QUANTIFICATION OF CryIA(c) δ-ENDOTOXIN IN TRANSGENIC BT COTTON: CORRELATING INSECT SURVIVAL TO DIFFERENT PROTEIN LEVELS AMONG PLANT PARTS AND VARIETIES

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Abstract

Clear differences in bollworm and fall armyworm survival and development were observed when these larvae were fed transgenic Bt cotton leaves from 17 commercially available varieties. A quantification assay (ELISA) was used to quantify the levels of δ-endotoxin in two of these varieties (cv. DP 451B/RR & cv. NuCOTN 33B; Delta & Pine Land Co., Scott, MS) throughout the growing season. Differences in the amount of δ-endotoxin present in various plant parts and leaves of these two Bt varieties were correlated with larval survival and development. Larvae that were fed on cv. DP 451B/RR completed development faster and exhibited better survivorship than those larvae fed cv. NuCOTN 33B, while lower levels of δ-endotoxin were detected in cv. DP 451B/RR compared to cv. NuCOTN 33B.

Introduction

The first transgenic CryIA(c) *Bacillus thuringiensis* (Bt) cotton variety was commercialized in 1996 and numerous advancements in insect control with transgenic technology have been developed. Where once a single variety contained a single insecticidal gene, growers can now choose from over 25 transgenic varieties. These varieties can contain the CryIA(c) Bt gene (Bollgard®; Monsanto Ag. Co., St. Louis, MO), herbicide resistance genes, and Bt varieties stacked or pyramided with a herbicide resistance gene. With the current number of transgenic cotton varieties being developed annually, evaluation of which varieties best suit specific geographical regions and growing conditions can become tedious and thus may be compromised. The advent of commercialized CryIA(c) δ-endotoxin quantification systems will allow more routine evaluations of different Bt varieties. As with any foliar insecticide and herbicide, research must be performed to determine the efficacy of every variety to ensure the best recommendation to growers.

To prolong the use of current transgenic insect control, resistance management guidelines have been developed. Primarily based on modeling data, these recommendations suggest that planting non-Bt cotton may serve as a refuge for Bt susceptible Lepidoptera and thus delay resistance (Caprio 1994). These models also rely on a high-dose strategy for insects such as the tobacco budworm, *Heliothis virescens*, but delaying resistance in intrinsically tolerant insects such as the cotton bollworm, *Helicoverpa zea*, are much debated. Recently, laboratory studies have shown that temporal mating can potentially occur among tobacco budworm and pink bollworm, *Pectinophora gossypiella*, populations from non-Bt and Bt cotton, since development is delayed for resistant larvae feeding on Bt cotton (Liu et al. 1999, Peck et al. 1999). Therefore, if current Bt varieties express different levels of CryIA(c) δ-endotoxin, then further reproductive isolation of populations of intrinsically tolerant Lepidoptera may occur, complicating recommendations.

Although genetic transformation events often modify the agronomics of the transgenic variety compared to the corresponding parental variety (i.e. plant maturity and mean height) (D&PL Seed Research and Agronomic Services; Scott, MS), few studies have been published that examine differential expression of toxin among different plant parts and varieties. Greenplate (1999) developed a quantification bioassay for the tobacco budworm that showed that CryIA(c) δ-endotoxin levels decrease among squares and bolls throughout the growing season. While numerous studies have shown that intrinsically tolerant Lepidoptera (i.e. bollworms, armyworms, and loopers) widely differ in their susceptibility to the δ-endotoxin (Adamczyk et al. 1998, Jenkins et al. 1992, Luttrell et al. 1999, MacIntosh et al. 1990), none have tried to correlate larval survival and development to the amount of δ-endotoxin present in commercial varieties. This study was initiated to determine if differences exist in bollworm survival and fall armyworm development among various Bt plant parts and varieties, and if such differences could be correlated to δ-endotoxin concentrations.

Materials and Methods

Research Field Plots

Eight non-Bt varieties and 17 Bt varieties were planted on May 24, 1999 near Elizabeth, MS. Plots consisted of 4 rows (40 inch centers) x 100 ft. Treatments were arranged in a split-plot design within a randomized complete block with 4 replications (main plot, soil type; sub-plot, variety). All plots were non-irrigated. Insecticide applications were made on all varieties for non-lepidopterous insects throughout the season. Only non-Bt varieties received a single application of foliar insecticide to control a natural infestation of tobacco budworms and cotton bollworms.

Larval Survival and Development among Bt Varieties Cotton Bollworms-Survival Study

To insure that healthy larvae would be used in all tests, collections were made from field corn near Stoneville, MS in early May of 1999. Approximately 300 larvae were collected and reared to pupation on artificial diet. Rearing of adults and egg harvesting were conducted as described in Adamczyk

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et al. (1998). The F_1 generation of neonates was used in all tests.

