ABSTRACT

MARCUS, MARIA ADALITA. Fitness studies and cross resistance evaluations of an eastern North Carolina cotton bollworm strain (*Helicoverpa zea*) (Boddie) tolerant to the *Bacillus thuringiensis* delta endotoxin Cry1Ac. (Under the direction of Dr. J. R. Bradley Jr. and Dr. F. L. Gould)

A component of insect resistance management is the use of alternative insecticides to delay the onset of resistance to one type or class of toxin. Because cotton bollworm (*Helicoverpa zea*) larvae have been found to survive on Bollgard plants in the field, concern has been raised over the possible development of resistance to Cry1Ac. A Cry1Ac tolerant bollworm strain (XYZ) was initially collected from Bt cotton plants in eastern NC in summer 2002 and selected against Cry1Ac for 12 generations. Dose-mortality bioassays were conducted to determine response to selection, the highest LC_{50} recorded was 884.9 µg/ml at generation F_{12}. Cross resistance of this resistant strain was evaluated against Bt endotoxins Cry1Ab, Cry1F, and Cry2Ab. Cross paired matings were made for susceptible (HZ 02) and resistant (XYZ) bollworm strains to obtain F_1 neonates for testing against a five fold serial dilution insect diet blend with a concentration range of 0.32-1000 µg/ml for the Cry1Ac, Cry1Ab, Cry1F, and Cry2Ab toxins. Mortality and weights were assessed after a 10 day incubation period at 27°C and 14:10 L:D photophase. Based on mortality and growth results there was evidence of cross resistance for *H. zea* to Cry1Ab, negative cross resistance to Cry1F, and no cross resistance to Cry2Ab. Cotton plant tissue and surface treated diet bioassays were performed to determine the extent of cross resistance to the novel insecticidal protein Vip3A, in Cry1Ac tolerant tobacco budworm (*Heliothis virescens*) strains YHD2, KCBhyb, and CXC, and bollworm strain XYZ. Control *H. virescens* strain YDK and *H.zea* strain HZ 02 are susceptible to the delta endotoxin. All insect strains were

subjected to cotton plant tissue and surface treated diet assays containing the vegetative insecticidal protein, Vip3A. Purified Vip3A and Cry1Ac proteins were used in surface treated diet assays, the plant tissue assays included three types of insecticidal expression Vip3A, Cry1Ac, or Cry1Ac+Cry2Ab. Control material did not contain either of the insecticidal proteins. Surface treated diet evaluations indicate the budworm resistant strain, YHD2, had lower survival and lower average larval weight on Vip3A than the control strain, YDK. However, the KCBhyb strain which has been previously found to have cross-resistance had somewhat lower mortality and average higher weight than YDK on the Vip3A surface treated diet. The resistant *H. zea* strain XYZ, had lower mortality than HZ 02, the control strain, for the surface treated assay. However, HZ 02 had higher average weights than XYZ on Vip3A. Plant tissue bioassays based on mortality, leaf area consumption, and weight data showed the Cry1Ac tolerant budworm strains were not significantly different in mortality from the susceptible YDK strain when compared on cotton varieties expressing Vip3A protein. Similar findings in mortality and weight were observed for the H. zea control strain, HZ 02, compared to resistant strain XYZ based on plant tissue assay. Our preliminary results from both plant and diet bioassays indicate there is no strong cross resistance of the Cry1Ac resistance *H. virescens* or *H. zea* strains to Vip3A. Based on the results from this study, there are a number of insecticidal alternatives available to delay evolution of resistance in cotton bollworm and tobacco budworm to Bt cotton.

Widespread use of transgenic cotton Bollgard has raised concern for development of resistance in cotton bollworm (*Helicoverpa zea*). If there were a fitness cost present in individuals carrying the allele for Cry1Ac tolerance, a delay in resistance development

could be enhanced. Fitness comparisons between a Bt tolerant (XYZ) and control (HZ 02) bollworm strain were made through exposure to a technical grade pyrethroid, growth on unadulterated insect diet, and growth on secondary plant compound, gossypol. Intergenerational growth responses on an unadulterated diet were measured through larval weight for both *H zea* strains. Significant differences in weight between the two strains were not found. For the pyrethroid evaluations, third instar larvae of both strains over several generations were treated topically with 1 µl of technical grade cypermethrin and allowed to incubate at 27°C for 72 hours at which time mortality was assessed. Results indicate there were no statistically significant differences between the strains within generation. In the gossypol evaluations, first instar larvae were exposed to a diet incorporated blend of varying concentrations and allowed to incubate at 27°C and 14:10 L:D photoperiod. After 10 days larvae were weighed to assess growth and mortality was recorded. Mortality and growth results suggested no differences between the two strains. Fitness costs for cotton bollworm are not apparent for Cry1Ac resistant individuals. This information may be used in developing strategies for managing resistance to transgenic Bt crops.

FITNESS STUDIES AND CROSS RESISTANCE EVALUATIONS OF AN EASTERN NORTH CAROLINA COTTON BOLLWORM STRAIN (*HELICOVERPA ZEA*) (BODDIE) TOLERANT TO THE *BACILLUS THURINGIENSIS* DELTA-ENDOTOXIN CRY1AC

by

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APPROVED BY:

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BIOGRAPHY

Maria Adalita Ruiz Sims was born 20 December 1974 in Phoenix, Arizona. She is the eldest child of Ms. Marta Isabella Ruiz and Mr. Emmett Sims. Ms. Ruiz has been the primary parent of Maria and her two younger siblings, Hollis Ruiz and Sara Mc Near. Maria graduated from Sunnyside High School, Tucson, AZ in 1993. She attended the University of Arizona and in May of 1998 received her Bachelor of Science degree in Ecology and Evolutionary Biology.

Maria developed an interest in agricultural entomology and insecticide resistance management while managing the pink bollworm *Bt* resistance laboratory under the guidance of Dr. Timothy J. Dennehy at the Department of Entomology at the University of Arizona. During her time in Tucson she met Mr. Matthew Werner Marcus, an alumnus of the university in Geological Engineering. The two were married in Tucson, Arizona on 6 January 2002 and moved to Raleigh, NC in April 2002.

In August of 2002 Maria was accepted into the graduate program in the Department of Entomology at North Carolina State University under the direction of Drs. J. R. Bradley Jr., Fred L. Gould, and John W. Van Duyn.

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"If I appear great it is because I have stood on the shoulders of giants."

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

Chapter I

Chapter II

LIST OF FIGURES

Page

Chapter I

Chapter II

CHAPTER ONE

CROSS RESISTANCE EVALUATIONS OF CRY1AC TOLERANT HELIOTHINE STRAINS TO ENDOTOXINS CRY1AB, CRY1F, CRY2AB AND THE NOVEL VEGETATIVE INSECTICIDAL PROTEIN VIP3A

Abstract

A component of insect resistance management is the use of alternative insecticides to delay the onset of resistance to one type or class of toxin. Because cotton bollworm (*Helicoverpa zea*) larvae can survive on Bollgard plants in the field, concern has been raised over the possible development of resistance to Cry1Ac. A Cry1Ac tolerant bollworm strain (XYZ) was initially collected from Bt cotton plants in eastern NC in summer 2002 and selected against Cry1Ac for 12 generations. Dose-mortality bioassays were conducted to determine response to selection, the highest LC_{50} recorded was 884.9 µg/ml at generation F_{12}. Cross resistance of this resistant strain was evaluated against Bt endotoxins Cry1Ab, Cry1F, and Cry2Ab. Cross paired matings were made for susceptible (HZ 02) and resistant (XYZ) bollworm strains to obtain F_1 neonates for testing against a five fold serial dilution insect diet blend with a concentration range of 0.32-1000 µg/ml for the Cry1Ac, Cry1Ab, Cry1F, and Cry2Ab toxins. Mortality and weights were assessed after a 10 day incubation period at 27°C and 14:10 L:D photophase. Based on mortality and growth results there was evidence of cross resistance for *H. zea* to Cry1Ab, negative cross resistance to Cry1F, and no cross resistance to Cry2Ab. Cotton plant tissue and surface treated diet bioassays were performed to determine the extent of cross resistance to the novel insecticidal protein Vip3A, in Cry1Ac tolerant tobacco budworm (*Heliothis virescens*) strains YHD2, KCBhyb, and CXC, and bollworm strain XYZ. Control *H. virescens* strain YDK and *H.zea* strain HZ 02 are susceptible to the delta endotoxin. All insect strains were subjected to cotton plant tissue and surface treated diet assays containing the vegetative insecticidal protein, Vip3A. Purified Vip3A and Cry1Ac proteins were used in surface treated diet assays, the

plant tissue assays included three types of insecticidal expression Vip3A, Cry1Ac, or Cry1Ac+Cry2Ab. Control material did not contain either of the insecticidal proteins. Surface treated diet evaluations indicate the budworm resistant strain, YHD2, had lower survival and lower average larval weight on Vip3A than the control strain, YDK. However, the KCBhyb strain which has been previously found to have cross-resistance had somewhat lower mortality and average higher weight than YDK on the Vip3A surface treated diet. The resistant *H. zea* strain XYZ, had lower mortality than HZ 02, the control strain, for the surface treated assay. However, HZ 02 had higher average weights than XYZ on Vip3A. Plant tissue bioassays based on mortality, leaf area consumption, and weight data showed the Cry1Ac tolerant budworm strains were not significantly different in mortality from the susceptible YDK strain when compared on cotton varieties expressing Vip3A protein. Similar findings in mortality and weight were observed for the H. zea control strain, HZ 02, compared to resistant strain XYZ based on plant tissue assay. Our preliminary results from both plant and diet bioassays indicate there is no strong cross resistance of the Cry1Ac resistance *H. virescens* or *H. zea* strains to Vip3A. Based on the results from this study, there are a number of insecticidal alternatives available to delay evolution of resistance in cotton bollworm and tobacco budworm to Bt cotton.

Introduction

Since 1996, Bollgard ® (Monsanto) cotton has been commercially available to U.S. cotton growers. This genetically modified organism (GMO) expresses an efficacious insecticidal protein, Cry1Ac, throughout its plant parts. Due to Bollgard's widespread use, resistance management specialists have been monitoring its effectiveness over time in controlling two of the most important cotton pests, cotton bollworm (*Helicoverpa zea*) (Mahaffey et al. 1995) and tobacco budworm (*Heliothis virescens*) (Gould et al. 1995). Despite a very low frequency of resistant genotypes in the field (Jackson et al. 2003), bollworm larvae have a 5-25% rate of survival on Bollgard plants (Gore et al. 2003). In addition, previous studies have indicated that a bollworm strain with genetic tolerance to Bollgard (Cry1Ac) has a low level of cross resistance to Bollgard II (Cry1Ac and Cry2Ab) plants grown in the greenhouse (Jackson et al. 2000). Because some bollworm larvae can survive on Bollgard cotton the high dose criteria for resistance management of transgenic crops is not applicable to this pest species (Stone and Sims 1993). Because the inheritance of resistance in cotton bollworm can be characterized as dominant (Burd et al. 2003), we investigated the survivorship of heterozygotes at varying concentrations of Cry1Ac toxin. Historically, there have been examples of cross resistance in other lepidopterans to various Bt toxins (Gould et al. 1992, Tabashnik et al. 2000, Liu et al. 2001), and the possibility remains of resistance developed in response to selection with one Bt toxin conferring resistance to all Bt toxins (Gould 1998). A component of resistance management strategies is the use of alternative insecticides to delay the onset of resistance to one class or type of insecticide (Bauer 1995, Tabashnik 1994).

Vip3A is a toxic protein produced by *Bacillus thuringiensis* and the structural gene for its production has been transferred to and expressed in a new line of transgenic cotton from Syngenta, VipCot™. Vegetative insecticidal proteins (VIP) are exotoxins produced during the vegetative growth phase of the soil bacterium *Bacillus thuringiensis*. Whereas, production of the δ endotoxins in Bollgard are restricted to the sporulation stage of bacterial growth (USDA/APHIS 2005, Estruch et al. 1996). The Vip3A protein has a wide spectrum of activity against major economically important lepidopteran pests (Estruch et al. 1996). Furthermore, the Vip3A protein shares no structural or sequence homology with the other δ endotoxins. In addition to these physical dissimilarities, Vip3A possess a different mode of action in its formation of a unique pore channel in the insect gut wall and a protein activation site not homologous to that of Cry1 (Shotkoski et al. 2003). As a result, Vip3A is relevant to insect resistance management as a potential tool for delaying insect resistance in heliothines to transgenic Bt crops (Bradley et al. 2004).

Although there have been cross comparison studies of this novel insecticide with other heliothine pests (Liao et al. 2002), there remain no studies on the potential of cross resistance to Vip3A in strains of *Helicoverpa zea* that are tolerant Cry1Ac.
We initiated a set of experiments to determine the extent of cross resistance between Cry1Ac and Vip3A in bollworm and tobacco budworm (*Heliothis virescens*) strains highly tolerant to the *Bacillus thuringiensis* endotoxin Cry1Ac. Experiments included tests of cotton plant leaf tissue as well as bioassays with artificial diets that were surface treated with *Bt* toxins.

Although our study focused on cross resistance between Cry1Ac and Vip3A we also examined cross resistance between Cry1Ac and the toxins Cry1F, Cry1Ab, and Cry2Ab.

Materials and Methods

Background Information on Insect Strains

***Helicoverpa zea*—**Two bollworm strains including one control and one Cry1Ac resistant strain were subjected to cross resistance testing. The Cry1Ac tolerant bollworm strain, XYZ, used in this study originated from 126 pupae. Large fourth and fifth instar bollworms collected from Bollgard® cotton plants in Martin and Edgecombe counties in eastern North Carolina in late summer 2002. Upon collection from the field, larvae were transported to the laboratory where they were placed individually into 30ml plastic cups containing artificial diet (Burton 1970) where they completed larval development and pupate. For sixteen subsequent generations first instar larvae were selected at increasing concentrations of Cry1Ac with a starting concentration of 0.5 µg/ml and a maximum concentration of 500 µg/ml of diet. Larvae were reared on diet containing toxin for 7 to 14 days. The susceptible control strain, HZ 02, is housed at the NCSU Insectary. This strain originated from 79 individual females collected from light traps in eastern North Carolina in August 2002. A bollworm strain originating from conventional cotton in proximity to the Bollgard was collected in 2002 as well. This strain originated from 19 pupae and was used as the susceptible control strain prior to the F_2 generation. Periodic dose-mortality bioassays were performed to determine the response to selection. The bioassay consisted of a five fold serial dilution using an insect diet blend with concentrations ranging from 0-1000 µg/ml Cry1Ac. Response to each concentration was

evaluated with 30 neonate individuals placed on the diet with a camelhair paintbrush and allowed to feed for 10 days at 27±2°C with a 14:10 light:day photo phase. Percent mortality was assessed and subsets of 15 individuals per concentration were weighed to determine growth rate.

Heliothis virescens—A total of four budworm strains originating from Dr. Fred Gould's laboratory including one control and three strains previously characterized as resistant to Cry1Ac were tested for cross resistance to Vip3A. The YDK control strain was established from a collection of tobacco budworm eggs from three adjacent counties in North Carolina in 1988 (Gould et al. 1995) and served as a susceptible control for the three other strains. Most recent evaluations of YDK indicated an LC_{50} value of 0.73 µg Cry1Ac/ml. The YHD2 strain is a subset of the susceptible control strain YDK that was selected on Cry1Ac. The YHD2 strain has developed a very high level of resistance to Cry1Ac (LC_{50} >2000 µg/ml), but the spectrum of cross resistance in this strain is very narrow (Gould et al. 1995). The CXC and the KCBhyb strains were collected from the field at the same time as the YHD2 strain but have a different history of selection with Bt toxins (Fuentes et al. 2002, 2003). Both strains have lower levels of resistance to Cry1Ac (LC_{50} values of 211.20 µg/ml and 137 µg/ml respectively) but their spectrum of cross resistance is broader.

Cross Resistance Evaluations of Cry1Ac tolerant *Helicoverpa zea* strain to δ-Endotoxins Cry1Ab, Cry1F, and Cry2Ab

The Cry1Ac tolerant strain of *Helicoverpa zea* was evaluated for cross resistance to the δ-endotoxins Cry1Ab, Cry1F, and Cry2Ab using a dilution series of toxin incorporated into artificial diet. Cross matings of 15 moths from each sex of both the

selected (RR) and control (SS) strains were used to obtain F1's for genetic testing. These crosses were conducted after nine generations of selection in March 2004. Cross matings for the toxins Cry1Ab, Cry1F, and Cry1Ac were conducted after eleven generations of selection in June 2004. The Cry1Ab, Cry1F, and Cry1Ac toxins were obtained in house from Mycogen. A five fold serial dilution of each of the three Cry1 toxins was used with concentrations ranging from 0-1000 µg/ml. Bioassays were performed with each of the four types of crosses: Selected ♀ x ♂, Selected ♀ x Control ♂, Control ♀ x Selected ♂, and Control ♀ x ♂. The material for testing Cry2Ab toxin was formulated differently than the Cry1 toxins. Lyophilized corn plant powder containing the protein Cry2Ab was quantified, purified, and provided by Monsanto Company. A corresponding corn powder without the Cry2 protein was used for the control. A five fold serial dilution bioassay incorporating the lyophilized powder was performed for each of the four types of genetic crosses. This bioassay ranged in concentration 0-200 µg/ml. Thirty neonate individuals were tested at each concentration for ten days at 27±2° C with a 14:10 light:day photophase. Percent mortality was assessed and subsets of fifteen individuals per concentration were weighed to determine growth rates.

