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Influence of Dual-Bt Protein Corn on Bollworm, *Helicoverpa zea* (Boddie), Survivorship on Bollgard II Cotton

M. B. Von Kanel,[1] J. Gore,[2,3] A. Catchot,[1] D. Cook,[2] F. Musser,[1] and M. Caprio[1]

[1]Department of Biochemistry, Molecular Biology, Entomology, and Plant Pathology, Mississippi State University, 100 Old Hwy 12, Mississippi State, MS 39762 (ben.vonkanel@bayer.com; Acathcot@entomology.msstate.edu; Fmusser@entomology.msstate.edu; mcaprio@entomology.msstate.edu), [2]Delta Research and Extension Center, Mississippi State University, P.O. Box 197, Stoneville, MS 38776 (jgore@drec.msstate.edu; dcook@drec.msstate.edu), and [3]Corresponding author, e-mail: jgore@drec.msstate.edu

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Abstract

Similar Cry proteins are expressed in both Bt corn, *Zea mays* L., and cotton, *Gossypium hirsutum* (L.), commercial production systems. At least one generation of corn earworm, *Helicoverpa zea* (Boddie), completes development on field corn in the Mid-South before dispersing across the landscape into other crop hosts like cotton. A concern is that Bt corn hybrids may result in selection for *H. zea* populations with a higher probability of causing damage to Bt cotton. The objective of this study was to determine the susceptibility of *H. zea* offspring from moths that developed on non-Bt and VT Triple Pro (VT3 PRO) field corn to lyophilized Bollgard II cotton tissue expressing Cry1Ac and Cry2Ab. Offspring of individuals reared on VT3 PRO expressing Cry1A.105 and Cry2Ab had a significantly higher LC_{50} two out of the three years this study was conducted. Excess larvae were placed on artificial diet and allowed to pupate to determine if there were any inheritable fitness costs associated with parental development on VT3 PRO corn. Offspring resulting from males collected from VT3 PRO had significantly lower pupal weight and longer pupal duration compared with offspring of individuals collected from non-Bt corn. However, offspring from females collected from VT3 PRO were not different from non-Bt offspring. Paternal influence on offspring in insects is not commonly observed, but illustrates the side effects of development on a transgenic plant expressing less than a high dose, 25 times the concentration needed to kill susceptible larvae.

Key words: risk assessment, resistance management, cotton insects, IPM-agricultural, genetically engineered traits

The greatest threat to the longevity of transgenic crops expressing Bt crystalline (Cry) proteins is the widespread evolution of resistance (Roush 1997). Bt cotton has been used to successfully manage a number of key lepidopteran pests, including tobacco budworm, *Heliothis virescens* (F.), and pink bollworm, *Pectinophora gossypiella* (Saunders). However, corn earworm, *Helicoverpa zea* (Boddie), is inherently more tolerant to the Bt proteins than tobacco budworm and pink bollworm, and larvae are often observed developing on Bt cotton (Mahaffey et al. 1995; Chitkowski et al. 2003).

There have been no confirmed field-evolved cases of Bt resistance in *H. zea* to date (Ali et al. 2006, Huang et al. 2011). This is partly owing to the implementation of the refuge strategy mandated by the U.S. Environmental Protection Agency (EPA; Caprio and Sumerford 2007, Huang et al. 2011) combined with pyramided Bt crops. A high dose was defined as a dose 25 times the concentration needed to kill susceptible larvae. For this strategy to be successful, three assumptions must be met: 1) alleles associated with resistance must be recessively inherited; 2) resistance alleles must be rare; and 3) mating among resistant and susceptible individuals must be random (Carriére and Tabashnik 2001). Criteria for one of these assumptions could possibly be in danger of being violated. Because survival on a sublethal dose delays developmental time (Horner and Dively 2003), assortative mating may be taking place at a higher frequency than expected (Liu et al. 2001) and, thereby, increasing the number of homozygous individuals carrying resistance alleles. The high-dose refuge strategy also assumes mortality of heterozygous individuals is high and that is not true for insect pests like *H. zea*, even on pyramided Bt corn hybrids (Reisig and Reay-Jones 2015).