To compare larval survival of cotton bollworms among 17 different Bt varieties, 15 leaves were selected from 15 different plants from each plot. In addition, leaves from 8 non-Bt varieties were used as controls. These leaves were harvested with ca. 0.5 cm of petiole remaining to prevent desiccation. The leaves were placed into a 9.2 cm Petri dish containing a moistened filter paper. One neonate was infested in each dish, and the dishes were covered with corresponding lids (60 neonates/variety). All dishes were held in an environmental chamber at 27 ± 1°C, 80% RH, and a photoperiod of 14:10 (L:D) h. Leaves were changed at 2 days after exposure (DAE) and assessed at 4 DAE. Larvae were considered dead if no movement was observed after being prodded with a blunt surgical probe. All data were log transformed and mean survivorship was analyzed using the LSMEANS option of PROC MIXED (Littell et al. 1996).

Fall Armyworms-Development Study

Fall armyworms, *Spodoptera frugiperda*, were used in the larval development study because this species is intrinsically tolerant to the CryIA(c) δ-endotoxin and can be easily reared to pupation on Bt varietal tissue (Adamczyk et al. 1998). As with cotton bollworms, every effort was made to utilize healthy insects. Larvae were obtained from the USDA-ARS laboratory at Mississippi State, MS. This colony is routinely outcrossed with wild, pheromone trapped males to insure genetic diversity and traits present in field individuals. Larval and adult rearing as well as egg harvesting were conducted as described by Adamczyk et al. (1998).

In a preliminary experiment, two Bt varieties widely differed in their effect on cotton bollworm survival (cv. NuCOTN 33B and cv. DP 451B/RR, Delta & Pine Land Co., Scott, MS). Therefore, these two varieties were closely examined to determine if differences existed in fall armyworm development when fed either variety. A non-Bt variety was used as a control (cv. DP 5415, Delta & Pine Land Co., Scott, MS). From each plot, 30 different leaves from 30 different plants were selected as described above. One neonate was placed in each dish along with a moistened filter paper, and the dishes were covered with corresponding lids (120 neonates/variety). Leaves were changed every 48 h until pupation. Larval weights were recorded at 16 DAE. Pupal weights and time to pupation also were recorded. All data were log transformed and mean survivorship was analyzed using the LSMEANS option of PROC MIXED (Littell et al. 1996).

Correlating Differences in Larval Survival and Development to δ-Endotoxin Levels in Two Bt Varieties : Sample Preparations

Leaves. Two Bt varieties that differed in larval survival and development of cotton bollworms and fall armyworms (cv. NuCOTN 33B & cv. DP 451B/RR) were analyzed to quantify the amount of δ-endotoxin present. In addition, a non-Bt variety (cv. DP 5415) was used as a control. From each plot, one leaf was randomly selected from 5 plants. A single sample was taken from each leaf using a standard 6 mm paper ticket punch. The 5 leaf samples were weighed and combined into a 1.5 ml Eppendorf® tube and homogenized in extraction buffer using a fitted pestle. Each sample was replicated twice.

An experiment was designed to quantify the amount of δ-endotoxin present in leaves from the same two Bt varieties (cv. NuCOTN 33B & cv. DP 451B/RR) throughout the growing season. The 4th true leaf from various plants in each plot was tagged on June 24, 1999 (Slant 'N Lock; A.M. Leonard, Piqua, OH) to ensure that the same leaf stage was selected for each individual sample date. One additional location (Macon Ridge Location of the Northeast Research Station, LSU Agricultural Center, B. R. Leonard) was added. The identical leaf sample preparation protocol was followed as above.

Bracts, Squares, Flowers, Bolls. The same protocol was used for sample preparation, except that the homogenization step was modified. For uniformity during extraction, a high-speed homogenizer was used. This apparatus utilizes stainless steel beads (6 mm) to shear the tissue uniformly (Mini-Bead Beater®; Biospec Prod., Inc., Bartlesville, OK).

δ-Endotoxin Quantification Assay. To quantify the amount of δ-endotoxin present for each variety, a commercial quantification plate kit was utilized (EnviroLogic, Inc.; Portland, ME). This "sandwich" Enzyme-linked ImmunoSorbent Assay (ELISA) utilizes a color development step where color production is proportional to CryIA(c) concentration in the sample extract. Therefore, quantification of δ-endotoxin is determined spectrophotometrically (Benchmark®; Bio-Rad, Hercules, CA). The proper standard curve, dilution factors, and calculations were conducted. Means were analyzed using the LSMEANS option of PROC MIXED (Littell et al. 1996).