Larval weight data were converted to log weight and analyzed with SAS two-way ANOVA with strain and concentration as fixed variables. Mortality data were analyzed using the Probit procedure in SAS Version 8.0.

Vip3A Surface Treated Diet Bioassays

Evaluations of the *H. virescens* and *H. zea* strains were conducted in the lab in September 2004. The Cry1Ac tolerant tobacco budworm strains, YHD2 and KCBhyb

were tested, as well as the susceptible control strain YDK. The Cry1Ac tolerant bollworm strain XYZ (F_{13}) and the susceptible strain HZ 02 were subjected to testing.

Approximately 0.2 ml of corn-soy blend insect diet (Burton 1970) was injected into 2 ml conical bottom plastic vials. The surface area of each vial was treated with 15 µl of test solution and permitted to dry for one hour. Toxin concentrations for the bollworm and budworm tests (0-400 µg/ml Vip3A) were generated by a two-fold serial dilution of a lyophilized sample of a purified protein, Vip3A. As a diagnostic control, tobacco budworm strains were concurrently tested with a two-fold serial dilution diet surface treated assay incorporating Cry1Ac (MVPII Mycogen) with a concentration range of 0-100 µg/ml Cry1Ac; cotton bollworm strains were tested with a five fold serial dilution with a concentration range of 0-1000 µg/ml Cry1Ac. The Vip3A protein powder sample was reconstituted and serially diluted using a 200mM ammonium carbonate buffer pH 9.5; the Cry1Ac toxin was diluted with distilled water. Single, newly hatched neonates were transferred to the treated vials with a fine camel hair paintbrush. Four small holes were made into the plastic vial caps 24 hours after set up to ensure proper gas exchange. Fifty individuals were tested at each concentration. Test conditions were 27±2°C and a light:dark photoperiod of 14:10 hours. Overall mortality was assessed six days after treatment and a subset of 30 individuals were weighed at this time. Larval weight data were converted to log weight and analyzed with SAS Version 8.0 two-way ANOVA with strain and concentration as fixed variables.

VipCot™ Cotton Plant Tissue Evaluations for *Helicoverpa zea* and *Heliothis virescens*

Plant tissue leaves used in this study were obtained from field grown cotton plants from a field test plot in eastern North Carolina. Plant varieties with insecticidal properties used were Bollgard and Bollgard II (Monsanto), and Vip203 and Vip102 (Syngenta). A very similar non-Bt cotton variety (Coker 312) was used as a control. The Vip cotton varieties were planted on May 20, 2004. The Bollgard plants were planted May 19, 2004. For this study, youngest leaves were collected from the terminal region of the cotton plant. Both the control and selected *Helicoverpa zea* strains, HZ 02 and XYZ respectively, were evaluated on September 9, 2004. The three *Heliothis virescens* strains, YDK, CXC, and KCBhyb were evaluated August 25, 2004.

Field collected cotton leaves were sealed in plastic ziplock bags and placed in coolers. They were immediately transported to the laboratory at NCSU. Upon arrival, leaf disks were punched out of the leaves with a cork borer, diameter 1.5 cm (area= 1.766 cm^2). Three leaf disks were placed into small clear plastic snug fitting Fisher brand Petri dishes (60 mm diameter, 15 mm height). Approximately ½ ml of distilled water was added to a single circle of filter paper placed inside each dish to help retain moisture. A single newly hatched neonate per Petri dish was placed onto the leaf disks with a camelhair paintbrush. Petri dishes were bound by rubber bands in groups of five and were held at 27±2°C and at 14:10 light:day photoperiod. After five days, mortality was scored and all live larvae were weighed. Amount of plant tissue consumed was assessed by assigning each ¼ circle eaten a value of one and multiplying that value by 0.4415 cm^2 (¼ area of the circle).

Results

Response to Selection of *Helicoverpa zea* Strain Originating from Bollgard Cotton

Survivorship percentages for each generation from the neonate stage to pupation for the selected strain are presented in Table 1 along with the toxin concentration at which each generation was selected. Overall survival generally remained low, not exceeding 50%, excluding the F_{11} and F_{15} generations. Population size and mortality of the XYZ selected strain resulting from ongoing laboratory selection with Cry1Ac was not recorded until the F_4 generation. Prior to the fourth generation, the XYZ strain was subjected to concentrations of MVPII ranging from 0.5 to 1µg/ml. Survival fluctuated from generations F_4 to F_{10}. Larvae from the F_{10} generation were the first to be treated at 500µg/ml. Survivorship in the F_{11} generation rapidly increased and was the highest observed value of 78.3%. In the F_{12} generation, survivorship decreased tremendously to 4.66%. After the F_{12} generation, survivorship remained between 20 and 43%.

For each generation, mean larval weights for the concentration 1.6µg/ml and LC_{50} values from the Cry1Ac concentration mortality bioassays for both the selected and control strains are given in Figure 1 and Table 2. Mean larval weights between the resistant and control bollworm strain differed significantly at the concentration 1.6µg/ml for generations F_4, F_5, and F_{11} in Figure 1. Resistance ratios in Table 2 ranged from 0.686 fold in the F_5 generation to 98.7 fold at generation F_{11}. Excluding the F_{11} and F_{12} generations, resistance ratios remained under 20.3 fold. Overall, the selected strain had significantly greater LC_{50} values than the control strain. One exception was observed at generation F_5, where the control LC_{50} (21.99 µg/ml) was greater than the selected strain LC_{50} (15.09 µg/ml). Through generations F_3 – F_6, LC_{50} values for the selected strain

fluctuated. After the F_6 generation, LC_{50} values for the XYZ selected strain increased considerably. One outlier to this increase was observed in the F_{11} generation. The LC_{50} for generation F_{10} (655.9 µg/ml) declined to LC_{50} 119.4 µg/ml for F_{11}. LC_{50} value then increased considerably to 884.9 µg/ml in the next generation, F_{12}.

Cross Resistance Evaluations in *Helicoverpa zea* to δ-Endotoxins Cry1Ab, Cry1F, and Cry2Ab

The LC_{50} values for Cry1Ac from the four genetic crosses of the control and selected strain (Table 3) show that although the RR ♀ x ♂ cross did not yield 95% fiducial limits, the reported value (119.4 µg/ml) lies within the upper and lower range for both the heterozygotic crosses. The SS ♀ x ♂ cross LC_{50} (1.209 µg/ml), was significantly lower and not within the limits range of the other three genotypic crosses. Larval growth ratios for the four types of crosses (Figure 2) varied over the Cry1Ac concentrations 0.32–1000 µg/ml. Progeny from the RR ♀ x ♂ cross had greater growth than the other three genotypes at the higher concentrations 8 and 40 µg/ml. Growth ratios for the RR ♀ x SS ♂ cross at concentration 200 µg/ml was approximately three times greater than other strains with survivors at the same concentration. Overall, the SS ♀ x RR ♂ cross had the highest growth ratio at the low concentration 0.32 µg/ml, but this value steadily decreased with increasing concentrations. Progeny from the SS ♀ x ♂ cross did not survive at 40 and 200 µg/ml, whereas there were survivors from the other three crosses. Log transformed weight data analyzed with SAS two way ANOVA revealed there was not a significant interaction effect between strain and concentration ($F=1.67$; $df=13$; $P=0.0645$). However, strain had a significant effect as did concentration on larval weight ($P<0.0001$).

The LC_{50} results for genetic crosses SS ♀ x ♂ and RR ♀ x ♂ to Cry1Ab (Table 4) have significantly larger ranges in upper and lower confidence intervals. The SS ♀ x RR ♂ cross is the only genotype to differ significantly from the RR ♀ x ♂ cross. The RR ♀ x SS ♂ cross did not yield fiducial limits, but the LC_{50} value differs significantly from both the homozygotic selected and control crosses. Although the SS ♀ x ♂ cross has the lowest LC_{50} (72.81µg/ml), it did not differ significantly from the RR ♀ x ♂ cross because fiducial limits for both strains overlapped. Both the RR ♀ x ♂ and RR ♀ x SS ♂ crosses had higher LC_{50}s than SS ♀ x ♂ and SS ♀ x RR ♂. Growth ratios (Figure 3) for the four crosses did not differ greatly from each other at lower concentrations of 0.32 and 1.6 µg/ml. However, the RR ♀ x ♂ cross had consistently greater growth ratios than the other three genotypes for concentration 8 µg/ml and higher. Log transformed weight data analyzed with SAS two way ANOVA showed a significant interaction effect between strain and concentration ($F=6.82$; $df=16$; $P<0.0001$). Strain and concentration had significant effects on larval weight ($P<0.0001$).

SAS PROBIT results from mortality concentration testing done with the Cry1F toxin (Table 5) showed *H. zea* SS ♀x ♂ cross had a LC_{50} value significantly higher than either RR ♀ x ♂ cross or RR ♀x SS ♂ cross. There was not a significant difference in the SS ♀x ♂ cross LC_{50} (455.8 µg/ml) compared to SS ♀x RR ♂ cross LC_{50} (185.7 µg/ml). Larval growth ratios (Figure 4) for both crosses with control female parents in concentration 40 µg/ml were greater than both crosses with selected female parents. The growth ratio for RR ♀ x ♂ cross approached a value of one for the lowest Cry1F concentration 0.32 µg/ml. In the case of the RR ♀ x SS ♂ cross for that same concentration, larval growth had exceeded growth on the control. The SS ♀x ♂ cross

had greater growth ratios than the other three crosses for higher concentrations 8 and 40 µg/ml. Additionally, SS ♀x ♂ cross retained the only survivor at 1000 µg/ml. Log transformed weight data analyzed with SAS two way ANOVA shows a significant interaction effect between strain and concentration (F=1.87; df=15; P<0.0235). Strain had a significant effect on weight as did concentration (P<0.0001).

Testing conducted using the toxins Cry1Ac, Cry1Ab, and Cry1F occurred at F_{11} generation for the selected *H. zea* strain XYZ. Paired matings made for Cry2Ab evaluations were done during generation F_9. The LC_{50} for progeny from RR ♀ x ♂ cross on Cry2Ab did not differ significantly from the progeny of SS ♀ x ♂ cross (Table 6). However, the progeny of the RR ♀ x ♂ cross was significantly different from the heterozygote crosses. The opposite was true for the SS ♀ x ♂ cross, because it did not differ significantly from the two heterozygote crosses. Oddly, larval growth on Cry2Ab diet for concentrations 0.064 and 0.32 µg/ml (Figure 5) was greater than growth of their counterparts tested on control diet. At concentration 0.064 µg/ml, the progeny of SS ♀ x RR ♂ and SS ♀ x ♂ were approximately 9-11 times larger than larvae on control diet. For the two lowest concentrations tested, the RR ♀ x ♂ genotype was at a maximum four times larger than average larvae found on the control diet. Growth of the RR ♀ x ♂ cross was markedly inhibited by Cry2Ab at concentration 1.6 and 8 µg/ml. Of the four insect crosses tested, the RR ♀ x SS ♂ cross was most affected by the toxin, having the lowest growth proportion value for all concentrations tested. The SS ♀x ♂ and SS ♀x RR ♂ crosses were least affected by the Cry2Ab toxin. Log transformed weight data analyzed with SAS two way ANOVA shows a significant interaction effect between strain and

concentration (F=4.79; df=12; *P*<0.0001). Strain had a significant effect on weight as did concentration (*P*<0.0001).

The Cry2Ab protein at the concentration 1.6 µg/ml (Figure 6) had the greatest effect of all proteins tested on inhibiting larval weight gain for all four crosses. Greater percent mortality (Figure 7) for the Cry1Ac resistant strain was observed at lower concentrations of Cry2Ab toxin compared to the other three toxins. Additionally, toxins Cry1F and Cry1Ab did not have higher mortality than Cry1Ac against the resistant strain except at very high concentrations. The control strain had higher percent mortality at low concentrations of Cry1Ac compared to its performance against the other three toxins.

Cross Resistance Evaluations to Novel Insecticide Vip3A

Helicoverpa zea

Surface Treated Diet Bioassay—Percent mortality for Cry1Ac treated diet (Figure 8) showed resistant strain XYZ was less affected than susceptible strain HZ 02. With an exception observed at 200 µg/ml, HZ 02 had greater mortality than XYZ. Percent mortality for Vip3A (Figure 9) for both strains, XYZ and HZ 02, went from an increasing to a decreasing trend when they reached higher concentrations of 25 and 50 µg/ml. Strain HZ 02 had higher mortality than XYZ for Vip3A concentrations 25-200 µg/ml, but not for the highest concentration tested (400 µg/ml). Overall, the HZ 02 strain had greater mean log weights (Figure 10) than the XYZ strain for the Vip3A assay. An exception to this trend was observed in the control diet, where XYZ larvae were significantly larger than HZ 02 larvae. Both strains did not differ significantly from each other at concentrations of 25-200 µg/ml, but they did differ significantly at 400 µg/ml.

Plant Leaf Tissue Bioassay—Percent mortality (Figure 11) was greater for XYZ than HZ 02 for all the plant varieties in this study. Furthermore, in none of the varieties XYZ was exposed to did percent mortality fall below 20%. The HZ 02 strain was significantly different in mean larval weight (Figure 12) from the XYZ strain on the Non-Bt, Bollgard II, Vip102, and Vip203 varieties. There was no significant difference between the two strains for the Bollgard variety. The mean leaf tissue consumed (Figure 13) by the Cry1Ac resistant strain XYZ was not significantly different from the susceptible strain HZ 02 for the Bollgard and Bollgard II varieties. However, there were significant differences between the two strains for the Non-Bt, Vip102 and Vip203 varieties. In addition, consumption of leaf material by HZ 02 was greater than XYZ for these same varieties.

After conducting a two way ANOVA analysis with SAS Version 8.0, a significant interaction effect ($P<0.0001$) for insect strain and plant variety was determined. Insect strain and plant variety ($P<0.0001$) were both significant factors on weight.

Heliothis virescens

Surface Treated Diet Bioassay—Percent mortalities for Cry1Ac and Vip3A (Figures 14 and 15 respectively) showed YDK was highly susceptible to Cry1Ac and the YHD2 strain was least affected. Comparing the mean larval log weights for Vip3A diet bioassay (Figure 16), KCBhyb generally had the highest weights, and YDK had higher weights than YHD2. There was no significant difference among the three strains for concentrations 50 and 100 µg/ml. KCBhyb and YDK were the only strains that had survivors at the higher concentrations of 200 and 400 µg/ml. There was no significant

difference in log weights between KCBhyb and YDK at the 400 µg/ml Vip3A concentration, but the two strains did differ at 200 µg/ml.

Plant Leaf Tissue Bioassay—In the cotton leaf tissue assays (Figure 17), the CXC, KCBhyb, and YDK strains did not exceed 50% mortality on the Cot 102 or Cot 203 varieties. The YDK strain was very susceptible to the active protein (Cry1Ac) in Bollgard cotton. With regard to the average larval weight of the three strains (Figure 18), YDK and CXC were larger than KCBhyb for the control non-Bt. However, the KCBhyb strain had the highest mean weight values for the Bollgard and Cot 203 varieties. The three strains did not differ significantly from each other on Cot 102. The leaf consumption (Figure 19) of the susceptible strain YDK on Bollgard cotton leaf was lower than consumption by the CXC and KCBhyb strains. YDK consumption was comparable or significantly greater than the two resistant strains for both the Cot 102 and Cot 203 varieties.

TWO WAY ANOVA. There was a significant interaction effect ($P<0.002$) for insect strain and plant variety on larval weight in a two way ANOVA analysis with SAS Version 8.0 Insect strain had no significant effect on weight, but plant variety did.

Discussion

The dose-mortality assays indicate that the XYZ strain that was initially collected from Bollgard cotton was responsive to further selection with Cry1Ac. Originally, this strain was selected at a concentration of 0.5 µg/ml Cry1Ac. Over several generations the concentration was increased to 500 µg/ml, a 1000 fold difference. Although we can see there were fluctuations over time in the weight of the XYZ strain in Figure 1 at a given concentration of 1.6 µg/ml, the selected strain consistently produced larvae that grew

faster than those of the control strain, HZ 02. There is adequate evidence from the LC_{50} data that the tolerance level of the selected strain for Cry1Ac has increased from the generation initially tested to generation 12, an 8.7 fold difference. These findings are similar to Burd's (2001) observations with a similar strain of bollworm. In both cases the actual resistance ratio fluctuates up and down over generations.

Given the results from the growth ratio comparisons and the LC_{50} values for the Cry1Ab toxin, cross resistance is highly likely in the XYZ strain. The selected strain had greater gains in larval weight than the control strain at all concentrations of toxin. The LC_{50} comparisons revealed the control strain and the two heterozygote crosses were more susceptible to the Cry1Ab toxin than the selected strain. Interestingly, the results of this evaluation point to a recessive mode of inheritance to Cry1Ab for this strain.

Based on the growth comparison data in Figure 4 and the LC_{50} data for Cry1F, there appears to be a preliminary argument for negative cross resistance in the Cry1Ac-selected strain of bollworm to this toxin. The resistant strain was the most susceptible of the four genotypes to this toxin; more so than the two heterozygotic crosses. With regard to growth and PROBIT analysis, the susceptible homozygous control strain was least affected by the Cry1F toxin.