Structured cotton refuges were eliminated in 2008 because research across the southern United States demonstrated that alternate hosts provide a substantial natural refuge to effectively delay resistance to Bt proteins (Jackson et al. 2007). Additionally, the commercialization of cotton varieties with two distinct Bt proteins provides added benefits for resistance management. Structured refuges remain an integral part of resistance management in corn production, especially in cotton-producing regions of the United States.

Table 1. Commercialized dual-gene Bt cotton and corn cultivars with activity against *H. zea*

Crop/cultivar	Lepidoptera active traits	Refuge requirement
Cotton		
Bollgard II	Cry1Ac + Cry2Ab	NA
Widestrike	Cry1Ac + Cry1F	NA
TwinLink	Cry1Ab + Cry2Ae	NA
Corn		
VT Double and VT Triple Pro	Cry1A.105* + Cry2Ab	20%
SmartStax	Cry1A.105* + Cry2Ab + Cry1F	20%
Agrisure Viptera	Cry1Ab + Vip3A	20%

Note: Similar Cry proteins are expressed in both cropping systems.

*Cry1A.105 is a chimeric protein structurally similar to both Cry1Ac and Cry1F.

In regions of the United States where Bt cotton is grown, corn varieties expressing a single protein have a refuge requirement of 50% of the total corn acreage. Pyramided corn varieties (expression of multiple insecticidal proteins targeting a specific group of pests; e.g., Lepidoptera) require a 20% non-Bt refuge (Que et al. 2010).

Currently, the same or similar Cry proteins are used in both Bt corn and cotton (Table 1). Van Rie et al. (1989) developed the basic model for binding sites of Cry proteins in the insect midgut. In theory, Cry1Aa binds to only receptor A. Cry1Ab binds to receptors A and B. Cry1Ac can bind to receptors A, B, and C. Cry proteins share structural similarities that may compromise the efficacy of one or more of these toxins. Cross resistance in *H. zea* to Bt has only been observed in isolated studies and at low levels (Burd et al. 2003). However, deployment of similar Cry proteins in two crop hosts for *H. zea* increases this risk of selection for resistance, especially when multiple generations develop on Bt corn before transitioning into cotton. Allele segregation of individual corn kernels within an ear may result in an array of variability in Cry protein expression (Horner et al. 2003), making selection difficult to quantify. Kernels may (and often do) express less than what is considered a high-dose. In pyramided varieties, allele segregation can also cause kernels to express one, both, or no toxin at all (Horner et al. 2003). Storer et al. (2001) determined that larvae have the ability to detect Bt-expressing kernels and feed on kernels expressing little or no toxin until developing into less susceptible instars.

Resistant allele frequency in *H. zea* is believed to be <0.001 (Carriére and Tabashnik 2001) and major genes conferring resistance have not been discovered in this species (Bates et al. 2005, Sumerford et al. 2013). This would suggest that multiple alleles each having a minor effect will be involved in resistance evolution and as such, changes in susceptibility will occur gradually over time (Caprio and Sumerford 2007). Most resistance monitoring programs utilize methods aimed at capturing the increase of a single resistance allele. There are variations in technique, but the most generally used method is to subject larvae to a diagnostic concentration incorporated into artificial diet (Bates et al. 2005). A discriminatory concentration allows for numerous individuals to be evaluated, but has limitations in the ability to detect resistance alleles that are minor, extremely rare, or recessive (Hawthorne et al. 2002). The objective of this study was to evaluate the influence of feeding on Genuity™ Yieldgard® VT Triple Pro® (VT3 PRO) corn (Monsanto Company, St. Louis, MO) on *H. zea* susceptibility of F1 generation to lyophilized Bollgard II® (Monsanto Company, St. Louis, MO) cotton leaf tissue at a range of concentrations. The goal of this research was to detect subtle changes in susceptibility that are associated with an inherent ability to develop on a transgenic host.

