

ABSTRACT

JACKSON, RYAN EVERETT. The Influence of Transgenic Cottons Expressing One or Two *Bacillus thuringiensis* Proteins Against *Helicoverpa zea* (Lepidoptera: Noctuidae) and Factors Affecting *B. t.* Resistance in *H. zea*. (Under the direction of J. R. Bradley, Jr. and J. W. Van Duyn)

The commercialization of transgenic cottons, *Gossypium hirsutum* (L.), that contain a gene from the soil bacterium *Bacillus thuringiensis* var. *kurstaki* (Berliner) that encodes the Cry1Ac δ -endotoxin has provided a novel control option for certain lepidopteran pests. However, susceptibility of bollworm, *Helicoverpa zea* (Boddie), to these *B. t.* cottons has been much lower than that of tobacco budworm, *Heliothis virescens* (Fab.). Because control of bollworm with *B. t.* cottons has not been absolute compared to that of tobacco budworm, transgenic cottons expressing two *B. t.* proteins (Cry1Ac + Cry2Ab) have been developed to provide greater control of bollworm. To substantiate this, various field, greenhouse, and laboratory studies were designed to investigate the influence of single-gene and dual-gene *B. t.* cottons, Bollgard[®] and Bollgard II, respectively, against bollworm and to determine the elements affecting resistance evolution to the Cry1Ac and Cry2Ab δ -endotoxins. Three cotton genotypes, Bollgard, Bollgard II, and a conventional sister line, were evaluated for their comparative susceptibility against bollworm in North Carolina field studies from 1999-2001. The impact of supplemental pyrethroid oversprays for bollworm control on yield for each genotype was also evaluated. Comparisons of the three untreated genotypes averaged across three years demonstrated that both *B. t.* genotypes successfully reduced the percentage of terminals, squares, and bolls infested with live larvae or subsequent damage as compared to the conventional variety. The Bollgard II line had fewer bolls infested with live larvae and less terminal, square, and boll damage compared to the commercial Bollgard variety. Comparisons of pyrethroid-treated and untreated genotypes

revealed that the addition of a pyrethroid to conventional cotton reduced the percentage of terminals with a live larva. However, the pyrethroid-treated *B. t.* genotypes significantly reduced larval survival in the terminal region below that of the pyrethroid-treated conventional variety. Averaged across insecticide regimes, the Bollgard genotype had less terminal feeding damage than the conventional variety, but more terminal damage than the Bollgard II line. Both *B. t.* cottons had reduced larval survival on squares and bolls and sustained less boll damage than the conventional variety; however, the Bollgard II line had significantly lower percentages of bolls infested with live larvae and less boll damage than Bollgard. Square damage was lessened by the addition of a pyrethroid to the conventional variety but square damage in the treated conventional variety remained higher than in the untreated Bollgard variety. In addition, the pyrethroid-treated Bollgard genotype exhibited a reduction in square damage below that of the untreated Bollgard, but was similar to that of pyrethroid-treated and untreated Bollgard II genotype. Pyrethroid-treated subplots of the conventional variety had significantly higher seed cotton yields than the untreated subplots in four of seven trials where bollworm numbers were high enough to cause significant yield reductions. Pyrethroid-treated and untreated subplots of Bollgard and Bollgard II genotypes produced similar yields in each year*location combination.

To determine the effect of Bollgard II cottons on feral and Cry1Ac-tolerant bollworms, greenhouse studies were designed to evaluate the efficacy of Bollgard II and Bollgard cottons on both a feral and Cry1Ac-selected bollworm strain in 1999. In 2000, an additional greenhouse experiment was designed to compare the efficacy of three transgenic cottons expressing either the Cry1Ac endotoxin alone, the Cry2Ab endotoxin alone, or both the Cry1Ac and Cry2Ab endotoxins against a feral and a Cry1Ac-selected bollworm strain.

Results from testing only the feral strain suggested that the Bollgard II genotype was more effective than the Bollgard variety in reducing larval survival, superficial fruit damage, and fruit penetration. Results from the second 1999 greenhouse study that evaluated both a feral and a Cry1Ac-selected bollworm strain demonstrated that when averaged across bollworm strains, the Bollgard II genotype outperformed the Bollgard variety with respect to larval survival and fruit penetration by bollworm. Also, the Cry1Ac-selected bollworm strain displayed increased larval survival, superficial fruit damage, and fruit penetration as compared to the feral strain when averaged across genotypes. Results from the 2000 study confirmed 1999 results as the Bollgard II genotype significantly reduced fruit penetration by bollworm below that of the Bollgard variety when averaged across strains; however, the single Cry2Ab-producing genotype performed similarly to both Bollgard and Bollgard II with respect to fruit penetration. The Cry1Ac-selected bollworm strain displayed significantly greater larval survival and superficial fruit damage on the Bollgard variety as compared to the feral strain, but no differences among larval strains were evident for other genotypes. Also, when averaged across genotypes, the Cry1Ac-selected bollworm strain penetrated a higher proportion of cotton fruit in comparison to the wild strain.

To determine the contribution of dual-gene cottons to *B. t.* resistance management, Bollgard and Bollgard II cottons were compared with the conventional sister line in field experiments to quantify production of bollworm and bolls damaged by bollworm in North Carolina in 2000-2001. The relative numbers of bollworms that were capable of successfully completing development on each genotype were estimated under untreated conditions in 2000 and both pyrethroid-treated and untreated conditions in 2001. Averaged across two years, the untreated Bollgard II genotype significantly reduced the numbers of large larvae

(L4-L5) produced per hectare below that of the Bollgard and conventional varieties. With regard to the numbers of damaged fruit per hectare, both *B. t.* genotypes reduced fruit damage below that of the conventional variety, and the Bollgard II line had less damaged fruit than the Bollgard variety. The numbers of adults produced per hectare were significantly reduced by both *B. t.* genotypes, with no significant differences between Bollgard and Bollgard II cottons. In 2001, the numbers of larvae produced per hectare and subsequent feeding damage were dramatically reduced by both *B. t.* cottons, with a further reduction exhibited by the Bollgard II genotype as compared to the commercial Bollgard variety when averaged across insecticide regimes. Both pupal and adult production on a per hectare basis were significantly lowered by the two *B. t.* genotypes as compared to the conventional variety and averaged across insecticide regimes. No significant differences were apparent between the Bollgard and Bollgard II genotypes with regard to pupal and adult production. Averaged across genotypes, pyrethroid oversprays caused a significant reduction in larval, pupal, and adult production, as well as damaged fruit.

To determine the factors affecting resistance evolution to Cry1Ac and Cry2Ab δ -endotoxins, a laboratory experiment was designed to estimate the frequency of major *B. t.* resistance genes in the general bollworm population, to determine the presence and contribution of minor *B. t.* resistance genes, and to determine inheritance of resistance to Cry1Ac and Cry2Ab δ -endotoxins. Female bollworm moths were collected from light traps at various locations near the Tidewater Research Station in Washington Co., NC, in 2001. Progeny from these moths (lines) were screened for growth rate and survival on regular artificial diet and on diets containing 5.0 $\mu\text{g/ml}$ of either Cry1Ac or Cry2Ab *Bacillus thuringiensis* Berliner toxin to isolate individuals carrying *B. t.* resistance genes from within

a field population of bollworms. Lines that performed within the upper and lower quartiles on each *B. t.* diet were reared to the adult stage and reciprocal crosses were performed to determine inheritance of resistance and the influence of maternal vigor effects on larval performance. Out of 2,244 genomes tested, none were found to possess major *B. t.* resistance genes. However, differences in larval growth rates among specific reciprocal crosses demonstrated that there is significant quantitative genetic variation for resistance to both the Cry1Ac and Cry2Ab toxins. Results from these crosses also suggested that inheritance of resistance to both Cry1Ac and Cry2Ab toxins was partially dominant. Stable carbon isotope analyses indicated that individuals whose maternal parent developed as a larva on a C₃ host or on a C₄ host performed similarly on diets containing Cry1Ac and Cry2Ab toxins.

**THE INFLUENCE OF TRANSGENIC COTTONS EXPRESSING ONE OR TWO
BACILLUS THURINGIENSIS PROTEINS AGAINST *HELICOVERPA ZEA*
(LEPIDOPTERA: NOCTUIDAE) AND FACTORS AFFECTING *B. T.*
RESISTANCE IN *H. ZEA***

by

RYAN EVERETT JACKSON

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirement for the Degree of
Doctor of Philosophy

DEPARTMENT OF ENTOMOLOGY

Raleigh

2002

APPROVED BY:

Co-chair of Advisory Committee

Co-chair of Advisory Committee

BIOGRAPHY

Ryan Everett Jackson was born on 12 May 1975 in Greenwood, MS. He is the older of two children of Everett Emmons and Nancy Murphy Jackson. Ryan lived in Greenwood until he was graduated from Greenwood High School in 1993. He attended Mississippi Delta Community College for two years and then proceeded to Mississippi State University, graduating in 1997 with a Bachelor of Science degree in Agricultural Pest Management.

Ryan was accepted to the graduate program in the Department of Entomology at Mississippi State University under the direction of Dr. Henry N. Pitre. During his stay at Mississippi State University, Ryan was married to Catherine Rogan Gillespie on 9 May 1998. Upon completion of his Master's research, "Influence of Roundup Ready Soybean and Roundup Ultra Herbicide on Pest and Beneficial Insects," he received his Master of Science degree in Entomology in 1999.

In June of 1999, Ryan and Rogan moved to North Carolina where Ryan accepted a graduate position in entomology at North Carolina State University in pursuit of a Ph.D. degree under the supervision of Dr. J. R. Bradley, Jr. and Dr. J. W. Van Duyn. His research focused on the effects of Bollgard and Bollgard II cottons against bollworm, *Helicoverpa zea*, and factors influencing *B. t.* resistance development in bollworm.

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to those with whom I worked closely for completion of my degree at North Carolina State University. First of all, I would like to offer thanks to my major advisors, Dr. J. R. Bradley, Jr and Dr. John W. Van Duyn, for giving me the opportunity to participate in their program. I will always have great appreciation for the learning experience provided by them. I would also like to thank Dr. Fred Gould and Dr. Clyde Sorenson for their knowledge, assistance and contributions toward my research. Enough thanks cannot be given to Wayne Modlin, Andrew Summerlin, Tony Burd, Margery Ambrose, Ryan Kurtz, Rogan Jackson, Phil Threatt, and Carlton Purvis for technical assistance in the field and laboratory.

Finally, I would like to thank my parents for their support throughout my graduate career. From emotional support to financial assistance, my parents have always been there for me, and I cannot thank them enough. Most of all, I would like to thank my wife for her unbelievable patience and support. She has been by my side for over four years and has provided words of encouragement all along. I would like to offer her a sincere thank you for everything that she has meant to me.

TABLE OF CONTENTS

	Page
LIST OF TABLES	vi
LIST OF FIGURES	xi
I. FIELD PERFORMANCE OF BOLLGARD AND BOLLGARD II COTTON GENOTYPES AGAINST BOLLWORM, <i>HELICOVERPA ZEA</i>, (LEPIDOPTERA: NOCTUIDAE)	1
Abstract.....	2
Introduction.....	4
Materials and Methods.....	5
Results.....	7
Discussion.....	11
Literature Cited.....	19
II. PERFORMANCE OF FERAL AND CRY1AC-SELECTED <i>HELICOVERPA ZEA</i> (LEPIDOPTERA: NOCTUIDAE) STRAINS ON TRANSGENIC COTTONS EXPRESSING ONE OR TWO <i>BACILLUS THURINGIENSIS</i> PROTEINS UNDER GREENHOUSE CONDITIONS	32
Abstract.....	33
Introduction.....	35
Materials and Methods.....	36
Results.....	38
Discussion.....	41
Literature Cited.....	47

III. ESTIMATED PRODUCTION OF *HELICOVERPA ZEA* FROM BOLLGARD AND BOLLGARD II COTTONS AND IMPLICATIONS FOR RESISTANCE

MANAGEMENT.....57

 Abstract.....58

 Introduction.....59

 Materials and Methods.....61

 Results.....63

 Discussion.....65

 Literature Cited.....71

IV. GENETIC VARIATION FOR RESISTANCE IN *HELICOVERPA ZEA* TO CRY1AC AND CRY2AB ENDOTOXINS.....79

 Abstract.....80

 Introduction.....81

 Materials and Methods.....82

 Results.....84

 Discussion.....87

 Literature Cited.....92

LIST OF TABLES

	Page
Chapter I	
Table 1	Mean (SE) percentage of terminals containing heliothine eggs, live larvae, and damage for three cotton genotypes averaged across locations and years (1999-2001) in North Carolina.....23
Table 2	Mean (SE) percentage of squares with live larvae or bollworm damage averaged across locations and years (1999-2001) in North Carolina.....24
Table 3	Mean (SE) percentage of bolls with live larvae (L4-L5) or bollworm damage averaged across locations and years (1999-2001) in North Carolina.....25
Table 4	Mean (SE) percentage of terminals containing heliothine eggs and damage for three cotton genotypes averaged across pyrethroid-treated and untreated subplots and two locations in North Carolina in 2001.....26
Table 5	Mean (SE) percentage of terminals containing live bollworm larvae for pyrethroid-treated and untreated subplots of three cotton genotypes averaged across two locations in North Carolina in 2001.....27
Table 6	Mean (SE) percentage of squares with a live bollworm larva for three cotton genotypes averaged across pyrethroid-treated and untreated subplots and two locations in North Carolina in 2001.....28

Table 7	Mean (SE) percentage of squares sustaining bollworm damage for pyrethroid-treated and untreated subplots of three cotton genotypes averaged across two locations in North Carolina in 2001.....	29
Table 8	Mean (SE) percentage of bolls containing a live bollworm larva (L4-L5) and damage for three cotton genotypes averaged across pyrethroid-treated and untreated subplots and two locations in North Carolina in 2001.....	30
Table 9	Mean yields expressed in kg seed cotton per hectare for pyrethroid-treated and untreated subplots for each year*location*genotype combination in North Carolina from 1999-2001.....	31

Chapter II

Table 1	Mean (SE) percentage of bollworm larval survival, superficially damaged fruit, and fruit penetration at seven days after infestation averaged across two runs for three cotton genotypes at ~80 days after planting in the greenhouse, Wake Co., NC. 1999.....	50
Table 2	Mean (SE) percentage of bollworm larval survival at seven days after infestation of a feral and a Cry1Ac-selected bollworm strain onto three cotton genotypes averaged across two runs at ~100 days after planting in the greenhouse, Wake, Co., NC, 1999.....	51

Table 3	Mean (SE) percentage of superficially damaged fruit at seven days after infestation of a feral and a Cry1Ac-selected bollworm strain onto three cotton genotypes averaged across two runs at ~100 days after planting in the greenhouse, Wake Co., NC, 1999.....	52
Table 4	Mean (SE) percentage of bollworm-penetrated fruit at seven days after infestation of a feral and a Cry1Ac-selected bollworm strain onto three cotton genotypes averaged across two runs at ~100 days after planting in the greenhouse, Wake Co., NC, 1999.....	53
Table 5	Mean (SE) percentage of bollworm larval survival of a feral and a Cry1Ac-selected bollworm strain within each of three cotton genotypes averaged across two evaluation dates at ~100 days in the greenhouse, Washington Co., NC, 2000.....	54
Table 6	Mean (SE) percentage of superficially damaged fruit of a feral and a Cry1Ac-selected bollworm strain within each of three cotton genotypes averaged across two evaluation dates at ~100 days in the greenhouse, Washington Co., NC, 2000.....	55
Table 7	Mean (SE) percentage of fruit penetration by a feral and a Cry1Ac-selected bollworm strain for three cotton genotypes averaged across two evaluation dates at ~100 days in the greenhouse, Washington Co., NC, 2000.....	56

Chapter III

Table 1	Estimated mean (SE) numbers of live bollworm larvae (L4-L5), damaged bolls, and successfully-emerged adults produced on a per hectare basis by untreated conventional, Bollgard, and Bollgard II cotton genotypes averaged across three locations in North Carolina from 2000-2001.....	74
Table 2	Estimated mean (SE) numbers of live bollworm larvae (L4-L5) per hectare produced by conventional, Bollgard, and Bollgard II cotton genotypes under pyrethroid-treated and untreated conditions averaged across three sample dates in Edgecombe Co., North Carolina in 2001.....	75
Table 3	Estimated mean (SE) numbers of bollworm-damaged bolls per hectare in conventional, Bollgard, and Bollgard II cotton genotypes under pyrethroid-treated and untreated conditions averaged across three sample dates in Edgecombe Co., North Carolina in 2001.....	76
Table 4	Estimated mean (SE) numbers of bollworm pupae per hectare produced by conventional, Bollgard, Bollgard, and Bollgard II cotton genotypes under pyrethroid-treated and untreated conditions averaged across three sample dates in Edgecombe Co., North Carolina in 2001.....	77

Table 5	Estimated mean (SE) numbers of bollworm adults per hectare produced by conventional, Bollgard, and Bollgard II cotton genotypes under pyrethroid-treated and untreated conditions averaged across three sample dates in Edgecombe Co., North Carolina in 2001.....	78
---------	--	----

Chapter IV

Table 1	Ordinal rating scale for <i>Helicoverpa zea</i> larval instar size after seven days on artificial diet.....	95
Table 2	Correlation coefficients and <i>P</i> -values for various comparisons among bollworm families selected for larval growth and survival on Cry1Ac, Cry2Ab, and NBT artificial diets.....	97

LIST OF FIGURES

	Page
Chapter IV	
Figure 1	Distribution of average ratings for 7-day old larvae of <i>Helicoverpa zea</i> female lines on Cry1Ac, Cry2Ab, and NBT diets. Mean ratings are approximately 4.4, 4.9, and 9.4, which correspond to a late 2 nd instar, early 3 rd instar, and mid 4 th instar, respectively.....96
Figure 2	Correlation graphs for all comparisons among Cry1Ac, Cry2Ab, and NBT families with average corrected ratings plotted for each family.....98
Figure 3	Correlation graph comparing percent survival for bollworm families tested on Cry1Ac and Cry2Ab artificial diets.....99
Figure 4	Correlation graphs for percent survival versus average corrected ratings for families tested on Cry1Ac and Cry2Ab artificial diets.....100
Figure 5	Mean larval weight in milligrams for <i>Helicoverpa zea</i> resistant lines (RxR), control lines (SxS), and reciprocal crosses between these lines (RxS and SxR) taken after ten days on 5.0 µg/ml of Cry1Ac-containing and Cry2Ab-containing diets, respectively.....101
Figure 6	Histogram showing frequency of larval weights (log _e) for upper quartile lines, lower quartile lines, and reciprocal F ₁ crosses between these lines taken after ten days on 5.0 µg/ml of Cry1Ac diet. Percent survival for each group is shown on each graph.....102

Figure 7	Histogram showing frequency of larval weights (\log_e) for upper quartile lines, lower quartile lines, and reciprocal F_1 crosses between these lines taken after ten days on 5.0 $\mu\text{g/ml}$ of Cry2Ab diet. Percent survival for each group is shown on each graph.....	103
Figure 8	Correlation graphs for average ratings for families on Cry1Ac, Cry2Ab, and NBT artificial diets versus ^{13}C levels.....	104

CHAPTER I

FIELD PERFORMANCE OF BOLLGARD AND BOLLGARD II COTTON GENOTYPES AGAINST BOLLWORM, *HELICOVERPA ZEA*, (LEPIDOPTERA: NOCTUIDAE)

Abstract

Two transgenic *Bacillus thuringiensis* var. *kurstaki* (Berliner) cottons, Bollgard® (Monsanto Agric. Co., St. Louis, MO) and Bollgard II, along with the conventional sister line, were evaluated for their comparative susceptibility against bollworm, *Helicoverpa zea* (Boddie), in North Carolina field studies from 1999-2001. Field studies were designed to determine if the Bollgard II genotype exhibited an increased efficacy against bollworm compared to its commercial Bollgard sister line. The impact of supplemental pyrethroid oversprays for bollworm control on yield for each genotype was also evaluated. Comparisons of the three untreated genotypes averaged across three years demonstrated that both *B. t.* genotypes successfully reduced the percentage of terminals, squares, and bolls infested with live larvae or subsequent damage as compared to the conventional variety. The Bollgard II line had fewer bolls infested with live larvae and less terminal, square, and boll damage compared to the commercial Bollgard variety. Comparisons of pyrethroid-treated and untreated genotypes revealed that the addition of a pyrethroid to conventional cotton reduced the percentage of terminals with a live larva. However, the pyrethroid-treated *B. t.* genotypes significantly reduced larval survival in the terminal region below that of the pyrethroid-treated conventional variety. Averaged across insecticide regimes, the Bollgard genotype had less terminal feeding damage than the conventional variety, but more terminal damage than the Bollgard II line. Both *B. t.* cottons had reduced larval survival on squares and bolls and sustained less boll damage than the conventional variety; however, the Bollgard II line had significantly lower percentages of bolls infested with live larvae and less boll damage than Bollgard. Square damage was lessened by the addition of a pyrethroid to the conventional variety but square damage in the treated conventional variety remained

higher than in the untreated Bollgard variety. In addition, the pyrethroid-treated Bollgard genotype exhibited a reduction in square damage below that of the untreated Bollgard, but was similar to that of pyrethroid-treated and untreated Bollgard II genotype. Pyrethroid-treated subplots of the conventional variety had significantly higher seed cotton yields than the untreated subplots in four of seven trials where bollworm numbers were high enough to cause significant yield reductions. Pyrethroid-treated and untreated subplots of Bollgard and Bollgard II genotypes produced similar yields in each year*location combination.

