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TEMPORAL EXPRESSION PROFILES AND BIOACTIVITY OF SINGLE (BOLLGARD®) AND DUAL-TOXIN (BOLLGARD II®) BT COTTON

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Abstract

Laboratory studies were conducted using ELISA and bioassays (*Helicoverpa zea*) to investigate temporal Cry protein expression in field-grown single ('Bollgard') and dual-toxin ('Bollgard II') Bt cotton. In single-toxin Bt cotton, Cry1Ac levels changed significantly as the season progressed, exhibiting ca. 3-fold difference in Cry1Ac expression between the highest date (6.2 ppm—12 June) and the lowest date (1.7 ppm-- 6 August). In subsequent bioassays for single-toxin Bt cotton (7 DAI), there were also significant differences among dates with ca. 2-fold difference in larval weights between the highest date (37.0 mg—6 August) and the lowest date (18.4 mg —26 June). When comparing results from ELISA to those from *H. zea* bioassays, Cry1Ac levels were inversely related to mean larval weight across all dates, in that the highest levels of toxin expression resulted in the lowest larval weights among treatments, and vise versa. For dual-toxin Bt cotton, Cry1Ac responded similarly to those from single-toxin Bt cotton, exhibiting ca. 3-fold difference between the highest date (6.43 ppm—12 June) and the lowest date (2.19 ppm—10 July). Cry2Ab levels, however, only exhibited ca. 1.5-fold difference in toxin expression throughout the season (14.6 - 24.6 ppm). In bioassays for dual-toxin Bt cotton, there were no significant differences in weights of *H. zea* among dates at 7 DAI (15.6 - 20 mg). Because the insertion of the *cry2Ab* gene had no deleterious effect on the performance of the *cry1Ac* gene in dual-toxin Bt cotton, enhanced efficacy can be attained from the additive effects of both toxins. In our studies, the presence of the Cry2Ab protein played a significant role in reducing the variation in susceptibility of *H. zea* larvae feeding on transgenic Bt cotton tissue. Therefore, cottons containing two toxins of *B. thuringiensis* can potentially be valuable tools in resistance management, as well as provide enhanced season-long protection against *H. zea*.

Introduction

Transgenic Bt cotton expressing the Cry1Ac δ-endotoxin from *B. thuringiensis* var. *kurstaki* (i.e., 'Bollgard', Monsanto Company, St. Louis, MO), offers a unique and innovative alternative to traditional chemical insecticides. Bollgard varieties have successfully supplemented integrated pest management programs throughout cotton producing areas in the United States since 1996. Grower acceptance of Bollgard in Mississippi, for example, has increased from 42% of cotton acreage in its first year of commercial availability (Layton 1997) to 85% in 2003 (USDA 2003). Of all upland cotton grown in the U.S. in 2003, 49% of total acreage was planted to varieties containing the Bollgard gene (USDA 2003).

Numerous experiments conducted since the development of Bollgard cotton have proven its effectiveness against tobacco budworm (*Heliothis virescens*) and moderate populations of bollworm (*Helicoverpa zea*) (Benedict et al. 1993, Mascarenhas et al. 1994). However, studies have shown that bollworm larvae are not as susceptible to Cry1Ac as tobacco budworm (Stone and Sims 1993, MacIntosh et al. 1990). As a result, control of bollworm in Bollgard cotton is less dependable than that of tobacco budworm. Mahaffey et al. (1995) reported damaged squares (14%) in Bt cotton due to large infestations of *H. zea*. Supplemental insecticide applications have been routinely required for control of *H. zea* in Bollgard cotton across the southeastern U.S. (Leonard et al. 1997, Roof and Durant 1997, Smith 1998). Layton et al. (2003) conducted a 7-year survey of the performance of Bt cotton in Mississippi, and reported that the average number of insecticide sprays required to control caterpillar pests in Bollgard cotton fields in Mississippi ranged from 0.33 (1996) to 1.22 (1998). Greenplate (1998) reported temporally declining levels of Cry1Ac as one factor that may allow significant survival of bollworms in Bollgard cotton and subsequently lead to economic damage. The apparent lack of susceptibility in a small percentage of *H. zea* larvae also increases the potential of selection for resistance to Cry1Ac in this pest.