Results

Cotton Bollworms-Survival Study

Although there were no significant differences observed in larval survival (avg. 92.19%, stdev. 2.04) among the 8 non-Bt varieties ($F=0.50$; df=7,14; $P=0.82$), significant differences were observed in larval survival (avg. 34.31%, stdev. 12.51) among the 17 Bt varieties ($F=1.96$; df=16,32; $P=0.05$).

Significant differences were observed between the two closely examined Bt varieties [cv. NuCOTN 33B (18.3%) & cv. DP 451B/RR (55.0%)] (t=1.97; df=48, *P*=0.05).

Fall Armyworms-Development Study

Control data validate that the experimental design and quality of insects was adequate. As in Adamczyk et al. (1998), larval and pupal weights were significantly higher for larvae reared on a non-Bt variety compared to a Bt variety (*P*<0.0001). In addition, time to pupation for larvae reared on cv. DP5415 was significantly less than for those larvae reared on cv. NuCOTN 33B or cv. DP 451B/RR.

There were significant differences in larval and pupal weights, and time to pupation between Bt varieties. Mean larval weights at 16 DAE were significantly higher for those larvae fed cv. DP 451B/RR (66.7 mg) than for those fed cv. NuCOTN 33B (32.0 mg) (t=4.23, df=141, *P*<0.0001) (Fig. 1). Likewise, mean pupal weights were significantly higher for those larvae fed cv. DP 451B/RR (169.2mg) than cv. NuCOTN 33B (151.4mg) (t=2.39, df=130, *P*<0.0182) (Fig. 2). In addition, mean time to pupation was significantly less for those larvae fed cv. DP451B/RR (29.0 d) than cv. NuCOTN 33B (32.1 d) (t=4.29, df=130, *P*<0.0001) (Fig. 3).

Quantification of δ-Endotoxin

Leaves. Significant differences in the amount of δ-endotoxin present in leaves from the two varieties was observed for all sample dates including the sample sent from Louisiana. Higher levels of δ-endotoxin were detected in cv. NuCOTN 33B than cv. DP 451B/RR leaves (Fig. 4).

Bracts, Squares, Flowers, Bolls. The amount of δ-endotoxin present in various plant parts was numerically lower in cv. DP 451B/RR than cv. NuCOTN 33B. In addition, significant differences in δ-endotoxin levels for bracts and bolls were observed between the two Bt varieties (Fig. 5).

Discussion

Assuming that all Bt varieties express similar levels of CryIA(c) δ-endotoxin appears to be inaccurate. Precise factors that affect δ-endotoxin expression among plants appears to be limited at this time. Our study shows that the level of δ-endotoxin decreases in leaves throughout the growing season, and that the trend in δ-endotoxin levels between the two Bt varieties was not geographically isolated. High mortality was observed in larval bioassays involving leaves collected late in the season (data not shown), but very low-levels of δ-endotoxin were detected. This discrepancy was attributed to low nutritional value in these late-season leaves compared to early-season leaves because control mortality using non-Bt leaves was also extremely high (>95%). Thus, solely relying on larval bioassays to assess the effectiveness of δ-endotoxin may not always be accurate unless proper controls and high-quality insects are used.

By not providing a high-dose strategy to control the intrinsically tolerant Lepidoptera (i.e. armyworms and bollworms), managing resistance to these insects may be further complicated by differential expression of δ-endotoxin among plant parts and varieties. Although complex interactions are involved, Peck et al. (1999) showed that delays in larval development time for tobacco budworms feeding on Bt cotton compared to a non-Bt refuge may increase or decrease the rate of resistance development. In another study involving the pink bollworm, *Pectinophora gossypiella*, researchers showed that Bt-resistant larvae feeding on Bt cotton take 5 to 6 d longer to develop into moths compared to Bt-susceptible larvae feeding on non-Bt cotton (Liu et al. 1999). Our data show that development of the fall armyworm was increased by three days when fed cv. NuCOTN 33B compared to cv. DP 451B/RR, which was ample time for these populations to segregate in the laboratory. The implications of differential expression among Bt cotton varieties expressing the same δ-endotoxin clearly needs to be further examined.

Because of the relative ease in conducting this quantification assay, the utility of using these systems for providing information to the grower concerning varietal choices may be more common in the future. With over 25 commercially available transgenic Bt cotton varieties to choose from, more information to determine which variety offers the best insect control is clearly needed.

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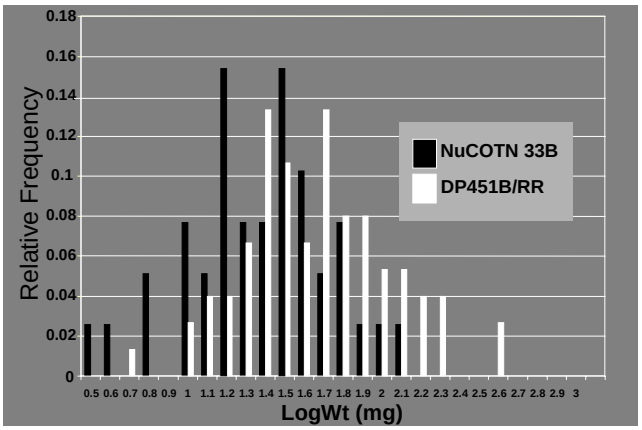

Figure 1. Larval weights at 16 DAE for fall armyworms fed cv. DP 451B/RR or cv. NuCOTN 33B leaves.

