PRODUCTION AND CHARACTERIZATION OF BT CRY1AC RESISTANCE IN BOLLWORM, *HELICOVERPA ZEA* (BODDIE)

Except where reference is made to the work of others, the work described in this dissertation is my own or was done in collaboration with my advisory committee. This dissertation does not include proprietary or classified information.

Konasale Jayaramu Anilkumar

Certificate of approval:

Arthur G. Appel Professor Entomology and Plant Pathology	William J. Moar, Chair Professor Entomology and Plant Pathology
Nannan Liu Associate Professor Entomology and Plant Pathology	Sakuntala Sivasupramaniam Senior Research Scientist Monsanto Company, St. Louis, MO
James T. Bradley Professor Biological Sciences	George T. Flowers Interim Dean Graduate School

PRODUCTION AND CHARACTERIZATION OF BT CRY1AC RESISTANCE IN BOLLWORM, *HELICOVERPA ZEA* (BODDIE)

Konasale Jayaramu Anilkumar

A Dissertation

Submitted to

the Graduate Faculty of

Auburn University

in Partial Fulfillment of the

Requirements for the

Degree of

Doctor of Philosophy

Auburn, Alabama
August 9, 2008

PRODUCTION AND CHARACTERIZATION OF BT CRY1AC RESISTANCE IN BOLLWORM, *HELICOVERPA ZEA* (BODDIE)

Konasale Jayaramu Anilkumar

_____
Signature of Author

_____
Date of Graduation

DISSERTATION ABSTRACT

PRODUCTION AND CHARACTERIZATION OF BT CRY1AC RESISTANCE IN BOLLWORM, *HELICOVERPA ZEA* (BODDIE)

Konasale Jayaramu Anilkumar

Doctor of philosophy, August 9, 2008
(M.Sc. University of Agricultural Sciences, Bangalore, India, 2002)
(B.Sc. University of Agricultural Sciences, Bangalore, India, 2000)

201 Typed Pages

Directed by William J. Moar

Laboratory-selected *Bt*-resistant colonies are important tools for elucidating *Bt* resistance mechanisms and helping to determine appropriate resistance management strategies for *Bt* crops. Here, two laboratory populations of *Helicoverpa zea* (AR and MR), resistant to *Bt* Cry1Ac, were established by selection with either Cry1Ac activated toxin (AR) or MVP II (MR) from an unselected parent strain (SC). Stable and high level resistance was achieved in AR but not in MR. AR was only partially cross-resistant to MVP II suggesting that MVP II does not have the same Cry1Ac selection pressure as Cry1Ac toxin against *H. zea* and that proteases may be involved with resistance. AR was highly cross-resistant to Cry1Ab toxin. AR was not cross-resistant to

Cry2Aa2, Cry2Ab2-expressing corn leaf powder, Vip3A and cypermethrin. Toxin binding assays showed no significant differences, indicating that resistance was not linked to a reduction in binding.

In response to selection, heritability values for AR increased in generations 4 to 7 and decreased in generations 11 to 19. While rearing on Cry1Ac treated diet, AR had significantly increased pupal mortality, a male-biased sex ratio, and lower mating success compared to SC. AR males had significantly more mating costs compared to females. AR had significantly higher fitness costs in involving larval mortality, weight, and period; pupal weight, period, and mortality compared to SC. Cry1Ac-resistance was not stable in AR in the absence of selection.

In laboratory experiments with field-cultivated *Bt* and non-Bt cotton squares AR significantly outperformed SC. However, AR could not complete larval development on *Bt* cotton. Additionally, a significantly lower percentage of AR larvae reached pupation on non-Bt compared with SC. Diet incorporation bioassays indicated Cry1Ac was significantly more lethal to SC compared to AR; however, no differential susceptibility was observed in strains for gossypol. Combinations of Cry1Ac with gossypol, cotton and corn powder were synergistic against AR, but not against SC. These results may help understand the inability of AR to complete development on *Bt* cotton.

These results 1) highlight the need to choose carefully the form of *Bt* protein used in experimental studies, 2) support the lack of success of selecting, and maintaining Cry1Ac-resistant populations of *H. zea* in the laboratory, and 3) aid in understanding why this major pest of cotton and corn has not yet evolved *Bt* resistance.