To address the apparent weaknesses of Bollgard, Monsanto Company has developed cotton containing the *cry1Ac* gene found in the original Bollgard cotton plus an additional gene that encodes for Cry2Ab toxin from *B. thuringiensis* var. *kurstaki* (i.e., 'Bollgard II'). Although Bollgard II was developed to enhance control of bollworm as well as occasional Lepidopteran pests, Cry2 proteins are less effective against bollworm than Cry1 proteins on a per unit basis (Sims 1997). However, Greenplate et al. (2000) and Adamczyk et al. (2001b) reported that in various Bollgard II cotton tissues, Cry2Ab protein is expressed at significantly higher levels than Cry1Ac. Therefore, greater overall bioactivity may result from additive effects from Cry1Ac

and the higher-expressed Cry2Ab in Bollgard II cotton, particularly if the insertion of the *cry2Ab* gene has no deleterious effect on the performance of the *cry1Ac* gene. Bollgard II was approved by the U.S Environmental Protection Agency (Washington DC, USA) in December 2002 and was made available to growers in limited amounts for the 2003 growing season. More varieties containing both *cry* genes are expected to be available to cotton producers for 2004.

Preliminary studies have investigated efficacy of Bollgard II cotton in the laboratory (Stewart et al. 2001), greenhouse (Jackson et al. 2000), and in the field (Stewart et al. 2000, Ridge et al. 2000). Stewart et al. (2001) found only 2% of bollworms surviving to 15 d on Bollgard II flowers compared to 16% survival on Bollgard flowers. A significant reduction in percent damaged fruit was noted in Bollgard II compared with Bollgard genotypes (Jackson et al. 2000). Stewart et al. (2000) reported higher larval stunting and pupation delay in fall and beet armyworms feeding on Bollgard II cotton when compared with Bollgard. Under low population levels, Ridge et al. (2000) also found fewer bollworms and soybean loopers in Bollgard II plots compared to Bollgard plots. These preliminary data indicate that Bollgard II varieties can reinforce IPM programs in cotton production throughout the United States, particularly in areas where heavy, predictable moth flights occur on an annual basis.

Providing improved efficacy against target Lepidoptera, reduction of chemical insecticide input, and preservation of yields, transgenic Bt technology will continue to be a valuable tool in the future of cotton IPM. However, the long-term benefits of Bt cotton may be at risk due to the possible evolution of resistance to Bt toxins in target pests (Luttrell and Caprio 1996, Caprio 1994). Results from modeling predict that the greatest overall benefits of "stacking" Cry toxins (e.g., Cry1Ac + Cry2Ab) are achieved when no cross-resistance occurs, resistance to each toxin is rare and recessive, and an effective refuge strategy utilizing non-Bt plants is implemented (Gould 1998). Gould et al. (1992) found that a tobacco budworm strain resistant to Cry1Ac exhibited low levels of cross-resistance to Cry2A. One factor that can contribute to the evolution of resistance to Bt toxins is reduced toxin-induced mortality of individuals heterozygous for resistance alleles (Gould and Tabashnik 1998). If Cry2Ab in dual-toxin Bt cotton is expressed at sufficient levels to improve bioactivity against economically important Lepidoptera throughout the susceptible times of the season, Bollgard II can be a highly effective tool for resistance management.

Temporal declines in toxin expression among transgenic Bt cottons can contribute to reduced toxin-induced mortality of heterozygotes and thus, an increase in resistance alleles. Adamczyk et al. (2001b) showed a decline in Cry1Ac levels in terminal leaves of Bollgard cotton as the growing season progressed. In Cry1Ac expression studies, Greenplate et al. (1998) also reported a significant reduction in seasonal toxin levels in various tissues of Bollgard cotton. Because temporal reductions in Bt toxin levels may increase resistance alleles in a population, research should be conducted to determine whether the same temporal patterns observed in Bollgard cotton exist with Cry1Ac and/or Cry2Ab in Bollgard II cotton. Implications involving efficacy provided by both ("stacked") genes should be also investigated to determine whether temporal expression profiles of Bt toxins translate to bioactivity.