The results from the evaluations with Cry2Ab toxin do not support an argument for cross resistance to this toxin in the XYZ strain. The low LC_{50} values for both the control and the selected strain indicate there were no significant differences between the two. For the larval growth ratio comparisons, we see that the crosses where the maternal parent was taken from the control strain did better than progeny descended from a mother who had been selected previously. Such differences in growth rate may be due to

maternal effects (Lambert et al. 1998, Rossiter et al 1990); the Cry1Ac protein fed on in the prior generation may have had a detrimental effect on the ability of the current generation to handle the Cry2Ab toxin as well as the control counterparts. The rate at which the resistant strain decreases at the lower concentrations is worthy of taking note as well since there appears to be no difference in growth ratios between larvae on 0.064 and 0.32 µg/ml.

Perhaps the mechanism of resistance in this strain is overall reduced receptor binding sites in the brush border membrane of the insect midgut (Schnepf et al. 1998) or the ability for the resistant strain to quickly repair damaged columnar cells in the gut (Martinez-Ramirez et al. 1999) since there is little difference in growth at sublethal doses. Due to the high morality and low larval weights in the control, it is plausible that the amount of secondary compounds in the plant may have had an effect on growth ratios. The resistant strain originated from a smaller number of moths than the control and may have lost its ability to appropriately digest such types of compounds, or there may be a fitness cost associated with one of the secondary plant compounds (Carrière et al. 2004).

The overall levels of control by each delta endotoxin on the resistant and control strains and their respective crosses are shown in Figures 6 and 7. The toxin that had the greatest inhibition of weight gain at a low dose of 1.6 µg/ml was Cry2Ab. The Cry1Ac toxin was more effective than the Cry1Ab toxin at minimizing growth. The Cry1F toxin was the least effective of the four toxins, especially for progeny that descended from control mothers. The percent mortality for the resistant strain showed Cry2Ab had the greatest effect on survivorship for the XYZ resistant strain but had similar results for the other three toxins. Reduction in survivorship for the control strain, HZ 02, was impacted

more by Cry1Ac than Cry2Ab. Survivorship for the control was affected more by Cry2Ab, than Cry1Ab. Similarly, Cry1Ab produced greater mortality than the Cry1F toxin.

The evidence gathered from this study does not support a definitive argument for strong cross resistance to the novel insecticidal protein Vip3A for strains of cotton bollworm and tobacco budworm that are highly tolerant to the δ endotoxin Cry1Ac. For the cotton bollworm, in surface treated diet bioassays performance of XYZ and HZ 02 on Vip3A were comparable for both mortality and log weight data. Larval log weight of HZ 02 was greater than or equal to that of XYZ, with an exception at the highest concentration tested (400 μg/ml) and at the control concentration. With regards to the mortality data, XYZ had lower survivorship than the HZ 02 strain at low concentrations. This scenario was reversed at the concentration 25 μg/ml and continued until 400 μg/ml, where HZ 02 survivorship was greater. A possible explanation for this discrepancy may be due to methodology. Larvae that are subjected to diet that is surface treated may be able to avoid the toxin once the surface layer is punctured (Liao et al. 2002). Given this potential scenario, the larvae may have consumed enough Vip3A protein to limit weight, but not enough to cause death. For the tobacco budworm, in the surface treated diet bioassays for Vip3A, we observed definite differences both in mortality and in larval log weight between the highly susceptible strain YDK and the highly Cry1Ac tolerant strain YHD2. Although YHD2 had lower mortality than YDK for Cry1Ac, it was markedly more susceptible to Vip3A than YDK in terms of mortality. In contrast, the KCBhyb strain performed better on the Vip3A surface treated diet bioassay than the YDK control

strain. This is not too surprising since the KCBhyb strain is cross resistant to Cry2Aa. However, the difference between the YDK and KCBhyb was not large.

The evidence we gathered from the plant tissue bioassays do not support the preliminary finding of moderate cross resistance to Vip3A in the KCBhyb strain. Based on leaf consumption and average larval weight, for the bollworm we found that the susceptible strain, HZ 02, performed better on plants with the Vip3A protein than did the resistant strain XYZ. The control strain HZ 02 consumed significantly more leaf material than did XYZ for both VipCot varieties Cot 102 and Cot 203. However, Cot 203 appears to be more effective than the Cot 102 variety since it had a significantly greater effect at limiting growth and consumption for both the strains. There is an apparent difference between the two strains for the control plant variety with regard to weight and consumption. As previously hypothesized, there may be a fitness cost associated with the resistant strain with regard to secondary plant compounds in the plant tissues. However growth ratio comparisons of these two strains on Bollgard plants demonstrate that larval weight growth of the XYZ strain is greater than HZ 02. This indicates that XYZ is less impacted by the Cry1Ac protein that HZ 02. Percent mortality was higher for XYZ than HZ 02 for all the cotton varieties tested. The difference in survivorship between the two bollworm strains was smallest for Bollgard II, demonstrating the overall effectiveness of the Cry2Ab protein on both strains. For the tobacco bollworm, with regard to the consumption, mortality, and weight data for the two VipCot varieties Cot 102 and Cot 203, YDK performed comparably or greater than its two resistant counterparts CXC and KCBhyb. Differences in the promoter for the Cot 102 and Cot 203 varieties (Bradley et al. 2004, Boets et al. 2004) may explain variations in strain response amongst the two

plant varieties. Due to its dissimilar mode of action and the data presented herein Vip3A promises to be an effective tool in insect resistance management. However, further investigation into the response levels of KCBhyb and its potential for cross resistance at higher levels of Vip3A are warranted.

References

Bauer, L. S. 1995. Resistance: A threat to the insecticidal crystal proteins of *Bacillus thuringiensis*. Florida Entomol. 78(3): 414-443.

Boets, A., G. Arnaut, J. Van Rie, and N. Damme. 2004. U. S. Patent # 6,706,860.

Bradley, J. R., J. W. Van Duyn, and R. E. Jackson. 2004. VipCot: Field performance in North Carolina under conditions of high bollworm populations. *In* 2004 Proc. Beltwide Cotton Conference. January 5-9. San Antonio, Texas. 1362-1364.

Burd, A. D., F. Gould, J. R. Bradley, J. W. Van Duyn, and W. J. Moar. 2003. Estimated frequency of nonrecessive *Bt* resistance genes in bollworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) in eastern North Carolina. J. Econ. Entomol. 96(1): 137-142.

Burd, A. D. 2001. The influence of environmental factors on susceptibility of Bt cottons to bollworm, *Helicoverpa zea*, and factors affecting resistance to Bt toxins for bollworm. Ph.D. dissertation, North Carolina State University. Raleigh, North Carolina.

Burd, A. D., J. R. Bradley, J. W. Van Duyn, and F. Gould. 2000. Resistance of bollworm, *Helicoverpa zea*, to Cry1Ac toxin. *In* 2000 Proc. Beltwide Cotton Conference. January 4-8. San Antonio, Texas. 923-926.

Burton, R. L. 1970. A low-cost artificial diet for the corn earworm. J. Econ. Entomol. 63(6): 1969-1970.

Carrière, Y., C. Ellers-Kirk, R. Biggs, D. M. Higginson, T. J. Dennehy, and B. E. Tabashnik. 2004. Effects of gossypol on fitness costs associated with resistance to Bt cotton in pink bollworm. J. Econ. Entomol. 97(5): 1710-1718.

Estruch, J. J., G. W. Warren, M. A. Mullins, G. J. Nye, J. A. Craig, and M. G. Koziel. 1996. Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against Lepidopteran insects. Proc. Natl. Acad. Sci. USA. 93: 5389-5394.

Fuentes, J. L., F. L. Gould, and M. J. Adang. 2003. Dual resistance to *Bacillus thuringiensis* Cry1Ac and Cry2Aa toxins in *Heliothis virescens* suggests multiple mechanisms of resistance. Appl. Environ. Microbiol. 69 (10): 5898-5906.

Fuentes, J. L., F. L. Gould, and M. J. Adang. 2002. Altered glycosylation of 63- and 68-kilodalton microvillar proteins in *Heliothis virescens* correlates with reduced Cry1 toxin binding, decreased pore formation, and increased resistance to *Bacillus thuringiensis* Cry1 toxins. Appl. Environ. Microbiol. 68 (11): 5711-5717.

Gore, J., B. R. Leonard, and R. H. Gable. 2003. Distribution of bollworm, *Helicoverpa zea* (Boddie), injured reproductive structures on genetically engineered *Bacillus thuringiensis* var. *kurstaki* Berliner cotton. J. Econ. Entomol. 96(3): 699-705.

Gould, F. 2003. Bt-resistant management-theory meets data. Nat. Biotechnol. 21(12): 1450-1451.

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

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(6): 1545-1559.

Gould, F., A. Martinez-Ramirez, A. Anderson, J. Ferre, F. Silva, and W. Moar. 1992. Broad-spectrum resistance to *Bacillus thuringiensis* toxins in *Heliothis virescens*. Proc. Natl. Acad. Sci. USA. 89: 7986-7990.

Jackson, R. E., J. R. Bradley Jr., J. W. Van Duyn, and A. D. Burd. 2003. *Bt* Resistance evolution in the *Helicoverpa zea* population in eastern North Carolina. *In* 2003 Proc. Beltwide Cotton Conference. January 6-10. Nashville, Tennessee. 1168-1176.

Jackson, R. E., J. R. Bradley, A. D. Burd, and J. W. Van Duyn. 2000. Field and greenhouse performance of bollworm on Bollgard II cotton genotypes. *In* Proc. Beltwide Cotton Conference. 1048-1051.

Kurtz, R. W., F. L. Gould, J. R. Bradley Jr., and J. W. Van Duyn. 2004. Helicoverpa zea fitness on Bt corn and cotton in eastern North Carolina: Potential effects of alternate host crops and pyramided Bt plants. *In* 2004 Proc. Beltwide Cotton Conference. January 5-9. San Antonio, Texas. 1430-1434.

Lambert, A. L., J. R. Bradley Jr., F. Gould, and J. W. Van Duyn. 1998. Bollworm (*Helicoverpa zea*): adaptation to *Bt* toxin? *In* 1998 Proc. Beltwide Cotton Conference. January 5-9. San Diego, California. 1033-1037.

Liao, C., D. Heckel, and R. Akhurst. 2002. Toxicity of *Bacillus thuringiensis* insecticidal proteins for *Helicoverpa armigera* and *Helicoverpa punctigera* (Lepidoptera: Noctuidae), major pests of cotton. J. Invertebr. Pathol. 80: 55-63.

Liu, Y. -B., B. E. Tabashnik, S. K. Meyer, and N. Crickmore. 2001. Cross resistance and stability of resistance to *Bacillus thuringiensis* toxin Cry1C in diamondback moth. Appl. Environ. Microbiol. 67(7): 3216-3219.

Mahaffey, J. S., J. R. Bradley, Jr., and J. W. Van Duyn. 1995. Bt cotton: Field performance in North Carolina under conditions of unusually high bollworm populations. *In* 1995 Proc. Beltwide Cotton Conference. January 4-7. San Antonio, Texas. 795-798.

Marcus, M. A., J. R. Bradley, F. L. Gould, and J. W. Van Duyn. 2004. Fitness evaluations of *Helicoverpa zea* (Boddie) from Bollgard cotton in subsequent generations. *In* 2004 Proc. Beltwide Cotton Conference. January 5-9. San Antonio, Texas. 1390-1394.

Martinez-Ramirez, A. C., F. Gould, and J. Ferré. 1999. Histopathological effects and growth reduction in a susceptible and a resistant strain of *Heliothis virescens* (Lepidoptera: Noctuidae) caused by sublethal doses of pure Cry1A crystal proteins from *Bacillus thuringiensis*. Biocontrol Science and Technology. 9: 239-246.

Rossiter, M. C., W. G. Yendol, and N. R. Dubois. 1990. Resistance to *Bacillus thuringiensis* in gypsy moth (Lepidoptera: Lymantriidae): Genetic and environmental causes. J. Econ. Entomol. 83(6): 2211-2218.

Schnepf, E., N. Crickmore, J. Van Rie, D. Lereclus, J. Baum, J. Feitelson, D. Zeigler, and D. Dean. 1998. *Bacillius thuringiensis* and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62(3): 775-806.

Shotkoski, F., E. Chen, V. Mascarenhas, and R. Boykin. 2003. Vip: A novel insecticidal protein with broad spectrum Lepidopteran activity. In 2003 Proc. Beltwide Cotton Conferences. January 6-10. Nashville, Tennessee. 89-93.

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(4): 989-994.

Tabashnik, B. E., F. Gould, and Y. Carrière. 2004. Delaying evolution of insect resistance to transgenic crops by decreasing dominance and heritability. J. Evol. Biol. 17: 904-912.

Tabashnik, B. E., Y. -B. Liu, R. A. deMaagd, and T. J. Dennehy. 2000. Cross-resistance of pink bollworm (*Pectinophora gossypiella*) to *Bacillus thuringiensis* toxins. Appl. Environ. Microbiol. 66(10): 4582-4584.

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

USDA/APHIS Environmental Assessment. 2005. Syngenta petition 03-155-01p for determination of nonregulated status for Lepidopteran resistant event COT102. U. S. Department of Agriculture Animal and Plant Health Inspection Service Biotechnology Regulatory Services. www.aphis.usda.gov/brs/aphisdocs/03_15501p.pdf

Zhao, J. -Z., J. Cao, Y. Li, R. Roush, E. Earle, and A. Shelton. 2003. Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance evolution. Nat. Biotechnol. 21(12): 1493-1497.

Table 1. Total number of neonates of *Helicoverpa zea* strain XYZ exposed to toxin each generation, total number of pupae to survive, concentration of Cry1Ac toxin (µg/ml) each generation was selected and percent survival.

Generation	Total # neonates selected	Total # pupae survived	Cry1Ac (µg/ml)	%Survival
F4	1386	146	10	10.53391
F5	828	201	40	24.27536
F6	3887	527	100	13.55801
F7	1732	546	100	31.52425
F8	2956	387	100	13.09202
F9	1362	587	100	43.09838
F10	819	145	500	17.70452
F11	912	714	500	78.28947
F12	1008	47	500	4.662698
F13	1323	266	500	20.10582
F14	2016	638	500	31.64683
F15	630	401	500	63.65079
F16	945	404	500	42.75132

Table 2. Intergenerational LC_{50} values with corresponding upper and lower 95% fiducial limits for *Helicoverpa zea* strains Selected (XYZ) and Control (HZ 02) to Cry1Ac, slope values and resistance ratios.

	Control Strain				Selected Strain				
Generation	LC_{50}[a]	Lower[b]	Upper	Slope[c]	LC_{50}	Lower	Upper	Slope	RR[d]
F3	10.05	6.111	20.33	0.0857	101.7	77.16	140	0.0112	10.1194
F4	9.151	6.376	17.53	0.1259	40.87	NC	NC	0.0133	4.466179
F5	21.99	16.37	31.09	0.0415	15.09	6.411	36.53	0.0472	0.686221
F6	24.57	18.66	34.09	0.0498	92.66	66.63	136.1	0.0107	3.771266
F8	24.11	18.12	33.5	0.0487	490.5	365.6	691.2	0.0022	20.34426
F9	38.32	NC	NC	0.0291	387.6	255.1	647.7	0.0028	10.11482
F10	54.44	NC	NC	0.0238	655.9	528.7	845.3	0.002	12.04813
F11	1.209	NC	NC	0.2573	119.4	NC	NC	0.0047	98.75931
F12	15.71	7.846	38.09	0.0754	884.9	NC	NC	0.0012	56.32718

[a/] Toxin incorporated Cry1Ac (µg/ml)

[b/] Lower and upper 95% Fiducial Limits SAS Probit (SAS 2001 Version 8.2)

[c/] Slope calculated by SAS Probit

[d/] Resistance Ratio = LC_{50} selected / LC_{50} control

[e/] NC, not calculated by SAS Probit due to poor fit to log/probit model

Table 3. PROBIT LC_{50} values of *Helicoverpa zea* strains Selected (XYZ F_{11}) and Control (HZ 02) to δ-endotoxin Cry1Ac (µg/ml), 95% upper and lower fiducial limits, and slope.

Strain	LC_{50} (µg/ml)	Lower[a]	Upper	Slope
Selected ♀ x Selected♂	119.4	NC [b]	NC	0.0047
Selected ♀ x Control ♂	49.58	17.65	471.3	0.0167
Control ♀ x Selected ♂	138.3	74.66	479.2	0.0075
Control ♀ x Control ♂	1.209	NC	NC	0.2573

[a] Upper and Lower 95% Fiducial Limits.
[b] NC, not calculated by SAS Probit because of poor fit to log/probit model.

Table 4. PROBIT LC_{50} values of *Helicoverpa zea* strains Selected (XYZ F_{11}) and Control (HZ 02) to δ-endotoxin Cry1Ab (µg/ml), 95% upper and lower fiducial limits, and slope.

Strain	LC_{50} (µg/ml)	Lower[a]	Upper	Slope
Selected ♀ x Selected ♂	530.5	287.9	1345	0.0017
Selected ♀ x Control ♂	332.1	NC[b]	NC	0.0036
Control ♀ x Selected ♂	159.8	133.4	196.4	0.0124
Control ♀ x Control ♂	72.81	35.58	358.3	0.0183

[a] Upper and Lower 95% Fiducial Limits.
[b] NC, not calculated by SAS Probit because of poor fit to log/probit model.

Table 5. PROBIT LC_{50} values of *Helicoverpa zea* strains Selected (XYZ F_{11}) and Control (HZ 02) to δ-endotoxin Cry1F (μg/ml), 95% upper and lower fiducial limits, and slope.