ACKNOWLEDGMENTS

It has been a great pleasure working with faculty, staff and students at the Auburn University, during my tenure as a doctoral student; I thank all of them for their help, suggestions and camaraderie. I greatly acknowledge efforts of William J. Moar, my adviser for helping me to develop as a scientist/good citizen through his guidance, motivation and insight. I sincerely thank other members of my committee, Arthur Appel, Nannan Liu, James Bradley, and Sakuntala Sivasupramaniam and my outside reader, Mark Liles, for their time and genuine interest. My sincere thanks to Edzard van Santen for providing insight and helping me in statistical analysis. I thank Moar's lab members Nathan Burkett, Megan Leach, Emily Boydson, and Jayadevi Chandrashekar for helping me in taking care of my insects while I was away from lab. I am grateful to my parents Jayaramu and Sowbhagya who helped me see this through from start to finish, to whom I dedicate this dissertation. I especially thank my good friend, Pratap Simha, and other friends at Auburn and elsewhere for several years of laughter, camaraderie, and stimulating, if not always intellectual, conversation. Finally, I profoundly thank my wife, Thara, for her unfailing love, her gentle but persistent encouragement, her kindness and her patience kept me sane and happy all the while.

Style manual of journal used: Chapter 1, Journal of Economic Entomology; Chapter 2, Applied and Environmental Microbiology; Chapter 3, Journal of Economic Entomology; Chapter 4, Journal of Economic Entomology

Computer software used: Microsoft® office 2003, 2007, SAS for Windows® version 9.1, Sigma Plot® version 10.0, SPSS version 14.0

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 2

CHAPTER 3

CHAPTER 1:

THE RESEARCH INTRODUCTION, OBJECTIVES AND REVIEW OF LITERATURE

1.1 Introduction

Currently registered insect-protected plants are genetically transformed to express insecticidal proteins from *Bacillus thuringiensis* and are referred to as *Bt* crops. Since their commercial introduction into the US in 1996, growers have rapidly adopted *Bt* crops as an effective tool to increase yield by effectively controlling insect pests. *Bt* crops constitutively produce insecticidal toxins throughout the life of the plant providing excellent control of primary target insects and are a very important component of integrated pest management practices (Shelton et al. 2002).

Bt cotton (Bollgard®) was the first large scale commercialized Bt transgenic crop in the US (Perlak et al. 1990) and has been cultivated on approximately 52% (2.8 m Ha) of the total US cotton acreage in 2006 (Brookes and Barfoot 2006). The adoption of Bt cotton has helped US farmers increase their income by $50/Ha and reduce insecticide use by 841.5 metric tons of insecticides in 2001 (James 2002). However, the success of Bt cotton may be short lived if the target pest(s) develop resistance due to the widespread crop plantings and prolonged exposure to Bt toxins. Concerns regarding the development of Bt resistance by the target pests are so great that an insect resistance management

(IRM) plan is mandated by EPA (Environmental Protection Agency) as part of the registration package for Bollgard® as well as other Bt crops (Bates et al. 2005).

In the US, Bollgard® expresses Bt Cry1Ac protoxin, and is cultivated primarily to control tobacco budworm (TBW) *Heliothis virescens* (F.) pink bollworm (PBW) *Pectinophora gossypiella* (Saunders), and, to a lesser extent, cotton bollworm (CBW) *Helicoverpa zea* (Boddie). Many TBW and PBW strains derived from laboratory selection have demonstrated their ability to adapt to Cry1Ac. Results from these studies have contributed greatly to insect resistant management (IRM) policy making discussed above that have, arguably, helped to delay *Bt* resistance development in cotton (Gould 1998, 2000). Interestingly, studies with laboratory-selected resistant strains of these two species have shown that resistance characteristics and mechanisms are related to both toxin and species (Tabashnik et al. 2003b). The TBW resistant strain YHD2 with 10,100-fold resistance to Cry1Ac failed to survive, but PBW resistant strain AZP-R with little over 3,000-fold resistance to Cry1Ac could complete its development on *Bt* cotton, even though both species have similar susceptibilities to Cry1Ac (MacIntosh et al. 1990, Tabashnik et al. 2003b, Sivasupramaniam et al. 2008). Although Bt resistance was at least partly due to an altered cadherin-like protein for both species, the mutation in TBW lead to a single amino acid change (Xie et al. 2005) while an 8 amino acid deletion occurred in PBW (Morin et al. 2003).