Materials and Methods

Leaf Tissue Collection

During the 2011 growing season, cotton leaf-tissue samples were collected from Delta & Pine Land® 0924B2RF (Monsanto Company, St. Louis, MO) cotton (expressing Cry1Ac and Cry2Ab) and Delta & Pine Land® 174RF (non-Bt) cotton. Cotton was grown according to standard agronomic practices and pest management recommendations, with the exception that no insecticides were used with activity against Lepidoptera. Leaf tissue was collected during approximately the third week of flowering. One cotton leaf from each plant was selected from the third most upper node for a total of 500 leaves for each cultivar. Leaves were placed in 3.785-liter Ziploc® bags and then put in a −84°C freezer for 72 h. After 72 h, lyophilized leaf tissue was finely ground into powder that would pass through a 40-mesh sieve, and then kept at −84°C until needed for bioassays.

Insect Rearing

H. zea larvae were collected from a VT3 PRO hybrid corn (DKC67-88, Monsanto Company, St. Louis, MO) expressing Cry1A.105 and Cry2Ab, and its non-Bt near isoline (DKC 67-86, Monsanto Company, St. Louis, MO). Multiple collections were made each year in 2011, 2012, and 2013. Each collection consisted of approximately 600 larvae (300 from each corn genotype). Only third-instar larvae or larger were collected to maximize their selection to Bt and to minimize mortality from handling. Larvae were placed in 36-ml Solo® (Bio-Serv®, Frenchtown, NJ) cups containing a soy-protein, wheat-germ-based artificial diet with matching lids. Cups containing larvae were kept in a rearing facility maintaining 25°C, 80% RH, and a photoperiod of 16:8 (L:D) h. All other rearing was done under these environmental conditions. Larvae were monitored daily for pupation. Pupae were removed from individual cups to determine gender. Females were identified by the presence of a ventral, V-shaped suture near the tip of the abdomen. Males were identified by two rectal pads on the ventral tip of the abdomen (Ditman and Cory 1931). Pupae were then placed in empty 36-ml cups. Lids were labeled with the sex of the pupae and respective corn hybrid from which larvae were collected. Pupae were monitored daily for adult eclosion to arrange the following parental crosses: 1) $NBt_F \times NBt_M$, 2) $NBt_F \times$ VT3 PRO_M, 3) VT3 $PRO_F \times NBt_M$, and 4) VT3 $PRO_F \times$ VT3 PRO_M. The capitalized abbreviation of each parental cross corresponds to the corn hybrid the larvae were collected from with the subscript denoting the sex. Cohorts of 50 moths (25 males and 25 females) were placed in identical 3.79-liter cardboard containers with matching lids with the respective parental cross labeled on the outside of each bucket and fed a 10% sugar-water solution. The center of each lid was removed so that only the rim remained. Cotton cloth was placed over each bucket and fastened into place by a lid to serve as an oviposition substrate. Eggs were collected daily and new cloths were applied to every bucket. Collected egg sheets were kept individually in 1.83-liter Ziploc® (S.C. Johnson & Johnson, Inc., Racine WI) bags until larvae hatched for use in bioassays.

H. zea Bioassays

For bioassays, 0.5 ml of warm soy flour-wheat germ-based artificial diet (Product No. 9915B, Frontier Agricultural Sciences, Newark,

DE) was added to every well in a 128-well bioassay tray (Product No. BAW128, Frontier Agricultural Sciences, Newark, DE). Diet was allowed to harden before application of powder slurry. A stock solution of powder slurry was made for each cotton variety by diluting 10 mg of leaf powder with 200 ml of a 0.02% agar (Product No. 7060, Frontier Agricultural Sciences, Newark, DE; Greenplate et al. 2003). Eight concentrations of powder slurry were developed from each stock solution. They included 0.03, 0.08, 0.25, 0.74, 1.11, 2.22, 3.35, and 6.67 mg of leaf powder per ml of 0.02% agar. Fifty microliters of one concentration was applied to the diet surface of each well and a total of 16 wells were used for each concentration per tray (Greenplate et al. 2003). Assays were replicated based on the availability of larvae from parental crosses and offspring from each cross were assayed a minimum of two times each year. Overlay concentrations were allowed to dry under a laminar-flow hood (Agnew-Higgins, Inc, Garden Grove, CA) before one *H. zea* neonate (<24 h after hatching) from one of the crosses was placed in each well. Cells were covered with perforated, clear 16-well lids (P.E. film, Bio-Serv®, Frenchtown, NJ). Trays were placed in a rearing chamber maintained at 25°C, 80% RH, and a photoperiod of 16:8 (L:D) h. Mortality ratings were taken 7 d later. For the purpose of this study, mortality was defined as larvae that failed to molt to the second instar (weighing less than 10 mg) within 7 d or larvae that failed to respond to a probe (Siegfried et al. 2000).