Quantitative diagnostic tools such as ELISA (enzyme-linked immunosorbent assay) have been used to detect differences in Cry1Ac expression levels in leaves among Bollgard varieties (Adamczyk et al. 2001a). The same methods can be used to quantify Cry1Ac and Cry2Ab levels in Bollgard II cotton. Bioassays can then be implemented with Bollgard and Bollgard II tissues to determine whether observed differences in Cry toxin levels translate into bioactivity. Expression and additional bioactivity of the Cry2A protein should especially be investigated to evaluate its performance as a second toxin in dual-toxin Bt cotton. Although there may be significant differences in toxin expression levels between given dates, the time of the season with the lowest toxin expression can still provide sufficient Bt protein to serve as an effective dose. Therefore, ELISA and bioassay techniques can be used to investigate the aforementioned resistance management considerations, as well as implications regarding enhanced efficacy of Bollgard II cotton. Scouting protocols can then be modified, if necessary, to better correspond with times of the season when toxin expression is lowest.

Determining the temporal pattern of Cry protein expression in Bollgard and Bollgard II cotton plants will help us better understand potential strengths or weaknesses related to time of the growing season. With the above considerations in mind, the objectives of our research were as follows:

1) Determine how toxin levels differ over time in terminal leaves of Bollgard and Bollgard II cottons using enzyme-linked immunosorbent assay
2) Use *H. zea* bioassays to evaluate patterns in bioactivity of Bt cotton leaves throughout the susceptible stages of the growing season
3) Investigate the relationship between Bt toxin expression and growth inhibition of *H. zea* larvae

Materials and Methods

Terminal leaves of NuCOTN 33B ('Bollgard', Cry1Ac only) and NuCOTN 33BII ('Bollgard II', Cry1Ac + Cry2Ab) were collected from field-grown cotton at the Plant Science Research Center in Starkville, Mississippi. Both genotypes were planted on April 26, 2001 in a randomized complete block design with three replications, with plot size of 16 rows (38 in spacing) x 50 ft. The soil type is a well-drained Leeper silty clay loam, and plots were furrow-irrigated twice during the

growing season to supplement natural rainfall. Every two weeks (6/12, 6/26, 7/11, 7/25, 8/6, 8/21, 9/4), the third uppermost unfurled leaf was collected from 33 randomly selected plant terminals per plot. Terminal leaves were then stored at –80 C until lyophilization could be performed. Leaves for each date and genotype were freeze-dried with a Genesis 25LL lyophilizer (Virtis Company, Inc., Gardiner, NY 12525) and pulverized into a fine powder with an 8-oz blender. Tissue samples were then stored at –20 C until immunoassays could be conducted.

Enzyme-Linked Immunosorbent Assay

Commercial quantification plate kits (Envirologix Inc., Portland, ME) were utilized to quantify the amount of Cry1Ac in Bollgard and Bollgard II cotton and Cry2Ab in Bollgard II cotton. The technique used to quantify Bt toxins in this experiment utilizes enzyme-linked immunosorbent assay (ELISA), as described by Adamczyk et al. (2001b). This is a commercially available test specific for each protein (i.e., Cry1 or Cry2) that uses a color development step in which color is proportional to the amount of antigen (i.e. Bt protein) present in the sample extract. Optical densities were quantified using a Thermomax® microplate reader (Molecular Devices, Inc., Sunnyvale, CA), then converted to concentrations using SOFTmax Pro®2.1.1 computer software (Molecular Devices, Inc.). 20 mg tissue was used for each date/genotype, and the entire extraction process was replicated five times. Differences in Cry1Ac and Cry2Ab levels were analyzed using Proc GLM, and means were separated using Fisher's protected LSD (SAS Institute 1998).

