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Authors: Adamczyk, John J., and Gore, Jeffrey

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Development of bollworms, *Helicoverpa zea*, on two commercial Bollgard® cultivars that differ in overall Cry1Ac levels

John J. Adamczyk, Jr. and Jeffrey Gore

USDA, ARS, Southern Insect Management Research Unit, Stoneville, MS
jadamczyk@ars.usda.gov

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Abstract

Research was conducted to quantify the development of the corn earworm (= bollworm), *Helicoverpa zea* (Boddie), on two different transgenic cotton cultivars (DP 50B and NuCOTN 33B) that contained different levels of the Cry1Ac endotoxin from the soil bacterium, *Bacillus thuringiensis* Berliner. Using a field cage, an inverse relationship between the amount of Cry1Ac among cultivars versus the weight of bollworm larvae was observed. Larvae that were recovered from the DP 50B cultivar expressing lower Cry1Ac weighed significantly more than larvae collected from the higher expressing NuCOTN 33B cultivar. Cotton plants from NuCOTN 33B were measured as expressing 300% more Cry1Ac than DP 50B plants. The distribution of larval weights indicates that more late-instars (> 200 mg) were collected from the lower expressing DP50B cultivar than the higher expressing NuCOTN 33B cultivar. Within a single population, bollworm larvae were highly variable in their development when feeding on Bollgard® cotton. Possible reasons and consequences for this variation are discussed.

Keywords: Lepidoptera, endotoxin, transgenic, *Bacillus thuringiensis*, genetically-modified organism, plant incorporated protectant

Introduction

Since commercialization in 1996, transgenic cotton plants containing a modified form of the *cry1Ac* gene from the soil bacterium, *Bacillus thuringiensis* Berliner, (Bt) (Bollgard®, Monsanto Co., St. Louis, MO) have been used extensively to manage lepidopteran pests. However, some heliothines are not adequately controlled with this technology (Bacheler and Mott 1997; Smith 1998). Although this technology is highly effective against the tobacco budworm, *Heliothis virescens*, (Williams 2000), supplemental foliar insecticide applications to control the corn earworm (= bollworm), *Helicoverpa zea* (Boddie), have been used extensively in Bollgard® fields (Jenkins *et al.* 1992; Burd *et al.* 1999).

Season-long expression differences among Bollgard® cultivars can vary as much as 200-300% throughout the season (Adamczyk *et al.* 2001; Adamczyk and Meredith 2004), and plant structures, such as terminal leaves, express more Cry1Ac compared to certain flower structures (Greenplate 1999; Greenplate *et al.* 2000; Adamczyk *et al.* 2001; Gore *et al.* 2001). These Cry1Ac expression differences among plant structures and cultivars can create spatial variability in survival and development of lepidopterans, such as the bollworm. Factors that have been proposed to influence the level of expressed Cry1Ac among Bollgard® cultivars are still not fully understood, but site-of-gene insertion, demethylation of the protein, and genetic background effects have been implicated (Finnegan *et al.* 1998; Sachs *et al.*1998; Adamczyk and Meredith 2004). The purpose of this research was to quantify the development of bollworms on two Bollgard® cultivars with different levels (low vs. high) of Cry1Ac expression.

Materials and Methods

Plots

Cotton cultivars (cv. NuCOTN 33B and cv. DP 50B, Delta and Pineland Co., Scott, MS) were planted on May 8, 2003 in a 0.051 hectare (0.125 acre) field cage located in Stoneville, MS. These cultivars were chosen because of inherent differences in the amounts of Cry1Ac expressed. NuCOTN 33B expresses significantly more Cry1Ac than DP 50B (Adamczyk and Sumerford 2001). On July 1, 2003, the entire cage was enclosed with a nylon mesh, and no natural infestations of bollworms were observed at that time. Two row plots (5.0 m) were arranged in a randomized complete block design with 4 replications. All plots were maintained according to local agronomic practices. No insecticides were applied after the cage was enclosed with mesh.