Although TBW and PBW have been extensively studied with regards to Bt resistance, similar studies have not been conducted with CBW even though it has a naturally higher tolerance (Stone and Sims 1993, Luttrell et al. 1999, Sivasupramaniam et

al. 2008) to Cry1Ac than the other two target species, which can result in occasional completion of larval development on Bollgard® (Jackson et al. 2004). This increased tolerance increases the likelihood that resistance could evolve. Similar concerns have also been expressed in relation to *Helicoverpa armigera* (Hübner), the primary target pest of *Bt* cotton in the Old World and validated with laboratory selection experiments. *H. armigera* (Akhurst et al. 2003) is known to have similar susceptibility (LC_{50}=10µg/g) to Cry1Ac as *H. zea* (MacIntosh et al. 1990) and studies conducted with Cry1Ac-Sel (13-fold resistance, Fan et al. 2000) and BX strain (57-fold resistance, Akhurst et al. 2003) showed 25 and 58% survival on *Bt* cotton, respectively. Resistance in *H. armigera* is inherited as a partial recessive character (Bird and Akhurst 2004, Kranthi et al. 2006) contrary to the observed recessive inheritance in PBW and TBW (Tabashnik et al. 2004). Hence, it remains to be seen as to how CBW responds to Cry1Ac-selection, the inheritance of developed resistance and the fitness costs, if any, associated with the evolution of resistance.

Selection experiments cited above on PBW, TBW and *H. armigera* have been conducted with either MVP II or Cry1Ac crystals (containing protoxin). Because Bollgard® expresses *Bt* Cry1Ac solubilized protoxin that is at least partly activated to toxin within cotton tissue (Gao et al. 2006), we hypothesize that selection experiments using MVP II or Cry1Ac crystals may not adequately reflect resistance selection that is taking place *in planta*. Several physiological processes (solubilization and proteolysis) must occur before the *Bt* protein present in *Bt* protoxin is toxic to insects, and at least one of these processes (proteolysis) has been documented to be associated with Bt resistance

(Oppert et al. 1997). Therefore, we proposed to conduct selection experiments, in parallel with both MVP II as well as Cry1Ac toxin. Even though selection using Bollgard® tissues would be more realistic, proprietary research restrictions with Bollgard® limited our scope of research.

1.2 The research goal and specific objectives

The present study has been undertaken with the following objectives. 1) To select for resistance to the Cry1Ac protein using MVP II and activated Cry1Ac toxin; 2) To characterize the biochemical and molecular mechanisms of resistance such as alteration in binding, and altered proteolysis; 3) To ascertain the possible fitness costs associated with resistance development; 4) To document cross resistance to other Bt Cry proteins and other relevant insecticides belonging to different classes/groups; 5) To study the survivorship of resistant and susceptible strains on Bt and non-Bt cotton squares; and 6) To investigate the interactions of Cry1Ac and plant secondary metabolites such as gossypol on resistant and susceptible straisn.

1.3 Review of literature

Reviews of literature pertaining to cotton bollworm, *Helicoverpa zea* (Boddie), *Bt* toxins, and *Bt* resistance in various insects has been reviewed and presented.

1.3.1 Cotton bollworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae)

1.3.1.1 Biology

H. zea is known to be highly polyphagous and is an economic pest of crops such as corn (corn ear worm), cotton (cotton bollworm), tomato (tomato fruit worm), pepper, bean, eggplant, alfalfa, sorghum, and soybean (Bergvinson 2005).

Eggs are deposited singly on leaves, fruiting structures, and corn silk. The shape varies from slightly dome-shaped to a flattened sphere, and measures about 0.5 to 0.6 mm in diameter and 0.5 mm in height. Eggs are creamy white in color when laid and turn black before hatching, after 3-4 days of incubation at room temperature (25-27 ^{0}C) (Ellsworth and Bradley 1992).

Larval duration is about 15 D with 5-7 instars at room temperature (25-27 ^{0}C). Larvae feed on flower buds, fruits, bolls or pods (Gore et al. 2003). Larval color ranges from green, brown and red, to black depending on their host and genetic makeup, and have distinct, longitudinal stripes running down the body (Archer and Bynum 1994). Larval exoskeleton is roughened by numerous minute spines which is an identifying character to distinguish it from *H. virescens* larvae (Bailey et al. 2001). First instar larvae are not cannibalistic, and therefore, several larvae may feed together initially. However, as larvae mature they become very aggressive, killing and cannibalizing other larvae (Kolodny-Hirsch and Harrison 1982, Ellsworth and Bradley 1992, Archer and Bynum 1994, 1998).

Mature larvae leave the feeding site and drop to the ground, where they burrow into the soil and pupate. The larva prepares a pupal chamber 5 to 10 cm below the soil surface and the duration of the pupal period is about 7 - 10 days (Butler 1976).

The forewings of the moths usually are yellowish brown in color, and often bear a small dark spot centrally. The small dark spot is especially distinct when viewed from below. The forewing also may bear a broad dark transverse band distally, but the margin of the wing is not darkened. The hind wings are creamy white basally and blackish

distally, and usually bear a small dark spot centrally. The adult longevity is about a week. The sex ratio is 1:1.2 (male: female) and fecundity is about 500-2000 eggs per female (Geraud et al. 1996).