Bioassays

Helicoverpa zea (Boddie) was used as the model insect to investigate the relationship between bioactivity and toxin expression. *H. zea* were obtained as eggs from colonies maintained by USDA-ARS Genetics and Precision Agriculture Unit (Mississippi State, MS) and reared to eclosion. Once hatched, neonate larvae were individually placed into 29.5-ml diet cups containing the appropriate treatment. Treatments consisted of ca. 10 ml meridic diet containing terminal leaf powder from one of the aforementioned seven dates derived from either Bollgard or Bollgard II genotypes. Lyophilized leaf tissue was incorporated into meridic diet (45-50 C) using a standard blender. Based on preliminary bioassays (Akin, unpublished data), it was determined that lower amounts of tissue should be used in the Bollgard II assays in order to avoid excessive mortality and stunting. Dosages of transgenic cotton tissue included 1200 µg/ml for Bollgard tissue and 900 µg/ml for Bollgard II tissue. Bioassays were conducted as a RCB design with 6 replications (10 larvae per rep) including an untreated check (plain diet with no tissue). Larvae were stored at 27 C, 60% RH, and 14:10 L/D. Individual larval weights (mg) were recorded at 7 days after infestation (DAI), and subjected to Proc GLM using Fisher's protected LSD for mean separation (SAS Institute 1998).

Results

In single-toxin Bt cotton, Cry1Ac levels varied significantly as the season progressed, exhibiting ca. 3.5-fold difference in Cry1Ac expression between the highest date (6.2 ppm—12 June) and the lowest date (1.7 ppm-- 6 August) ($F=59.37$; df=6, 28; $p<0.0001$) (Figure 1). In the subsequent bioassay with single-toxin cotton, there were also significant differences among dates with ca. 2-fold difference in larval weights between the highest date (37.0 mg—6 August) and the lowest date (18.4 mg —26 June) at 7 DAI ($F=5.07$; df=6, 303; $p<0.0001$) (Figure 5). When comparing results from ELISA to those from *H. zea* bioassay, Cry1Ac levels corresponded strongly to mean growth inhibition across all dates, in that the highest levels of toxin expression resulted in the highest reduction in mean larval weight, and vise versa (Figure 6).

For dual-toxin Bt cotton, Cry1Ac levels responded similarly to those from single-toxin Bt cotton (Figure 3). There was ca. 3-fold difference in Cry1Ac levels between the highest date (6.43 ppm—12 June) and the lowest date (2.19 ppm—10 July) in Bollgard II cotton ($F=20.16$; df=6, 28; $p<0.0001$) (Figure 2). The similarity in seasonal expression profiles of Cry1Ac in the near-isogenic lines of Bollgard and Bollgard II indicates that the *cry1Ac* gene in each genotype performed similarly. This suggests that the insertion of the *cry2Ab* gene had little or no effect on the performance of the *cry1Ac* gene in dual-toxin Bt cotton. This is an important consideration in that if the activity of Cry1Ac and Cry2Ab are independent and additive, the presence of Cry2Ab protein can significantly enhance season-long control and help to delay resistance to Bt toxins.

As expected, levels of Cry2Ab protein were much higher than those of Cry1Ac in Bollgard II cotton (Figure 8). There were significant differences in Cry2Ab expression among dates ($F=4.40$; df=6, 28; $p=0.0030$) (Figure 4), as was the case with Cry1Ac in Bollgard II. However, Cry2Ab levels appeared to be more stable than those of Cry1Ac, as there was only ca. 1.5-fold difference in expression throughout the season between the lowest and highest dates (14.6 - 24.6 ppm) (Figure 4). In bioassays with dual-toxin Bt cotton, there were no significant differences in larval weights between dates ($F=0.83$; df=6, 270; $p=0.5462$) (Figure 7). These results indicate that the presence of the more stable Cry2Ab protein played a significant role in reducing the seasonal variation in growth inhibition of *H. zea* larvae that feed on dual-toxin Bt cotton tissue.

Overall toxicity of Bollgard and Bollgard II against *H. zea* was also evaluated. Across all dates, higher levels of larval stunting were observed in larvae that fed on diet incorporated with Bollgard II than for larvae that fed on diet containing Bollgard tissue (Table 1). This result was evident regardless of Bollgard II treatments containing only 75% of the amount of tissue used for Bollgard. Therefore, increased overall toxicity can be expected in *H. zea* feeding on terminal leaves of Bollgard II

cotton compared to Bollgard, as observed by Stewart et al. (2001) and others. Although treatments containing Bollgard II cotton tissue caused more stunting than those containing Bollgard, both single and dual-toxin Bt cottons caused significant growth inhibition of *H. zea* compared to the untreated check (97.6 mg) at 7 DAI, causing 74% weight reduction in Bollgard and 82% weight reduction in Bollgard II.