To observe parental influence on fitness costs in offspring, excess progeny from parental crosses were placed in 36-ml Solo® cups containing a soy-protein, wheat-germ-based artificial diet (Bio-Serv®, Frenchtown, NJ) with matching lids. Larvae were maintained as previously described. Larvae were monitored daily until pupation. Pupae were recovered from diet cups, weighed, and sexed. Sex of pupae was determined by the same procedure formerly described. Pupae were placed in empty Solo® cups with the sex labeled on each lid and monitored daily for adult eclosion to record pupal duration.

Analyses

Assay results were pooled across multiple collections within each year owing to the difficulty in obtaining sufficient larvae to carry out replicated assays from one generation of a single collection. Data were analyzed separately for each year. Only first generation (F1) progeny were used for all assays. Concentration-mortality data were developed by evaluating F1 progeny survivorship on eight Bt overlay concentrations and eight non-Bt concentrations. Data were analyzed using Probit analysis (PROC PROBIT, SAS Institute 2012). Mean LC_{50} values were calculated and separated by nonoverlapping fiducial limits. Pupal weight and pupal duration were recorded from one collection of progeny in 2012. These data were analyzed with a mixed model analysis of variance (PROC MIXED, SAS Institute 2012). Sex and the origin of male and female adults were included as fixed effects. Means were separated using Fisher's Protected LSD ($\alpha = 0.05$).

Results and Discussion

Based on results of these experiments, the larval host of male and female moths can influence *H. zea* susceptibility to Bt proteins. In 2011 and 2012, the VT3 $PRO_F \times$ VT3 PRO_M homozygous cross (mating females and males collected from the same corn genotype) had an elevated LC_{50} value compared with the reciprocal crosses (mating females collected from VT3 PRO with males collected from non-Bt and vice versa; Table 2). The VT3 PRO homozygous cross was significantly different than the non-Bt homozygous cross in 2012, but not in 2011. In 2013, no progeny resulting from any cross displayed statistically higher LC_{50} values. However, the LC_{50} values generated for the VT3 $PRO_F \times$ VT3 PRO_M and $NBt_F \times NBt_M$ crosses are unreliable because there was no response to the range of concentrations of lyophilized tissue, as indicated by the lack of a significant slope. The actual LC_{50} could not be generated because less than 50% mortality was observed at the highest concentration tested.

Larval mortality varied for each cross among years, as expected. Numerous studies have documented the variation in the susceptibility of *H. zea* (Siegfried et al. 2000; Woodward et al. 2001; Ali et al. 2006; Ali and Luttrell 2009). Measuring actual shifts in susceptibility is difficult to determine with *H. zea* because of the wide range of responses observed when Luttrell et al. (1999) first documented baseline susceptibility to Bt. Additionally, if *H. zea* susceptibility is governed by multiple minor genes, then confirmation of whether a decrease in Bt susceptibility is owing to a buildup of minor resistance genes or the natural variation of *H. zea* tolerance to Bt is difficult. However, Tabashnik et al. (2008) used long-term monitoring data (cited above) to suggest that field-evolved resistance had occurred with *H. zea* in the southern United States. These data suggest that assortative mating of populations emerging from Bt corn can decrease insect susceptibility to Bt cotton, as indicated by the reciprocal crosses in all three years. If it is assumed that resistant moths oviposited onto non-Bt corn at collection sites during 2011 and 2013, the susceptibility of those offspring should increase in the absence of Bt expression owing to the instability of resistance alleles in a natural population (Bird and Akhurst 2004). Genes associated with resistance in Lepidoptera are thought to be maternally inherited (Bird and Akhurst 2006). Progeny from reciprocal crosses could then be expected to display some evidence of inherited resistance if the female parents were truly resistant. This was not seen in these experiments, given the following observations with regard to progeny pupal weight and duration.