Strain	LC_{50} (μg/ml)	Lower[a]	Upper	Slope
Selected ♀ x Selected ♂	100.4	80.46	128.5	0.0158
Selected ♀ x Control ♂	158.1	130.1	198.6	0.0113
Control ♀ x Selected ♂	185.7	119.7	419.6	0.0088
Control ♀ x Control ♂	455.8	360.5	605.1	0.0039

[a] Upper and Lower 95% Fiducial Limits.
[b] NC, not calculated by SAS Probit because of poor fit to log/probit model.

Table 6. PROBIT LC_{50} values of *Helicoverpa zea* strains Selected (XYZ F_9) and Control (HZ 02) to δ-endotoxin Cry2Ab (µg/ml), 95% upper and lower fiducial limits, and slope.

Strain	LC_{50} (µg/ml)	Lower[a]	Upper	Slope
Selected ♀ x Selected ♂	3.333	2.191	5.019	0.19
Selected ♀ x Control ♂	8.126	6.449	11.19	0.2151
Control ♀ x Selected ♂	15.18	8.148	55.39	0.0999
Control ♀ x Control ♂	5.161	3.256	9.375	0.2761

[a] Upper and Lower 95% Fiducial Limits.
[b] NC, not calculated by SAS Probit because of poor fit to log/probit model.

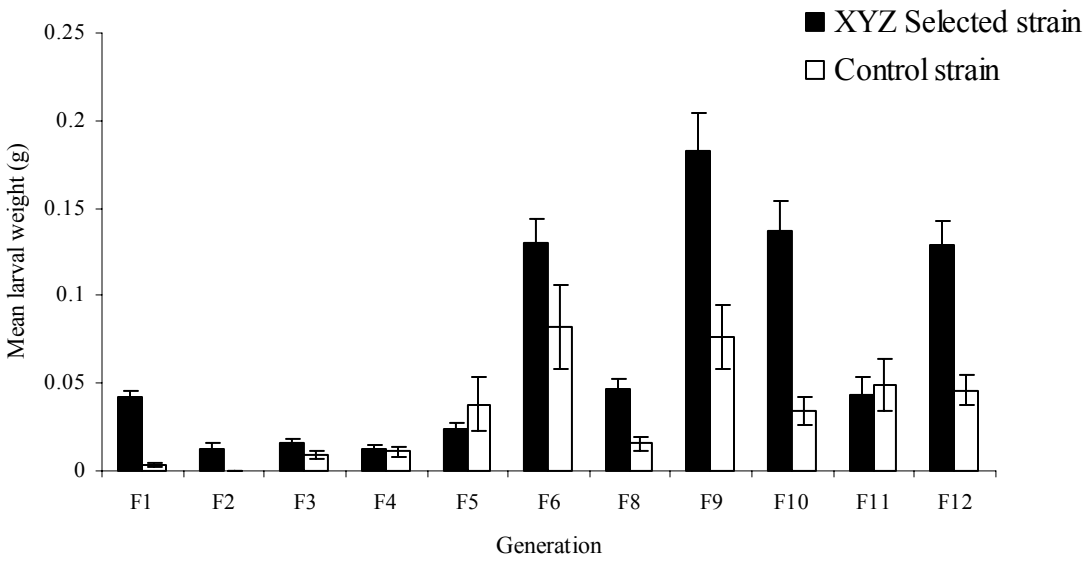

Figure 1. Intergenerational mean larval weights with SEM bars for 2002 *Helicoverpa zea* strains Selected (XYZ) and Control (HZ 02) for concentration 1.6µg/ml Cry1Ac.

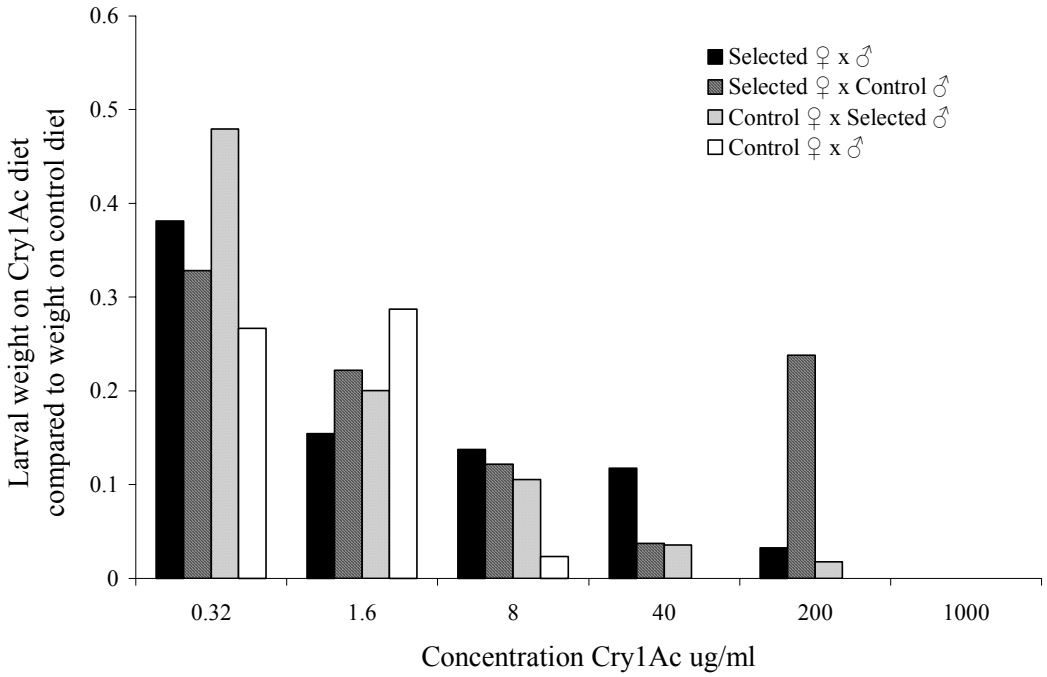

Figure 2. Larval growth ratio comparisons to toxin Cry1Ac for *Helicoverpa zea* crosses: Selected ♀ x ♂, Selected ♀ x Control ♂, Control ♀ x Selected ♂, Control ♀ x ♂.

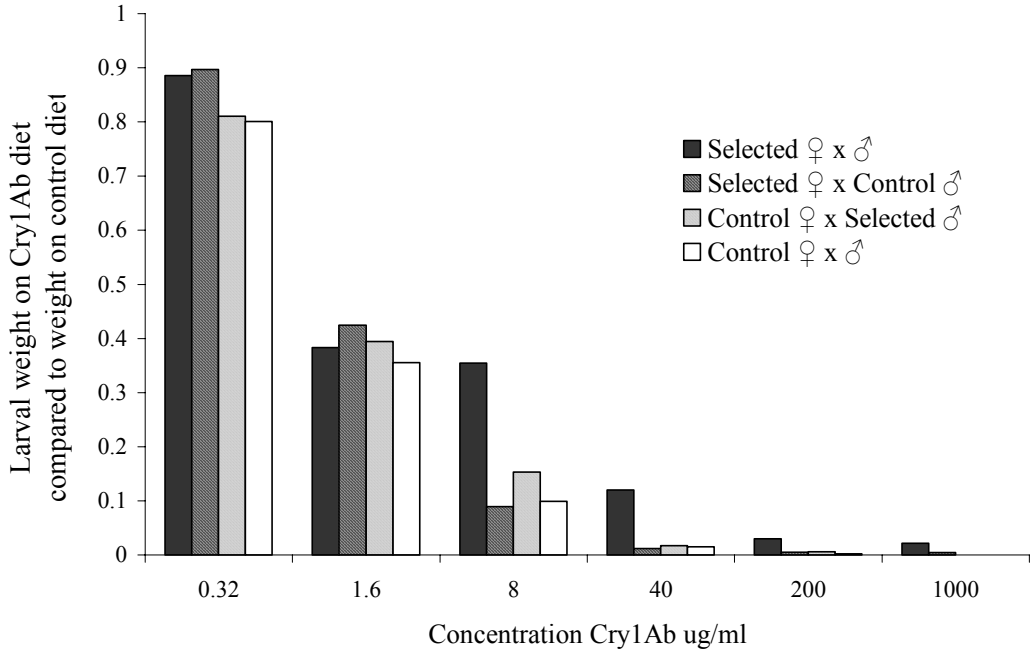

Figure 3. Larval growth ratio comparisons to toxin Cry1Ab for *Helicoverpa zea* crosses: Selected ♀ x ♂, Selected ♀ x Control ♂, Control ♀ x Selected ♂, Control ♀ x ♂.

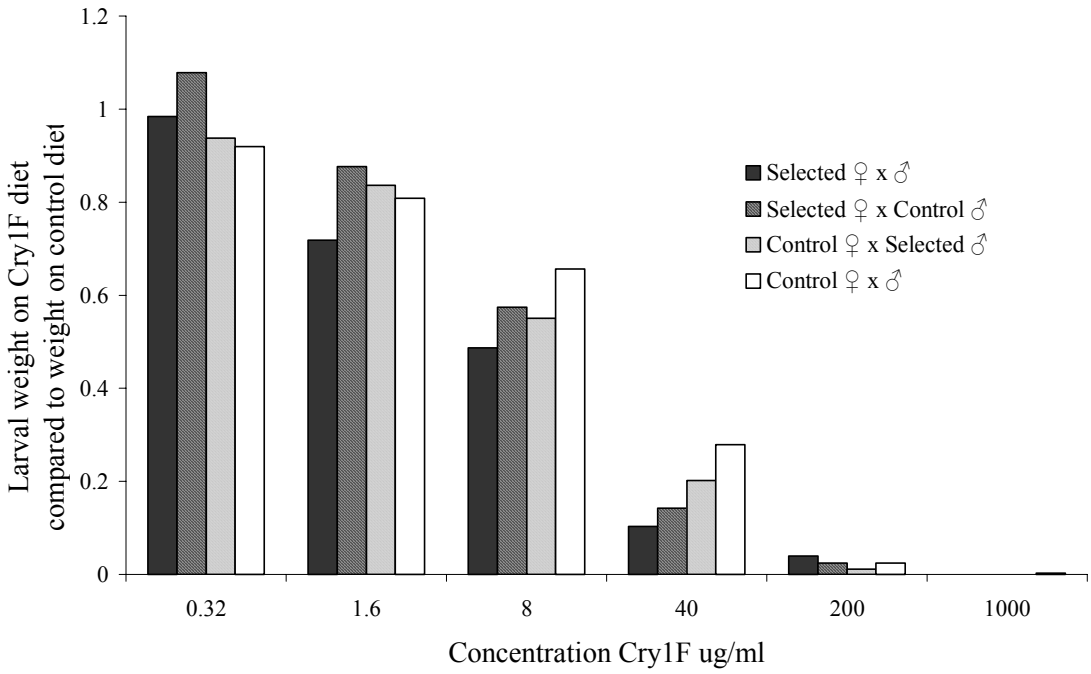

Figure 4. Larval growth ratio comparisons to toxin Cry1F for *Helicoverpa zea* crosses: Selected ♀ x ♂, Selected ♀ x Control ♂, Control ♀ x Selected ♂, Control ♀ x ♂.

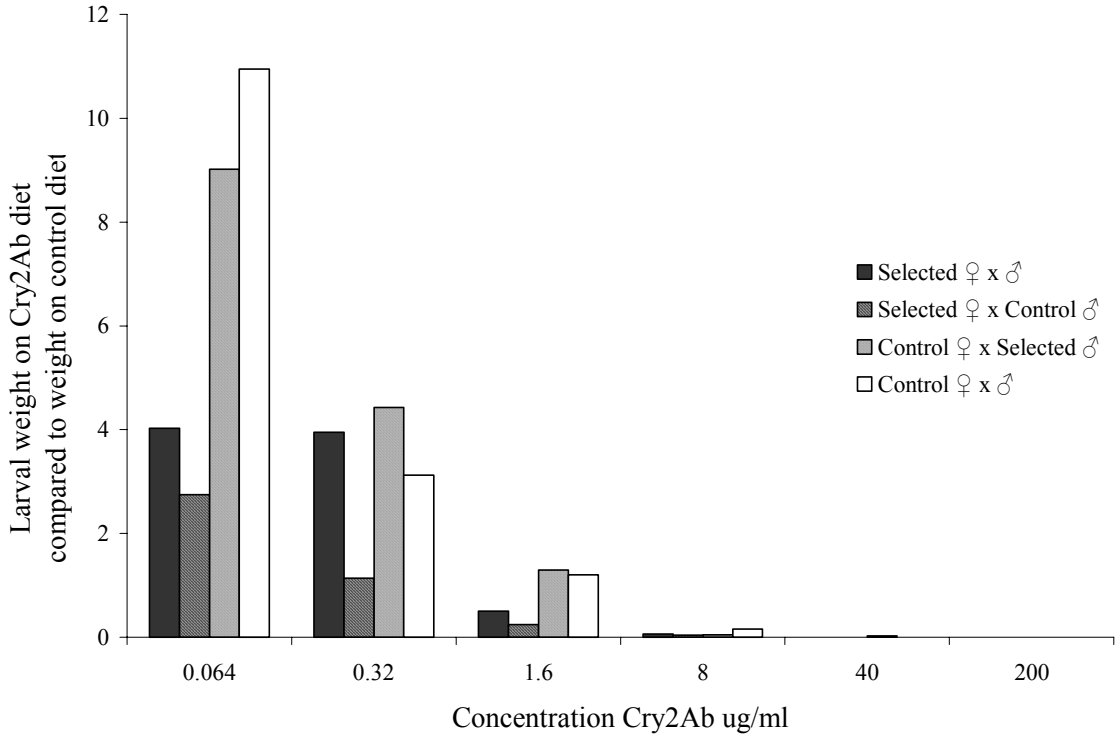

Figure 5. Larval growth ratio comparisons to toxin Cry2Ab for *Helicoverpa zea* crosses: Selected ♀ x ♂, Selected ♀ x Control ♂, Control ♀ x Selected ♂, Control ♀ x ♂.

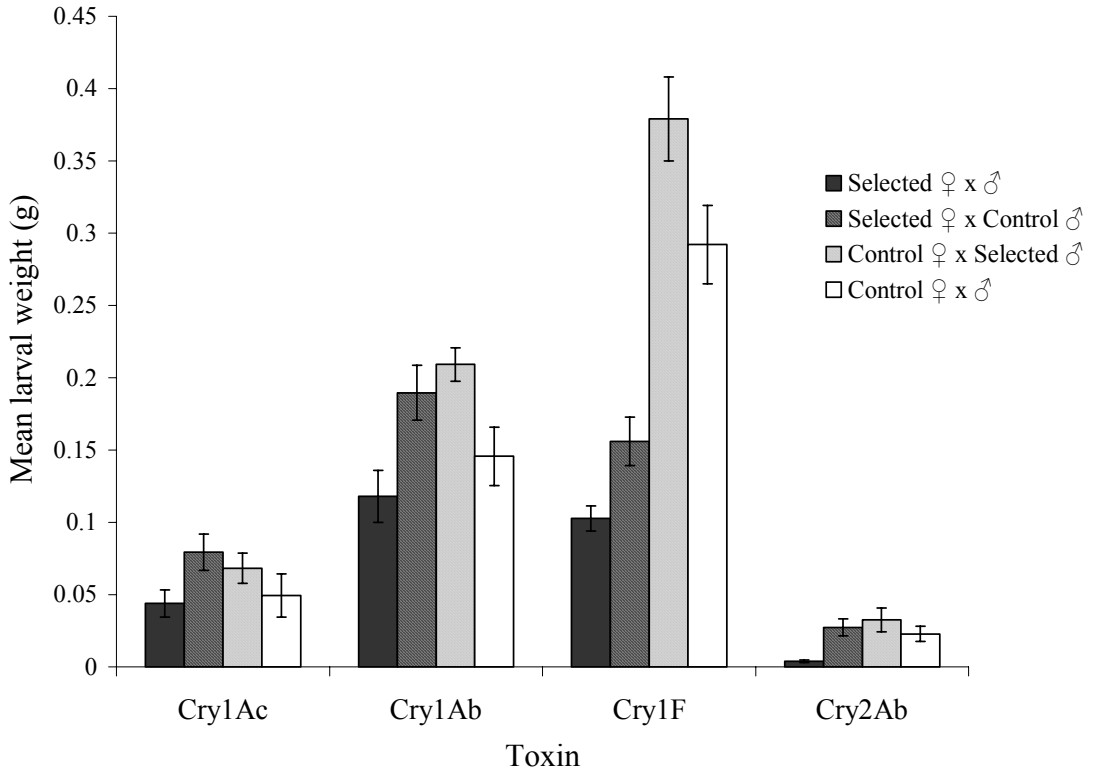

Figure 6. Mean larval weights and SEM bars of *Helicoverpa zea* crosses tested against the δ-endotoxins Cry1Ac, Cry1Ab, Cry1F, and Cry2Ab at concentration 1.6 µg/ml.