The availability of transgenic Bollgard[®] cottons that contain a gene from the soil bacterium *Bacillus thuringiensis* var. *kurstaki* that encodes for the Cry1Ac δ -endotoxin has provided a novel alternative for management of certain lepidopteran pests. Two major lepidopteran pests of cotton in North Carolina that are affected by this technology are the bollworm, *Helicoverpa zea* (Boddie), and the tobacco budworm, *Heliothis virescens* (Fab.). Although control of tobacco budworm with transgenic cottons has been absolute, bollworm susceptibility to the Cry1Ac protein has been much lower and considerably more variable. For example, Cry1Ac LC₅₀ values for bollworm populations were 4 to 60 times higher than those of tobacco budworm populations (Stone and Sims 1993). Field trials conducted in North Carolina confirmed that supplemental insecticide oversprays were frequently required to achieve satisfactory bollworm control and avoid yield reductions in Bollgard cottons (Lambert et al. 1996, 1997; Mahaffey et al. 1994, 1995). Survival of a portion of the bollworm population on Bollgard cottons may also be partially explained by the significant drop in the average levels of Cry1Ac protein in cotton fruit at approximately 80 days after planting (Greenplate 1999; Greenplate et al. 2001), which is coincident with the major bollworm flight into North Carolina cotton. Bollworm survival on Bollgard cottons is not only an economic problem, but also causes concern for resistance evolution; thus, further advances in *B. t.* cotton technology to decrease bollworm survival are needed to address these problems.

Bollgard II cottons produce two *B. t.* endotoxins, Cry1Ac and Cry2Ab, whereas, commercially available Bollgard varieties produce only the Cry1Ac endotoxin. These dual-gene cottons produce approximately the same level of the Cry1Ac protein as the single-gene Bollgard varieties, but are further protected by the Cry2Ab protein (Greenplate et al. 2000b;

Adamczyk et al. 2001). With the probable increased field control of bollworm due to the additional Cry toxin as compared to Bollgard varieties, Bollgard II cottons may be the new standards with respect to control of heliothines in cotton. Furthermore, they are expected to delay *B. t.* resistance evolution in bollworm.

Reported herein are the results of field studies designed to evaluate Bollgard and Bollgard II cottons for performance against bollworm and for agronomic productivity as measured by yield under North Carolina conditions.

Materials and Methods

Field studies were conducted at the Upper Coastal Plain Research Station, Edgecombe Co., NC, in 1999-2001; the Tidewater Research Station, Washington Co., NC, in 1999-2000; the Central Crops Research Station, Johnston Co., NC, in 2000; and at C. A. Martin Farm, Martin Co., NC, in 1999. Each test site represented a randomized complete split-plot design with four replicates. Whole plots consisted of cotton genotypes and subplots were unsprayed or sprayed with a pyrethroid insecticide. Whole plots were 12 rows by 45ft in 1999, but were 16, 20, and 24 rows by 50ft for DP50 (conventional), DP50B (Bollgard), and DP50BX (Bollgard II) genotypes, respectively, in 2000-2001. Area designated to whole plots for each genotype varied in order to increase the probability of collecting large bollworm larvae from Bollgard and Bollgard II lines. Subplots consisted of various numbers of unsprayed rows and 4 rows that were treated with a pyrethroid as needed for supplemental bollworm control.

All cotton genotypes were planted on 20 May in Martin Co., 21 May in Washington Co., and 24 May in Edgecombe Co. in 1999; on 15 May in Edgecombe Co., 17 May in Johnston Co., and 18 May in Washington Co. in 2000; and 2 May in Edgecombe Co. in

2001. Aldicarb (Temik[®] 15 G, Aventis CropScience, Research Triangle Park, NC) was applied in-furrow at planting at 0.84 kg a. i./hectare for control of early season insect pests in each test. Acephate (Orthene[®] 97 PE, Valent USA Corp., Walnut Creek, CA) was applied at 0.84 kg a. i./hectare as a mid-season overspray for control of tarnished plant bugs and stink bugs and to eliminate arthropod natural enemies. Supplemental bollworm control within appropriate subplots was accomplished by applications of cypermethrin (Ammo[®] 2.5 EC, FMC Corp., Philadelphia, PA), lambda cyhalothrin (Karate[®] Z 2.08 CS, Syngenta Crop Protection, Inc., Greensboro, NC), or cyfluthrin (Baythroid[®] 2 2.0 EC, Bayer Corp., Kansas City, MO). Pyrethroid applications consisting of cypermethrin at 0.112 kg a. i./hectare and lambda cyhalothrin at 0.045 kg a. i./hectare were sprayed at Martin Co. (4 and 16 August, respectively) and at Washington Co. (6 and 19 August, respectively), whereas cyfluthrin at 0.056 kg a. i./hectare was sprayed at Edgecombe Co. (5 August) in 1999. Lambda cyhalothrin at 0.045 kg a. i./hectare was applied to appropriate subplots for supplemental bollworm control at Johnston and Edgecombe counties (19 July and 7 August) and at Washington Co. (27 July and 9 August) in 2000, as well as at Edgecombe Co. (10 and 16 August) in 2001. Weed control, fertilization, plant growth regulation, and defoliation followed the recommendations of the North Carolina Cooperative Extension Service.

Assessments of bollworm eggs, live larvae, and damage were made in the terminal region of cotton plants, whereas live larvae and damage were evaluated on squares and bolls. Fifty terminals or squares were examined per plot on the respective sample dates. Bolls were sampled at either 50 or 100 per plot on a given sample date. Egg, larval, and damage ratings were made only in the untreated subplots in 1999 and 2000, but 2001 ratings were made in both pyrethroid-treated and untreated subplots. Yields were determined by picking the entire

lengths of the two middle rows of each subplot using a mechanical cotton picker. Yields were converted to kg seed cotton/ha prior to analysis.

All egg, live bollworm larvae, and damaged fruit numbers were converted to percentages and subjected to arcsine square root transformation prior to analysis. These data, along with yield data, were then subjected to ANOVA using PROC GLM (SAS Institute 1990), and means for each treatment were separated ($P < 0.05$) using Fisher's Protected Least Significant Difference test in SAS.

Results

Heliothine egg deposition onto cotton terminals was consistent as no differences were found among the three untreated cotton genotypes averaged across three seasons ($F = 0.05$; $df = 2, 10$; $P = 0.9498$) (Table 1). With respect to the percentage of terminals containing a live bollworm larva, both *B. t.* genotypes outperformed the conventional variety by significantly reducing larval numbers in the terminal region ($F = 37.30$; $df = 2, 10$; $P < 0.0001$) (Table 1). Bollgard and Bollgard II genotypes reduced the percentage of terminals containing a live larva by 3X and 7X, respectively, as compared to the conventional variety, with no differences evident between *B. t.* genotypes. The percentage of terminals sustaining bollworm damage was significantly reduced by both Bollgard genotypes as compared to the conventional variety ($F = 39.20$; $df = 2, 10$; $P < 0.0001$) (Table 1). Furthermore, the Bollgard II line significantly lowered the percentage of damaged terminals below that of the Bollgard genotype. When compared to the conventional variety, a 2.5X and 4.3X reduction in the percentage of terminals sustaining damage was exhibited by Bollgard and Bollgard II genotypes, respectively.

The percentage of squares containing a live bollworm larva was significantly reduced by both Bollgard genotypes as compared with the conventional variety, with no significant differences among *B. t.* lines ($F=121.79$; $df=2, 10$; $P<0.0001$) (Table 2). Both Bollgard genotypes significantly lowered the percentage of squares sustaining bollworm damage as compared to the conventional variety ($F=180.63$; $df=2, 10$; $P<0.0001$) (Table 2); however, as with terminal ratings, the percentage of squares suffering damage by bollworm was significantly reduced by the Bollgard II genotype as compared to the Bollgard variety. The reduction in the percentage of squares containing a live larva was ~10X for both Bollgard lines, while the percentage of squares sustaining damage was reduced 5X and 10X for Bollgard and Bollgard II genotypes, respectively, as compared to the conventional variety.

Both *B. t.* genotypes significantly reduced the percentage of bolls with a late-instar (L4-L5) larva ($F=65.80$; $df=2, 10$; $P<0.0001$) and bollworm damage ($F=113.60$; $df=2, 10$; $P<0.0001$) as compared to the conventional variety (Table 3). Bollgard II further reduced the percentage of bolls with a live larva and damage below that of the Bollgard variety. The percentage of bolls containing a live larva was reduced 6X and 29X by Bollgard and Bollgard II genotypes, respectively, as compared to the conventional variety. The Bollgard and Bollgard II genotypes reduced the percentage of bolls with bollworm damage below that of the conventional variety by 6X and 37X, respectively.

In 2001, both pyrethroid-treated and untreated subplots were evaluated for egg, live larvae, and damage numbers. Heliothine egg deposition in the terminal region was uniform across all treatment combinations as no significant differences in the percentage of terminals containing eggs were found ($F=3.54$; $df=2, 2$; $P=0.2202$) (Table 4). However, Table 5 shows a significant genotype*insecticide interaction for the percentage of terminals containing a

live larva ($F=31.35$; $df=2, 2$; $P=0.0309$). Seven and one-half percent of the terminals in the untreated conventional variety were infested with live bollworm larvae. The percentage of cotton terminals with live larvae was reduced to 1.5% by applying a pyrethroid insecticide to the conventional cotton variety. The pyrethroid-treated conventional variety was similar to the untreated Bollgard and Bollgard II genotypes in the percentage of terminals infested with larvae, but significantly differed from the pyrethroid-treated Bollgard and Bollgard II lines. No significant differences were evident among the pyrethroid-treated and untreated Bollgard genotype with regard to the percentage of terminals with live larvae. Differences in terminal damage among treatment combinations were significantly influenced by the various cotton genotypes averaged across insecticide treatments and locations ($F=84.17$; $df=2, 2$; $P=0.0117$) (Table 4). The conventional variety sustained 24.5% bollworm damage to cotton terminals; however, terminal damage caused by bollworm larvae was significantly reduced to 8.9% and 5.3% by Bollgard and Bollgard II genotypes, respectively. The commercial Bollgard variety lowered terminal damage by 64% compared to the conventional variety; the Bollgard II line reduced terminal damage by 79% versus the conventional variety and 40% versus the Bollgard variety.

The percentage of squares containing a live bollworm larva was significantly reduced by both *B. t.* genotypes when compared to the conventional variety ($F=19.09$; $df=2, 2$; $P=0.0498$) (Table 6). No statistical differences were detected between *B. t.* lines with respect to the percentage of squares infested with a live larva. However, Table 7 illustrates a significant genotype*insecticide interaction for the percentage of squares bearing bollworm damage ($F=60.34$; $df=2, 2$; $P=0.0163$). The untreated conventional variety sustained 45.3% damage to squares and this damage was reduced to 14.0% by the addition of a pyrethroid

overspray. The untreated Bollgard variety sustained approximately half as much damage as the pyrethroid-treated conventional variety. As with the pyrethroid-treated and untreated conventional variety, a pyrethroid overspray to the Bollgard genotype significantly lowered the percentage of squares with bollworm damage when compared to the untreated Bollgard variety. However, no differences were evident between the pyrethroid-treated and untreated Bollgard II lines with respect to the percentage of squares sustaining bollworm damage. Also, no statistical differences were found among the untreated Bollgard II genotype and the pyrethroid-treated Bollgard and Bollgard II lines with regard to the percentage of squares with bollworm feeding damage.

The percentage of bolls with a late-instar bollworm larva (L4-L5) was primarily affected by the various cotton genotypes tested ($F=870.40$; $df=2, 2$; $P=0.0011$) (Table 8). Approximately 5.5% of bolls from the conventional variety contained a late-instar bollworm larva when averaged across insecticide regimes. Both Bollgard and Bollgard II genotypes reduced the percentage of bolls with a live larva to less than 0.4%, with the Bollgard II line exhibiting a further reduction in larval survival (0.05%) when averaged across pyrethroid-treated and untreated subplots. Cotton genotype was also the primary factor influencing the percentage of bolls with bollworm damage ($F=56.66$; $df=2, 2$; $P=0.0173$) (Table 8). The conventional variety sustained bollworm damage to 23.2% of its bolls when averaged across insecticide treatments. However, the commercial Bollgard variety significantly lowered the percentage of bolls with bollworm damage to 2.5%, an 89% reduction in boll damage when averaged across pyrethroid-treated and untreated subplots and compared to the untreated conventional variety. The percentage of bolls with bollworm damage was further reduced to

0.3% by Bollgard II lines, a 99% and 88% reduction when averaged across insecticide regimes and compared to the conventional and Bollgard varieties, respectively.

A significant year*location*genotype*insecticide ($F=8.30$; $df=12, 42$; $P<0.0001$) interaction characterized 1999-2001 yield data; therefore, yields expressed in kg seedcotton/ha for pyrethroid-treated and untreated subplots are shown for each year*location*genotype combination in Table 9. Yields in pyrethroid-treated subplots of the conventional variety were significantly higher than the untreated subplots in 1999 at C. A. Martin Farms ($F=25.78$; $df=1, 42$; $P<0.0001$) and the Upper Coastal Plain Research Station ($F=5.39$; $df=1, 42$; $P=0.0237$). In 2000, treated subplots produced higher yields than the untreated subplots of the conventional variety at the Tidewater ($F=18.61$; $df=1, 42$; $P<0.0001$) and Upper Coastal Plain Research Stations ($F=161.46$; $df=1, 42$; $P<0.0001$). Pyrethroid-treated and untreated subplots of Bollgard and Bollgard II genotypes produced similar yields in each year*location combination.

Discussion

Low to moderate bollworm numbers characterized the 1999-2001 field seasons in North Carolina; thus, numbers of eggs, live larvae, and damaged fruit were representative of infestation levels typically encountered in the region. Distribution of heliothine eggs in the terminal region of cotton plants was uniform across test sites averaged across three years, since oviposition has not been affected by *B. t.* cottons (Lambert et al. 1996, 1997). The percentages of terminals containing a live larva and subsequent damage were significantly reduced by Bollgard and Bollgard II genotypes below that of the conventional variety. Although no differences were detected between *B. t.* genotypes with respect to larval survival, the Bollgard II line significantly lowered terminal damage compared to the Bollgard

variety. Perhaps larval survival was similar between Bollgard genotypes because the Cry endotoxins for each are expressed at a high level in the youngest tissues of the terminals (Greenplate et al. 2000a). Therefore, bollworm larvae feeding on either *B. t.* genotype would likely ingest a high level of Cry endotoxin(s) leading either to larval death or movement from the terminal region (Gore et al. 2001). On the contrary, a significant decrease in terminal damage was demonstrated by the Bollgard II genotype as compared to the Bollgard variety. These results apparently occurred because less Bollgard II terminal tissue must be consumed compared to the Bollgard variety for larvae to acquire either a repellent or toxic dose of the Cry proteins.

As with larval measurements in cotton terminals, the percentages of squares with a live larva and subsequent damage were reduced by both *B. t.* genotypes below that of the conventional variety averaged across three years. The Bollgard II genotype sustained significantly less feeding damage on squares as compared to the Bollgard variety, but no differences in larval survival on squares were evident between *B. t.* cottons because survival was so low on both. As in the terminal region, squares of the *B. t.* genotypes express a relatively high level of the Cry endotoxin(s) that should cause larval death or increased larval movement about the plant after larval feeding. With the additional production of the Cry2Ab endotoxin, the Bollgard II genotype expresses a significantly higher level of endotoxin per unit area of square tissue compared to the Bollgard variety. Additionally, total lepidopteran activity does not differ between Bollgard II terminals and squares as shown in Bollgard varieties (Penn et al. 2001). Therefore, less square tissue would need to be ingested to achieve a toxic dose of Cry proteins, and this factor may have resulted in the reduced square damage with the Bollgard II genotype.

Both *B. t.* genotypes reduced the percentage of bolls with a live bollworm larva or feeding damage below that of the conventional variety over the three-year period. As with terminal and square damage, both larval survival and damage on bolls were lower in the Bollgard II line than in the Bollgard variety. Reduced larval survival and damage on bolls in Bollgard II cottons may be explained by the overall increase (3.5X) in lepidopteran activity for the dual gene technology as compared to the single toxin varieties as demonstrated by Penn et al. (2001). Farrar and Bradley (1985) demonstrated that the majority of bollworm eggs are deposited within the upper one-third of the plant canopy and larval movement down the plant proceeds with larval age. Gore et al. (2001) reported that larval movement in *B. t.* cottons was increased above that of conventional varieties; as young larvae move down the plant, they feed briefly on leaves and squares. With the increased lepidopteran activity demonstrated by Bollgard II lines, a significantly higher proportion of larvae apparently die before reaching the size necessary to feed on bolls. Thus, it was expected that the percentage of bolls infested and boll damage would be less in Bollgard II than in the Bollgard variety.

In 2001, both pyrethroid-treated and untreated subplots within each genotype were sampled to quantify egg and larval populations, as well as terminal and fruit damage. Heliothine egg deposition in the terminal region was consistent across all treatment combinations of genotype and insecticide regime. However, a genotype*insecticide interaction occurred for the percentage of terminals with a live larva. The untreated conventional variety exhibited the highest percentage of terminals with living larvae, and the application of a pyrethroid significantly reduced larval survival by 5X. The pyrethroid application to the conventional variety also lowered larval survival in terminals to the level observed in the untreated Bollgard and Bollgard II genotypes. However, the pyrethroid-

treated *B. t.* genotypes further reduced the percentage of terminals with a live larva below that of the pyrethroid-treated conventional variety. Since pyrethroid oversprays are very effective against bollworm (Bradley 1996), especially larvae located in the terminal region, it was expected that the pyrethroid-treated conventional variety would exhibit similar levels of larval survival to that of the untreated *B. t.* genotypes. However, the combination of the *B. t.* genotypes and the pyrethroid-oversprays eliminated surviving larvae in the cotton terminals. The percentage of terminals sustaining bollworm damage was characterized by a genotype effect and was significantly reduced by both Bollgard genotypes below that of the conventional cotton when averaged across insecticide regimes. Bollgard II cottons further diminished the level of terminal damage as compared to the Bollgard variety. The reduction in terminal damage demonstrated by the Bollgard II genotype can be explained by the increased toxin production as compared to the Bollgard variety.