There was a significant interaction between the origin of female and male adults ($F = 15.32$; df = 1, 421; $P = 0.01$) for the pupal weight of progeny. Offspring from the VT3 $PRO_F \times NBt_M$ parental cross had the highest mean pupal weight compared with all other crosses (Fig. 1). Pupae from the $NBt_F \times$ VT3 PRO_M reciprocal cross had significantly lower pupal weight than the $NBt_F \times NBt_M$ cross, but higher than pupae from VT3 $PRO_F \times$ VT3 PRO_M. Progeny from VT3 $PRO_F \times$ VT3 PRO_M had the lowest pupal weight of all crosses. Similarly, an interaction between the origin of female and male adults ($F = 7.24$; df = 1, 345; $P = 0.01$) had an impact on pupal duration of their respective offspring. Offspring from $NBt_F \times NBt_M$ cross had the shortest pupal duration compared with all other crosses (Fig. 2). Pupae from VT3 $PRO_F \times$ VT3 PRO_M took longer to emerge than VT3 $PRO_F \times NBt_M$ pupae, but had a shorter duration compared with pupae from the $NBt_F \times$ VT3 PRO_M cross. Progeny from $NBt_F \times$ VT3 PRO_M cross had the longest pupal duration.

Offspring from parental crosses were affected by parental development on VT3 PRO corn. In both crosses consisting of males collected from VT3 PRO, the offspring had significantly lower mean pupal weight (Fig. 1). Similarly, offspring of males collected from VT3 PRO also had significantly longer mean pupal duration. These results do not conform to observations typically associated with inheritance mechanisms of fitness costs. As with resistance alleles, most fitness costs are recessively inherited (Gassman et al. 2009) and are often linked maternally (Wu et al. 2009). We suggest that non-genetic paternal effects (seminal fluids or nuptial gift) are influencing differences in F1 pupal weight and pupal duration. And

Table 2. Dose–mortality curves for progeny resulting from reciprocal and backcrosses of *H. zea* adults reared on non-Bt and VT3 PRO field corn from 2011 to 2013

Parental cross	χ^2	$P < 0.05$	Slope	LC_{50} (95% fiducial limits)[a]
2011				
$NBt_F \times NBt_M$	5.82	0.56	0.88[1]	1.04 (0.59–1.43)AB
$NBt_F \times$ VT3 PRO_M	4.88	0.77	0.28[1]	0.56 (0.19–1.03)B
VT3 $PRO_F \times NBt_M$	7.85	0.44	0.56[1]	0.50 (0.22–0.86)B
VT3 $PRO_F \times$ VT3 PRO_M	4.90	0.77	0.28[1]	3.65(1.06–15.59)A
2012				
$NBt_F \times NBt_M$	15.51	0.63	0.66[1]	0.75(0.53–1.02)B
$NBt_F \times$ VT3 PRO_M	6.11	0.30	0.98[1]	1.03(0.42–1.61)B
$PRO_F \times NBt_M$	10.56	0.22	0.66[1]	0.76(0.48–1.12)B
VT3 $PRO_F \times$ VT3 PRO_M	7.43	0.76	0.55[1]	2.73(1.67–4.75)A
2013				
$NBt_F \times NBt_M$	96.53	<0.01	0.66	>6.67[b]
$NBt_F \times$ VT3 PRO_M	2.0	0.74	1.12[1]	0.63(0.34–0.92)A
VT3 $PRO_F \times NBt_M$	11.89	0.68	0.55[1]	1.58(0.85–2.34)A
VT3 $PRO_F \times$ VT3 PRO_M	131.92	<0.01	0.64	>6.67[2]

Means within a year followed by the same letter are not significantly different according to Fisher's Protected LSD ($\alpha = 0.05$).

[a] Slope is significant at $\alpha = 0.05$.

[b] The actual LC_{50} could not be generated because less than 50% mortality was observed at the highest concentration tested.