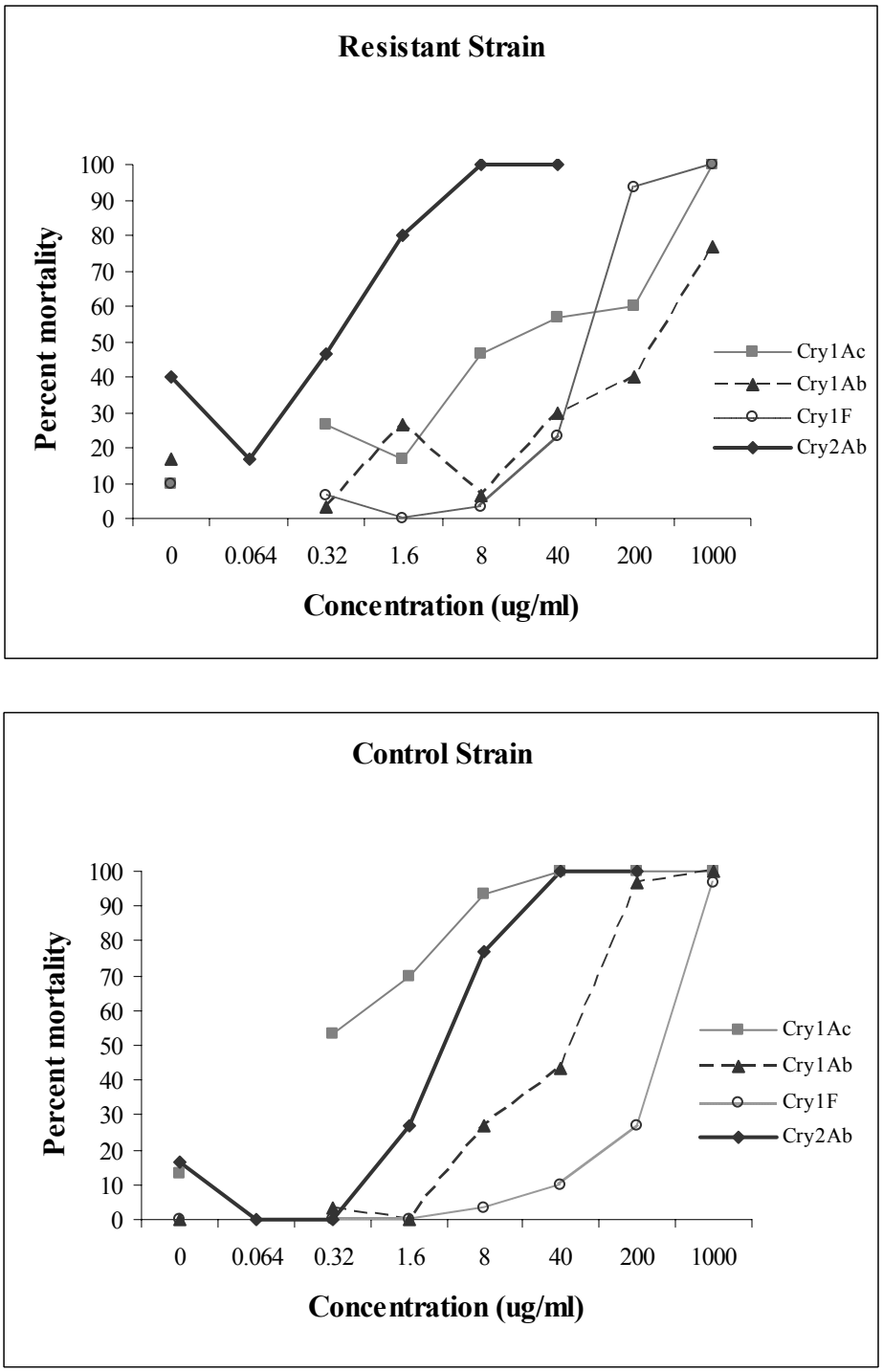

Figure 7. Percent mortality of *Helicoverpa zea* Resistant (Selected ♀ x ♂) and Control (Control ♀ x ♂) strains at varied concentrations for the four δ-endotoxins: Cry1Ac, Cry1Ab, Cry1F and Cry2Ab.

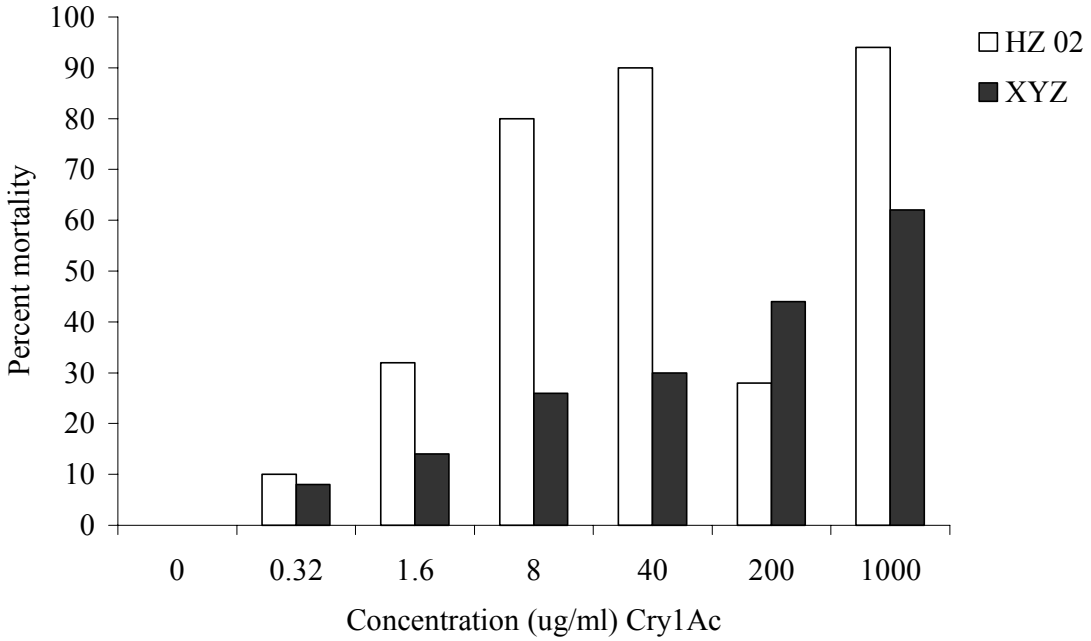

Figure 8. Percent mortality of *Helicoverpa zea* strains HZ 02 and XYZ against δ-endotoxin Cry1Ac for surface treated diet bioassay.

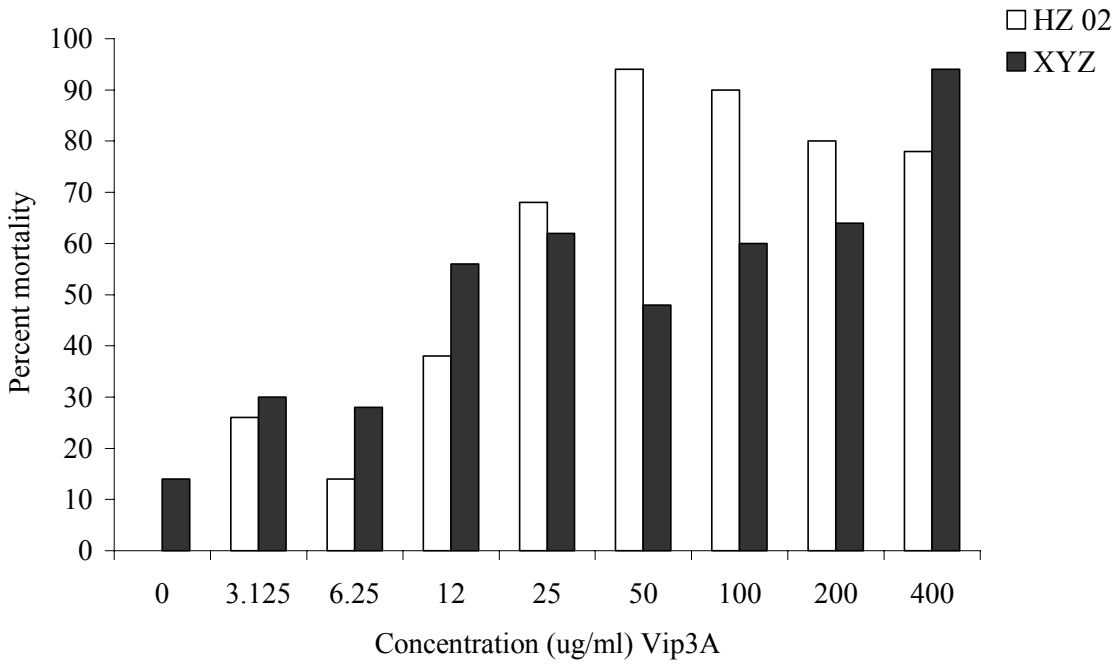

Figure 9. Percent mortality of *Helicoverpa zea* strains HZ 02 and XYZ against Vip3A protein for surface treated diet bioassay.

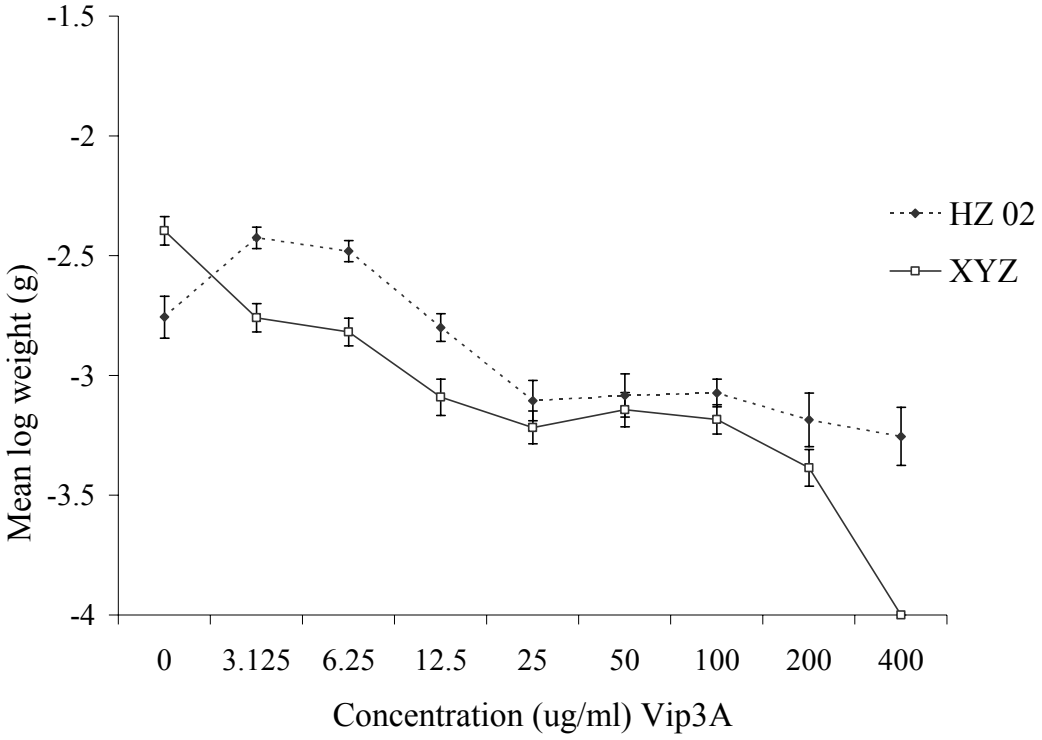

Figure 10. Logweight with SEM bars of *Helicoverpa zea* strains HZ 02 and XYZ against Vip3A protein for surface treated diet bioassay.

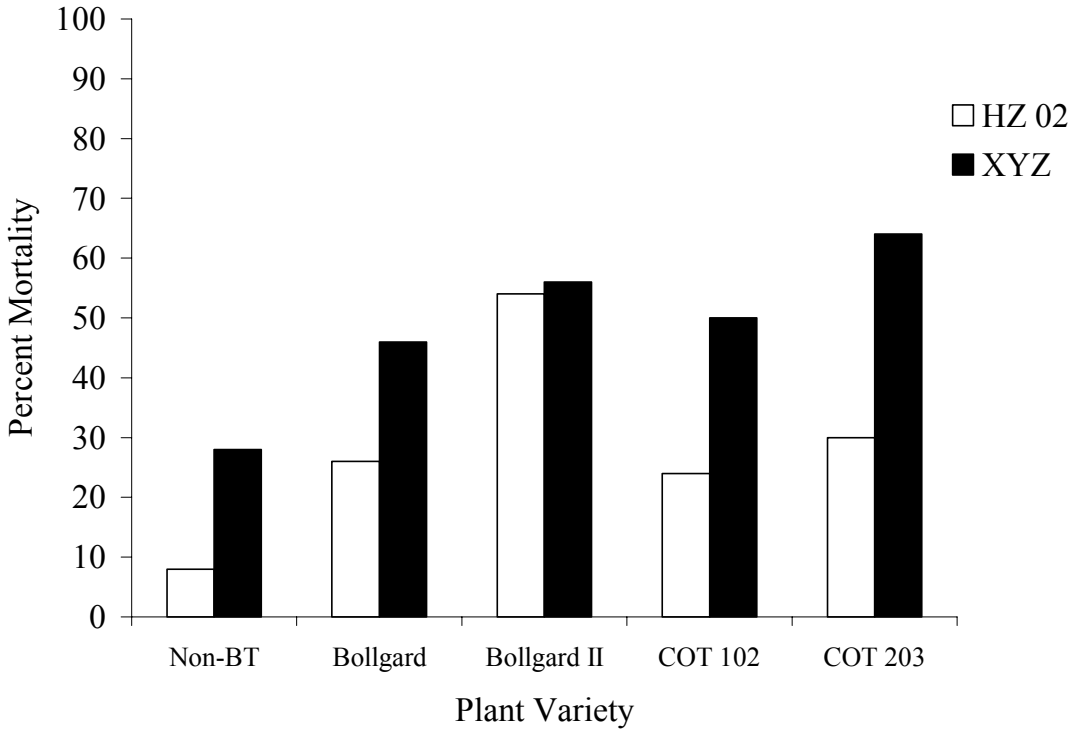

Figure 11. Percent mortality of *Helicoverpa zea* strains HZ 02 and XYZ (5 DAT) for cotton plant leaf assay.

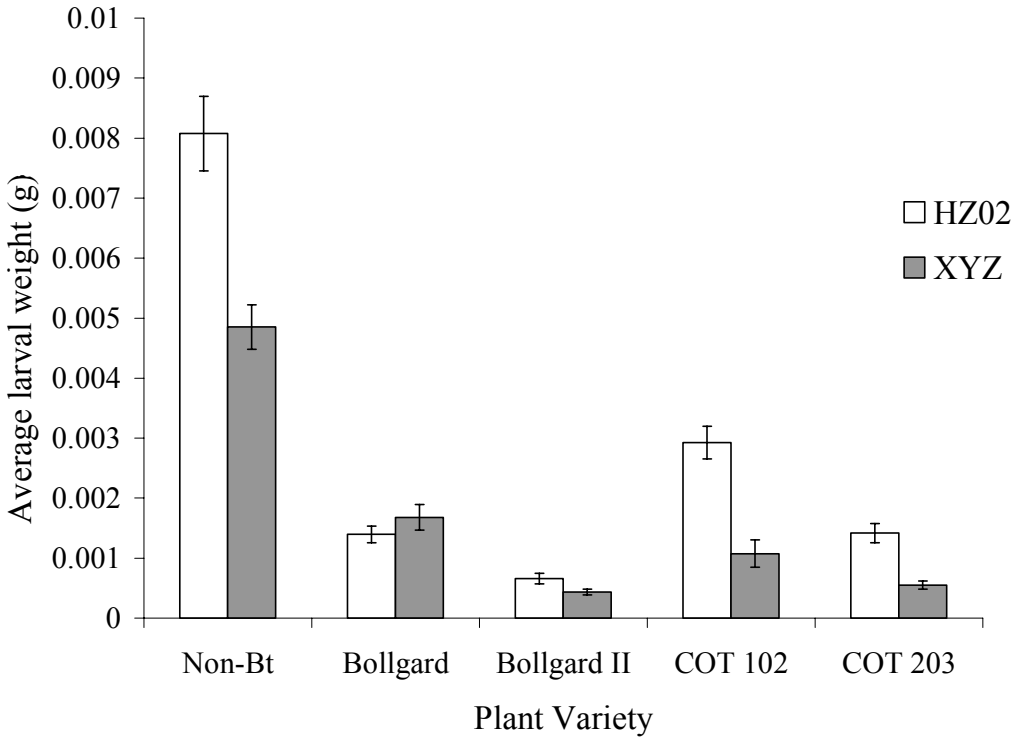

Figure 12. Average larval weight (g) and SEM five days after placement onto leaf material of *Helicoverpa zea* strains HZ 02 and XYZ.