Both *B. t.* lines significantly lowered larval bollworm survival as compared to the conventional variety averaged across insecticide regimes. The similarity in larval survival in the single and dual toxin genotypes may be explained by the relatively high expression of the Cry endotoxins in the squares for both genotypes. Although expression of Cry endotoxins is higher in squares of the Bollgard II genotype, toxin production by the Bollgard variety is adequate to cause larval movement or death. The percentage of squares sustaining bollworm feeding damage was typified by a genotype*insecticide regime interaction. The untreated conventional variety had the highest percentage of squares with feeding damage (45.3%), but the addition of a pyrethroid overspray to the conventional variety reduced the level of square damage to 14.0 %. However, the untreated Bollgard variety had only 7.3% square damage, and the pyrethroid-treated Bollgard variety, along with both treated and untreated Bollgard II

genotype, sustained even lesser amounts of square damage. Unlike larval survival in the terminal region, the untreated Bollgard variety significantly reduced the percentage of squares sustaining bollworm feeding damage below that of the pyrethroid-treated conventional variety. The difference may have been a result of reduced coverage of the pyrethroid overspray on squares compared to that in the exposed terminal region. A reduction in coverage could have resulted in some squares having little to no insecticide, whereas, the Bollgard varieties produced its internal insecticide in every square on the plant. Coverage with the pyrethroid-overspray, however, was sufficient enough to reduce the percentage of squares with damage in the pyrethroid-treated Bollgard variety compared to that of the untreated Bollgard. A portion of the susceptible bollworm larvae, as well as those that may carry *B. t.* resistance alleles, have demonstrated the ability to complete larval development on Bollgard cottons because Bollgard varieties do not express a “high dose” of Cry1Ac endotoxin with respect to the level necessary to prevent resistance development (Scientific Advisory Panel 1998). Therefore, survivors in the untreated Bollgard cottons could have caused feeding damage to squares, whereas, most larvae surviving the Cry1Ac toxin in the pyrethroid-treated Bollgard variety would have been eliminated by the pyrethroid overspray. Moreover, the addition of a second Cry endotoxin in the Bollgard II genotype appeared to have the same effect in the reduction of square damage as the pyrethroid overspray to the Bollgard genotype.

Both larval survival and subsequent boll damage were significantly reduced by both *B. t.* genotypes in 2001; however, the Bollgard II genotype had lower larval survival and damage than the commercial Bollgard variety. As previously mentioned, the reduction of larval survival and subsequent boll damage by Bollgard II cottons may be explained by the

overall increase (3.5X) in lepidopteran activity for the dual gene cottons as compared to the single toxin varieties as demonstrated by Penn et al. (2001).

Pyrethroid-treated subplots of the conventional variety out-yielded the untreated subplots with respect to kg seed cotton/ha in 1999 at C. A. Martin Farms and the Upper Coastal Plain Research Station. In 2000, treated subplots again out-produced the untreated subplots of the conventional variety at the Tidewater and Upper Coastal Plain Research Stations. Year*location combinations where the pyrethroid-treated subplots yielded higher than the untreated subplots were those subjected to moderate or moderately high bollworm numbers. Conversely, year*location combinations with no evident differences between pyrethroid-treated and untreated conventional subplots occurred under conditions of low or moderately low numbers of bollworm.

Pyrethroid-treated and untreated subplots of Bollgard and Bollgard II genotypes produced similar yields in each year*location combination due to the low numbers of survivors within each *B. t.* genotype*insecticide regime combination. Yield differences between pyrethroid-treated and untreated Bollgard II cottons at the UCPRS in 2000 were due to inefficiencies in harvest methods that were complicated by high seed cotton yields; furthermore, the 2001 yield differences in pyrethroid-treated versus untreated Bollgard II cottons may have been due to the same inefficiencies, but it was also likely that stink bugs and plant bugs were contributing to a portion of the yield loss in the untreated Bollgard II cottons. It should also be noted that bollworm numbers in these tests did not approach levels considered to be high by North Carolina standards. Mahaffey et al. (1994, 1995) reported much higher fruit losses to bollworm and yield reductions in conventional and Bollgard cotton in the mid-1990's. Since that time, several structural changes in the North Carolina

agroecosystem are thought to have influenced a reduction in the mean abundance of bollworm; among these are a decrease in corn acreage, an increase in cotton acreage, and the planting of Bollgard cotton to >50% of the total cotton acreage in the 2000 and 2001 seasons.

Results from these studies cast doubts on the added value of Bollgard II cottons to the farmers since Bollgard provides absolute control of tobacco budworm and adequate bollworm control, particularly when oversprayed with a pyrethroid. Bacheler (unpublished data) reported that Bollgard fields averaged 0.99 late-season applications with insecticides active against bollworm in 2001. The apparent value of Bollgard II is its potential to delay resistance evolution in heliothines. While we did not measure yield differences between Bollgard and Bollgard II in this three-year study, there were reductions in numbers of surviving bollworms in Bollgard II. Survival of bollworm larvae in the Bollgard II genotype was negligible, particularly when oversprayed with a pyrethroid.

Acknowledgments

The authors express appreciation to Cotton, Inc. for providing a graduate research assistantship for the senior author, to Monsanto Agric. Co. for providing partial project funding, and to Delta and Pine Land Co. for providing cotton seed. Sincere thanks Wayne Modlin, Andrew Summerlin, Phil Threatt, Tony Burd, and Rogan Jackson for technical assistance.

Literature Cited

Adamczyk, J. J., Jr., K. Bew, L. C. Adams, and D. D. Hardee. 2001. Evaluation of Bollgard[®] (cv. DP50BII) in the Mississippi delta: field efficacy against various Lepidoptera while profiling season-long expression of cry1Ac and cry2Ab. p. 835-836. In Proc. Beltwide Cotton Conf., Anaheim, CA. 9-13 Jan. 2001. Natl. Cotton Counc. Am., Memphis, TN.

Bradley, J. R., Jr. 1996. Major developments in management of insect and mite pests in cotton. pp. 1-13. *In*: E. G. King, J. R. Phillips and R. T. Coleman (eds.) The Cotton Foundation. Memphis, TN.

Farrar, R. R., Jr., and J. R. Bradley, Jr. 1985. Within-plant distribution of *Heliothis* spp. (Lepidoptera: Noctuidae) eggs and larvae on cotton in North Carolina. *Env. Entomol.* 14: 205-209.

Gore, J., B. R. Leonard, D. R. Cook, and R. H. Jones. 2001. Dispersal of bollworm larvae on Bollgard[®] and non-Bollgard cotton cultivars. p. 851-854. *In* Proc. Beltwide Cotton Conf., Anaheim, CA. 9-13 Jan. 2001. Natl. Cotton Counc. Am., Memphis, TN.

Greenplate, J. T. 1999. Quantification of *Bacillus thuringiensis* insect control protein cry1Ac over time in Bollgard cotton fruit and terminals. *J. Econ. Entomol.* 92: 1377-1383.

Greenplate, J. T., S. R. Penn, J. W. Mullins, and M. Oppenhuizen. 2000a. Seasonal cry1Ac levels in DP50B; the “Bollgard basis for Bollgard II.” 2000. p. 1039-1040. *In Proc. Beltwide Cotton Conf.*, San Antonio, TX. 4-8 Jan. 2000. Natl. Cotton Counc. Am., Memphis, TN.

Greenplate, J. T., S. R. Penn, Z. Shappley, M. Oppenhuizen, J. Mann, B. Reich, and J. Osborn. 2000b. Bollgard II efficacy: quantification of total lepidopteran activity in a 2-gene product. p. 1041-1043. *In Proc. Beltwide Cotton Conf.*, San Antonio, TX. 4-8 Jan. 2000. Natl. Cotton Counc. Am., Memphis, TN.

Greenplate, J. T., W. Mullins, S. R. Penn, and K. Embry. 2001. Cry1Ac levels in candidate commercial Bollgard[®] varieties as influenced by environment, variety, and plant age: 1999 gene equivalency field studies. p. 790-793. *In Proc. Beltwide Cotton Conf.*, Anaheim, CA. 9-13 Jan. 2001. Natl. Cotton Counc. Am., Memphis, TN.

Lambert, A. L., J. R. Bradley, Jr., and J. W. Van Duyn. 1996. Effects of natural enemy conservation and planting date on the susceptibility of *Bt* cotton to *Helicoverpa zea* in North Carolina. p. 931-935. *In Proc. Beltwide Cotton Conf.*, Nashville, TN. 9-12 Jan. 1996. Natl. Cotton Counc. Am., Memphis, TN.

Lambert, A. L., J. R. Bradley, Jr., and J. W. Van Duyn. 1997. Interactions of *Helicoverpa zea* and *Bt* cotton in North Carolina. p. 870-873. *In Proc. Beltwide Cotton Conf.*, New Orleans, LA. 7-10 Jan. 1997. Natl. Cotton Counc. Am., Memphis, TN.

Mahaffey, J. S., J. S. Bacheler, J. R. Bradley, Jr., and J. W. Van Duyn. 1994. Performance of Monsanto's transgenic *B.t.* cotton against high populations of lepidopterous pests in North Carolina. p. 1061-1063. *In* Proc. Beltwide Cotton Conf., San Diego, CA. 5-8 Jan. 1994. Natl. Cotton Counc. Am., Memphis, TN.

Mahaffey, J. S., J. R. Bradley, Jr., and J. W. Van Duyn. 1995. *B. t.* cotton: field performance in North Carolina under conditions of unusually high bollworm populations. p. 795-798. *In* Proc. Beltwide Cotton Conf., San Antonio, TX. 4-7 Jan. 1995. Natl. Cotton Counc. Am., Memphis, TN.

Penn, S. R., B. Reich, J. Osborn, K Embry, and J. T. Greenplate. 2001. Quantification of lepidopteran activity in a 2-gene product: a 2-year summary of Bollgard II[®]. p. 830-832. *In* Proc. Beltwide Cotton Conf., Anaheim, CA. 9-13 Jan. 2001. Natl. Cotton Counc. Am., Memphis, TN.

SAS Institute. 1990. SAS/STAT User's Guide, Vol. 2. SAS Institute, Cary, NC, 795 pp.

Scientific Advisory Panel, Subpanel on *Bacillus thuringiensis* (Bt) Plant-Pesticides, February 9-10, 1998. 1998. Transmittal of the final report of the FIFRA Scientific Advisory Panel, Subpanel on *Bacillus thuringiensis* (Bt) Plant-Pesticides and Resistance Management, Meeting held on February 9-10, 1998. (Docket Number: OPPTS-00231).

Stone, T. B., and S. R. Sims. 1993. Geographic susceptibility of *Heliothis virescens* and *Helicoverpa zea* (Lepidoptera: Noctuidae) to *Bacillus thuringiensis*. J. Econ. Entomol. 86: 989-994.

Table 1. Mean (SE) percentage of terminals containing heliothine eggs, live larvae, and damage for three cotton genotypes averaged across locations and years (1999-2001) in North Carolina.

Genotype	Percent Egg ^a	Percent Live Larvae ^a	Percent Damage ^a
Conventional (DP50)	4.75 (0.638) a	5.54 (0.689) a	23.50 (1.793) a
Bollgard (DP50B)	5.00 (0.605) a	1.79 (0.416) b	9.57 (0.975) b
Bollgard II (DP50BX)	5.32 (0.814) a	0.82 (0.264) b	5.50 (0.669) c

^a/ Means within the same column followed by the same letter are not significantly different according to Fisher's LSD procedure ($P \leq 0.05$).

Table 2. Mean (SE) percentage of squares with live larvae or bollworm damage averaged across locations and years (1999-2001) in North Carolina.

Genotype	Percent Live Larvae ^a	Percent Damage ^a
Conventional (DP50)	5.89 (0.517) a	21.58 (2.318) a
Bollgard (DP50B)	0.59 (0.167) b	4.05 (0.576) b
Bollgard II (DP50BX)	0.56 (0.262) b	2.28 (0.760) c

^a/ Means within the same column followed by the same letter are not significantly different according to Fisher's LSD procedure ($P \leq 0.05$).

Table 3. Mean (SE) percentage of bolls with live larvae (L4-L5) or bollworm damage averaged across locations and years (1999-2001) in North Carolina.

Genotype	Percent Live Larvae ^a	Percent Damage ^a
Conventional (DP50)	7.04 (0.695) a	31.73 (1.603) a
Bollgard (DP50B)	1.13 (0.186) b	5.01 (0.472) b
Bollgard II (DP50BX)	0.24 (0.094) c	0.87 (0.271) c

^a/ Means within the same column followed by the same letter are not significantly different according to Fisher's LSD procedure ($P \leq 0.05$).

Table 4. Mean (SE) percentage of terminals containing heliothine eggs and damage for three cotton genotypes averaged across pyrethroid-treated and untreated subplots and two locations in North Carolina in 2001.

Genotype	Percent Egg ^a	Percent Damage ^a
Conventional (DP50)	8.25 (0.772) a	24.50 (3.186) a
Bollgard (DP50B)	7.63 (0.712) a	8.88 (1.303) b
Bollgard II (DP50BX)	8.38 (0.917) a	5.25 (0.911) c

^a/ Means within the same column followed by the same letter are not significantly different according to Fisher's LSD procedure ($P \leq 0.05$).

Table 5. Mean (SE) percentage of terminals containing live bollworm larvae for pyrethroid-treated and untreated subplots of three cotton genotypes averaged across two locations in North Carolina in 2001.

Genotype	Insecticide Regime	Percent Live Larvae ^a
Conventional (DP50)	Untreated	7.50 (1.918) a
Conventional (DP50)	Pyrethroid-treated	1.50 (0.627) b
Bollgard (DP50B)	Untreated	0.50 (0.327) bc
Bollgard II (DP50BX)	Untreated	0.25 (0.250) bc
Bollgard (DP50B)	Pyrethroid-treated	0.00 (0.000) c
Bollgard II (DP50BX)	Pyrethroid-treated	0.00 (0.000) c

^a/ Means within the same column followed by the same letter are not significantly different according to LSMeans procedure ($P \leq 0.05$).

Table 6. Mean (SE) percentage of squares with a live bollworm larva for three cotton genotypes averaged across pyrethroid-treated and untreated subplots and two locations in North Carolina in 2001.

Genotype	Percent Live Larvae ^a
Conventional (DP50)	4.50 (1.103) a
Bollgard (DP50B)	0.25 (0.171) b
Bollgard II (DP50BX)	0.00 (0.000) b

^a/ Means within the same column followed by the same letter are not significantly different according to Fisher's LSD procedure ($P \leq 0.05$).

Table 7. Mean (SE) percentage of squares sustaining bollworm damage for pyrethroid-treated and untreated subplots of three cotton genotypes averaged across two locations in North Carolina in 2001.

Genotype	Insecticide Regime	Percent Damage ^a
Conventional (DP50)	Untreated	45.25 (3.272) a
Conventional (DP50)	Pyrethroid-treated	14.00 (1.363) b
Bollgard (DP50B)	Untreated	7.25 (2.505) c
Bollgard (DP50B)	Pyrethroid-treated	1.25 (0.648) d
Bollgard II (DP50BX)	Untreated	0.50 (0.327) d
Bollgard II (DP50BX)	Pyrethroid-treated	0.25 (0.250) d

^a/ Means within the same column followed by the same letter are not significantly different according to LSMeans procedure ($P \leq 0.05$).

Table 8. Mean (SE) percentage of bolls containing a live (L4-L5) bollworm larva and damage for three cotton genotypes averaged across pyrethroid-treated and untreated subplots and two locations in North Carolina in 2001.

Genotype	Percent Live Larvae ^a	Percent Damage ^a
Conventional (DP50)	5.54 (0.762) a	23.16 (2.105) a
Bollgard (DP50B)	0.39 (0.113) b	2.48 (0.445) b
Bollgard II (DP50BX)	0.05 (0.040) c	0.30 (0.092) c

^a/ Means within the same column followed by the same letter are not significantly different according to Fisher's LSD procedure ($P \leq 0.05$).

Table 9. Mean yields expressed in kg seed cotton per hectare for pyrethroid-treated and untreated subplots for each year*location*genotype combination in North Carolina from 1999-2001.

Genotype	Insecticide	UCPRS ^a			TRS ^b		CAM ^c	CCRS ^d
		1999	2000	2001	1999	2000	1999	2000
Conventional	Treated	1917*	3869*	3145	1207	2334*	2587*	4055
Conventional	Untreated	1505*	1614*	2929	872	1569*	1686*	4172
Bollgard	Treated	2100	4269	3628	1311	2773	2746	4569
Bollgard	Untreated	2355	4374	3682	1214	2752	2520	4604
Bollgard II	Treated	2401	3621*	4655*	1402	3426	2611	4311
Bollgard II	Untreated	2563	4062*	4118*	1342	3191	2505	4545

* / Indicates a significant difference between treated and untreated subplots for a given genotype according to the LSMeans procedure ($P \leq 0.05$).

^a/ Upper Coastal Plain Research Station, Edgecombe Co., NC.

^b/ Tidewater Research Station, Washington Co., NC.

^c/ C. A. Martin Farms, Martin Co., NC.

^d/ Central Crops Research Station, Johnston Co., NC.

CHAPTER II

**PERFORMANCE OF FERAL AND CRY1AC-SELECTED *HELICOVERPA ZEA*
(LEPIDOPTERA: NOCTUIDAE) STRAINS ON TRANSGENIC COTTONS
EXPRESSING ONE OR TWO *BACILLUS THURINGIENSIS*
SSP. *KURSTAKI* PROTEINS UNDER
GREENHOUSE CONDITIONS**

Abstract

Bollgard[®] (Monsanto Co., St. Louis, MO) and Bollgard II cottons that express either one or two *Bacillus thuringiensis* (Berliner) proteins, respectively, were evaluated along with either the conventional sister line or a *B. t.* line expressing only the Cry2Ab endotoxin in 1999-2000 North Carolina greenhouse experiments for efficacy against either a feral strain of bollworm, *Helicoverpa zea* (Boddie), or both a feral and a Cry1Ac-selected bollworm strain. Efficacy of Bollgard II and Bollgard cottons was determined for both strains in 1999. In 2000, a greenhouse study was designed to compare the efficacy of three transgenic cottons expressing either the Cry1Ac endotoxin alone, the Cry2Ab endotoxin alone, or both the Cry1Ac and Cry2Ab endotoxins against a feral and a Cry1Ac-selected bollworm strain. Results from testing only the feral strain suggested that the Bollgard II genotype was more effective than the Bollgard variety in reducing larval survival, superficial fruit damage, and fruit penetration. Results from the second 1999 greenhouse study evaluating both a wild and a Cry1Ac-selected bollworm strain demonstrated that when averaged across bollworm strains, the Bollgard II genotype significantly reduced larval survival and fruit penetration by bollworm compared to the Bollgard variety. Also, the Cry1Ac-selected bollworm strain displayed increased larval survival, superficial fruit damage, and fruit penetration compared to the feral strain when averaged across genotypes. Results from the 2000 study confirmed 1999 results as the Bollgard II genotype significantly reduced fruit penetration by bollworm below that of the Bollgard variety when averaged across strains; however, the single Cry2Ab-producing genotype performed similarly to both Bollgard and Bollgard II with respect to fruit penetration. The Cry1Ac-selected bollworm strain exhibited significantly greater larval survival and superficial fruit damage on the Bollgard variety compared to the

feral strain, but no differences among larval strains were evident for other genotypes. Also, when averaged across genotypes, the Cry1Ac-selected bollworm strain penetrated a higher proportion of cotton fruit in comparison to the wild strain.

Transgenic cottons that produce the Cry1Ac endotoxin derived from *Bacillus thuringiensis* ssp. *kurstaki* Berliner have been widely planted since the 1996 commercialization of Bollgard[®] (Monsanto Agric. Co., St. Louis, MO) cotton varieties. These cottons have provided producers with a unique, innovative, and environmentally sound method of insect pest management where heliothines such as tobacco budworm, *Heliothis virescens* (Fab.), and bollworm, *Helicoverpa zea* (Boddie), are prevalent pests. However, recent laboratory and field studies have demonstrated that these Cry1Ac-based transgenic cottons do not provide absolute control of the bollworm and that supplemental insecticide oversprays are often required on these cottons to prevent yield losses (Burd et al. 1999; Lambert et al. 1996, 1997; Mahaffey et al. 1994, 1995). With the knowledge that Bollgard cottons are not “high dose” with respect to bollworm, many scientists have developed concern for resistance evolution to this technology in bollworm (Tabashnik 1994; Gould 1998).