Summary

Bollgard II cotton can prove to be a valuable tool in resistance management, as well as provide enhanced season-long control of Lepidopteran pests. Due to the reduced variation in susceptibility and increased toxicity provided by Cry2Ab, and low levels of cross-resistance between Cry1Ac and Cry2Ab (Tabashnik et al. 2002, Gould et al. 1992), Bollgard II cotton should be considered a highly effective resistance management tool in cotton IPM across the U.S.

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Table 1. Mean weight of *H. zea* across all dates (F=30.99; df = 1, 585; p<0.0001).

Genotype	Dosage (µg/ml)	Mean larval weight (+SEM)
Bollgard	1200	25.7 ± 1.15 **a**
Bollgard II	900	17.9 ± 0.71 **b**

Means in the same column not followed by a common letter are significantly different (Proc GLM, SAS Institute 1998).

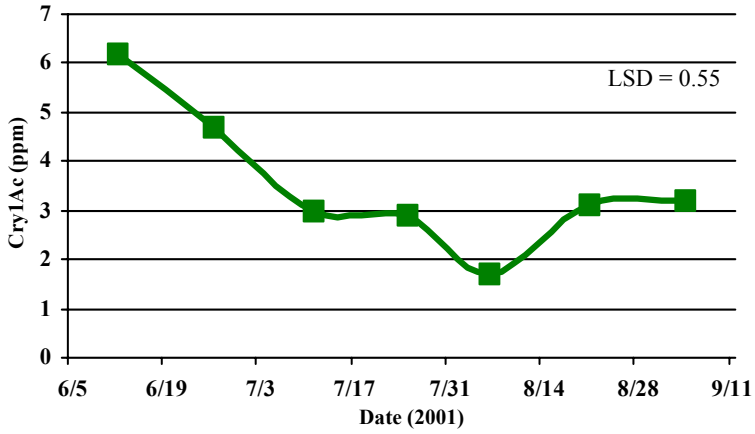

Figure 1. Seasonal Cry1Ac expression levels in terminal leaves of single-toxin Bt cotton ($F=59.37$; $df=6, 28$; $p<0.0001$) [SAS Institute, 1998].

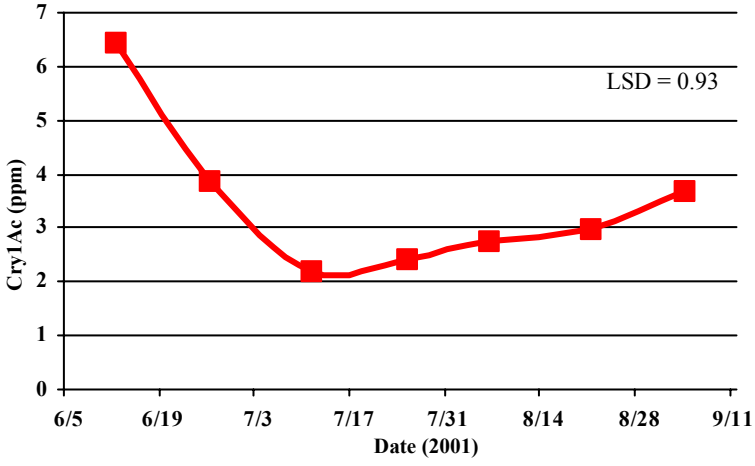

Figure 2. Seasonal Cry1Ac expression levels in terminal leaves of dual-toxin Bt cotton ($F=20.16$; $df=6, 28$; $p<0.0001$) [SAS Institute, 1998].