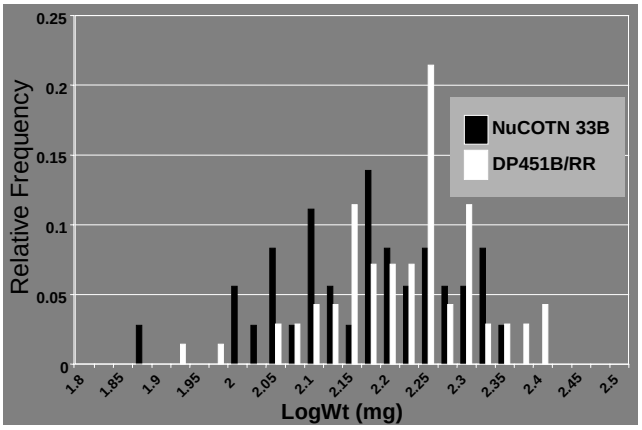

Figure 2. Pupal weights for fall armyworms fed cv. DP 451B/RR or cv. NuCOTN 33B leaves.

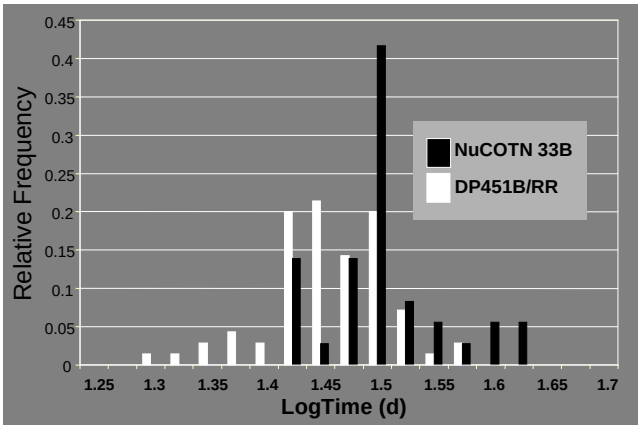

Figure 3. Time to pupation for fall armyworms fed cv. DP 451B/RR or cv. NuCOTN 33B leaves.

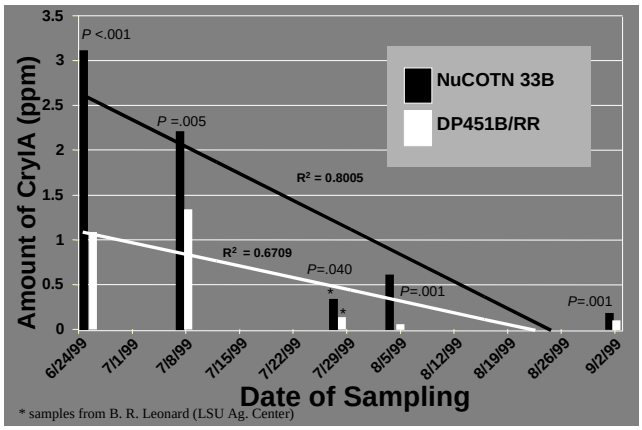

Figure 4. CryIA δ-endotoxin levels in leaves of two Bt varieties from two locations.

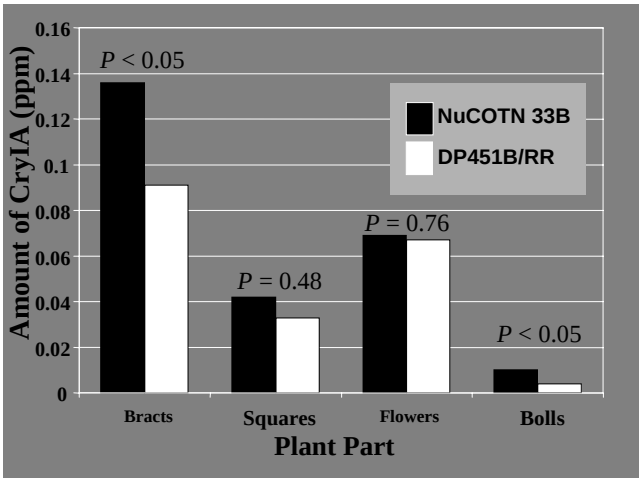

Figure 5. CryIA δ-endotoxin levels in various plant parts from two Bt varieties.