Egg Infestations

Bollworm eggs were inoculated once (July 14, 2003) on the caged cotton plants to ensure that observations were only made on one developing population. Because of a limited supply of eggs, only two replicates per cultivar were utilized to ensure an adequate infestation level. Eggs were obtained from a laboratory colony main-

tained at the USDA, ARS, Southern Insect Management Research Unit located in Stoneville, MS. All eggs were harvested at the same time, thus representing the same age (1 day old). The egg inoculation was made by modifying the technique described in McWilliams (1979) for inoculating heliothine eggs to soybean plants. In brief, eggs were suspended in a 0.25% solution of xanthan gum (Sigma-Aldrich, Inc., www.sigmaaldrich.com). Xanthan gum powder was dissolved in slightly heated (40° C) double-distilled water using a stir plate. Eggs were added directly to the stirring solution until a homogenous suspension was obtained. The suspended eggs were dispensed onto plants using a repeating 50 ml pipette in 500 µl aliquots. The pipettor was calibrated to deliver 15 eggs/500 µl. Eggs were transported to the field cage and dispensed at a rate of 15 eggs/row ft (450 eggs/plot) within an hour of their suspension in xanthum gum. Eggs were placed directly to the adaxial surface of the largest terminal leaf of cotton plants that had been flowering for about 1 week.

Recovered Larvae

After 14 days, all plants within a plot were visually examined for bollworm larvae. Larvae were collected from fruiting structures (i.e. flower bud, flower, or boll), placed in 29 ml plastic cups, and transported to the laboratory where they were weighed within 1 h after arrival. Mean weights of larvae were analyzed using REML-ANOVA, and means were separated according to Fisher's Protected LSD (PROC MIXED, SAS Institute 2001; Littell *et al.* 1996).

Bt Quantification

Quantification of the levels of Cry1Ac present in the two cotton cultivars was made using a commercially available kit (Envirologix, Inc., www.envirologix.com/). On July 15, 2003, healthy plants (5) were selected from each plot for Cry1Ac quantification. Terminal leaves from the plants were used because this tissue accurately reflects overall expression differences among cultivars (Adamczyk and Sumerford 2001). Tissue was excised from the lobed region of a terminal leaf by placing the tissue underneath the attached cap of a 0.5 ml microcentrifuge tube. Closing the cap produced a uniform circular sample of about 4.8 mg that was contained within the microcentrifuge tube. This procedure also minimized desiccation of the leaf samples. The five individual leaf samples per plot were placed into a plastic bag and transported to the laboratory in a cooler with ice. Within 1 hour, the 5 samples/plot were combined into an individual 2.0 ml 96 deep-well microtiter plate (BioSpec Products, Inc., www.biospec.com/) containing two 6.4 mm stainless steel ball-bearings (BioSpec). Cry1Ac extraction buffer (1.0 ml) (EnviroLogix) was then added to each well. The tissue was then homogenized for 30 s using a Mini-Beadbeater-96™ (BioSpec). The microtiter plate was then centrifuged at 3,000 rpm for 5 min at 4° C (Avanti™ J-20XP, Beckman Coulter, Inc., www.beckman.com). For each sample, a 20 µl aliquot was place in an individual 1.1 ml 96 deep-well microtiter plate containing 500 µl of Cry1Ac extraction buffer (EnviroLogix) (1:26 dilution). The microtiter plate was covered with a corresponding silicone-based lid (BioSpec) and placed on an orbital shaker for 1 min. at 300 rpm. A commercial quantification plate kit then was utilized to quantify the amount of Cry1Ac present for each cultivar/plot (EnviroLogix). Samples were plotted against a standard curve with Cry1Ab calibrators supplied in the kit. A simple conversion was used to express values as "Cry1Ac" as dictated by the kit protocol. The amount of Cry1Ac was expressed as parts per million (ppm) after accounting for the proper dilution factors. Mean expression of Cry1Ac was analyzed using REML-ANOVA, and means were separated according to Fisher's Protected LSD (PROC MIXED, SAS Institute 2001; Littell *et al.* 1996).