The bollworm/budworm complex was the top insect pests of cotton in 2004 damaging 1.23% of the 2004 US crop. Almost 82% of crop was infested with the complex of which 94% were bollworms (Source: M.R. Williams, 2004. www.msstate.edu/Entomology/Cotton.html). Interestingly, the primary reason bollworm is the current predominant lepidopteran "pest" is due to the high adoption of *Bt* cotton that severely suppressess the budworm population. The heliothine complex probably costs cotton farmers worldwide approximately $3 billion annually; which includes yield loss and control costs, excluding labor costs for sprays (James 2002).

1.3.1.2 Distribution

H. zea is widely distributed in southern Canada, Mexico and USA in North America; and Argentina, Bolivia, Brazil, Chile, Colombia, Ecuador, Falkland Islands, French Guiana, Guyana, Peru, Surinam, Uruguay, and Venezuela in South America. It is active throughout the year in tropical and sub-tropical climates; however, it is restricted to summer months in higher latitudes (Bergvinson 2005).

1.3.1.3 Management of *H. zea*

Before the advent of *Bt* cotton *H. zea* was treated primarily with synthetic insecticides belonging to most major classes of insecticides such as carbaryl, esfenvalerate, permethrin, spinosad, cyhalothrin, cyfluthrin, bifenthrin, zeta-cypermethrin, methomyl, lambda-cyhalothrin, and thiodicarb (Luttrell et al. 1994, Casida and Quistad 1998).

Widespread insecticide resistance to permethrin, methomyl (Hsu and Yu 1991), cyhalothrin (Brown et al. 1997, 1998), cypermethrin (Brown et al. 1997) and other insecticides (Leeper and Raffa 1986, Leonard et al. 1988, McCutchen et al.1989, Adb-Elghafar et al. 1993, Ernst and Dittrich 1992) and rising concerns regarding environmental hazards have urged scientists to search for alternatives to conventional insecticides (Gould 1991). As a result of rigorous research exploration, biological and microbial insecticides namely *Bt* (Ali and Young 1996, Lambert et al. 1996), and nuclear polyhedrosis virus (NPV) (Bell and Hayes 1994), were commercialized. Although these insecticides are eco-friendly and effective, they were short lived because of both biotic and abiotic environmental factors. These factors, plus the difficulties faced in the control of these pests which feed internally have led to ingeneous research resulting in the advent of the insect-protected plants in 1996 (Bergvinson 2005). These insect-protected plants are transformed to express insecticidal proteins derived from a soil bacterium, *Bacillus thuringiensis* Berliner (de Maagd et al. 1999a). These plants express the insecticidal toxins in all tissues and throughout its life under the influence of the constitutive promoters (Cannon 2000).

1.3.2 *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae)

B. thuringiensis (Bt) is a rod shaped aerobic, spore-forming common soil bacterium. Bt was first identified in 1901 by Ishiwata as a pathogen of silk worm which causes 'sotto' disease and hence it was named *Bacillus sotto* (Ishiwata 1901, Federici 2005). Later, it was isolated from dead Mediterranean flour moth larvae *Ephestia kunhniella* Keller in 1911 and named as *Bacillus thuringiensis* (after Thuringia, Germany) by Berliner

(Berliner 1915). Bt produces an array of insecticidal compounds belonging to different families such as crystal (Cry) proteins (delta-endotoxins), cytolytic proteins, vegetative insecticidal proteins, beta-exotoxin, zwittermycin, spore, etc. (de Maagd et al. 2003). Among these insecticidal compounds, the most widely studied group are delta-endotoxins (Cry proteins).

Cry proteins (Schnepf 1995) are produced during sporulation and are often toxic to insects (Höfte and Whiteley 1989, de Maagd et al. 2003). The first recorded *Bt* trials for insect control were conducted in Hungary in the 1920's and Yugoslavia in the early 1930's to control the European corn borer, *Ostrinia nubilalis* (Hübner) (Beegle and Yamamoto 1992). The success of these trials led to the first commercial formulation by Laboratoire Libec in France. The product, Sporeine, was available in 1938 (Lord 2005) and later in other parts of the world as spray formulations for managing insect pests (Beegle and Yamamoto 1992, Tabashnik 1994). These insecticides were environmentally safe and effective due to their high specificity and unique mode of action (Lambert and Peferoen 1992, Federici 2005).