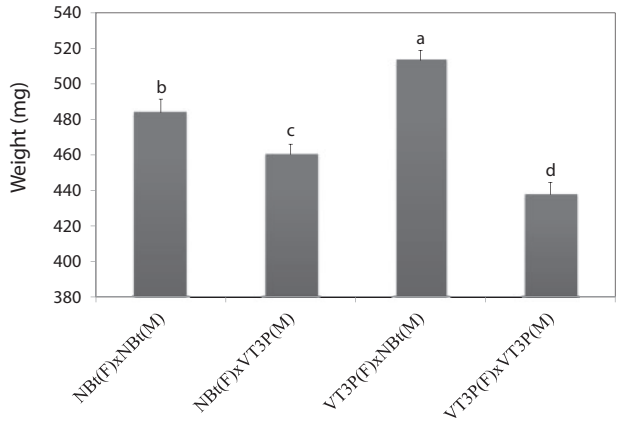

Fig. 1. Mean (SEM) pupal weight of F1 progeny resulting from parental crosses of larvae collected from VT3 PRO and non-Bt field corn. Bars sharing the same letter grouping are not significantly different according to Fisher's Protected LSD ($\alpha = 0.05$). Crosses are indicated by the source (NBt corn or VT3P corn) and the sex (M or F) of the moths used in the cross.

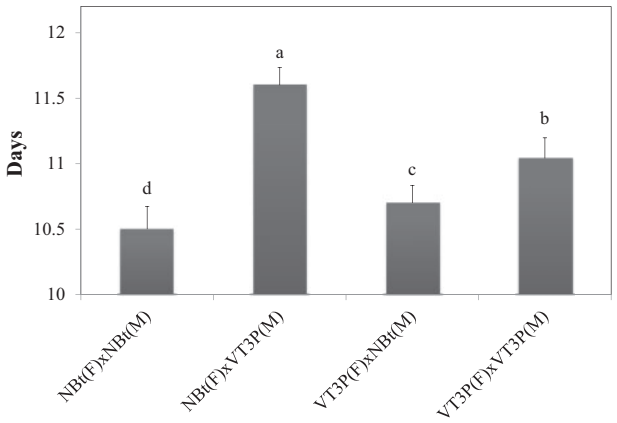

Fig. 2. Mean (SEM) pupal duration (days) of F1 progeny resulting from parental crosses of larvae collected from VT3 PRO and non-Bt field corn. Bars sharing the same letter grouping are not significantly different according to Fisher's Protected LSD ($\alpha = 0.05$). Crosses are indicated by the source (NBt corn or VT3P corn) and the sex (M or F) of the moths used in the cross.

as a result, these measurements were not taken into consideration at the onset of this experiment.

Assumptions can be made as to the origin and genetic background of the populations of *H. zea* collected over the course of this study, yet the hypotheses loosely support the results of these experiments. Alleles that affect *H. zea* survival with regard to Bt proteins can be owing to qualitative aspects or epigenetic effects, which may partially explain the results observed in 2013 with the reciprocal crosses. The concentrations of Bollgard II tissue evaluated were too low to accurately measure a response in some crosses, resulting in different responses between the homozygous crosses and the reciprocal crosses. Lack of a significant concentration–mortality relationship with moths reared on non-Bt corn suggests reduced susceptibility in that cohort. This complicates interpreting the implications of these data, but suggests that more research is needed to address *H. zea* susceptibility to Bt proteins. Assortative mating of adults emerging from VT3 PRO corn has the potential to decrease susceptibility of offspring to Bollgard II cotton. The degree of this susceptibility that makes sense in the context of actual square and boll damage remains in question.

The influence of paternal origin on offspring is an interesting theory and one that requires further investigation. The majority of fitness costs are recessive, although dominant alleles linked to disadvantages in fitness have been discovered (Gassmann et al. 2009). Non-genetic effects having deleterious effects on progeny represents another example of mechanisms preventing the increase of resistance alleles. Paternal effects may have a much larger impact on the evolution of resistance in *H. zea* than previously believed. Owing to the small sample size of this observation, further experimentation should be performed to determine the consequences of such interactions on the ecology of *H. zea*.

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