The invasive period of *H. zea* into North Carolina cottons coincides with the observed drop in average levels of Cry1Ac in cotton fruit (Greenplate 1999; Greenplate et al. 1998). Because of this, a significant proportion of the bollworm population is exposed to sublethal doses of the *B. t.* endotoxin produced by transgenic cottons. Reports from the Scientific Advisory Panel (1998) agree with suggestions that Bollgard cottons produced only a moderate dose of the Cry1Ac endotoxin with regard to the level considered necessary to prevent resistance development in bollworm. With the need for supplemental bollworm control in *B. t.* cottons coupled with the concern for resistance evolution in bollworm to the Cry1Ac endotoxin, new technologies with increased activity against the bollworm are desired by cotton producers. Such advances may be imperative for sustainability of the technology.

Obviously, higher efficacy *B. t.* cottons would better suit resistance management strategies as compared to current, single toxin Bollgard varieties.

The current study evaluates the efficacy of Bollgard II cottons producing two endotoxins, Cry1Ac and Cry2Ab, and the commercial, single-toxin Bollgard variety against feral and Cry1Ac-selected strains of bollworm.

Materials and Methods

Two greenhouse studies were conducted in greenhouse chambers at North Carolina State University, Wake Co., NC, in 1999, and a single study at the Tidewater Research Station, Washington Co., NC, in 2000. Each experiment was a randomized complete block design with 5 and 7 replicates in 1999 and 2000, respectively. In the first 1999 experiment, replicates consisted of two plants per cotton genotype. For the second 1999 experiment and the 2000 experiment, replicates included two plants per treatment combination of cotton genotype and bollworm strain. Distance between plants within blocks was 0.61 meters, whereas, blocks were separated by a 0.91-m space on tables.

Cotton genotypes consisting of conventional (DP50), Bollgard (DP50B), and Bollgard II (DP50BX) were planted in 1999. In 2000, however, a conventional genotype was not used; instead, a genotype expressing the Cry2Ab endotoxin alone (DP50X) was used in place of the conventional variety in combination with Bollgard and Bollgard II cottons. Cotton genotypes were planted on 29 June in 1999, and 27 June in 2000, into 11.36-liter pots containing BC5s (Bio-Comp, Inc., Edenton, NC) planting medium at one plant per pot. Aldicarb (Temik[®] 15 G, Aventis CropScience, Research Triangle Park, NC) was applied at 0.85 kg a. i./hectare to soil within pots at planting and as a side-dressing two weeks prior to bollworm infestation for the reduction of arthropod natural enemies and elimination of aphids

and whiteflies. Fertilization was accomplished through the application of Peters Professional[®] 20-10-20 water soluble fertilizer at the rate of 200 ppm as needed throughout plant growth.

Feral female bollworms were collected from light traps at various locations in eastern North Carolina. Females were placed individually into 0.3-ml clear plastic cups covered with cheesecloth as a substrate for oviposition. Moths were held in the laboratory at 27°C and cheesecloths were checked daily for the presence of eggs. Neonate larvae derived from the field-collected bollworm strain were tested alone on the various cotton genotypes in the first study, but served as the control strain in the second study in 1999, as well as in 2000.

A Cry1Ac-selected laboratory strain of bollworm was originally collected from a Cry1Ab-producing sweet corn hybrid at the Tidewater Research Station, Washington Co., NC, and Central Crops Research Station, Johnston Co., NC. Sampling for bollworm larvae consisted of collecting corn ears from randomly selected plants throughout a given field. Ears were then examined for surviving third-stage or larger larvae; this criterion was used to increase the probability of recovering bollworm larvae carrying resistance alleles. Larvae were transported to the laboratory and selected for tolerance to the Cry1Ac protein (MVP[®], Mycogen Corp., San Diego, CA) in artificial diet for thirteen generations in 1999, and for 2 generations in 2000 (Burd et al. 2000). The Cry1Ac-selected colonies were maintained at rearing facilities at North Carolina State University at 27-30°C, 55-60% relative humidity, and a photoperiod of 14:10 (L: D) h. Both the 1999 and 2000 Cry1Ac-selected strains exhibited ~100-fold resistance compared to the feral strain. Neonate larvae from the Cry1Ac-selected strain were compared with those from the field-collected strain to determine differences in survival on various *B. t.* cotton genotypes.

Five neonate larvae were introduced onto fruiting structures on each cotton plant using a fine-haired artist paint brush when plants reached 80 days in the first 1999 study, and 100 days in the second 1999 study and the 2000 experiment. Assessments of surviving larvae and fruit damage were made by examining whole plants at 7 days after infestation in 1999, and at 14 and 21 days after infestation in 2000. Each fruiting structure on each plant was given a damage rating of no damage, superficial damage (no penetration of carpal wall), or fruit penetration.

Surviving larvae and damage numbers were converted to percentages and transformed using arcsine prior to analysis. Data were then subjected to ANOVA using PROC GLM (SAS Institute 1990), and treatment means were separated ($P \leq 0.05$) using Fisher's Least Significant Difference test.

Results

The percentage of surviving larvae from the feral bollworm strain was significantly lowered by both Bollgard genotypes as compared to the conventional variety in 1999 ($F=112.55$; $df=2, 16$; $P<0.0001$) (Table 1). Larval survival at seven days after infestation was high (47.4%) on the conventional cotton variety; however, only 6% of the larval cohort survived on the Bollgard cotton, and no larvae were living on the Bollgard II genotype.

The percentage of fruit sustaining superficial bollworm damage was similar for the conventional and Bollgard varieties, but was significantly reduced by the Bollgard II genotype ($F=9.17$; $df=2, 16$; $P=0.0008$) (Table 6). In contrast, the percentage of fruit penetrated by bollworm was significantly lowered by the Bollgard variety (5.1%) as compared to the conventional variety (40.1%) ($F=92.98$; $df=2, 16$; $P<0.0001$); no fruit were penetrated in the Bollgard II genotype.

Comparison of a feral bollworm strain with a laboratory Cry1Ac-selected strain on various cotton genotypes in 1999 demonstrated that the Cry1Ac-selected bollworm strain exhibited a higher percentage of larval survival (23.7%) as compared to the feral bollworm strain (14.7%) when averaged across genotypes ($F=10.09$; $df=1, 40$; $P=0.0024$) (Table 2). Cotton genotypes also significantly affected larval survival with the commercial Bollgard genotype reducing the percentage of surviving larvae to 11.5% in comparison to 43.5% for the conventional variety ($F=67.14$; $df=2, 40$; $P<0.0001$); furthermore, the Bollgard II genotype significantly lowered the percentage of surviving bollworm larvae to 2.5% as compared to that of the Bollgard variety (Table 2).

The Cry1Ac-selected strain inflicted more superficial fruit damage compared to the feral bollworm strain when averaged across genotypes ($F=4.60$; $df=1, 40$; $P=0.0361$) (Table 3). The percentage of fruit sustaining superficial damage by the Cry1Ac-selected strain was 5.5% compared to 2.7% exhibited by the wild bollworm strain. Cotton genotype had no effect on the percentage of superficially damaged fruit with all genotypes performing similarly when averaged across bollworm strains ($F=0.13$; $df=2, 40$; $P=0.8774$) (Table 3).

As with larval survival and superficial fruit damage, the Cry1Ac-selected bollworm strain exhibited the ability to penetrate a higher percentage of fruit as compared to the feral strain when averaged across genotypes ($F=9.84$; $df=1, 40$; $P=0.0026$) (Table 4). The percentage of penetrated fruit was 15.3% for the Cry1Ac-selected strain, compared to 11.5% for the wild strain. Cotton genotype also significantly influenced the percentage of fruit penetrated by bollworm when averaged across bollworm strains ($F=142.22$; $df=2, 40$; $P<0.0001$) (Table 4). The Bollgard genotype significantly reduced the percentage of penetrated fruit to 9.3% as compared to 30.1% for the conventional variety. In addition, the

Bollgard II genotype further lowered the percentage of penetrated fruit (0.84%) below that of the Bollgard variety.

Comparison of a Cry1Ac-selected bollworm strain to a feral strain with respect to larval performance on selected cotton genotypes in 2000 indicated a genotype*strain interaction ($F=3.65$; $df=2, 60$; $P=0.0303$) for the percentage of surviving bollworm larvae. When comparing larval strains within each genotype, the Cry1Ac-selected strain displayed increased larval survival (5.0%) on the Bollgard variety as compared to that of the feral strain (0.0%) ($F=4.81$; $df=1, 12$; $P<0.0367$) (Table 5). No differences in bollworm strains were evident among the Bollgard II or DP50X genotypes.

As with larval survival, a genotype*strain interaction ($F=4.06$; $df=2, 60$; $P=0.0208$) characterized the percentage of fruit sustaining superficial damage. When comparing larval strains within each genotype, the percentage of superficially damaged fruit was higher for the Cry1Ac-selected strain (4.4%) compared to the feral strain (1.2%) on the Bollgard variety ($F=10.25$; $df=1, 12$; $P=0.0034$) (Table 6). Bollworm strains did not differ with respect to the percentage of superficially damaged fruit within the Bollgard II and DP50X genotypes.

The Cry1Ac-selected strain penetrated a higher percentage of fruit (3.2%) as compared to the feral strain (0.3%) when averaged across genotypes ($F=11.55$; $df=1, 60$; $P=0.0010$) (Table 7). Cotton genotype also affected the percentage of penetrated fruit when averaged across bollworm strains ($F=4.42$; $df=2, 60$; $P=0.0149$) (Table 7). The single toxin-producing lines, Bollgard and DP50X, performed similarly with respect to the percentage of penetrated fruit; however, the dual toxin genotype, Bollgard II, significantly reduced the percentage of fruit penetration as compared to the Bollgard variety. No statistical differences

existed among the Bollgard II and DP50X genotypes with respect to percentage of bollworm-penetrated fruit

Discussion

In testing the performance of a wild bollworm strain on various cotton genotypes in 1999, the Bollgard and Bollgard II genotypes provided a high level of control as compared to the conventional variety. The Bollgard II genotype outperformed the Bollgard variety with regard to the percentage of larval survival, superficial fruit damage, and fruit penetration. Greenplate et al. (2000) reported that the level of expression of the Cry1Ac endotoxin is similar among Bollgard and Bollgard II genotypes. Also, the Cry2Ab protein in Bollgard II cottons is expressed at a much higher level than the Cry1Ac protein. Therefore, the increased performance of the Bollgard II genotype is most likely due to the increased toxicity to bollworm from the addition of the Cry2Ab endotoxin. However, the increased performance of the Bollgard II genotype could be due to the reported drop in average levels of the Cry1Ac endotoxin in cotton fruit in the Bollgard variety at ~80 days (Greenplate et al. 1998). Since the Cry1Ac endotoxin level in the Bollgard variety was known to decrease significantly at this point, the test of the feral bollworm strain on various cotton genotypes in 1999 was initiated when cotton reached 80 days to determine if the Bollgard II genotype would demonstrate an increased performance against a wild population of bollworm. At that time, the Bollgard II genotype provided excellent control of bollworm at this late stage of growth of the cotton plant.

In comparing the performance of a laboratory Cry1Ac-selected bollworm strain to that of a feral strain on selected cotton genotypes in 1999, the Cry1Ac-selected strain outperformed the wild strain with respect to the percentage of surviving larvae, superficial

fruit damage, and fruit penetration when averaged across genotypes. The ~100-fold resistance to Cry1Ac-incorporated artificial diet exhibited by the Cry1Ac-selected strain provided an increased tolerance (ca. 4.8X) to the Bollgard variety as compared to the wild strain with respect to larval survival. The increased percentage of surviving larvae from the Cry1Ac-selected strain when averaged across genotypes was most likely caused by the pronounced increase of larval survival of the Cry1Ac-selected strain on the Bollgard variety, although larval survival was numerically higher for the Cry1Ac-selected strain on all genotypes.

When averaged across bollworm strains, the percentages of surviving larvae were much lower on the Bollgard and Bollgard II genotypes (11.5% and 2.5%, respectively) as compared to that of the conventional variety (43.5%). Also, larval survival was significantly reduced by the Bollgard II genotype compared to the Bollgard variety. A decline in larval survival in the *B. t.* genotypes below that of the conventional variety was expected, since numerous field studies have reported significant reductions in bollworm larval numbers in Bollgard cottons (Burd et al. 1999; Lambert et al. 1996, 1997; Mahaffey et al. 1994, 1995). However, the observed decreased larval survival of the Bollgard II genotype compared to the Bollgard variety was partially attributable to the increased survival of the Cry1Ac-selected strain on the Bollgard variety. Nevertheless, the Bollgard II genotype provided increased control of both larval strains over that of the commercially available Bollgard variety.

The elevated percentage of superficially damaged fruit due to the Cry1Ac-selected strain as compared to the wild bollworm strain was presumably caused by the ability of the Cry1Ac-selected strain to ingest a higher level of *B. t.* endotoxins produced by the Bollgard and Bollgard II genotypes and an increase in larval movement on *B. t.* cottons. Bollworm

larval movement on a cotton plant has been demonstrated to increase on Bollgard varieties as compared to conventional cottons (Gore et al. 2001). This is a natural phenomenon exhibited by bollworm larvae when fed an undesirable food source; movement increases as the insects attempt to locate a more suitable food source.

Although the percentage of superficially damaged fruit differed significantly among bollworm strains, cotton genotype had no measurable effect on superficial fruit damage when averaged across bollworm strains. The percentage of superficial fruit damage was 5.3, 3.6, and 3.3% for the conventional, Bollgard, and Bollgard II genotypes, respectively. This would be expected since plants must be fed upon for the *B. t.* toxin to be ingested; therefore, insects should produce similar amounts of surface damage on *B. t.* or non-*B. t.* plants.

As with larval survival and superficial fruit damage, the percentage of fruit penetrated by bollworm was significantly higher for the Cry1Ac-selected strain as compared to the feral strain. The increase in fruit penetration exhibited by the Cry1Ac-selected strain when averaged across genotypes was apparently caused by the 1.7X increase in fruit penetration displayed by the Cry1Ac-selected strain as compared to the feral strain on the Bollgard variety. The higher level of fruit penetration exhibited by the Cry1Ac-selected strain was directly related to the increased survival of this strain on the *B. t.* cottons as compared to that of the feral strain.

The percentage of penetrated fruit for each cotton genotype averaged across bollworm strains followed the same trend as larval survival among genotypes. This was expected because fruit penetration for each genotype should be directly related to larval survival on each genotype.

In the 2000 trial, the Cry1Ac-selected strain exhibited increased larval survival and superficial fruit damage compared to the wild strain on the Bollgard variety; however, no differences among larval strains were evident for the Bollgard II and DP50X genotypes with regard to larval survival and superficially damaged fruit. As with the 1999 study, the increased survival of the Cry1Ac-selected strain on the Bollgard variety was due to the ~100-fold increase in tolerance to Cry1Ac-incorporated artificial diet compared to the susceptible strain. No differences were expected in larval survival among bollworm strains for either the Bollgard II or DP50X genotypes since these cottons express the Cry2Ab endotoxin; therefore, both bollworm strains should have been equally susceptible to these genotypes. It would also be expected that the Cry1Ac-selected strain would superficially damage a higher percentage of the available fruit than the feral strain because of the ability of the Cry1Ac-selected strain to ingest a higher level of the Cry1Ac endotoxin.

The percentage of fruit penetrated by the Cry1Ac-selected bollworm strain was significantly higher than that of the feral strain when averaged across genotypes. This difference existed partially because of the increased survival of the Cry1Ac-selected strain on the Bollgard variety and increased tolerance of the selected strain to the Cry1Ac endotoxin; however, the Cry1Ac-selected strain also penetrated numerically more fruit than the feral strain on the DP50X genotype. This phenomenon may be attributable to infesting the neonates at ~100 days. Average Cry1Ac levels of the single gene Bollgard variety have been shown to drop significantly at ~80 days, so the level of Cry2Ab in the single gene DP50X genotype may have dropped after ~100 days as well. If this was the case, the dual toxins of the Bollgard II genotype demonstrated somewhat of an additive effect since this genotype remained effective against both bollworm strains at ~100 days.

Averaged across bollworm strains, cotton genotype significantly influenced the percentage of fruit penetration by bollworm larvae. The percentage of penetrated fruit did not differ among the Bollgard and DP50X genotypes, but differed significantly among Bollgard and Bollgard II genotypes. This was expected since larval survival and superficial damage was higher for the Bollgard variety due to the increased survival of the Cry1Ac-selected strain. As mentioned above, the percentage of penetrated fruit for the DP50X genotype did not differ from that of the Bollgard variety because of the apparent drop in average levels of the Cry2Ab endotoxin at ~100 days.

The results presented herein suggest that the commercialization of Bollgard II cottons would reduce bollworm survival and damage over that experienced by Bollgard varieties. With the likelihood of Cry1Ac resistance development in bollworm, the commercialization of dual toxin Bollgard II genotypes may be absolutely necessary for the sustainability of this technology as a first line of defense against heliothine pests.

Acknowledgments

The authors express appreciation to Cotton, Inc. for providing a graduate research assistantship for the senior author and to Monsanto Agric. Co. for providing partial project funding. Special thanks are given to Tony Burd and Margery Ambrose for technical assistance.

Literature Cited

Burd, A. D., J. R. Bradley, Jr., and J. W. Van Duyn. 1999. Performance of selected *Bt* cotton genotypes against bollworm in North Carolina. p. 931-934. *In Proc. Beltwide Cotton Conf.*, Orlando, FL. 3-7 Jan. 1999. Natl. Cotton Counc. Am., Memphis, TN.

Burd, A. D., J. R. Bradley, Jr., J. W. Van Duyn, and F. Gould. 2000. Resistance of bollworm, *Helicoverpa zea*, to CryIA(c) toxin. p. 923-926. *In Proc. Beltwide Cotton Conf.*, San Antonio, TX. 4-8 Jan. 2000. Natl. Cotton Counc. Am., Memphis, TN.

Gore, J., B. R. Leonard, D. R. Cook, and R. H. Jones. 2001. Dispersal of bollworm larvae on Bollgard[®] and non-Bollgard cotton cultivars. p. 851-854. *In Proc. Beltwide Cotton Conf.*, Anaheim, CA. 9-13 Jan. 2001. Natl. Cotton Counc. Am., Memphis, TN.

Gould, F. 1998. Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. *Annu. Rev. Entomol.* 43: 701-726.

Greenplate, J. T., G. P. Head, S. R. Penn, and V. T. Kabuye. 1998. Factors potentially influencing the survival of *Helicoverpa zea* on Bollgard cotton. p. 1030-1033. *In Proc. Beltwide Cotton Conf.*, San Diego, CA. 5-9 Jan. 1998. Natl. Cotton Counc. Am., Memphis, TN.

Greenplate, J. T., 1999. Quantification of *Bacillus thuringiensis* insect control protein cry1Ac over time in Bollgard cotton fruit and terminals. *J. Econ. Entomol.* 92: 1377-1383.

Greenplate, J. T., S. R. Penn, Z. Shappley, M. Oppenhuizen, J. Mann, B. Reich, and J. Osborn. 2000. Bollgard II efficacy: quantification of total lepidopteran activity in a 2-gene product. p. 1041-1043. *In Proc. Beltwide Cotton Conf.*, San Antonio, TX. 4-8 Jan. 2000. Natl. Cotton Counc. Am., Memphis, TN.

Lambert, A. L., J. R. Bradley, Jr., and J. W. Van Duyn. 1996. Effects of natural enemy conservation and planting date on the susceptibility of *Bt* cotton to *Helicoverpa zea* in North Carolina. p. 931-935. *In Proc. Beltwide Cotton Conf.*, Nashville, TN. 9-12 Jan. 1996. Natl. Cotton Counc. Am., Memphis, TN.

Lambert, A. L., J. R. Bradley, Jr., and J. W. Van Duyn. 1997. Interactions of *Helicoverpa zea* and *Bt* cotton in North Carolina. p. 870-873. *In Proc. Beltwide Cotton Conf.*, New Orleans, LA. 7-10 Jan. 1997. Natl. Cotton Counc. Am., Memphis, TN.