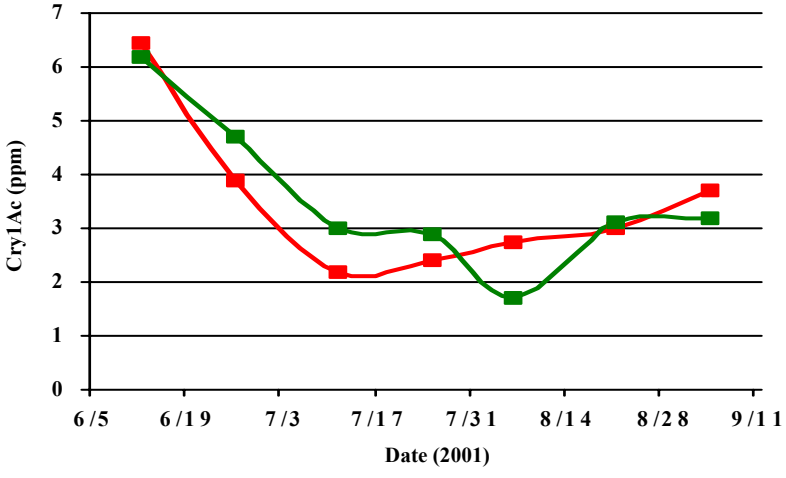

Figure 3. Seasonal Cry1Ac expression levels in terminal leaves of single (■) and dual-toxin (■) Bt cotton.

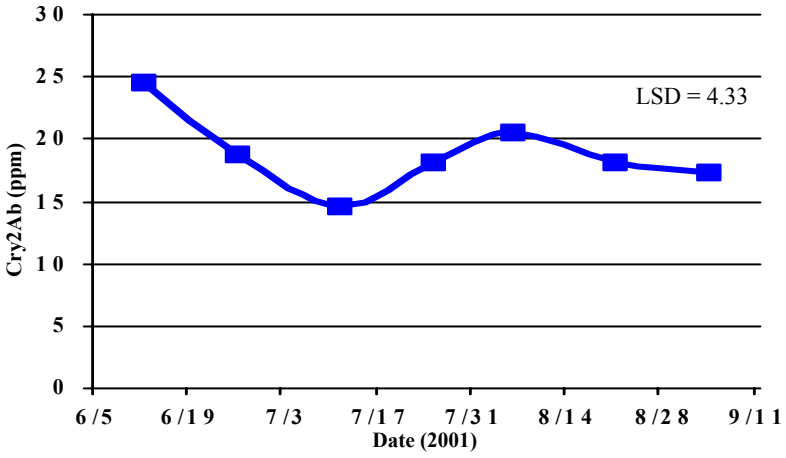

Figure 4. Seasonal Cry2Ab expression levels in terminal leaves of dual-toxin Bt cotton (F=4.40; df=6, 28; p=0.0030) [SAS Institute, 1998].

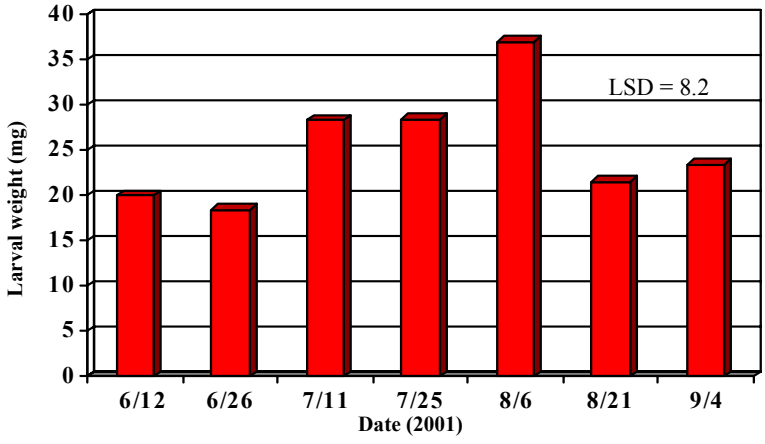

Figure 5. Mean larval weights of *H. zea* 7 d after infestation onto diet incorporated with terminal leaves of single-toxin Bt cotton (F=5.07; df=6, 303; p<0.0001) [SAS Institute, 1998].