Results and Discussion

There was an inverse relationship between the amount of Cry1Ac among cultivars versus the weight of bollworm larvae (Table 1). Cotton plants from NuCOTN 33B expressed 300% more Cry1Ac than DP 50B plants. Larvae that were recovered from the lower expressing DP 50B cultivar weighed significantly more ($P < 0.01$) than larvae collected from the higher expressing NuCOTN 33B cultivar. However, 30% more larvae were collected in the higher expressing NuCOTN 33B cultivar than the lower expressing DP 50B cultivar. Possible explanations for this discrepancy are sam-

Table 1. Mean level of Cry1Ac and weights of recovered bollworm larvae, *Helicoverpa zea,* from two commercial cultivars of Bollgard® cotton.

Cultivar	Amount of Cry1Ac (ppm) ± SE	Mean weight of larva (mg) ± SE
NuCOTN 33B	8.56 ± 0.704	54.47[1] ± 8.846
DP 50B	2.85 ± 0.832	99.92[2] ± 20.112
n =	30	
df =	1, 3	1, 53.4
F =	44.84	8.43
p =	0.007	0.005

[1] n = 33

[2] n = 23

pling error due to visual inspection of many plants (i.e. chance in finding the larvae) or late instar bollworms may have dropped from the plants to pupate in the soil sooner on the lower expressing DP 50B culivar as suggested by Adamczyk et al. (2001). Because damage ratings of all fruiting structures were not taken between the cultivars, explanations must remain speculative at this time. However, the distribution of larval weights indicates that more late-instars (> 200 mg) were collected from the lower expressing DP50B cultivar than the higher expressing NuCOTN 33B cultivar (Figure 1).

Clear differences in the performance of Bollgard® cotton cultivars against the bollworm were observed in this field cage experiment. These results corroborate the laboratory bioassay findings in Adamczyk *et al.* (2001) in which they showed that survival of bollworm larvae was significantly higher on Bollgard® cultivars that contained lower amounts of Cry1Ac compared to the higher expressing NuCOTN 33B cultivar. In another study, Clemens (2000) showed that larval mortality for the soybean looper, *Pseudoplusia includens*, was significantly higher when fed NuCOTN 33B versus DP 50B leaves in laboratory bioassays, although the level of Cry1Ac was not measured.

Some studies have indicated that conventional cotton cultivars may provide different levels of control against certain lepidopteran pests due to varying levels of naturally occurring toxins such as gossypol (Lukefahr *et al.* 1975; Stewart *et al.* 2001). However, Adamczyk *et al.* (2001) determined no differences in the survival of bollworm larvae between the non-transgenic parental lines of DP50B and NuCOTN 33B. Furthermore, Clemens (2000) also showed no differences in the survival of the soybean looper on the same non-transgenic parental lines. Therefore, differences in the weights of bollworm larvae on Bollgard® cultivars in the current study are most likely attributed to varying levels of Cry1Ac, however, differences in secondary plant compounds among the two cultivars cannot be ruled out.