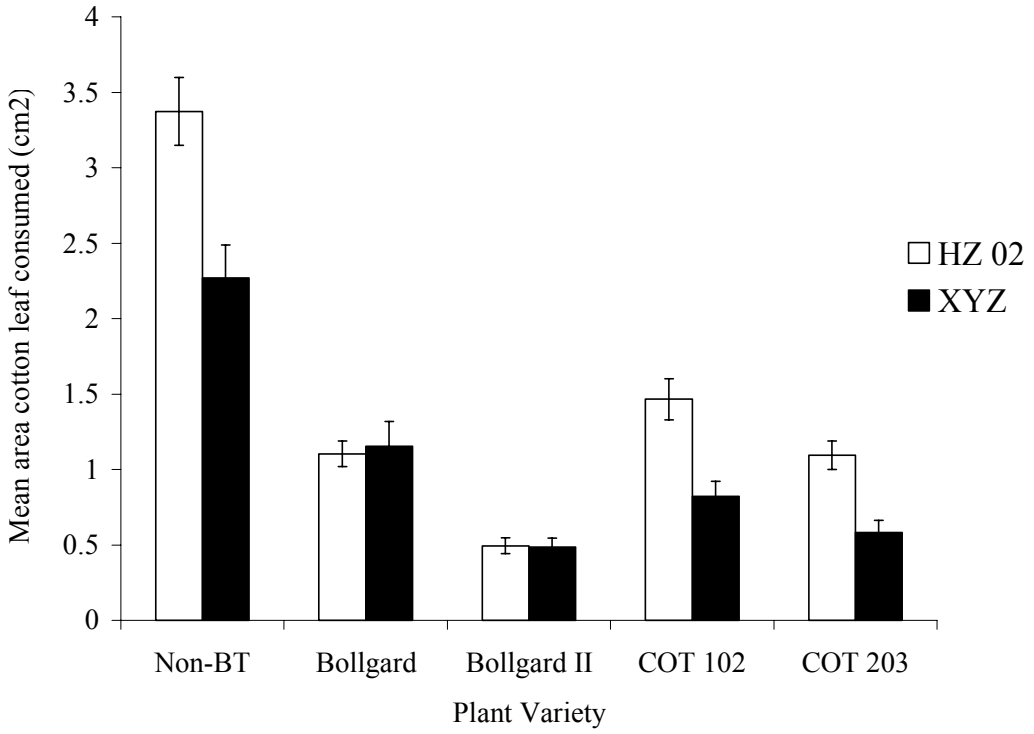

Figure 13. Approximate mean area of leaf tissue (cm^2) consumed by *Helicoverpa zea* strains HZ 02 and XYZ.

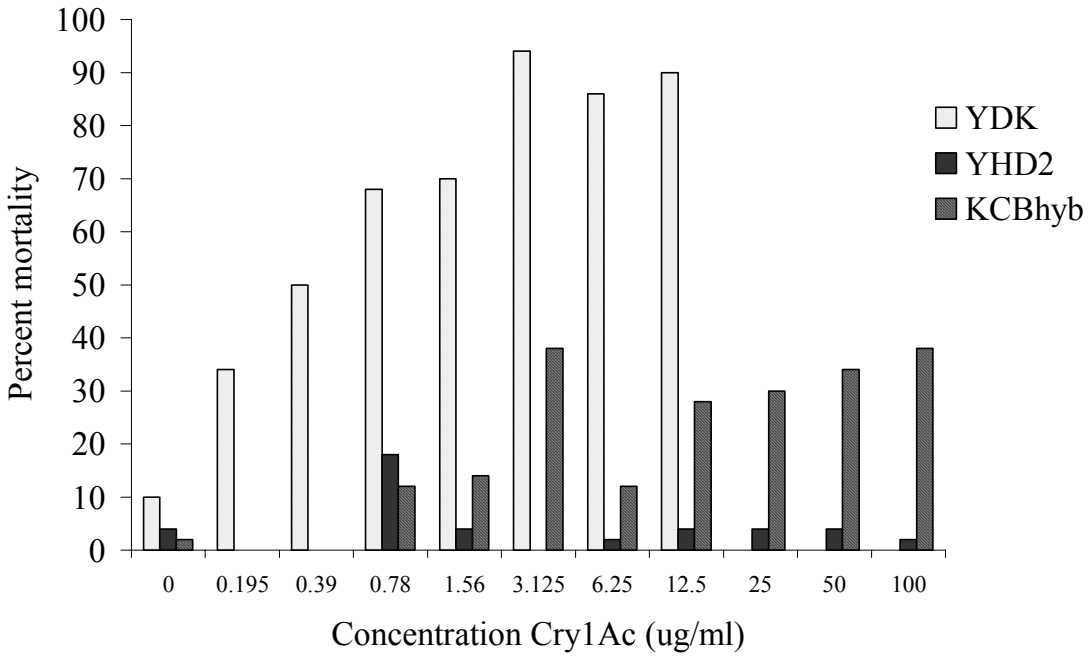

Figure 14. Percent mortality of *Heliothis virescens* strains KCBhyb, YHD2, and YDK for Cry1Ac surface treated diet bioassay.

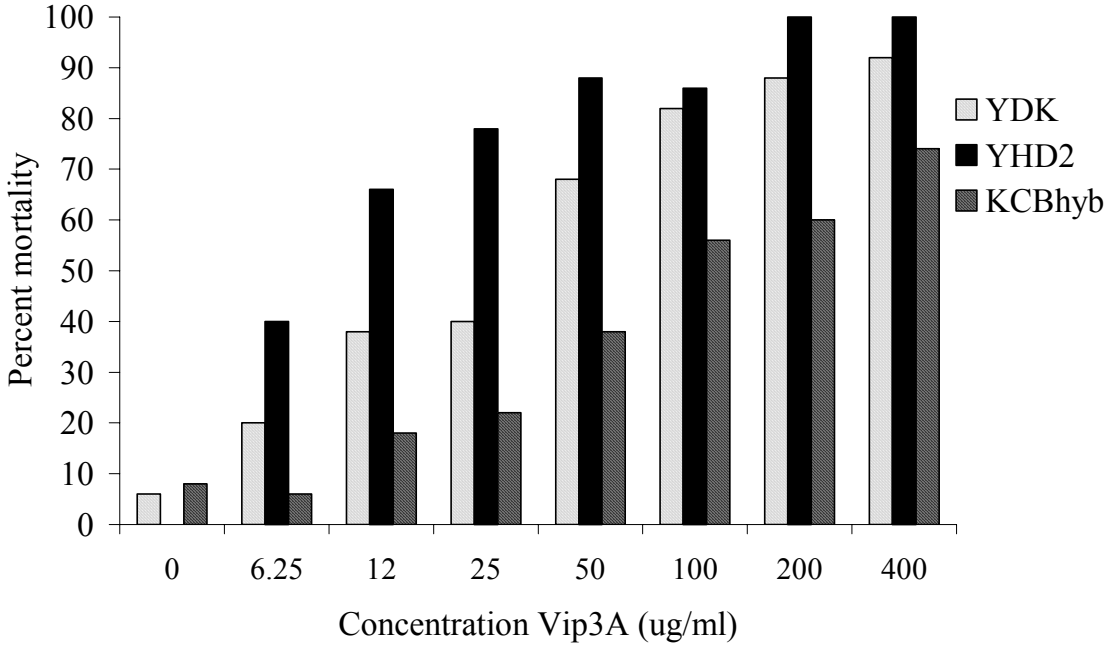

Figure 15. Percent mortality of *Heliothis virescens* strains KCBhyb, YHD2, and YDK for Vip3A surface treated diet bioassay.

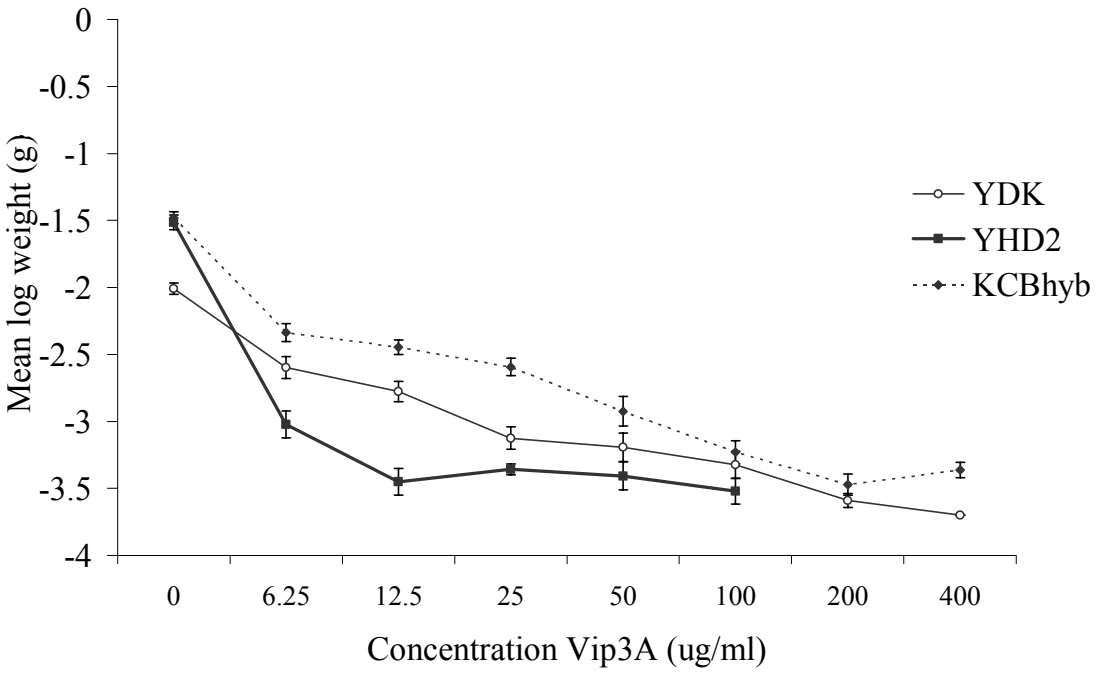

Figure 16. Mean logweight and SEM of *Heliothis virescens* strains KCBhyb, YHD2, and YDK for Vip3A surface treated diet bioassay.

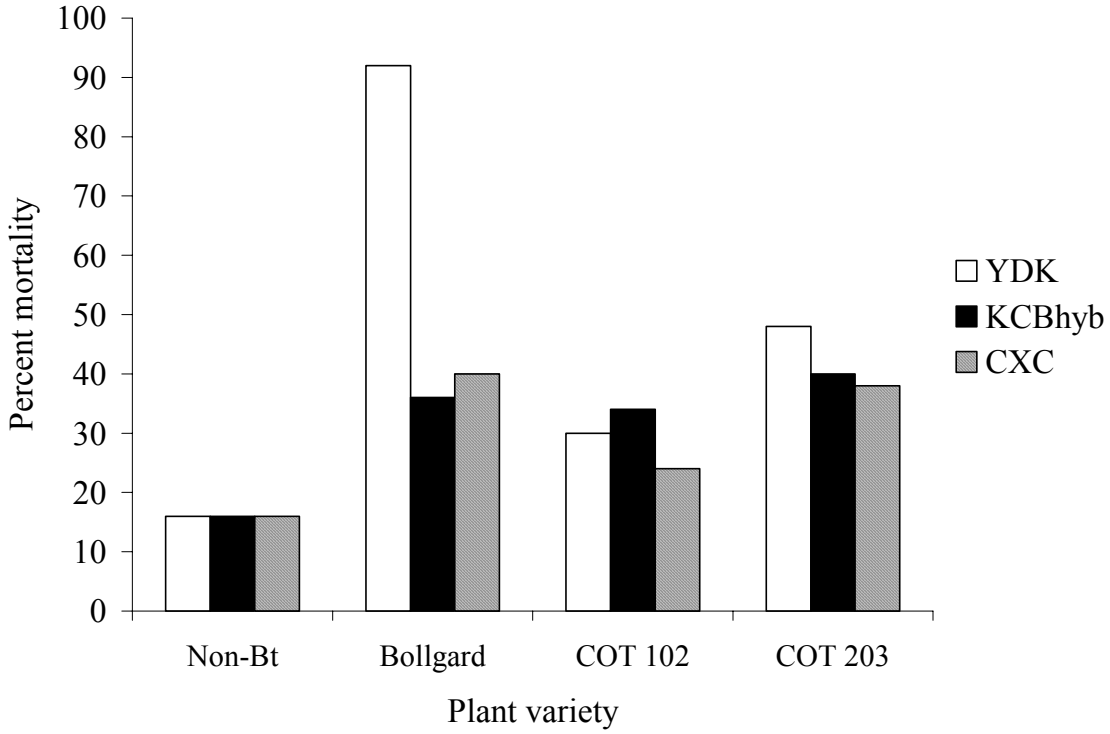

Figure 17. Percent mortality of *Heliothis virescens* strains CXC, KCBhyb, and YDK (5 DAT) for cotton plant leaf material.

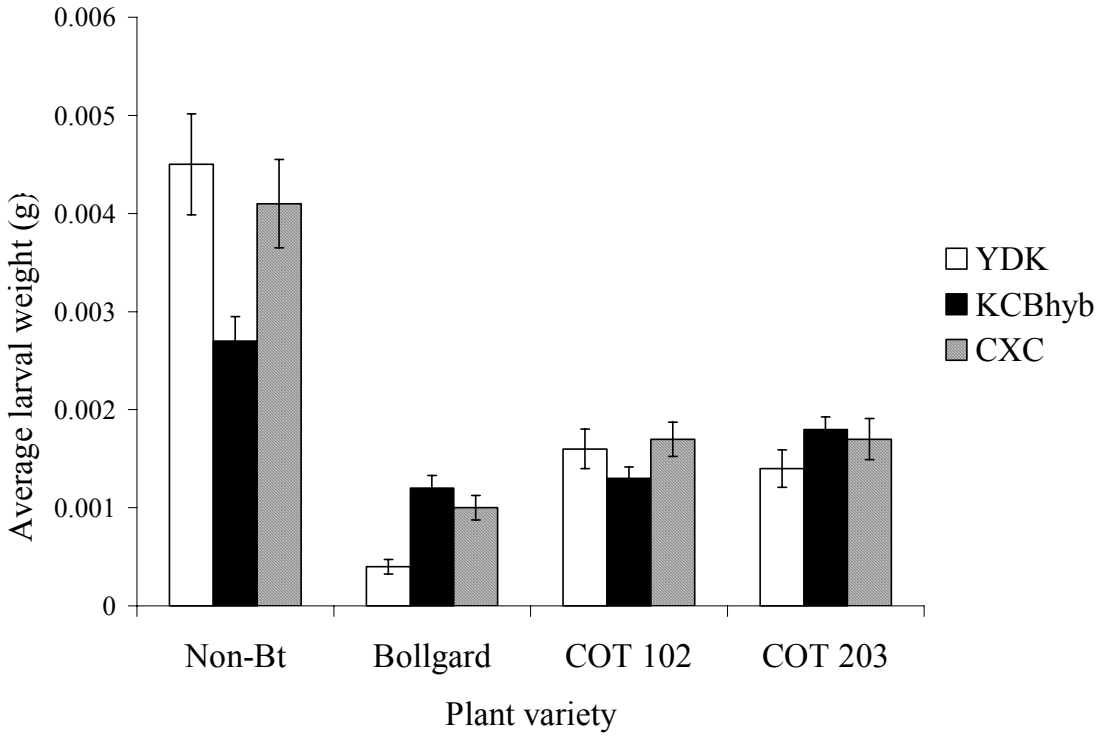

Figure 18. Average larval weight five days after placement onto leaf material of *Heliothis virescens* strains CXC, KCBhyb, and YDK.

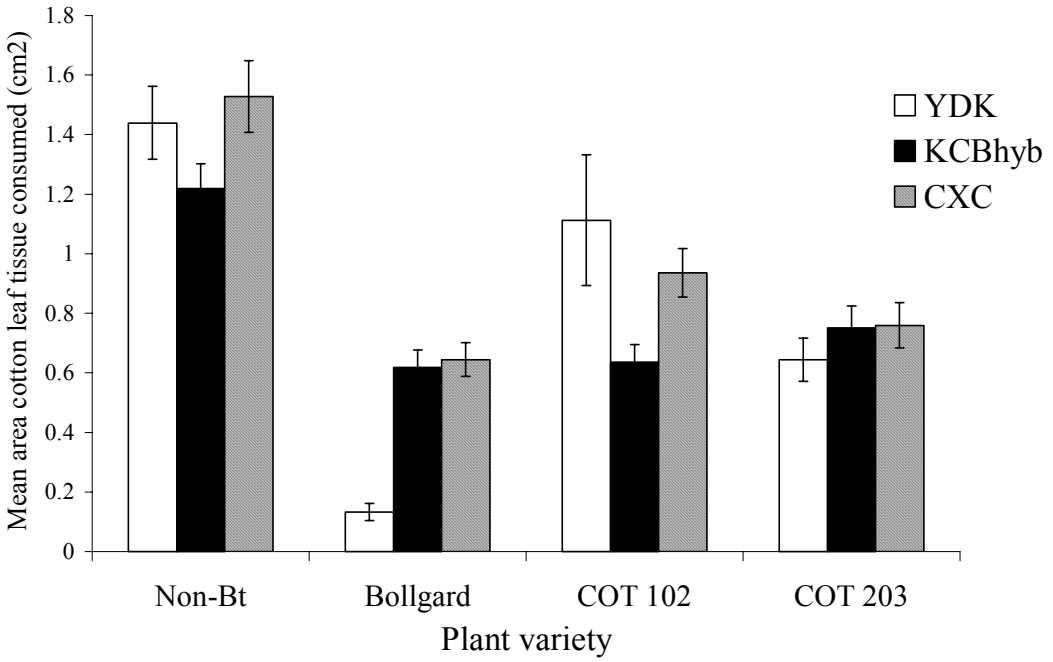

Figure 19. Approximate mean area of leaf tissue (cm^2) consumed by *Heliothis virescens* strains CXC, KCBhyb, and YDK.