Mahaffey, J. S., J. S. Bachelier, J. R. Bradley, Jr., and J. W. Van Duyn. 1994. Performance of Monsanto's transgenic *B.t.* cotton against high populations of lepidopterous pests in North Carolina. p. 1061-1063. *In Proc. Beltwide Cotton Conf.*, San Diego, CA. 5-8 Jan. 1994. Natl. Cotton Counc. Am., Memphis, TN.

Mahaffey, J. S., J. R. Bradley, Jr., and J. W. Van Duyn. 1995. *B. t.* cotton: field performance in North Carolina under conditions of unusually high bollworm populations. p. 795-798. *In Proc. Beltwide Cotton Conf.*, San Antonio, TX. 4-7 Jan. 1995. Natl. Cotton Counc. Am., Memphis, TN.

SAS Institute. 1990. SAS/STAT User's Guide, Vol. 2. SAS Institute, Cary, NC, 795 pp.

Scientific Advisory Panel, Subpanel on *Bacillus thuringiensis* (Bt) Plant-Pesticides, February 9-10, 1998. 1998. Transmittal of the final report of the FIFRA Scientific Advisory Panel, Subpanel on *Bacillus thuringiensis* (Bt) Plant-Pesticides and Resistance Management, Meeting held on February 9-10, 1998. (Docket Number: OPPTS-00231).

Tabashnik, B. E. 1994. Evolution of resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol. 39: 47-79.

Table 1. Mean (SE) percentage of bollworm larval survival, superficially damaged fruit, and fruit penetration at seven days after infestation averaged across two runs for three cotton genotypes at ~80 days after planting in the greenhouse, Wake Co., NC, 1999.

Genotype	% Larval Survival	% Superficially	
		Damaged Fruit	% Fruit Penetration
Conventional (DP50)	47.36 (6.876) a	14.09 (3.392) a	40.14 (6.258) a
Bollgard (DP50B)	6.00 (2.103) b	6.61 (1.245) a	5.10 (1.478) b
Bollgard II (DP50BX)	0.00 (0.000) c	2.28 (1.237) b	0.00 (0.000) c

Means within the same column followed by the same letter are not significantly different, Fisher's LSD, ($P \leq 0.05$).

Table 2. Mean (SE) percentage of bollworm larval survival at seven days after infestation of a feral and a Cry1Ac-selected bollworm strain onto three cotton genotypes averaged across two runs at ~100 days after planting in the greenhouse, Wake Co., NC, 1999.

Genotype	Feral	Cry1Ac-Selected	Mean
Conventional (DP50)	40.00 (5.620)	47.00 (5.482)	43.50 (3.915) a
Bollgard (DP50B)	4.00 (1.835)	19.00 (3.692)	11.50 (2.363) b
Bollgard II (DP50BX)	0.00 (0.000)	5.00 (2.460)	2.50 (1.279) c
Mean	14.67 (3.039) b	23.67 (3.241) a	

Means within the same column or row followed by the same letter are not significantly different, Fisher's LSD, ($P \leq 0.05$).

Table 3. Mean (SE) percentage of superficially damaged fruit at seven days after infestation of a feral and a Cry1Ac-selected bollworm strain onto three cotton genotypes averaged across two runs at ~100 days after planting in the greenhouse, Wake Co., NC, 1999.

Genotype	Feral	Cry1Ac-Selected	Mean
Conventional (DP50)	4.51 (2.384)	6.07 (2.235)	5.29 (1.618) a
Bollgard (DP50B)	2.28 (0.949)	4.86 (1.777)	3.57 (1.016) a
Bollgard II (DP50BX)	1.21 (0.603)	5.47 (1.504)	3.34 (0.869) a
Mean	2.67 (0.882) b	5.47 (1.059) a	

Means within the same column or row followed by the same letter are not significantly different, Fisher's LSD, ($P \leq 0.05$).

Table 4. Mean (SE) percentage of bollworm-penetrated fruit at seven days after infestation of a feral and a Cry1Ac-selected bollworm strain onto three cotton genotypes averaged across two runs at ~100 days after planting in the greenhouse, Wake Co., NC, 1999.

Genotype	Feral	Cry1Ac-Selected	Mean
Conventional (DP50)	27.63 (3.907)	32.56 (2.944)	30.10 (2.446) a
Bollgard (DP50B)	6.91 (2.028)	11.70 (1.984)	9.30 (1.452) b
Bollgard II (DP50BX)	0.00 (0.000)	1.68 (0.848)	0.84 (0.440) c
Mean	11.51 (2.101) b	15.32 (2.058) a	

Means within the same column or row followed by the same letter are not significantly different, Fisher's LSD, ($P \leq 0.05$).

Table 5. Mean (SE) percentage of bollworm larval survival of a feral and a Cry1Ac-selected bollworm strain within each of three cotton genotypes averaged across two evaluation dates at ~100 days in the greenhouse, Washington Co., NC, 2000.

Genotype	Feral	Cry1Ac-Selected
Bollgard (DP50B)	0.00 (0.000) b	5.00 (0.022) a
(DP50X)	0.00 (0.000) a	0.71 (0.007) a
Bollgard II (DP50BX)	0.00 (0.000) a	0.00 (0.000) a

Means within the same row followed by the same letter are not significantly different, Fisher's LSD, ($P \leq 0.05$).

Table 6. Mean (SE) percentage of superficially damaged fruit of a feral and a Cry1Ac-selected bollworm strain within each of three cotton genotypes averaged across two evaluation dates at ~100 days in the greenhouse, Washington Co., NC, 2000.

Genotype	Feral	Cry1Ac-Selected
Bollgard (DP50B)	1.19 (0.006) b	4.36 (0.009) a
(DP50X)	0.00 (0.000) a	0.65 (0.005) a
Bollgard II (DP50BX)	1.36 (0.006) a	1.36 (0.006) a

Means within the same row followed by the same letter are not significantly different, Fisher's LSD, ($P \leq 0.05$).

Table 7. Mean (SE) percentage of fruit penetration by a feral and a Cry1Ac-selected bollworm strain for three cotton genotypes averaged across two evaluation dates at ~100 days in the greenhouse, Washington Co., NC, 2000.

Genotype	Feral	Cry1Ac-Selected	Mean
Bollgard (DP50B)	0.85 (0.005)	4.93 (0.017)	2.89 (0.009) a
(DP50X)	0.00 (0.000)	4.10 (0.020)	2.05 (0.010) ab
Bollgard II (DP50BX)	0.00 (0.000)	0.45 (0.004)	0.22 (0.002) b
Mean	0.28 (0.002) b	3.16 (0.009) a	

Means within the same column or row followed by the same letter are not significantly different, Fisher's LSD, ($P \leq 0.05$).

CHAPTER III

**ESTIMATED PRODUCTION OF *HELICOVERPA ZEA* (LEPIDOPTERA:
NOCTUIDAE) FROM BOLLGARD AND BOLLGARD II COTTONS
WITH AND WITHOUT PYRETHROID OVERSPRAYS**

Abstract

Transgenic cottons, *Gossypium hirsutum* (L.), expressing either one or two *Bacillus thuringiensis* ssp. *kurstaki* Berliner proteins were compared with the conventional sister line in field experiments with regard to production of bollworm, *Helicoverpa zea* (Boddie), and bolls damaged by bollworm in North Carolina in 2000-2001. The relative numbers of bollworms that were capable of successfully completing development on Bollgard[®] (Monsanto Agric. Co., St. Louis, MO), Bollgard II, and conventional cottons were estimated under untreated conditions in 2000 and both pyrethroid-treated and untreated conditions in 2001. Averaged across two years, the untreated Bollgard II genotype significantly reduced the numbers of large larvae (L4-L5) produced per hectare below that of the Bollgard and conventional varieties. With regard to the numbers of damaged fruit per hectare, both *B. t.* genotypes reduced fruit damage below that of the conventional variety, and the Bollgard II line had less damaged fruit than the Bollgard variety. The numbers of adults produced per hectare were significantly reduced by both *B. t.* genotypes, with no significant differences between Bollgard and Bollgard II cottons. In 2001, the numbers of larvae produced per hectare and subsequent feeding damage were dramatically reduced by both *B. t.* cottons, with a further reduction exhibited by the Bollgard II genotype as compared to the commercial Bollgard variety when averaged across insecticide regimes. Both pupal and adult production on a per hectare basis were significantly lowered by the two *B. t.* genotypes as compared to the conventional variety and averaged across insecticide regimes. No significant differences were apparent between the Bollgard and Bollgard II genotypes with regard to pupal and adult production. Averaged across genotypes, pyrethroid oversprays caused a significant reduction in larval, pupal, and adult production, as well as damaged fruit.

Genetically altered cottons, *Gossypium hirsutum* (L.), expressing the Cry1Ac δ -endotoxin derived from the soil bacterium *Bacillus thuringiensis* spp. *kurstaki* were planted to approximately 67% of the total North Carolina cotton acreage in 2001. The primary targets of these *B. t.* cottons in North Carolina are the bollworm, *Helicoverpa zea* (Boddie), and to a lesser extent the tobacco budworm, *Heliothis virescens* (Fab.). While Bollgard cottons have provided unequivocal control of tobacco budworm, supplemental insecticide oversprays have often been required to provide adequate control of bollworm, as well as to prevent yield losses (Lambert et al. 1996, 1997; Mahaffey et al. 1994, 1995). Increased survival of bollworm in *B. t.* cottons as compared to tobacco budworm can be attributed to a higher natural tolerance of bollworm to the Cry1Ac protein (Stone and Sims 1993). Due to this increased tolerance of bollworm to *B. t.* cottons, an Environmental Protection Agency Scientific Advisory Panel (1998) reported that commercially available Bollgard cottons do not meet the “high dose” criterion for resistance management to *B. t.* cottons because the single toxin-producing varieties expressed only a moderate dose with respect to the level necessary to prevent rapid *B. t.* resistance evolution in bollworm when an effective refuge is employed.

The current EPA approved resistance management strategy for *B. t.* cottons is based on a high dose of the toxin and a non-*B. t.* cotton planted in proximity to *B. t.* cottons. The non-transgenic cottons serve to produce susceptible moths that reduce the probability of resistant moths mating with one another (Gould 1998). The SAP (1998) suggested that the production of 500 susceptible bollworm adults for each bollworm carrying at least one *B. t.* resistance allele would be required to successfully delay resistance development in bollworm to Bollgard cottons. Although Bollgard cotton varieties have been commercially available

since 1996, production of bollworm adults from *B. t.* and non-*B. t.* refuge cottons has not been reported.

Bollgard II cottons that produce two *B. t.* endotoxins, Cry1Ac and Cry2Ab, are currently under development. Laboratory studies have confirmed that these dual-gene cottons produce approximately the same level of the Cry1Ac protein as the single-gene Bollgard varieties, but the addition of the Cry2Ab protein provides additional protection against lepidopterous pests (Greenplate et al. 2000; Adamczyk et al. 2001). Stewart et al. (2001) demonstrated in a laboratory bioassay that leaf tissue from Bollgard II plants was more toxic to second-instar bollworm larvae than leaf tissue from Bollgard plants.

From a resistance management standpoint, pyramiding the two toxin-producing genes into a plant could increase the durability of the toxins 9.3X in comparison to serial introductions of the same toxins in the presence of a 5% refuge (Caprio 1998). Therefore, the pyramiding of genes encoding Cry1Ac and Cry2Ab expression in Bollgard II genotypes should increase the effectiveness of current refuge strategies for bollworm. With preliminary reports on the increased efficacy exhibited by Bollgard II cottons coupled with resistance management benefits, commercialization of the dual-toxin cottons may delay *B. t.* resistance development in bollworm and sustain this important technology.

The objectives of the study reported herein were to quantify bollworm damage to cotton fruit and to estimate production of large larvae, pupae, and adults in conventional, Bollgard and Bollgard II cottons under pyrethroid-treated and untreated conditions in field experiments.

Materials and Methods

Field studies were conducted at the Tidewater Research Station, Washington Co., NC, in 2000, and the Upper Coastal Plain Research Station, Edgecombe Co., NC, in 2000 and 2001. The experiment was designed as a randomized complete split-plot with four replicates. Whole plots were cotton genotypes that consisted of Bollgard II (DP50BX), Bollgard (DP50B), and a conventional sister line (DP50), which were 24, 20, and 16 rows, respectively, by 15.2 meters in length. Unequal plot sizes were established to increase the probability of estimating that proportion of the bollworm population completing development on each genotype. Fewer bollworms have been found on Bollgard II genotypes; therefore, larger whole plots were necessary to obtain useful numbers for analysis. Subplots consisted of 20, 16, and 12 untreated rows for Bollgard II, Bollgard, and conventional genotypes, respectively, and 4 rows that were treated with a pyrethroid as required for supplemental bollworm control.

Field trials were planted in 2000 on 15 and 18 May in Edgecombe and Washington counties, respectively, and on 2 May in Edgecombe Co., NC, in 2001. Aldicarb (Temik[®] 15G, Aventis CropScience, Research Triangle Park, NC) was applied in-furrow at planting at 0.85 kg a. i./hectare for control of early season insect pests. Acephate (Orthene[®] 97 PE, Valent USA Corp., Walnut Creek, CA) was applied at 0.85 kg a. i./hectare as a mid-season overspray for control of tarnished plant bugs and stink bugs, as well as to reduce arthropod natural enemies of bollworm. Two applications of lambda cyhalothrin (Karate[®] Z 2.08 CS, Syngenta Crop Protection, Inc., Greensboro, NC) at 0.045 kg a.i./hectare were made to appropriate subplots for supplemental bollworm control in early- to mid-August. A CO₂-powered backpack sprayer fitted with one TX-12 hollow cone nozzle per row delivering

113.2 liters per hectare at a CO₂ pressure of 3.9 kilograms per square centimeter was used to apply foliar insecticides. Weed control, fertilization, plant growth regulation, and defoliation were achieved as recommended by North Carolina State University.

The total number of large harvestable bolls were counted in a randomly selected area of 1.5 row meters per treatment per replicate, which provided a means of converting numbers of larvae to a per hectare basis. The total numbers of bollworm-damaged bolls and large fourth-to-fifth instar larvae were counted on a predetermined number of bolls per plot on various sample dates in mid-to-late August. Fourth-to-fifth instar bollworm larvae were collected and placed on fresh cotton bolls from the respective genotype in individual 30-ml plastic cups and transported to the laboratory. These larvae were reared on bolls from the respective genotypes until the prepupal stage. Prepupae were then placed into 30-ml plastic cups containing non-*B. t.* artificial diet that served as a medium to tunnel into for pupation. Numbers of successfully emerged bollworm adults from each genotype were counted and converted to a per hectare basis prior to analysis along with the total numbers of harvestable bolls, bollworm-damaged bolls, live fourth-to-fifth instar larvae, and pupae. Bollworm larvae, pupae, and adults, in addition to boll damage, were estimated in pyrethroid-treated subplots only in 2001.

Numbers of damaged bolls, large larvae, pupae, and adults were converted to a per hectare basis and subjected to a log transformation. All data were then subjected to ANOVA using PROC GLM (SAS Institute 1990), and means for each treatment were separated ($P \leq 0.05$) using Fisher's Protected Least Significant Difference test.

Results

Moderate bollworm populations in the non-*B. t.* cottons characterized the three experiments in 2000-2001 as compared to populations encountered by Mahaffey et al (1995). Larval production was statistically similar between the Bollgard and conventional variety, even though the Bollgard variety lowered the number of larvae per hectare by 2.8X as compared to the conventional variety ($F=19.67$; $df=2,4$; $P=0.0085$) (Table 1). However, the Bollgard II genotype reduced larval numbers by 9.0X and 25.6X as compared to the Bollgard and conventional varieties, respectively.

Both *B. t.* genotypes dramatically reduced bollworm-damaged bolls ($F=357.36$; $df=2,4$; $P<0.0001$) (Table 1); in addition, the Bollgard II genotype had significantly fewer damaged bolls than the Bollgard variety. The conventional cotton variety sustained an average of 317,175 damaged bolls per hectare, whereas, Bollgard and Bollgard II cottons had 55,708 and 5,540 damaged bolls per hectare, respectively. Bollgard and Bollgard II genotypes reduced the level of bollworm-damaged bolls by 5.7X and 57.2X, respectively, as compared to the conventional variety; furthermore, the number of damaged bolls was 10.1X fewer in the Bollgard II genotype as compared to the Bollgard variety.

Bollworm adult production was significantly reduced by both *B. t.* genotypes versus the conventional variety ($F=19.90$; $df=2,4$; $P=0.0083$) (Table 1); however, no differences were evident between Bollgard and Bollgard II genotypes. The commercially available Bollgard variety exhibited a 17.5X reduction in adult production as compared to the conventional variety, whereas, the Bollgard II genotype demonstrated a 79.2X reduction.

In 2001, estimates of bollworm production were made in both pyrethroid-treated and untreated subplots of each genotype. Both *B. t.* genotypes reduced the number of bollworm

larvae per hectare in comparison to the conventional variety when averaged across insecticide regimes ($F=31.85$; $df=2,6$; $P=0.0006$) (Table 2). In addition, the Bollgard II genotype lowered larval production below that of the Bollgard variety. Averaged across insecticide regimes, bollworm larval production was reduced 6.1X and 62.2X by Bollgard and Bollgard II genotypes, respectively, as compared to the conventional variety; the Bollgard II genotype also exhibited a 10.2X decrease in larval production as compared to the commercial Bollgard variety. The addition of a pyrethroid overspray significantly reduced larval production by 2.5X as compared to untreated cottons averaged across cotton genotypes ($F=15.06$; $df=1,9$; $P=0.0037$) (Table 2).

The number of damaged bolls per hectare was significantly reduced by both *B. t.* genotypes as compared to the conventional variety when averaged across insecticide regimes ($F=68.41$; $df=2,6$; $P<0.0001$) (Table 3). Bollgard II cottons had fewer damaged bolls than the Bollgard variety. Averaged across insecticide regimes, the number of bolls damaged per hectare by bollworm were reduced 6.0X and 40.7X by Bollgard and Bollgard II genotypes, respectively, as compared to the conventional variety. Bollgard II cottons had 6.7X less damage to bolls than the Bollgard variety. Averaged across genotypes, a pyrethroid overspray significantly reduced the number of damaged bolls by 2.3X as compared to untreated cottons ($F=6.53$; $df=1,9$; $P=0.0310$) (Table 3).

Bollworm pupal production was significantly lowered by both *B. t.* genotypes below that of the conventional variety when averaged across insecticide regimes ($F=45.08$; $df=2,6$; $P=0.0002$) (Table 4); there were no differences between *B. t.* genotypes. The reduction in the number of pupae produced per hectare by Bollgard and Bollgard II cottons was 21.3X and 92.5X as compared with the conventional variety. Production of bollworm pupae was also

significantly lowered by the addition of a pyrethroid overspray in comparison with non-treated cottons averaged across genotypes ($F=35.75$; $df=1,9$; $P=0.0002$) (Table 4). Averaged across genotypes, the pyrethroid application exhibited a 2.3X reduction in pupal production as compared to the cottons that were not sprayed with a lepidopterous-active insecticide.

Bollgard and Bollgard II genotypes also demonstrated a profound reduction in bollworm adult production when averaged across insecticide regimes ($F=28.59$; $df=2,6$; $P=0.0009$) (Table 5). Bollgard cottons lowered adult production 35.0X as compared with the conventional variety, whereas, the Bollgard II genotype exhibited an 86.2X reduction. The number of adults produced per hectare was 2.5X less for Bollgard II as compared to the commercial Bollgard variety. Pyrethroid-treated cottons produced 2.9X fewer bollworm adults as compared to untreated cottons averaged across genotypes ($F=7.84$; $df=1,9$; $P=0.0207$) (Table 5).

Discussion

Averaged across two years, the untreated conventional variety produced an estimated 19,562 large bollworm larvae (L4-L5) per hectare. The untreated Bollgard II genotype exhibited a significant reduction in large larval production in comparison to both the untreated conventional and Bollgard varieties. These reductions in larval production for the Bollgard II genotype was likely due to the increased toxicity of the dual-gene construct as compared to the single-gene varieties (Stewart et al. 2001). An increased toxicity of the Bollgard II genotype versus Bollgard varieties results from the high expression of the Cry2Ab endotoxin in addition to a level of Cry1Ac endotoxin production similar to that produced by the Bollgard variety (Greenplate et al. 2000).