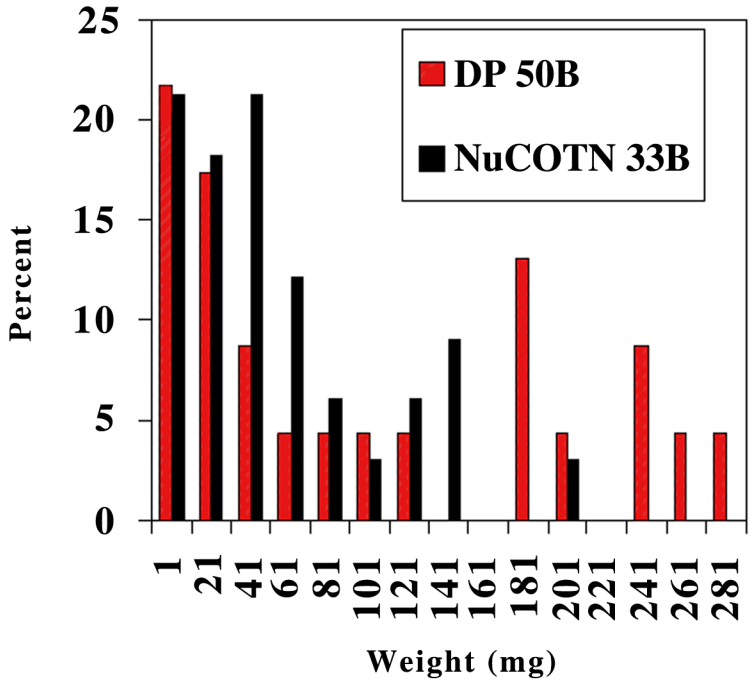

Figure 1. Distribution of weights of bollworm larvae recovered from two different Bollgard® varieties. Overall expression of cv. NuCOTN 33B was 300% greater than cv. DP 50B.

These finding may influence management strategies for controlling bollworms in Bollgard® cotton. Consultants currently assume that the occurrence of many different sizes of bollworm larvae in a particular field is indicative of multiple oviposition events over an extended period of time. Based on results from the current study, that may not always be the case. Bollworms from a single cohort of eggs were more variable in their development on the lower expressing DP50B than the higher expressing NuCOTN 33B. This could be due to more variability in Cry1Ac expression among plants from the DP50B cultivar, although plant-to-plant differences appear to be quite small (Adamczyk and Meredith 2004). Therefore, the overall expression levels in Bollgard® cultivars should be made available to growers and consultants to aid in using additional control tactics to manage bollworms in Bt cotton.

Differential expression of Cry1Ac among Bollgard® cultivars may complicate current resistance monitoring strategies. Luttrell *et al.* (2004) showed that bollworm larvae collected from Bollgard® cotton typically had higher LC_{50} values compared to bollworm larvae collected from a non-Bt host. Additionally, LC_{50} values were highly variable among bollworm populations collected from Bollgard® cotton. When comparing dose-mortality lines for different populations of bollworms collected from Bollgard® cotton, the mean level of Cry1Ac in a particular cultivar should be noted because the selection pressure may be higher in a cultivar such as NuCOTN 33B compared to DP 50B (Gould and Tabashnik 1998).

Regardless of Bollgard® cultivar, the weights of recovered bollworm larvae were highly variable. Larval stage ranged from first instars to 5-6th instars. Because a natural population of bollworms was not present in the field cage before the netting was erected, this variation could only be associated with the inoculated population. Greenplate (1999) and Adamczyk et al. (2001) showed that the level of Cry1Ac was significantly different among various cotton plant structures. In this current study, all eggs were deposited in the terminals of the cotton plant. Greenplate (1999) showed that the terminal portion of the Bollgard® plant contained significantly more Cry1Ac than any other plant structure. Furthermore, Gore *et al.* (2002) showed that neonate bollworms migrate from the terminals of Bollgard® plants and settle on fruiting structures lower in the plant canopy. Larvae may then feed on higher expressing flower buds (i.e. squares) or lower expressing flower anthers (Gore *et al.* 2001). Therefore, variation in bollworm development may be partially explained by the fact that larvae may require more time to develop when feeding on tissues that contain higher levels of Cry1Ac in addition to higher levels of naturally occurring toxins (e.g. gossypol in squares) (Lukefahr *et al.* 1975). Future research should explore this possibility. Luttrell *et al.* (1999) conducted dose-mortality studies for bollworms collected from different geographic regions, and showed that bollworm larvae are highly variable in their sensitivity to Cry1Ac. In addition, Sumerford *et al.* (2004) showed that sensitivity of bollworm larvae to Cry1Ac often is more variable within a given population than across populations collected

from different geographical regions. Thus, many factors could explain the variability of bollworm development from a single population on Bollgard® cotton, but the possible influence of each factor must remain speculative at this time.

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