CHAPTER TWO

FITNESS AND MATERNAL COSTS ASSOCIATED WITH THE PYRETHROID CYPERMETHRIN AND THE SECONDARY PLANT COMPOUND GOSSYPOL EVALUATED AGAINST A CRY1AC RESISTANT STRAIN OF COTTON BOLLWORM (*HELICOVERPA ZEA*)

Abstract

Widespread use of transgenic cotton Bollgard has raised concern for development of resistance in cotton bollworm (*Helicoverpa zea*). If there were a fitness cost present in individuals carrying the allele for Cry1Ac tolerance, a delay in resistance development could be enhanced. Fitness comparisons between a Bt tolerant (XYZ) and control (HZ 02) bollworm strain were made through exposure to a technical grade pyrethroid, growth on unadulterated insect diet, and growth on secondary plant compound, gossypol. Intergenerational growth responses on an unadulterated diet were measured through larval weight for both *H zea* strains. Significant differences in weight between the two strains were not found. For the pyrethroid evaluations, third instar larvae of both strains over several generations were treated topically with 1 µl of technical grade cypermethrin and allowed to incubate at 27°C for 72 hours at which time mortality was assessed. Results indicate there were no statistically significant differences between the strains within generation. In the gossypol evaluations, first instar larvae were exposed to a diet incorporated blend of varying concentrations and allowed to incubate at 27°C and 14:10 L:D photoperiod. After 10 days larvae were weighed to assess growth and mortality was recorded. Mortality and growth results suggested no differences between the two strains. Fitness costs for cotton bollworm are not apparent for Cry1Ac resistant individuals. This information may be used in developing strategies for managing resistance to transgenic Bt crops.

Introduction

Numerous factors are expected to affect the rate at which insects evolve resistance to toxins produced by the soil bacterium *Bacillus thuringiensis* (Bt). The major factors considered in most models of resistance evolution are: number of generations an insect is exposed to a Bt transgenic crop per year, proportion of the insect population exposed to Bt per generation, mortality of heterozygotes caused by toxin, larval and adult mobility, mating patterns, initial frequency of resistant alleles in population, fitness costs to individuals carrying resistance genes (Gould and Tabashnik 1998, Georghiou and Taylor 1977).

The high dose refuge strategy which is generally recommended to delay the development of resistance is dependant on low initial frequency of resistant alleles, extensive mating between resistant and susceptible adults, and effectively recessive inheritance of resistance in the field (Carrière and Tabashnik 2001). Bollgard cotton is toxic to cotton bollworm (*Helicoverpa zea*) but cannot be characterized as a high dose because a significant number of larvae survive on it under field conditions (Jackson et al. 2002, Mahaffey et al. 1995). It has been estimated 25% of Bollgard acreage in the US receives at least one insecticidal application for control of bollworm annually (Gore et al. 2003).

Although, initial frequencies of Bt resistance alleles in the bollworm to Cry1Ac are low, the resistance alleles examined have been characterized as dominant, or incompletely dominant (Burd et al. 2003). Given this scenario, the frequency of resistance alleles should increase rapidly unless a large refuge is present (Jackson et al. 2003b). Local alternate crop hosts, such as corn and soybean in the southeastern United States may provide substantial refugia for *H. zea* during the cotton production season (Jackson et al. 2003a), and *H. zea* migrating from other areas may also contribute to the refuge population (Gould et al. 2002). The frequency of resistance alleles has not increased in field populations of bollworm in

Eastern North Carolina (Jackson et al. 2002, Burd et al. 2003), despite evidence that *H. zea* has the genetic potential for resistance development (Burd et al. 2000).

In addition to presence of large refuges, the delay of resistance evolution in *H. zea* could, in part, be due to a fitness cost to individuals that have resistance alleles (Carrière and Tabashnik 2001). Unfortunately, available information on fitness costs for this pest remains limited. It has been shown secondary plant compounds like gossypol may contribute to an increase in dominance of fitness costs in pink bollworm (Carrière et al. 2002, 2004). Additionally, coupling the expression of Cry1Ab with that of a terpenoid like gossypol provided greater control of tobacco budworm (*Heliothis virescens*) than either compound alone in lab experiments (Sachs et al. 1996). The present study was initiated to provide more information on the fitness cost incurred by *H. zea* individuals carrying Bt resistance alleles. In the current study, intragenerational fitness of two North Carolina bollworm strains were evaluated under varied environmental conditions.

We tested for fitness costs by comparing a susceptible and Cry1Ac resistant bollworm strain in 1) larval growth on unadulterated artificial diet 2) response to technical grade cypermethrin 3) larval growth on artificial diet containing the secondary plant compound, gossypol. Tests with gossypol also included comparisons of growth of F_1 offspring to assess dominance of any fitness costs.

A fitness cost due to Bt resistance would be expected to decrease survival after exposure to pyrethroids, to decrease growth on non-toxin diet, and exhibit greater growth inhibition and mortality on gossypol. Our results indicate a more complex situation.

Materials and Methods

Cypermethrin Evaluations—Technical grade samples of the pyrethroid, cypermethrin (Chemserve) were used in all testing. The selected strain, XYZ, originated from third instar larvae found on Bollgard cotton in August 2002. A susceptible strain of *H. zea* collected from non-Bt cotton at the same time XYZ was collected was used as the control strain for generations (F_1-F_5). The original control strain was lost after the F_5 generation and was then replaced with the control strain, HZ 02, a strain obtained from light traps in August 2002 and reared at the NCSU Insectary. Larvae tested for response to cypermethrin were all reared on regular diet prior to treatment with cypermethrin, except for tests conducted in the F_1 and F_4 generations where the larvae of the resistant strain were reared on a diet containing Cry1Ac. Within 24 hours of individuals of each strain reaching third instar they were topically treated on the third abdominal terga with 1 µl of a cypermethrin solution diluted with acetone to concentrations ranging from 0.032-1.0 mg/ml. A subset of control larvae were treated with acetone alone. Each larva was placed back into its respective diet chamber and all larvae were held at 27ºC and 14:10 light:dark photoperiod. Number of replicate larvae per concentration ranged from 9-34 individuals in the 14 total bioassays that were conducted. Mortality was assessed 72 hours after treatment and LD_{50} values for each bioassay were calculated using SAS Version 8.0 Probit Analysis.

***Gossypol Evaluations*—**Fitness costs to Cry1Ac resistant *H. zea* in presence of the secondary plant compound gossypol were evaluated using a toxin incorporation diet bioassay. To obtain viable F_1 individuals for testing, we conducted genetic crosses of 15 moths from each sex of the selected and control strains, XYZ and HZ 02, respectively. The gossypol used was a 95% purified gossypol powder sample (Sigma Aldrich). Concentrations of 0.1% and 0.2%

of gossypol were incorporated into artificial diet (Burton 1970). Concentrations were computed by grams of test compound per 100 g of diet and a weight equivalent for acetic acid was used for the control. Newly hatched neonates, which are the most susceptible to gossypol of all larval stages, (Shaver and Parrott 1970), were placed on the diet with a fine camel hair paintbrush. Each concentration was evaluated with 50 individuals and incubated for 10 days at optimum temperature 29.4° C (Thomas 1991) with a 14:10 light:day photophase. Mortality was assessed and subsets of 30 individuals per concentration were weighed for growth.

Results

Cypermethrin Evaluations—Cypermethrin LD_{50} values (Table 1) for the selected and control strains were not significantly different except in the F_8 generation. In generations F_1 and F_4, in which a subset of larvae of the resistant strain were reared on Bt prior to exposure to the pyrethroid; the larvae reared on Bt diet had higher LD_{50}s than their non-Bt reared counterparts. However, these differences between diet treatments within strain were not significant. There were no significant differences in LD_{50}s between larvae of the resistant and control strains tested in a single generation. Generations $F_{5, 6, 9,}$ and $_{10}$ had the lowest LD_{50}s for the selected strain, but these values did not significantly differ from each other. There was no overall difference in the LD_{50}s for the two control strains used in this study, The NBT 02 (F_4) replicate had the highest LD_{50} value overall and differed significantly from HZ 02. The other two NBT 02 reps (F_1 and F_5) did not differ significantly from HZ 02.

Based on the mean larval log weight on Cry1Ac diet (Figure 1), the F_8 had the greatest resistance ratio value (RR=20.34) between the control and selected strain. The greatest difference in larval log weights on non toxin diet was observed for F_1, where

selected mean log weight was significantly higher than control strain mean log weight. This was also the case for F_4 and F_5, but not for the remaining generations. In general, mean log weights in the Cry1Ac selected strain were greater than those of the control strain.

Gossypol Evaluations—The control ♀ x ♂ cross had the highest mean weight value of the four different crosses for diet without gossypol (Table 2). The control ♀ x selected ♂ and selected ♀ x control ♂ crosses were similar to each other, but their mean weights were almost four times greater than that of the resistant ♀ x ♂ cross. At the 0.1% gossypol concentration, selected ♀ x control ♂ mean weight was the highest value by far. Mean weight for this cross was almost twice the value of the two crosses with control ♀ parents, and three times the size of selected ♀ x ♂ cross. At highest concentration gossypol tested, 0.2%; the selected ♀ x control ♂ cross had the highest mean weight overall. The other three crosses did not significantly differ among each other.

Larval growth on gossypol compared to growth on control diet (Figure 2) was greatest for the selected ♀ x control ♂ cross at 0.1% gossypol. Mean weight for this set was approximately 65% of the weight of larvae from the control. Crosses with a control ♀ as one of the parents were growth inhibited by the gossypol for both concentrations more than the two crosses where the mother was from the selected strain. Mortality (Figure 3) was low for all four crosses in the control and for concentration 0.1% gossypol. In the 0.2% concentration, mortality for selected ♀ x control ♂ was the only cross that did not exceed 50%.

A two way ANOVA SAS Version 8.0 was performed on the resultant weights of all strains tested. Strain and concentration were both significant factors on larval weight with

95% fiducial limits. Also, there was a significant interaction of strain and concentration affecting weight (F=11.5; df=3,2; $P<0.0001$).

Discussion

Predictions of the selected strain having higher mortality when exposed to a pyrethroid and reduced growth on unadulterated diet in comparison to that of the control strain were not borne out by our data. Results from the pyrethroid evaluations also suggest there is lack of fitness cost in our Bt resistant strain of *Helicoverpa zea*. Additionally, no indication is given to substantiate cross adaptation between the toxins. Aside from the results for the selected strain at F_8, there were no significant differences in LD_{50} values of the control and selected strains within a generation. The highest cypermethrin LD_{50}s were found in the F_4 generation, in which both the selected and control strains had increased in LD_{50} from the F_1 generation. Previous studies have shown there to be a decrease in cypermethrin resistance for field strains of *H. zea* (Campanhola et al. 1991, Marcus et al. 2004) once established in the lab. A non-Bt diet for maternal line of the F_4 generation was the only instance of parental rearing on a non toxin diet; this may have had a positive environmentally based maternal effect on the selected strain for that generation (Rossiter 1991). Another possible explanation for this observation may be an overall elevation in robustness from individuals the previous generation surviving an unexpected freeze in winter 2002. From the F_4-F_{10} tests, the LD_{50} values begin to decrease except for F_8. This generation had the highest Cry1Ac resistance ratio of generations tested. Pyrethroid resistance in *H. zea* is due to nerve insensitivity at the voltage gated sodium channels of the nerve cells (McCaffery 1998). The dissimilar mode of action cypermethrin has and findings reported here suggest supplemental

over sprays of pyrethroids to control *H. zea* in Bt crops will continue to be an effective tool in insecticide resistance management.

For each generation tested for larval growth on normal artificial diet the mean larval log weights of larvae from the selected strain were significantly greater or comparable to those of the control strain. Lambert et al. (1998) had similar results to those found in F_1 and F_4 when she found parents stressed on a Bt toxin diet had offspring larger than offspring from unstressed parents. Lambert et al. (1998) proposed stressed parents were able to produce more fit offspring and cites this as negative maternal effects (Kirkpatrick and Lande 1989, Rossiter 1991). Generations with substantially higher levels of Bt resistance did not possess significantly different sized offspring than the control. The robustness displayed by Bt resistant offspring may be genetically heritable. Rossiter et al. (1990) found variation in gypsy moth susceptibility to Bt and hypothesized such variation may have been based on differences in growth and development capabilities of offspring. Furthermore, to the extent that vigor is genetically based, Rossiter et al. (1990) concluded natural selection will not only favor resistant genotypes, but resistant genotypes that are more vigorous as well. Another possible explanation for why we saw no significant differences in larval weights for the control and resistant strains on non–toxin diet at the higher generations was proposed by Bourget et al. (2004). Bourget et al. (2004) has suggested fitness costs may be lowered by modifier alleles at other genes that minimize the deleterious effects of resistance alleles. In the absence of insecticides, fitness costs are thereby reduced resulting in equal fitness for resistant and susceptible individuals. Generation F_5 had the lowest resistance ratio and was the only test to meet the prediction of larger larvae from the control strain rather than selected strain on non-toxin diet.

The predication that in the gossypol evaluations we would find a greater decrease in growth in the selected strain than the control strain was not supported by our data. Mortality of the offspring from the selected ♀ x control ♂ was significantly lower than for other genotypes tested. Also, mean weights were significantly greater for this cross at both concentrations of gossypol that were tested. Although, mean weight of the resistant ♀ x ♂ was less on non toxin diet than control ♀ x ♂, the Bt resistant strain had larger offspring that fed on 0.2% gossypol. Based on the mean weight data and the growth ratio data it appears there may be a negative maternal effect (Rossiter 1991) at high concentrations of gossypol. The offspring that had a mother that fed on Bt grew larger than offspring from an unstressed mother. Since dominance of fitness costs can be altered by environment or genetics (Bourguet et al. 2000), it appears the RR and RS genotypes were all significantly larger than SS suggesting a genetic contribution from the Bt resistant strain in increasing robustness when exposed to gossypol. Given that the RS progeny performed better than the SS in both the mean weights and growth ratios; fitness costs associated with gossypol are recessive (Carrière et al. 2004, 2002) and do not increase in magnitude or dominance with the secondary plant compound.

The data herein provide information that can aid in the design of more effective resistance management strategies for *Helicoverpa zea* in North Carolina cotton producing areas by examining the impact fitness costs may have on vigor in Bt resistant cotton bollworm.

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References

Bourget, D., T. Guillemaud, C. Chevillon, and M. Raymond. 2004. Fitness costs of insecticide resistance in natural breeding sites of the mosquito *Culex pipens*. Evolution. 58(1): 128-135.

Bourguet, D., A. Genissel, and M. Raymond. 2000. Insecticide resistance and dominance levels. J. Econ. Entomol. 93(6): 1588-1595.

Burd, A. D., F. Gould, J. R. Bradley, J. W. Van Duyn, and W. J. Moar. 2003. Estimated frequency of nonrecessive *Bt* resistance genes in bollworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) in eastern North Carolina. J. Econ. Entomol. 96(1): 137-142.

Burd, A. D., J. R. Bradley, J. W. Van Duyn, and F. Gould. 2000. Resistance of bollworm, *Helicoverpa zea*, to Cry1Ac toxin. pp. 923-926. *In* C. P. Dugger and D. A. Richter (editors) 2000 Proc. Beltwide Cotton Conference. National Cotton Council, Memphis, TN.

Burton, R. L. 1970. A low-cost artificial diet for the corn earworm. J. Econ. Entomol. 63(6): 1969-1970.

Campanhola, C., B. F. McCutchen, E. H. Baehrecke, and F. W. Plapp, Jr. 1991. Biological constraints associated with resistance to pyrethroids in the tobacco budworm (Lepidoptera: Noctuidae). J. Econ. Entomol. 84(5): 1404-1411.

Carrière, Y., C. Ellers-Kirk, R. Biggs, D. M. Higginson, T. J. Dennehy, and B. E. Tabashnik. 2004. Effects of gossypol on fitness costs associated with resistance to Bt cotton in pink bollworm. J. Econ. Entomol. 97(5): 1710-1718.

Carrière, Y., T. J. Dennehy, C. Ellers-Kirk, D. Holley, Y.-B. Liu, M. A. Sims, and B. E. Tabashnik. 2002. Fitness costs, incomplete resistance, and management of resistance to Bt crops. *In* R. J. Akhurst, C. E. Beard, and P. Hughes (Eds.). *Biotechnology of* Bacillus thuringiensis *and its environmental impact*. Proc. 4th Pacific Rim Conference. Canberra, Australia.

Carrière, Y., and B. E. Tabashnik. 2001. Reversing insect adaptation to transgenic insecticidal plants. Proc. R. Soc. Lond. 268: 1475-1480.

Georghiou, G. P., and C. E. Taylor. 1977. Operational influences in the evolution of insecticide resistance. J. Econ. Entomol. 70(5):653-658.

Gore, J., B. R. Leonard, and R. H. Gable. 2003. Distribution of bollworm, *Helicoverpa zea* (Boddie), injured reproductive structures on genetically engineered *Bacillus thuringiensis* var. *kurstaki* Berliner cotton. J. Econ. Entomol. 96(3): 699-705.

Gould, F., N. Blair, M. Reid, T. L. Rennie, J. Lopez, and S. Micinski. 2002. *Bacillus thuringiensis* – Toxin resistance management: Stable isotope assessment of alternate host use by *Helicoverpa zea*. Proc. Natl. Acad. Sci. USA. 99(16): 581-586.

Gould F. and B. Tabashnik. 1998. Bt cotton resistance management. pp. 67-105. *In* M. Mellon and J. Rissler (editors). Now or never: Serious new plans to save a natural pest control. Union of Concerned Scientists

Jackson, R. E., J. R. Bradley Jr., and J. W. Van Duyn. 2003a. Quantification of *Helicoverpa zea* populations in eastern North Carolina crop environments: Implications for *Bt* resistance management. pp. 1017-1021. *In* D. A. Richter (editor). 2003 Proc. Beltwide Cotton Conference. National Cotton Council, Memphis, TN.