Similarly, the number of bolls sustaining bollworm damage was significantly reduced by both untreated *B. t.* genotypes as compared to the untreated conventional variety and averaged across two years. The Bollgard II genotype demonstrated a further reduction in damaged bolls per hectare when compared to the Bollgard variety. Reduced boll damage in Bollgard cotton, and presumably Bollgard II, was due to reduced numbers of larvae and because bollworm larvae infesting Bollgard cottons demonstrate significantly less boll feeding per larvae (Gore et al. 2002).

The untreated Bollgard and Bollgard II genotypes produced 1,115 and 247 bollworm adults per hectare, respectively, both of which were significantly less than the 19,562 produced by the untreated conventional variety averaged across two years. There were no significant differences between *B. t.* genotypes with regard to adult production because of the low numbers of bollworm adults produced by these genotypes. Both *B. t.* genotypes exhibited a significant reduction in adult production in comparison to the conventional variety because larval and pupal production were much higher in the conventional variety. In addition, many of the large larvae collected had already received a dose of endotoxin(s) high enough to induce mortality; thus, fewer pupae and adults were produced as compared to larvae in the *B. t.* genotypes. Although adult production did not statistically differ between *B. t.* cottons due to the low numbers produced by both *B. t.* genotypes, the Bollgard II genotype reduced the number of adults produced per hectare by 4.5X. This reduction was not statistically significant but could have major effects on *B. t.* resistance evolution in bollworm. When considered over the acreage currently planted to Bollgard cottons, the Bollgard II genotype would produce a dramatically lower number of bollworm adults as compared to the Bollgard variety; thus, resistance evolution in bollworm would be

significantly delayed. Based on reports from Jackson et al. (2002), the portion of the bollworm population that survives on Bollgard cottons may be those that carry minor *B. t.* resistance alleles; therefore, Bollgard II cottons may significantly delay resistance evolution in bollworm by reducing the number of individuals carrying these minor *B. t.* resistance alleles from the general bollworm population.

In 2001, both pyrethroid-treated and untreated subplots of each cotton genotype were compared with regard to bollworm production. Averaged across insecticide regimes, the conventional variety produced 17,981 large bollworm larvae per hectare. However, larval production was significantly reduced by both Bollgard and Bollgard II genotypes to 2,946 and 288 larvae per hectare, respectively. The Bollgard II genotype exhibited a significant reduction in bollworm larval numbers below that of the commercial Bollgard variety. Larval numbers were similar to those produced in 2000; however, in 2001, the Bollgard variety significantly lowered larval production on a per hectare basis in comparison to the conventional variety. Decreased larval numbers in the Bollgard variety was expected since various North Carolina field studies reported reduced numbers of bollworm larvae in Bollgard cottons as compared to a conventional variety (Lambert et al. 1996, 1997; Mahaffey et al. 1994, 1995). The further reduction of larval production in the Bollgard II genotype as compared to the Bollgard variety was likely due to the increased toxicity of the Bollgard II genotype caused by the combination of the Cry2Ab and Cry1Ac endotoxins (Stewart et al. 2001). Oversprays with lambda-cyhalothrin significantly lowered larval production when averaged across genotypes. Such was expected since pyrethroid insecticides have displayed a high efficacy against bollworm (Bradley 1996).

The number of bollworm-damaged bolls followed the same trend as larval production when averaged across insecticide regimes. Both *B. t.* cottons significantly reduced the level of boll damage below that observed in the conventional variety, and the Bollgard II genotype further reduced boll damage as compared to the Bollgard variety. Averaged across genotypes, subplots treated with lambda-cyhalothrin had less boll damage in comparison to the untreated subplots.

Numbers of bollworm pupae and adults produced per hectare were significantly lower in the *B. t.* genotypes in comparison to the conventional variety when averaged across insecticide regimes; no differences were evident between Bollgard and Bollgard II genotypes. Fewer pupae were produced in comparison with larval numbers since some of the large larvae had apparently ingested a chronically toxic dose of *B. t.* endotoxin(s). In addition, adult production was lower than pupal production, which most likely demonstrates a further effect of the *B. t.* endotoxins. Although Bollgard II genotypes did not exhibit a significant reduction in adult production as compared to the Bollgard variety due to the low numbers produced by each *B. t.* genotype, the 2.5X fewer adults produced by Bollgard II cottons could have a profound impact on *B. t.* resistance management in bollworm. Caprio (1998) reported that the dual-gene construct could effectively delay *B. t.* resistance evolution in bollworm 9.3X as compared to serial introductions of the single-gene varieties in the presence of a 5% refuge. The delay in resistance development in bollworm is related to adult production where Bollgard II demonstrates a benefit.

Oversprays with lambda-cyhalothrin significantly reduced pupal and adult production when averaged across genotypes. These results suggest that applications of lambda-cyhalothrin onto *B. t.* cottons may serve as an excellent additional *B. t.* resistance

management tool. The use of Bollgard II cottons in combination with oversprays of lambda-cyhalothrin clearly provides a more effective resistance management strategy than that presently available.

Although numbers of bollworms produced from the three cotton genotypes were elevated due to natural enemy disruption, ratios of production between the various genotypes should be similar to those in environments without disruptive insecticide oversprays (Hagerty et al. 2000). If natural enemies were conserved, the ratio of susceptible:resistant bollworms would likely be higher since larval reduction in *B. t.* cottons due to beneficial insects is higher than that of non-*B. t.* cottons (Lambert et al. 1997). Therefore, the results reported herein demonstrate that production ratios of bollworm adults in conventional versus Bollgard or Bollgard II cottons do not approach the desired 500:1 (susceptible: resistant) ratio proposed to be necessary to substantially delay resistance evolution in bollworm. Bollgard II cottons supplemented with pyrethroid oversprays presented the best option; however, lepidopterous-active insecticides are not likely to be applied to Bollgard II cottons due to its high efficacy against caterpillar pests, unless some other pest is targeted. These results suggest that moth production from the cotton refuge do not approach the numbers desired for effective resistance management. However, since bollworm adaptation to the Cry1Ac protein has not been observed over the six years of Bollgard use (Jackson et al. 2002), it is apparent that resistance development in bollworm has not occurred because of a fitness cost associated with larval development on *B. t.* cottons or because bollworm production from alternate crop hosts is more substantial than previously anticipated.

Acknowledgments

The authors express appreciation to Cotton, Inc. for providing a graduate research assistantship for the senior author and to Monsanto Agric. Co. for providing partial project funding. Special thanks are given to Wayne Modlin, Andrew Summerlin, and Phil Threatt for technical assistance.

Literature Cited

Adamczyk, J. J., Jr., K. Bew, L. C. Adams, and D. D. Hardee. 2001. Evaluation of Bollgard[®] (cv. DP50BII) in the Mississippi delta: field efficacy against various Lepidoptera while profiling season-long expression of cry1Ac and cry2Ab. p. 835-836. *In Proc. Beltwide Cotton Conf., Anaheim, CA. 9-13 Jan. 2001. Natl. Cotton Counc. Am., Memphis, TN.*

Bradley, J. R., Jr. 1996. Major developments in management of insect and mite pests in cotton. pp. 1-13. *In: E. G. King, J. R. Phillips and R. T. Coleman (eds.) The Cotton Foundation. Memphis, TN.*

Caprio, M. 1998. Evaluating resistance management strategies for multiple toxins in the presence of external refugia. *J. Econ. Entomol.* 91: 1021-1031.

Gore, J., D. R. Cook, M. M. Willrich, and B. R. Leonard. 2002. Bollworm/Bollgard interactions: implications for management. *In Proc. Beltwide Cotton Conf., Atlanta, GA. 8-12 Jan. 2002. Natl. Cotton Counc. Am., Memphis, TN.*

Greenplate, J. T., S. R. Penn, Z. Shappley, M. Oppenhuizen, J. Mann, B. Reich, and J. Osborn. 2000. Bollgard II efficacy: quantification of total lepidopteran activity in a 2-gene product. p. 1041-1043. *In Proc. Beltwide Cotton Conf., San Antonio, TX. 4-8 Jan. 2000. Natl. Cotton Counc. Am., Memphis, TN.*

Hagerty, A. M., S. G. Turnipseed, and M. J. Sullivan. 2000. Impact of beneficial arthropod conservation in *B. t.* and conventional cotton. p. 976-978. *In Proc. Beltwide Cotton Conf.*, San Antonio, TX. 4-8 Jan. 2000. Natl. Cotton Counc. Am., Memphis, TN.

Jackson, R. E., J. R. Bradley, Jr., F. Gould, and J. W. Van Duyn. 2002. Genetic variation for resistance to cry1Ac and cry2Ab in bollworm, *Helicoverpa zea*, in North Carolina. *In Proc. Beltwide Cotton Conf.*, Atlanta, GA. 8-12 Jan. 2002. Natl. Cotton Counc. Am., Memphis, TN.

Lambert, A. L., J. R. Bradley, Jr., and J. W. Van Duyn. 1996. Effects of natural enemy conservation and planting date on the susceptibility of *Bt* cotton to *Helicoverpa zea* in North Carolina. p. 931-935. *In Proc. Beltwide Cotton Conf.*, Nashville, TN. 9-12 Jan. 1996. Natl. Cotton Counc. Am., Memphis, TN.

Lambert, A. L., J. R. Bradley, Jr., and J. W. Van Duyn. 1997. Interactions of *Helicoverpa zea* and *Bt* cotton in North Carolina. p. 870-873. *In Proc. Beltwide Cotton Conf.*, New Orleans, LA. 7-10 Jan. 1997. Natl. Cotton Counc. Am., Memphis, TN.

Mahaffey, J. S., J. S. Bachelier, J. R. Bradley, Jr., and J. W. Van Duyn. 1994. Performance of Monsanto's transgenic *B.t.* cotton against high populations of lepidopterous pests in North Carolina. p. 1061-1063. *In Proc. Beltwide Cotton Conf.*, San Diego, CA. 5-8 Jan. 1994. Natl. Cotton Counc. Am., Memphis, TN.

Mahaffey, J. S., J. R. Bradley, Jr., and J. W. Van Duyn. 1995. *B. t.* cotton: field performance in North Carolina under conditions of unusually high bollworm populations. p. 795-798. *In* Proc. Beltwide Cotton Conf., San Antonio, TX. 4-7 Jan. 1995. Natl. Cotton Counc. Am., Memphis, TN.

SAS Institute. 1990. SAS/STAT User's Guide, Vol. 2. SAS Institute, Cary, NC, 795 pp.

Scientific Advisory Panel, Subpanel on *Bacillus thuringiensis* (Bt) Plant-Pesticides, February 9-10, 1998. 1998. Transmittal of the final report of the FIFRA Scientific Advisory Panel, Subpanel on *Bacillus thuringiensis* (Bt) Plant-Pesticides and Resistance Management, Meeting held on February 9-10, 1998. (Docket Number: OPPTS-00231).

Stewart, S. D., J. J. Adamczyk, Jr., K. S. Knighten, and F. M. Davis. 2001. Impact of Bt cottons expressing one or two insecticidal proteins of *Bacillus thuringiensis* Berliner on growth and survival of Noctuid (Lepidoptera) larvae. *J. Econ. Entomol.* 94: 752-760.

Stone, T. B., and S. R. Sims. 1993. Geographic susceptibility of *Heliothis virescens* and *Helicoverpa zea* (Lepidoptera: Noctuidae) to *Bacillus thuringiensis*. *J. Econ. Entomol.* 86: 989-994.

Table 1. Estimated mean (SE) numbers of live bollworm larvae (L4-L5), damaged bolls, and bollworm adults produced on a per hectare basis by untreated conventional, Bollgard, and Bollgard II cotton genotypes averaged across three locations in North Carolina. 2000-2001.

Genotype	No. Live Larvae per Hectare ^a	No. Damaged Bolls per Hectare ^a	No. Adults per Hectare ^a
Conventional (DP50)	19,562 (4,060) a	317,175 (39,330) a	19,562 (4,060) a
Bollgard (DP50B)	6,907 (1,408) a	55,708 (5,585) b	1,115 (341) b
Bollgard II (DP50BX)	762 (347) b	5,540 (985) c	247 (146) b

^a/ Means within the same column followed by the same letter are not significantly different, Fisher's Protected Least Significant Difference test ($P \leq 0.05$).

Table 2. Estimated mean (SE) numbers of bollworm larvae (L4-L5) per hectare produced by conventional, Bollgard, and Bollgard II cotton genotypes under pyrethroid-treated and untreated conditions averaged across three sample dates in Edgecombe Co., North Carolina. 2001.

Genotype	Insecticide Regime		Mean ^a
	Insecticide-treated	Untreated	
Conventional (DP50)	11,331 (2,587)	24,632 (5,845)	17,981 (3,419) a
Bollgard (DP50B)	756 (419)	5,138 (1,222)	2,946 (780) b
Bollgard II (DP50BX)	190 (190)	386 (261)	288 (159) c
Mean ^a	4,092 (1,213) b	10,052 (2,624) a	

^a/ Means within the same column or row followed by the same letter are not significantly different, Fisher's Protected LSD ($P \leq 0.05$).

Table 3. Estimated mean (SE) numbers of bollworm-damaged bolls per hectare in conventional, Bollgard, and Bollgard II cotton genotypes under pyrethroid-treated and untreated conditions averaged across three sample dates in Edgecombe Co., North Carolina. 2001.

Genotype	Insecticide Regime		Mean ^a
	Insecticide-treated	Untreated	
Conventional	89,752 (9,592)	195,528 (10,610)	142,640 (13,059) a
Bollgard	10,373 (1,820)	36,876 (4,146)	23,624 (3,541) b
Bollgard II	2,852 (779)	4,162 (1,168)	3,507 (700) c
Mean ^a	34,326 (7,362) b	78,855 (14,605) a	

^a/ Means within the same column or row followed by the same letter are not significantly different, Fisher's Protected LSD ($P \leq 0.05$).

Table 4. Estimated mean (SE) numbers of bollworm pupae per hectare produced by conventional, Bollgard, and Bollgard II cotton genotypes under pyrethroid-treated and untreated conditions averaged across three sample dates in Edgecombe Co., North Carolina. 2001.

Genotype	Insecticide Regime		Mean ^a
	Insecticide-treated	Untreated	
Conventional	10,994 (2,541)	24,632 (5,846)	17,813 (3,426) a
Bollgard	395 (267)	1,279 (414)	837 (258) b
Bollgard II	0 (0)	386 (261)	193 (134) b
Mean ^a	3,796 (1,194) b	8,766 (2,684) a	

^a/ Means within the same column or row followed by the same letter are not significantly different, Fisher's Protected LSD ($P \leq 0.05$).

Table 5. Estimated mean (SE) numbers of bollworm adults per hectare produced by conventional, Bollgard, and Bollgard II cotton genotypes under pyrethroid-treated and untreated conditions averaged across three sample dates in Edgecombe Co., North Carolina. 2001.

Genotype	Insecticide Regime		Mean ^a
	Insecticide-treated	Untreated	
Conventional	8,600 (2,114)	24,632 (5,846)	16,616 (3,469) a
Bollgard	214 (214)	737 (315)	475 (194) b
Bollgard II	0 (0)	386 (261)	193 (134) b
Mean ^a	2,938 (965) b	8,585 (2,698) a	

^a/ Means within the same column or row followed by the same letter are not significantly different, Fisher's Protected LSD ($P \leq 0.05$).

CHAPTER IV

GENETIC VARIATION FOR RESISTANCE TO CRY1AC AND CRY2AB PROTEINS IN *HELICOVERPA ZEA* (LEPIDOPTERA: NOCTUIDAE)

Abstract

Adult female bollworms, *Helicoverpa zea* (Boddie), were collected from light traps at various locations near the Tidewater Research Station in Washington Co., NC in 2001. Progeny from these moths (lines) were screened for growth rate and survival on regular artificial diet and on diets containing 5.0 µg/ml of either Cry1Ac or Cry2Ab *Bacillus thuringiensis* Berliner toxin to isolate individuals carrying *B. t.* resistance genes from within a field population of bollworms. Lines that performed within the upper and lower quartiles on each *B. t.* diet were reared to the adult stage and reciprocal crosses were performed to determine inheritance of resistance and the influence of maternal vigor effects on larval performance. Out of 2,244 genomes tested, none were found to possess major *B. t.* resistance genes. However, differences in larval growth rates among specific reciprocal crosses demonstrated that there is significant quantitative genetic variation for resistance to both the Cry1Ac and Cry2Ab toxins. Results from these crosses also suggested that inheritance of resistance to both Cry1Ac and Cry2Ab toxins was partially dominant. Stable carbon isotope analyses indicated that individuals whose maternal parent developed as a larva on a C₃ host or on a C₄ host performed similarly on diets containing Cry1Ac and Cry2Ab toxins.

The commercialization of transgenic cottons, *Gossypium hirsutum* (L.), expressing the Cry1Ac δ -endotoxin derived from the common soil bacterium, *Bacillus thuringiensis* ssp. *kurstaki* Berliner has offered producers a novel method of insect pest management. These genetically altered cottons provide unequivocal control of tobacco budworm, *Heliothis virescens* (Fab.), but control of bollworm, *Helicoverpa zea* (Boddie), is often incomplete. There is concern that this technology may decline in efficacy (Tabashnik 1994; Gould 1998) due to evolution of increasing tolerance of bollworm to the Cry1Ac endotoxin expressed by currently available *B. t.* cottons (Stone and Sims 1993).

In order for *B. t.* resistance evolution to be successfully prevented in field populations of bollworm, *B. t.* cottons must express a high dose of toxin to kill susceptible and heterozygous resistant insects, inheritance of resistance must be recessive or partially recessive, resistance alleles must be rare in the general bollworm population, or adequate refuges must be available for production of non-selected bollworms. Field studies from North Carolina reported moderate levels of surviving bollworm larvae in *B. t.* cottons that confirmed the suggestion from the Scientific Advisory Panel (1998) that *B. t.* cottons did not express a high dose of *B. t.* endotoxin relative to the natural level of bollworm tolerance (Lambert et al. 1996, 1997; Mahaffey et al. 1994, 1995). Burd et al. (2000) showed that bollworm resistance to the Cry1Ac endotoxin was inherited as a partially dominant trait, and the frequency of *B. t.* resistance alleles in the general bollworm population was determined to be higher than anticipated (Burd et al. in press). With various parameters of the “high dose strategy” for *B. t.* resistance management being violated by bollworm, it is imperative that appropriate resistance management strategies be operative for the *B. t.* technology to be sustainable.

The purpose of the current study was to determine if the frequency of *B. t.* resistance alleles in the general bollworm population had changed from 2000 to 2001 and to determine the effects of minor *B. t.* resistance genes on larval growth and survival in the presence of Cry1Ac and Cry2Ab toxins.

Materials and Methods

A total of 2,483 female bollworm moths were collected from light-traps at four locations in eastern North Carolina from August-October in 2001. Moths were then placed individually into a 237-ml clear plastic cup and covered with an oviposition substrate (cheesecloth) and held in rearing facilities at North Carolina State University at 27-30°C, 55-60% relative humidity, and a photoperiod of 14:10 light:dark hours. Cheesecloths were checked daily for the presence of eggs.

Upon larval hatch, 24 neonates from each female line were placed onto each of three different artificial diets; these diets were non-*B. t.* (NBT) (Gould et al. 1995), Cry1Ac-containing, and Cry2Ab-containing diets. The concentration of both *B. t.* proteins was 5.0 µg per milliliter of diet. Cry1Ac protein (MVP[®] II, Mycogen Corp., San Diego, CA) was obtained as a gift, but Cry2Ab protein was obtained through freeze-dried Cry2Ab-producing corn tissue powder. The concentration of Cry2Ab protein in the corn tissue powder was determined by a sensitive quantitative bioassay as described by Greenplate (1999). The artificial diet was poured into 24-well plastic bioassay plates (Corrigan and Company, Inc., Jacksonville, FL) and then refrigerated before use. A single neonate was placed into each diet well with a fine camel hair paintbrush, and plates were heat-sealed with Mylar[®] (Clear Lam Packaging, Elk Grove Village, IL) film. Insect pins were used to make holes in the Mylar film above each well to allow air exchange. Larvae were scored for survival and

developmental stage after seven days. Instar was determined based on head capsule and body size (Neunzig 1969). All growth stage values were converted to an ordinal ranking system (Table 1).

