Numerical differences in survival were observed for the SIMRU susceptible laboratory colony of bollworms exposed to the two Bt cottons in the study. We expect feral populations of bollworms with higher levels of tolerance than our laboratory colony to differ greatly in survival on cotton leaf disks with different Bt protein pyramids. Although Dipel DF contains a mixture of spores and toxins and is not fully representative of toxin combinations in cotton plants, readily available sources of Bt proteins are limited. Any survival of tobacco budworm on leaf disk assays would be cause for alarm and warrant further investigation. Quantification and correlation of the content of Bt protein(s) in leaf disks with insect growth and survival might help explain some of the variability in assays using leaf disks.

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