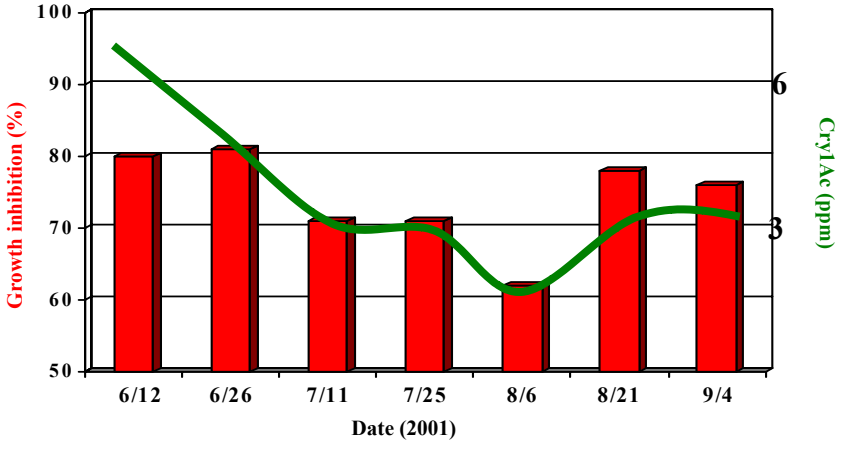

Figure 6. Growth inhibition (■) of *H. zea* (7 DAI) relative to the untreated check and seasonal Cry1Ac expression levels (–) of single-toxin Bt cotton.

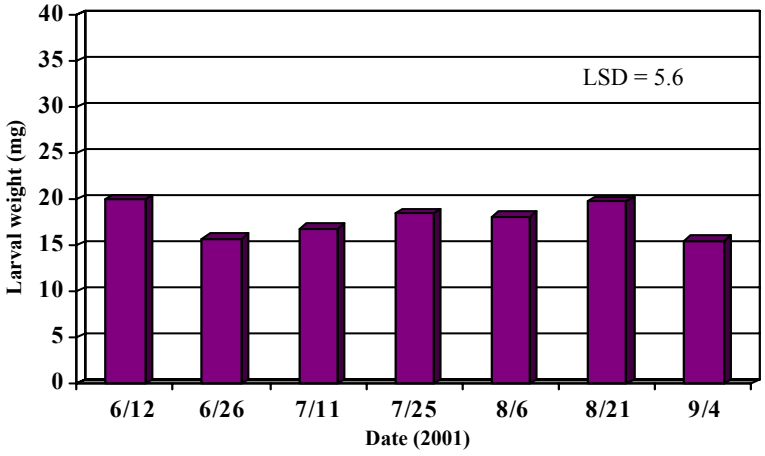

Figure 7. Mean larval weights of *H. zea* 7 d after infestation onto diet incorporated with terminal leaves of dual-toxin Bt cotton (F=0.83; df=6, 270; p=0.5462) [SAS Institute, 1998].

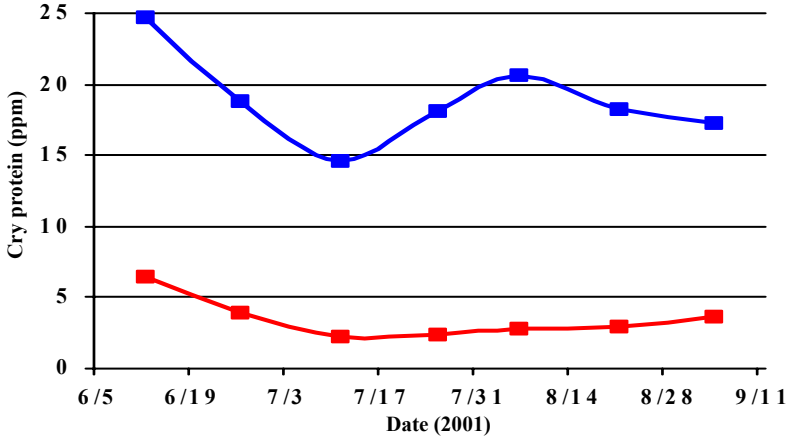

Figure 8. Seasonal Cry1Ac (■) and Cry2Ab (■) expression levels in terminal leaves of dual-toxin Bt cotton.