For those lines whose performance ranked in the upper or lower quartile for either of the *B. t.* diets, larvae from the NBT diet were reared to the adult stage. Reciprocal crosses were made between adults from lines that ranked in the highest and lowest quartiles for each diet. HxH and LxL crosses were also conducted. Neonates from successful crosses were assayed on the appropriate *B. t.* diet and weighed after 10 days.

Adult females whose progeny were tested in the original *B. t.* performance screen were placed individually into glass scintillation vials and frozen. One forewing from each moth was removed and placed into a 2.5ml plastic vial. The wing was then cut into three pieces and placed into a 0.2 cm², tared tin cup. The tin cup was then closed and formed into a small square that could fit into an autosampler. Samples to be analyzed were sent to the stable isotope research laboratory at the University of Georgia. Moth samples were analyzed using a gas chromatograph to combust each wing to form CO₂. The CO₂ was collected using a liquid-nitrogen trapping system and Pyrex breakseal tubes. Tubes were then loaded into a mass spectrophotometer for isotope analysis. Companion standards with known isotope ratios were tested along with the experimental samples. Data from the analysis were correlated to the average growth rating of the subsequent progeny on both Cry1Ac and Cry2Ab diets.

PROC UNIVARIATE was used to determine average ordinal ranking for each single female family on each of the three diets (SAS Institute 1990). Only survivors were used in determining the average ordinal ranking of each family. PROC CORR was also used to

determine any correlation between instar size and percent survivorship on any of the three diets. To control for vigor effects, average ratings for all families on either *B. t.* diet were corrected according to the highest rated family on NBT diet. Average ratings on *B. t.* diets were corrected according to the formula: Average Corrected Rating = [Average Rating (*B. t.*) / (Average Rating (NBT) / Highest Average Rating (NBT))]. Mean larval weights from various selection experiments were separated using Fisher's Protected Least Significant Difference test with PROC GLM (SAS Institute 1990).

Results

Distribution of average ratings for each female line on Cry1Ac-containing, Cry2Ab-containing, and NBT diet is shown in Figure 1. The average rating at seven days for all larvae on Cry1Ac-containing diet was 4.4, which ranged between late 2nd and early 3rd instar. The average rating for all larvae on Cry2Ab-containing diet was 4.9, which is almost an early 3rd instar. Larvae on NBT diet averaged between mid 4th and late 4th instar with an average rating of 9.4.

There was a significant correlation between average ratings of female lines on Cry1Ac and Cry2Ab diets ($P < 0.0001$) (Table 2) indicating that families with larger than average individuals on one *B. t.* diet had larger than average individuals on the other diet as well. There was no significant correlation between average ratings when comparing Cry1Ac versus NBT ($P = 0.2590$); however, a significant correlation did exist between average ratings when comparing Cry2Ab versus NBT ($P = 0.0044$).

Correlation analyses for average ratings that were corrected for vigor when comparing either *B. t.* diet versus NBT diet are illustrated in Table 2. The positive correlation between Cry1Ac and Cry2Ab diets remained when examining corrected ratings

($P < 0.0001$); however, the insignificant correlation ($P = 0.2590$) between ratings of families on Cry1Ac and NBT diets became a highly significant ($P < 0.0001$) negative correlation. In addition, the significant negative correlation ($P = 0.0044$) between ratings of families on Cry2Ab and NBT diets became increasingly significant ($P < 0.0001$) as a negative correlation. Correlation graphs of average corrected ratings for each diet comparison are illustrated in Figure 2.

There was also a significant positive correlation for percent survival of families when comparing Cry1Ac versus Cry2Ab ($P < 0.0001$) (Table 2 and Figure 3) indicating those families with higher survival on one *B. t.* diet had higher survival on the other *B. t.* diet as well. There was no significant correlation when comparing percent survival for families on either Cry1Ac or Cry2Ab versus those on NBT diet ($P = 0.1241$ and 0.3277 , respectively) (Table 2). A positive correlation was also found between percent survival and average ratings for families on Cry1Ac, Cry2Ab, and NBT diets ($P < 0.0001$ for each) (Table 2 and Figure 4) suggesting that as average family rating increased so did percent survival for families on these diets.

To determine if single female lines with growth in the highest 25% of all 561 single female lines on Cry1Ac and Cry2Ab were genetically different from lines with growth in the lowest 25%, lines within these ranges were used to perform reciprocal crosses. The upper quartile rating for growth on Cry1Ac-containing diet was 5.0 (early 3rd instar), while the lower quartile rating was 4.0 (late 2nd instar). Mean ratings for single female lines used to perform reciprocal crosses were 5.4 for upper quartile lines and undefined for lower quartile lines since lines that represented the lower quartile had no survivors on Cry1Ac-containing diet. The upper quartile growth rating for lines on Cry2Ab-containing diet was 6.0 compared

to 4.0 for lower quartile lines. Mean growth ratings for lines within these ranges used for reciprocal crosses were 6.0 for the upper quartile lines and 2.9 for the lower quartile lines.

Seven and eight successful crosses were obtained for Cry1Ac and Cry2Ab, respectively, for use in the reciprocal cross study. Successful crosses for Cry1Ac screening were as follows: 2 resistant crosses (RxR), 1 resistant & x susceptible % cross (RxS), 2 susceptible & x resistant % crosses (SxR), and 2 susceptible crosses (SxS). Cry2Ab crosses that were successful were: 2 resistant crosses, 2 resistant & x susceptible % crosses, 3 susceptible & x resistant % crosses, and 1 susceptible cross.

Average weights of larvae from RxR, RxS, SxR, and SxS crosses and percent survival after rearing on Cry1Ac-containing diet for 10 days are shown in Figure 5. The average (SE) larval weight for the RxR lines was 19.4 (0.99) milligrams compared to 6.3 (0.86) milligrams for the SxS lines. Average larval weights for the R & x S % and S & x R % crosses were 15.2 (1.51) and 12.7 (0.97) milligrams, respectively. Distributions of log weights for each cross on Cry1Ac-containing diet can be found in Figure 6. Average larval weights from crosses of RxR, RxS, SxR, and SxS crosses after rearing on Cry2Ab-containing diet are shown in Figure 5. The average (SE) larval weight for the RxR, RxS, SxR, and SxS crosses were 14.6 (1.38), 14.6 (1.07), 13.9 (0.78), and 7.95 (1.19), respectively. Figure 7 shows distributions of log weights for each cross on Cry2Ab-containing diet

A subsample of 74 female moths was analyzed to determine stable carbon isotope ratios for each moth. Approximately 34% of these individuals developed as larvae on a C₄ host. This C₄ host was most likely field corn, since this crop host is the most prevalent C₄ host in the areas in which adult females were collected. Correlation analyses for average ratings for families on Cry1Ac, Cry2Ab, and NBT diets versus ¹³C ratios of the

corresponding female parent are shown in Figure 8. Progeny of individuals that developed on C₄ hosts as larvae had similar average ratings on Cry1Ac and Cry2Ab diets as compared to progeny of those that developed as larvae on C₃ hosts. However, several families whose maternal parent developed on a C₃ host had an average rating of zero, which indicates that there were no survivors for that family. All C₃ and C₄ families on NBT diet performed similarly with respect to average ratings.

Discussion

Field-collected single female lines in 2000 were tested on Cry1Ac and on Cry2Aa by Burd et al. (in press). In that study, adults from the line that grew best on Cry1Ac were mated to adults from a line that performed very poorly on Cry1Ac, and progeny were tested for growth on Cry1Ac to confirm that the rare (0.00043) best-performing line had heritable Cry1Ac resistance. Although that work proved the existence of a rare major gene for resistance, no crosses were conducted to determine if smaller differences among other female lines were genetically based. Because Bollgard cottons do not produce a high dose for bollworm, larvae with minor resistance genes would be selected for and could, over time, decrease the efficacy of Bollgard cottons. The present study demonstrated that single female lines with growth in the highest 25% of all 561 single female lines exposed to *B. t.* toxins were genetically different from lines whose growth was in the lowest 25%. The fact that larvae from the SE x RI Γ crosses performed better than the larvae of the SxS crosses proved that the difference was not due to a maternal effect. This demonstration of heritable variation for minor resistance genes indicates that resistance monitoring efforts for bollworm and other pests exposed to moderate doses of *B. t.* toxins should not search only for major genes.

Accumulation of minor resistance genes within a bollworm population may result in a gradual decline of *B. t.* cotton efficacy.

A comparison of Figure 1 from the present study and the two related figures in Burd et al. (in press) show no substantial change in the shape of the growth distributions for single female families on either Cry1Ac or Cry2A. This indicates that if there has been an increase in the frequency of minor resistance genes in the general *H. zea* population it is too small to detect, despite screening over 500 females in each season. Furthermore, Burd et al. (in press) found only a single female line that did better than all other lines on Cry1Ac and Cry2Aa due to a genetic factor; we found no lines of this type. Had alleles for this major genetic factor increased substantially between the 2000 and 2001 season, we would have expected to find at least one such line. Thus, our data indicate that resistance to *B. t.* toxins in field populations of bollworm has not measurably increased.

The positive correlations between average ratings, average corrected ratings, and percent survival for families on Cry1Ac versus those on Cry2Ab confirm that families who performed well on one *B. t.* diet also did well on the other *B. t.* diet; these results agree with those produced by Burd et al. (in press). In addition, the significant positive correlations between percent survival and average ratings for families on the two *B. t.* diets (Figure 4) indicate that individuals possessing resistance alleles will grow larger and have higher survival rates when selected on the appropriate *B. t.* toxin, which also supports results produced by Burd (2001).

Based on correlation analyses from the average ratings and average corrected ratings, vigor effects did not influence the above average performance of certain families on either *B. t.* diet. A significant negative correlation, however, was evident between average corrected

ratings for families on both *B. t.* diets versus average ratings for families on NBT diet. These results suggest that those families that performed well on either *B. t.* diet demonstrated below average growth on the NBT diet. However, as seen in Figure 2, a few families influenced this significant negative correlation when using average corrected ratings; thus, there was an over compensation when the average ratings were corrected according to performance on NBT diet. Results from Burd et al. (2001) compared to those in this study indicate that performance on NBT diet have little or no effect on performance on either *B. t.* diet.

The reciprocal cross study (Figure 4) suggests that resistance to Cry1Ac toxin is dominantly or incompletely dominantly inherited, which is consistent with previous studies (Burd et al. 2000, in press). Results from the reciprocal cross study on Cry2Ab-containing diet (Figure 4) indicate that inheritance of resistance to Cry2Ab is dominant as well.

The stable carbon isotope analyses revealed that ~34% of the *H. zea* adults collected from late August through mid-September developed as larvae on C₄ hosts. This suggests that field corn may serve as a refuge for those adults emerging from *B. t.* cottons late in the season in North Carolina. Analyses also demonstrated that progeny from individuals that develop as larvae on a C₃ host are likely to perform similarly in the presence of a *B. t.* toxin as compared to individuals whose maternal parent developed as a larva on C₄ hosts. These results suggest that the *H. zea* adults that originated from C₃ hosts most likely developed on soybeans, peanuts, non-*B. t.* cotton, or broadleaf weeds, rather than on *B. t.* cotton.

Results provided herein may enable an improved quantification of parameters involved in modeling the evolution of *B. t.* resistance in bollworm. As suggested in this study and previous experiments, higher than anticipated resistance gene frequencies, in

addition to dominant or incompletely dominant inheritance, demand proper deployment of *B. t.* cottons and adequate refuges to sustain the usefulness of the *B. t.* technology.

Acknowledgments

The authors express appreciation to Cotton, Inc. for providing a graduate research assistantship for the senior author and to Monsanto Co. for providing partial project funding. Special thanks to Rogan Jackson, Margery Ambrose, Carlton Purvis, and Juan Cabrera for technical assistance.

Literature Cited

Burd, A. D., J. R. Bradley, Jr., J. W. Van Duyn, and F. Gould. 2000. Resistance of bollworm, *Helicoverpa zea*, to CryIA(c) toxin. p. 923-926. *In Proc. Beltwide Cotton Conf.*, San Antonio, TX. 4-8 Jan. 2000. Natl. Cotton Counc. Am., Memphis, TN.

Burd, A. D., F. Gould, J. R. Bradley, Jr., J. W. Van Duyn, and W. J. Moar. Estimated frequency of non-recessive *B.t.* resistance genes in bollworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), in eastern North Carolina. In press. *J. Econ. Entomol.*

Burd, A. D. 2001. The influence of environmental factors on susceptibility of *B.t.* cottons to bollworm, *Helicoverpa zea*, and factors affecting resistance to *B.t.* toxins for bollworm. Ph.D. Dissertation. 101 pp.

Gould, F., A. Anderson, A. Reynolds, L. Bumgarner, and W. Moar. 1995. Selection and genetic analysis of a *Heliothis virescens* (Lepidoptera: Noctuidae) strain with high levels of resistance to *Bacillus thuringiensis* toxins. *J. Econ. Entomol.* 88: 1545-1559.

Gould, F. 1998. Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. *Annu. Rev. Entomol.* 43: 701-726.

Greenplate, J. T. 1999. Quantification of *Bacillus thuringiensis* insect control protein Cry1Ac over time in Bollgard cotton fruit and terminals. *J. Econ. Entomol.* 92: 1377-1383.

Lambert, A. L., J. R. Bradley, Jr., and J. W. Van Duyn. 1996. Effects of natural enemy conservation and planting date on the susceptibility of *Bt* cotton to *Helicoverpa zea* in North Carolina. p. 931-935. *In Proc. Beltwide Cotton Conf.*, Nashville, TN. 9-12 Jan. 1996. Natl. Cotton Counc. Am., Memphis, TN.

Lambert, A. L., J. R. Bradley, Jr., and J. W. Van Duyn. 1997. Interactions of *Helicoverpa zea* and *Bt* cotton in North Carolina. p. 870-873. *In Proc. Beltwide Cotton Conf.*, New Orleans, LA. 7-10 Jan. 1997. Natl. Cotton Counc. Am., Memphis, TN.

Mahaffey, J. S., J. S. Bachelier, J. R. Bradley, Jr., and J. W. Van Duyn. 1994. Performance of Monsanto's transgenic *B.t.* cotton against high populations of lepidopterous pests in North Carolina. p. 1061-1063. *In Proc. Beltwide Cotton Conf.*, San Diego, CA. 5-8 Jan. 1994. Natl. Cotton Counc. Am., Memphis, TN.

Mahaffey, J. S., J. R. Bradley, Jr., and J. W. Van Duyn. 1995. *B. t.* cotton: field performance in North Carolina under conditions of unusually high bollworm populations. p. 795-798. *In Proc. Beltwide Cotton Conf.*, San Antonio, TX. 4-7 Jan. 1995. Natl. Cotton Counc. Am., Memphis, TN.

Neunzig, H. H. 1969. The biology of the tobacco budworm and the corn earworm in North Carolina with particular reference to tobacco as a host. North Carolina Agricultural Experiment Station. Tech. Bul. No. 196. 63 pp.

SAS Institute. 1990. SAS/STAT User's Guide, Vol. 2. SAS Institute, Cary, NC, 795 pp.

Scientific Advisory Panel, Subpanel on *Bacillus thuringiensis* (Bt) Plant-Pesticides, February 9-10, 1998. 1998. Transmittal of the final report of the FIFRA Scientific Advisory Panel, Subpanel on *Bacillus thuringiensis* (Bt) Plant-Pesticides and Resistance Management, Meeting held on February 9-10, 1998. (Docket Number: OPPTS-00231).

Stone, T. B., and S. R. Sims. 1993. Geographic susceptibility of *Heliothis virescens* and *Helicoverpa zea* (Lepidoptera: Noctuidae) to *Bacillus thuringiensis*. J. Econ. Entomol. 86: 989-994.

Tabashnik, B. E. 1994. Evolution of resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol. 39: 47-79.

Table 1. Ordinal rating scale for *Helicoverpa zea* instar size after seven days on artificial diet.

Larval Size	Ordinal Ranking
1 st instar	1
Early 2 nd instar	2
Mid 2 nd instar	3
Late 2 nd instar	4
Early 3 rd instar	5
Mid 3 rd instar	6
Late 3 rd instar	7
Early 4 th instar	8
Mid 4 th instar	9
Late 4 th instar	10
Early 5 th instar	11

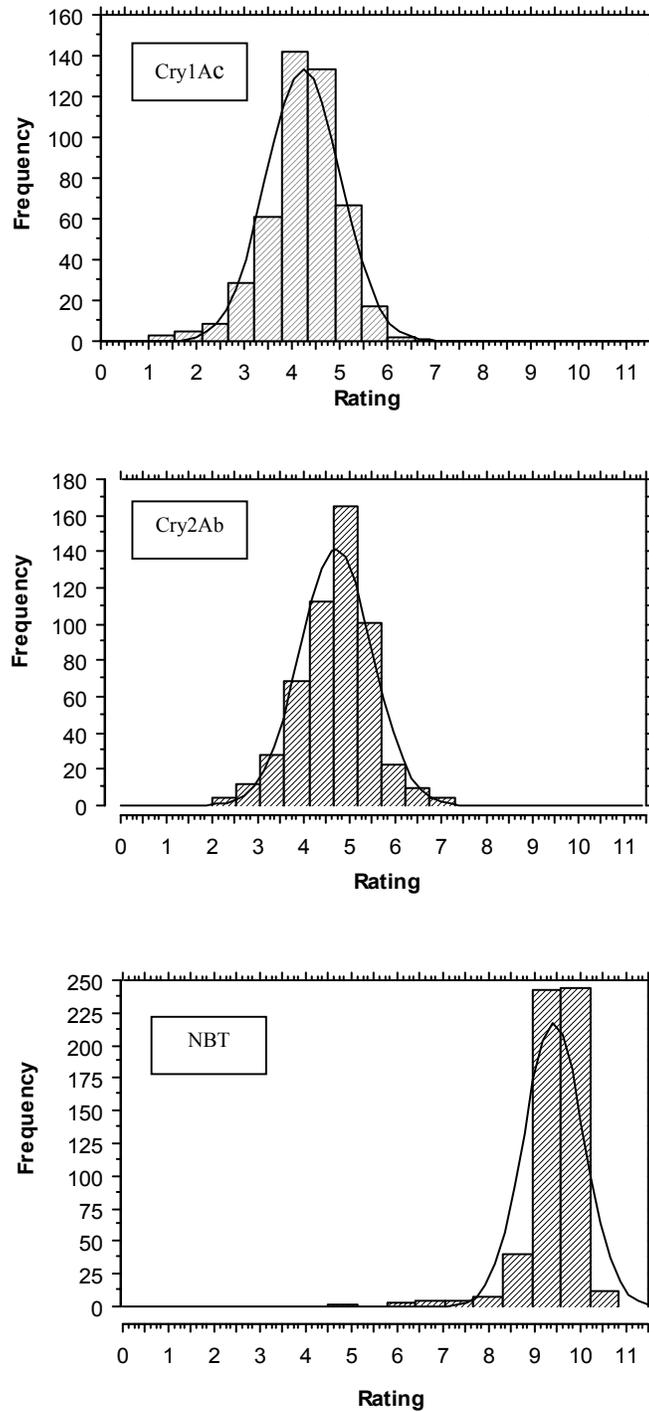


Figure 1. Distribution of average ratings for 7-day old larvae of *Helicoverpa zea* female lines on Cry1Ac, Cry2Ab, and NBT diets. Mean ratings are approximately 4.4, 4.9, and 9.4, which correspond to a late 2nd instar, early 3rd instar, and mid 4th instar, respectively.

Table 2. Correlation coefficients and *P*-values for various comparisons among bollworm families selected on Cry1Ac, Cry2Ab, and NBT artificial diets.