Jackson, R. E., J. R. Bradley Jr., J. W. Van Duyn, and A. D. Burd. 2003b. *Bt* Resistance evolution in the *Helicoverpa zea* population in eastern North Carolina. pp. 1168-1176. *In* D. A. Richter (editor). 2003 Proc. Beltwide Cotton Conference. National Cotton Council, Memphis, TN.

Jackson, R. E., J. R. Bradley Jr., and J. W. Van Duyn. 2002. Estimated production of *Helicoverpa zea* adults from Bollgard and Bollgard II cottons and implications for resistance management. *In* J. McRae and D. A. Richter (editors). 2002 Proc. Beltwide Cotton Conference. National Cotton Council, Memphis, TN.

Kirkpatrick, M. and R. Lande. 1989. The evolution of maternal characters. Evolution. 43(3): 485-503.

Lambert, A. L., J. R. Bradley Jr., F. Gould, and J. W. Van Duyn. 1998. Bollworm (*Helicoverpa zea*): adaptation to *Bt* toxin? pp.1033-1037. *In* C. P. Dugger and D. A. Richter (editors). 1998 Proc. Beltwide Cotton Conference. National Cotton Council, Memphis, TN.

Mahaffey, J. S., J. R. Bradley, Jr., and J. W. Van Duyn. 1995. Bt cotton: Field performance in North Carolina under conditions of unusually high bollworm populations. pp. 795-798. *In* C. P. Dugger and D. A. Richter (editors). 1995 Proc. Beltwide Cotton Conference. National Cotton Council, Memphis, TN.

Marcus, M. A., J. R. Bradley, F. L. Gould, and J. W. Van Duyn. 2004. Fitness evaluations of *Helicoverpa zea* (Boddie) from Bollgard cotton in subsequent generations. Pp. 1390-1394. *In* D. A. Richter (editor). 2004 Proc. Beltwide Cotton Conference. National Cotton Council, Memphis, TN.

McCaffery, A. R. 1998. Resistance to insecticides in heliothine Lepidoptera: A global view. Phil. Trans. R. Soc. Lond. *B*. 353: 1735-1750.

Rossiter, M. C. 1991. Environmentally-based maternal effects: A hidden force in insect population dynamics? Oecologia. 87: 288-294.

Rossiter, M. C., W. G. Yendol, and N. R. Dubois. 1990. Resistance to *Bacillus thuringiensis*: Genetic and environmental causes. J. Econ. Entomol. 83(6): 2211-2218.

Sachs, E. S., J. H. Benedict, J. F. Taylor, D. M. Stelly, S. K. Davis, and D. W. Altman. 1996. Pyramiding Cry1A(b) insecticidal protein and terpenoids in cotton to resist tobacco budworm (Lepidoptera: Noctuidae). Environ. Entomol. 25(6): 1257-1266.

SAS Institute. 1999. SAS/STAT User's guide: Version 8, Volume 3. SAS Institute. Cary, North Carolina.

Shaver, T. N. and W. L. Parrott. 1970. Relationship of larval age to toxicity of gossypol to bollworms, tobacco budworms, and pink bollworms. J. Econ. Entomol. 63(6): 1802-1804.

Thomas, W. M. 1991. Modeling the effect of temperature and gossypol concentration on developmental rate of *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae). J. Econ. Entomol. 84(2): 466-469.

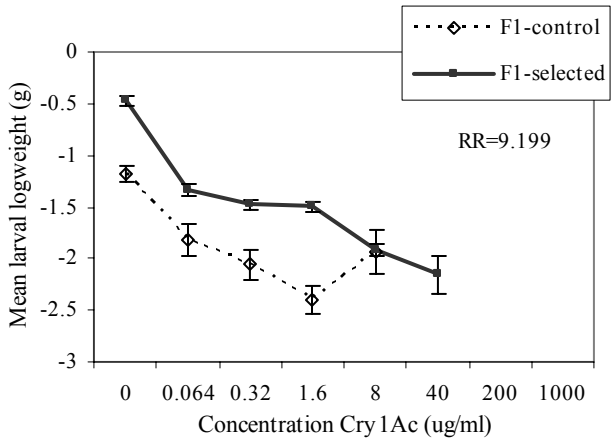
Table 1. Cypermethrin evaluations with LD_{50} values for Cry1Ac resistant selected strain XYZ (F_1-F_{10}) and susceptible control strains NBT 02 ($F_1 – F_5$) and HZ 02 (F_6-F_{10})

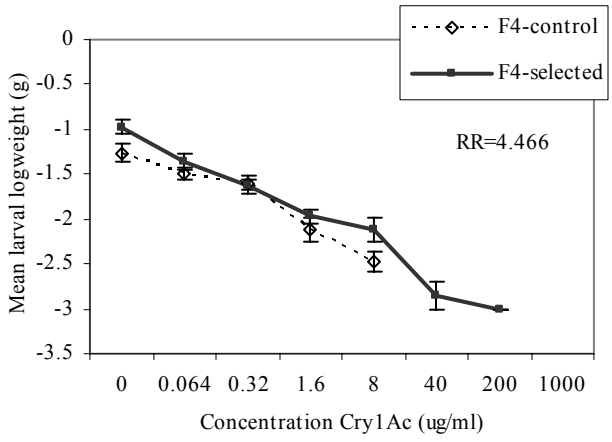
Selected strain						***Control Strain***		
Generation	Maternal diet	Larval diet	LD_{50} (mg/ml)	Lower	Upper	LD_{50} (mg/ml)	Lower	Upper
F1	Bollgard cotton	NBT	0.3106	0.25946	0.38111	0.3118	0.16822	0.41513
F1	Bollgard cotton	BT	0.42006	0.34298	0.53123			
F4	NBT	NBT	0.63845	0.46208	1.05362	0.52479	0.3145	1.03378
F4	NBT	BT	0.67529	0.52534	0.96922			
F5	BT	NBT	0.25004	0.22988	0.39695	0.34606	0.25394	0.48911
F6	BT	NBT	0.18977	0.12756	0.2423	0.13658	0.01987	0.26329
F8	BT	NBT	0.40887	0.32509	0.52172	0.15622	0.10839	0.19578
F9	BT	NBT	0.121756	0.082004	0.149145	0.103811	0.060486	0.13185
F10	BT	NBT	0.12012	0.03248	0.17325	0.092827	0.048188	0.120468

Table 2. Mean weight (SEM) of larvae in grams after 10 days of exposure to gossypol incorporated into corn soy blend insect diet.

	Percent weight of diet gossypol constitutes		
Strain	0 %	0.1 %	0.2 %
Selected ♀ x Selected♂	0.0444 c*	0.0317 c	0.0039 b
	(0.004)	(0.003)	(0.001)
Selected ♀ x Control ♂	0.1426 b	0.0934 a	0.0069 a
	(0.009)	(0.006)	(0.001)
Control ♀ x Selected ♂	0.1423 b	0.0459 b	0.0026 b
	(0.008)	(0.004)	(0.001)
Control ♀ x Control ♂	0.1854 a	0.0514 b	0.0017 b
	(0.013)	(0.006)	(0.001)

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





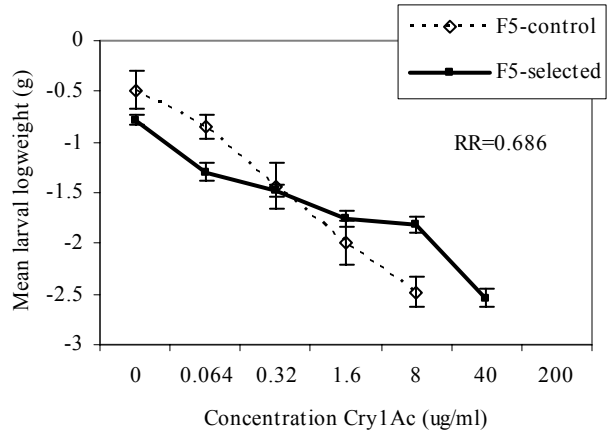
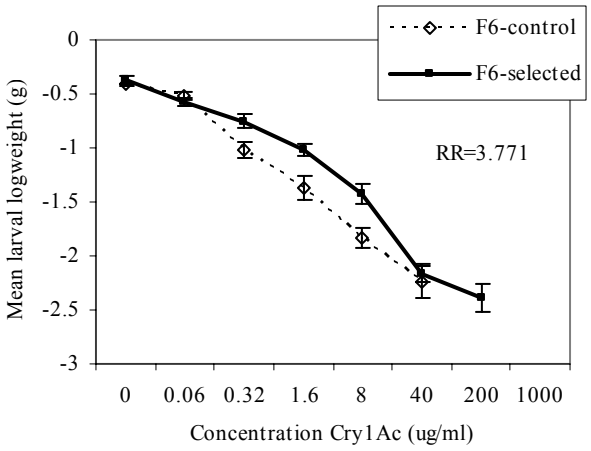
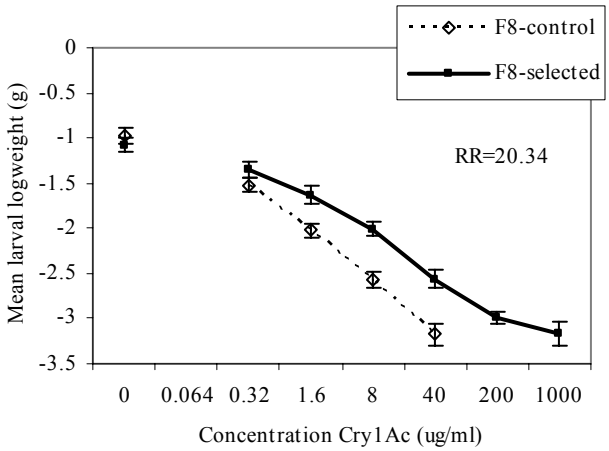


Figure 1. Mean larval log weight comparisons (SEM) for Cry1Ac resistant selected strain XYZ (F_1-F_{10}) and susceptible control strains NBT 02 (F_1–F_5) and HZ 02 (F_6-F_{10}). Resistance Ratios (RR) calculated from Cry1Ac LC_{50} value from selected strain divided by Cry1Ac LC_{50} value from control strain.





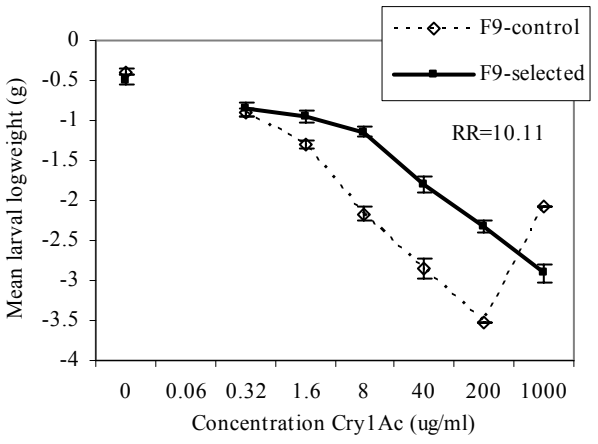


Figure 1. Continued.

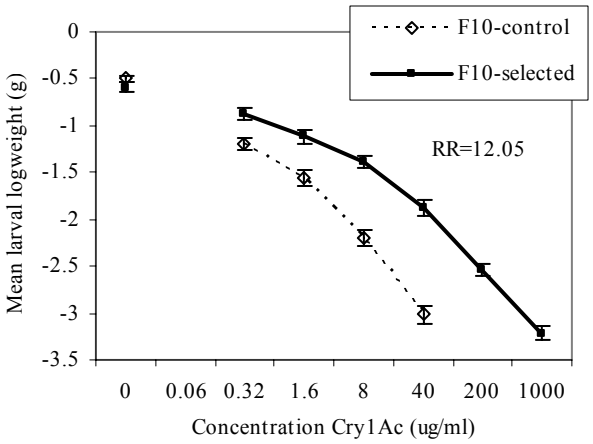


Figure 1. Continued.

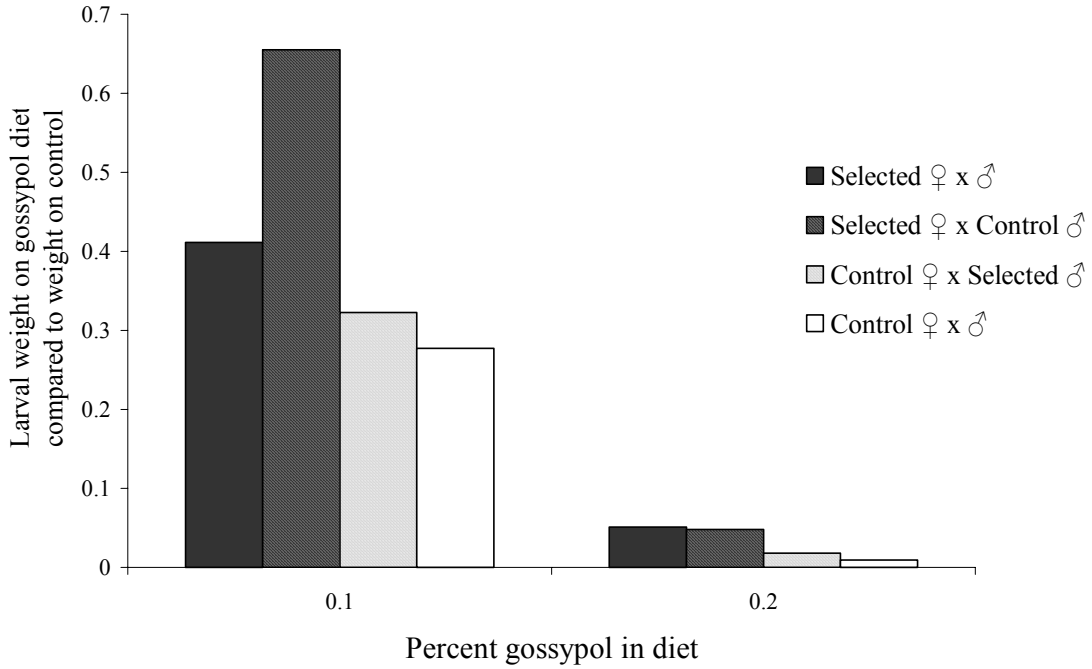


Figure 2. Larval growth ratio comparisons 10 days after exposure to gossypol incorporated diet for the *Helicoverpa zea* crosses: Selected ♀ x ♂, Selected ♀ x Control ♂, Control ♀ x Selected ♂, and Control ♀ x ♂.

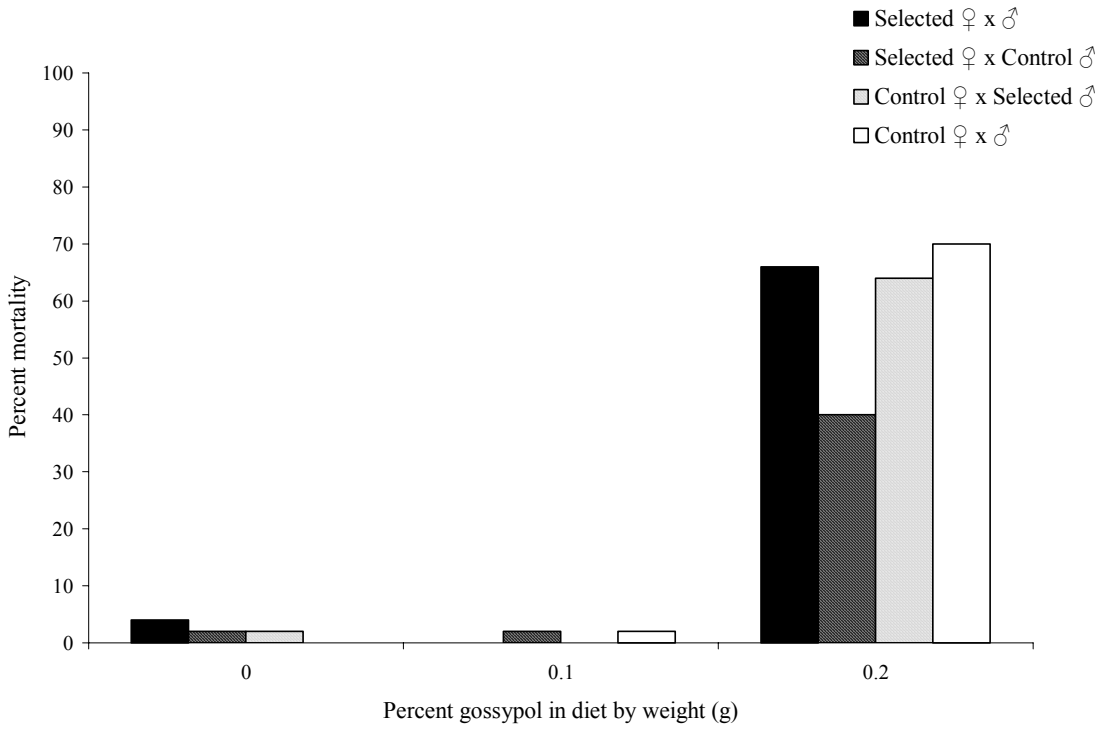


Figure 3. Percent mortality 10 days after larval exposure to gossypol incorporated diet for the *Helicoverpa zea* crosses: Selected ♀ x ♂, Selected ♀ x Control ♂, Control ♀ x Selected ♂, and Control ♀ x ♂.