Comparison			Correlation Coefficient ^a	<i>P</i> -value ^b
Average Ratings for Families on Cry1Ac	vs	Average Ratings for Families on Cry2Ab	0.31805	<.0001
Average Ratings for Families on Cry1Ac	vs	Average Ratings for Families on NBT	-0.05233	0.2590
Average Ratings for Families on Cry2Ab	vs	Average Ratings for Families on NBT	0.12393	0.0044
% Survival for Families on Cry1Ac	vs	% Survival for Families on Cry2Ab	0.48531	<.0001
% Survival for Families on Cry1Ac	vs	% Survival for Families on NBT	0.06513	0.1241
% Survival for Families on Cry2Ab	vs	% Survival for Families on NBT	0.04139	0.3277
% Survival for Families on Cry1Ac	vs	Average Ratings for Families on Cry1Ac	0.28357	<.0001
% Survival for Families on Cry2Ab	vs	Average Ratings for Families on Cry2Ab	0.41630	<.0001
% Survival for Families on NBT	vs	Average Ratings for Families on NBT	0.44970	<.0001
Average Corrected Ratings for Families on Cry1Ac	vs	Average Corrected Ratings for Families on Cry2Ab	0.45346	<.0001
Average Corrected Ratings for Families on Cry1Ac	vs	Average Ratings for Families on NBT	-0.43698	<.0001
Average Corrected Ratings for Families on Cry2Ab	vs	Average Ratings for Families on NBT	-0.35763	<.0001

^a/ Pearson Correlation Coefficient

^b/ Probability > |*r*| under Ho: Rho=0

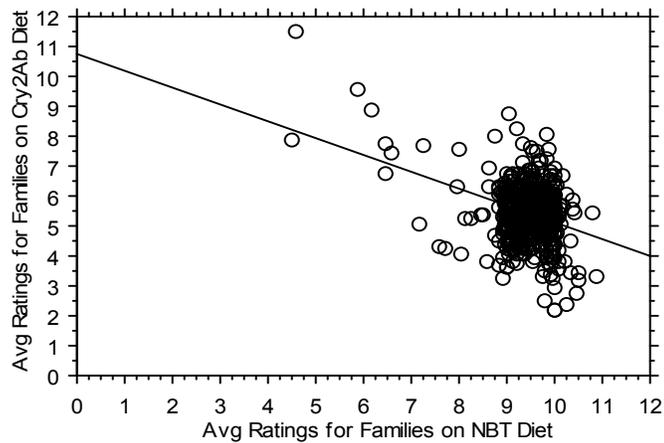
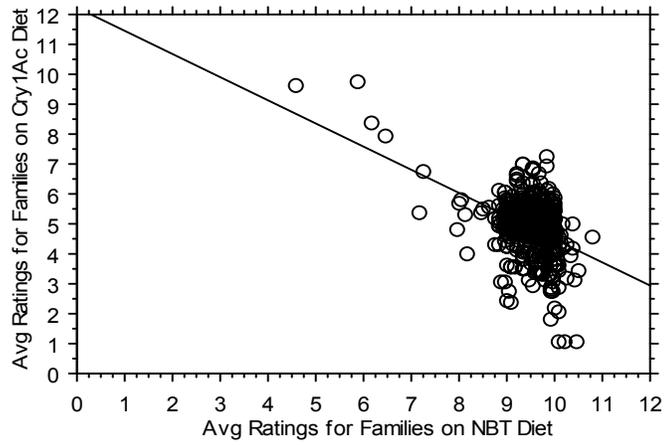
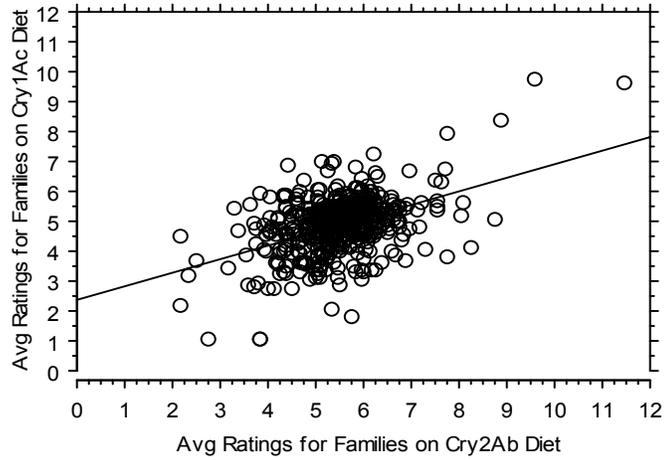


Figure 2. Correlation graphs for all comparisons among Cry1Ac, Cry2Ab, and NBT families with average corrected ratings plotted for each family.

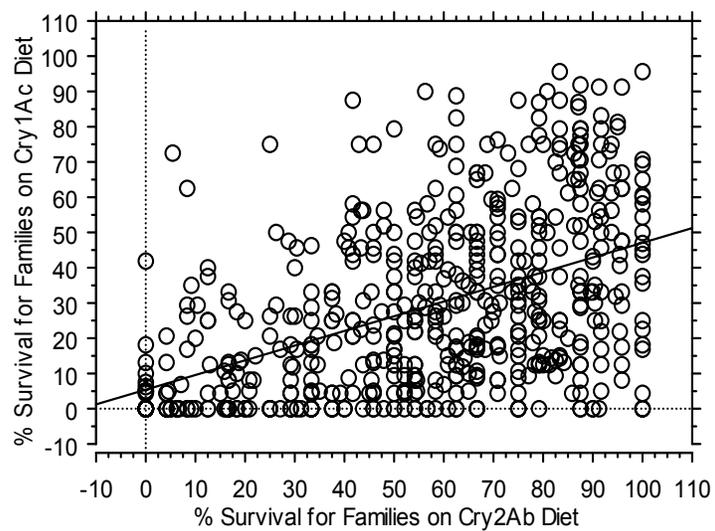


Figure 3. Correlation graph comparing percent survival for bollworm families tested on Cry1Ac and Cry2Ab artificial diets.

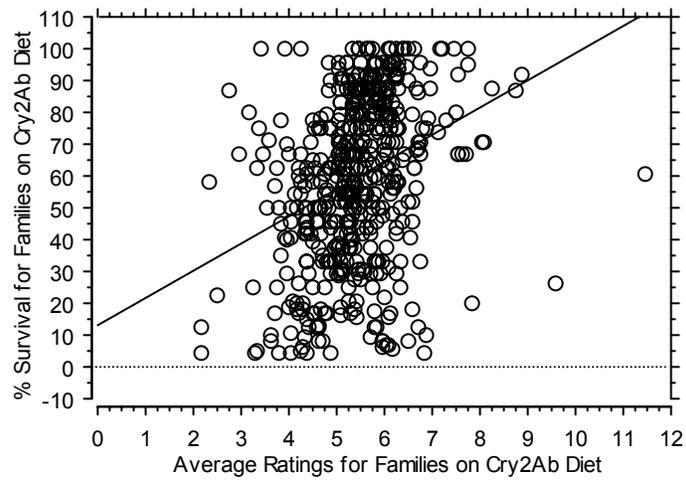
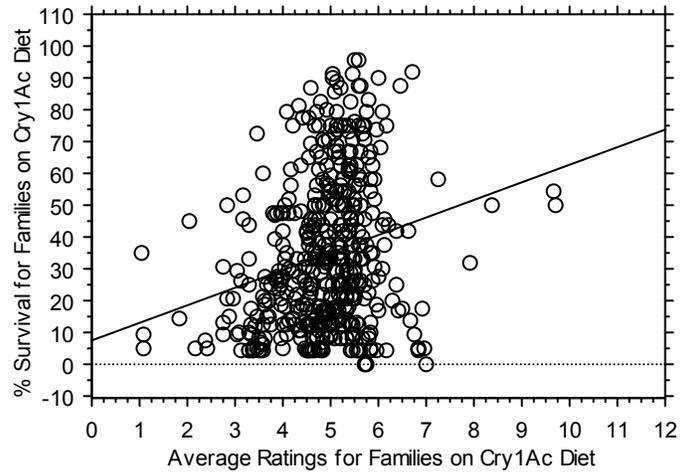


Figure 4. Correlation graphs for percent survival versus average corrected ratings for families tested on Cry1Ac and Cry2Ab artificial diets.

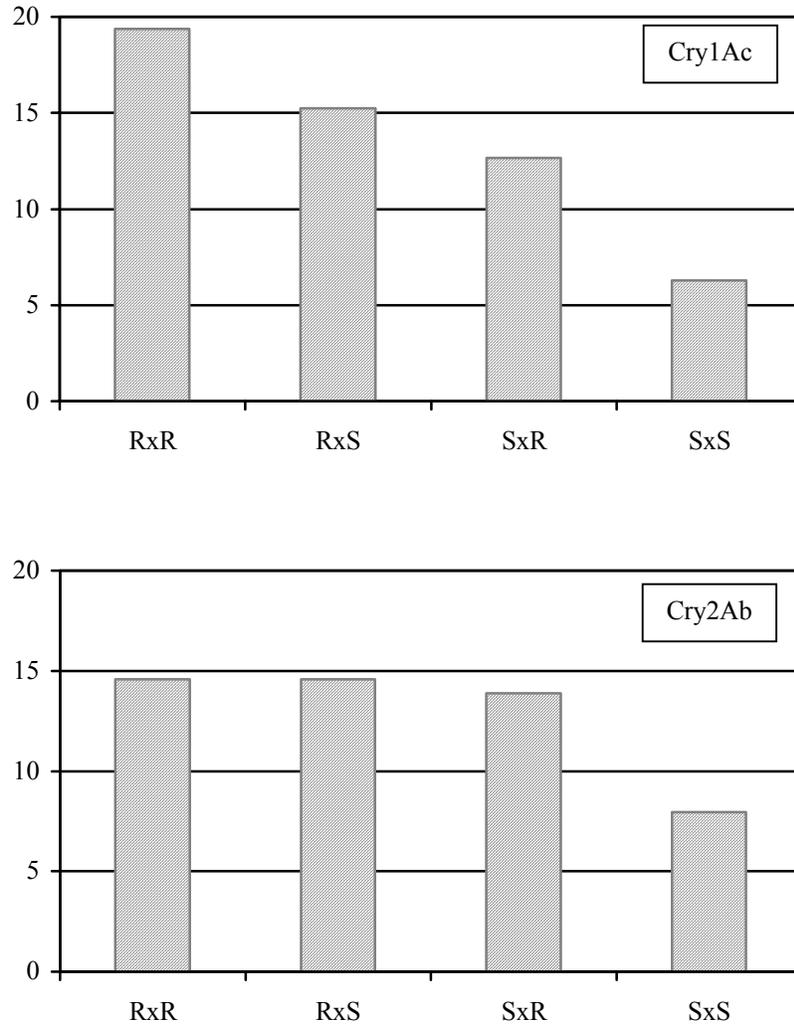


Figure 5. Mean larval weight in milligrams for *Helicoverpa zea* resistant line (RxR), control line (SxS), and reciprocal crosses between these lines (RxS and SxR) taken after ten days on 5.0 µg/ml of Cry1Ac-containing and Cry2Ab-containing diets, respectively.

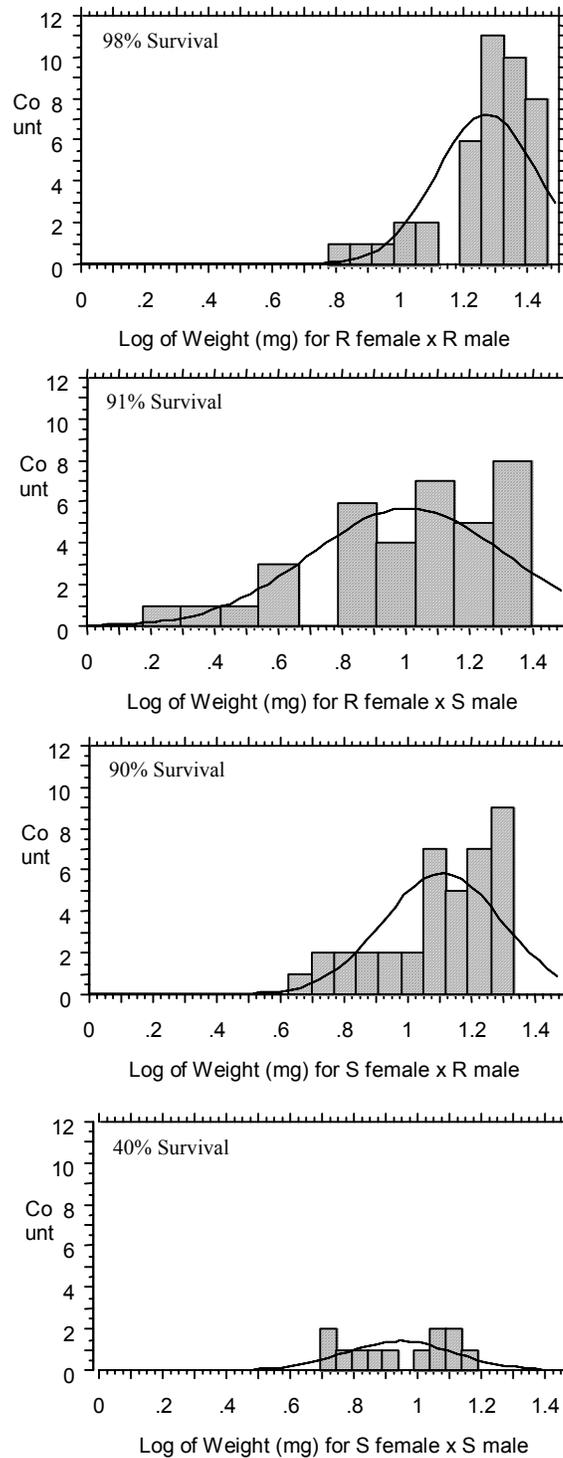


Figure 6. Histogram showing frequency of larval weights (\log_e) for upper quartile lines, lower quartile lines, and reciprocal F_1 crosses between these lines taken after ten days on 5.0 $\mu\text{g}/\text{ml}$ of Cry1Ac diet. Percent survival for each group is shown on each graph.

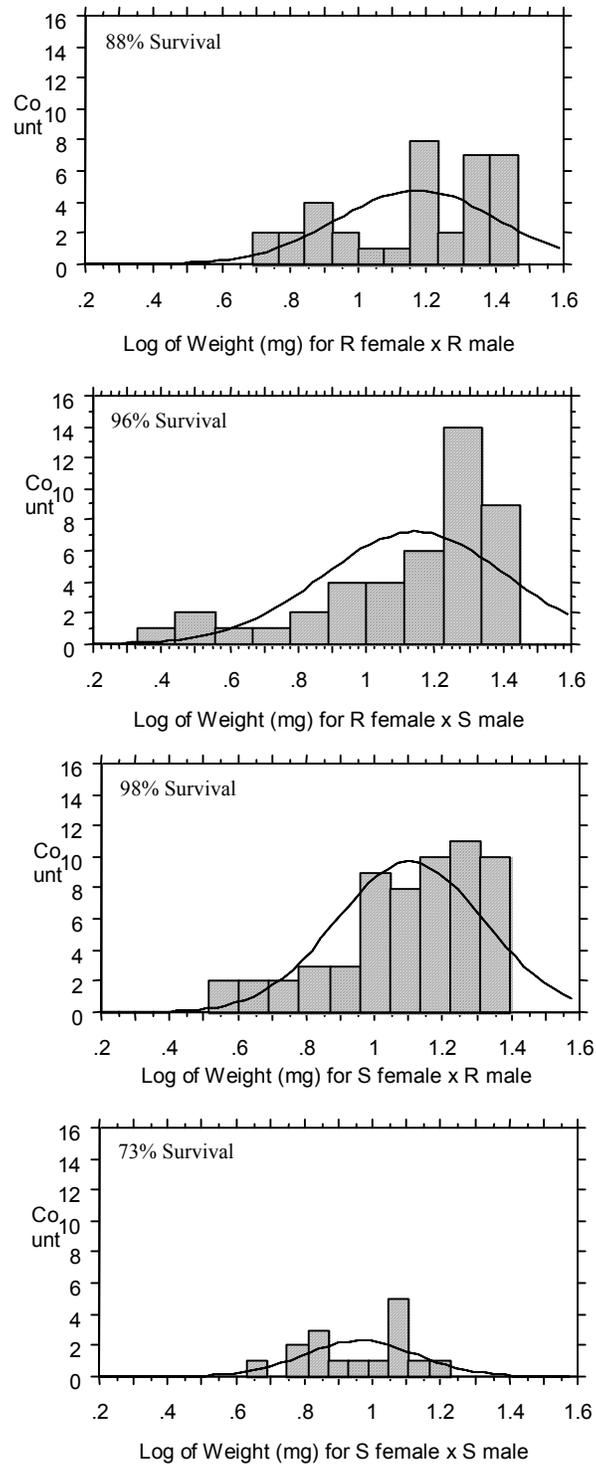


Figure 7. Histogram showing frequency of larval weights (\log_e) for upper quartile lines, lower quartile lines, and reciprocal F_1 crosses between these lines taken after ten days on 5.0 $\mu\text{g/ml}$ of Cry2Ab diet. Percent survival for each group is shown on each graph.

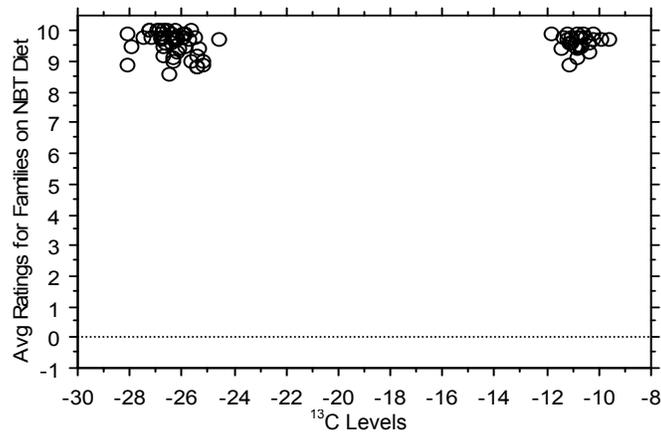
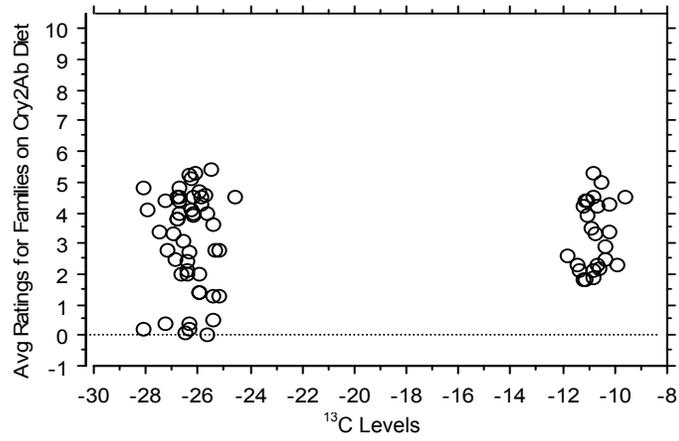
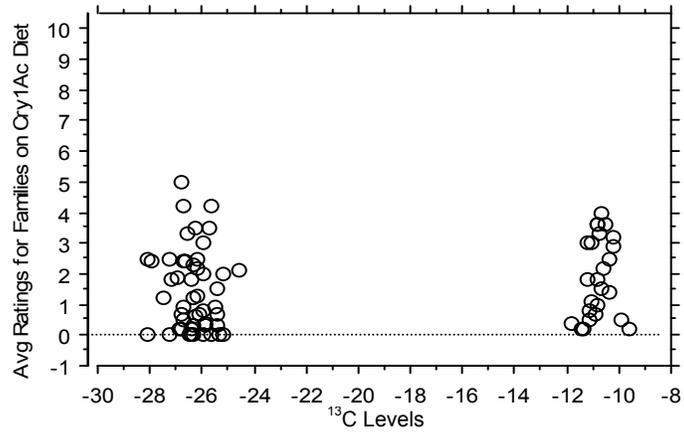


Figure 8. Correlation graphs for average ratings for families on Cry1Ac, Cry2Ab, and NBT artificial diets versus ^{13}C levels.