Bt is the most diverse species in the *B. cereus* group (*B. thuringiensis*, *Bacillus cereus*, *Bacillus anthracis*, and *Bacillus mycoides*, as well as the recently described *Bacillus pseudomycoides* and *Bacillus weihenstephanensis*), and its strains have been classified into 84 serovars (serovarieties), with over 800 strains (Lecadet et al. 1999, and Reyes-Ramirez and Ibarra 2005). The current nomenclature, classification, and range of toxicity to different insects can be found on the Bt Toxin Nomenclature webpage at http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/

1.3.2.1 Structure of Bt Crystal toxins

Bt Cry proteins (δ-endotoxins) consist of 3 domains (Li et al. 1991, Bravo 1997, Crickmore et al. 1998, de Maagd et al. 2003). Domain I is a seven α–helix bundle in which the central helix is completely surrounded by six outer helices (Schnepf et al. 1998). This domain is the most conserved and is important for channel formation in the membrane. Domain II consists of three antiparallel β -sheets sharing similar topology in a Greek key conformation, forming a β-prism (Rajamohan et al. 1996). This domain is the most divergent, plays an important role in receptor interaction (Cheng and Nickerson 1996, Jenkins and Dean 2000) and is the putative specificity-determining domain (Liang and Dean 1994). Finally, the third domain is a β –sandwich of two antiparallel β –sheets and is believed to be involved in toxin stability (Li et al. 1991, de Maagd et al. 1999b).

1.3.2.2 Bt toxin mode of action

Bt Cry proteins are stomach poisons primarily affecting mature columnar midgut epithelial cells after ingestion (Gill et al. 1992). A generally accepted model for Cry toxin action is that of a multistage process (Himeno et al. 1985, Haider and Ellar 1989). Within the crystal, insecticidal proteins interact through hydrogen bonding, disulfide linkages, and hydrophobic interactions (Choma and Kaplan 1990, 1992). In lepidopteran insects, insecticidal proteins are released in the alkaline gut and hydrolyzed to toxins by proteases (Höfte and Whiteley 1989). An unusual feature is that activation of the protoxin appears to occur by a sequential series of proteolytic cleavages, initiated at both termini and proceeding towards the center of the protein until finally the protease-stable toxin is generated (Choma et al. 1990). Twenty five -30 amino acids from the amino and

approximately half of the remaining protein from the carboxyl-terminal are proteolytically cleaved (Bravo et al. 2002).

Proteases such as trypsin (Pang et al. 1999), chymotrypsin and elastase are associated with the hydrolysis of the protoxins (Oppert 1999). Generally 130 -140 k Da protoxin is activated into 60-70 k Da proteins (Bravo 1997). Although proteolytic activation generates the proteinase-resistant activated toxin, exceptions have been reported in many insects. Intramolecular processing has been reported for several Cry toxins. Cry1Ab (Convents et al. 1991), Cry2Aa (Audtho et al. 1999), Cry4Aa (Yamagiwa et al. 1999), Cry4B (Zalunin et al. 1998) and Cry9Aa (Zalunin et al. 1998) are cleaved in domain I, while Cry11Aa (Dai and Gill, 1993), Cry1Ac (Choma et al. 1990) and Cry1Aa (Pang et al. 1999) are cleaved within domain II. The extra processing resulted in the production of a protein with less or no toxicity, which could increase the potential for *Bt* resistance development (Miranda et al. 2001). However, the extra nick in Cry3A renders it more soluble under neutral pH conditions making it more toxic to insects (Carroll et al. 1997).

Activated toxin binds to receptors present in brush border membranes (Hofmann et al. 1988, van Rie et al. 1990a). Later it undergoes conformational changes leading to oligomerization and enters the cell facilitated by pores formed in the membrane (Knowles 1994). This allows the movement of K^+ ions in to the gut lumen leading to an increase in hemolymph K^+ concentration resulting in gut pH changes. Ultimately, the affected cells are destroyed due to high gut pH and osmotic lysis resulting in insect death (Li et al. 1991, Lorence et al. 1995, Bravo 1997, Whalon and Wingerd 2003).

1.3.3 Insect midgut and response to *Bt* toxins

The midgut epithelium of larval lepidoptera consists of a highly folded pseudostratified epithelium separated from a framework of muscle and trachea by a thin basement membrane (Chapman 1998). Within the epithelium are three main cell types: cylindrical columnar cells exhibiting apical (lumenal) microvilli; pear shaped goblet cells; each with a central cavity that opens to the gut lumen through a valve, and small round stem cells that are located between the bases of the columnar and goblet cells. Stem cells undergo mitotic activity immediately prior to each molt (Baldwin and Hakim 1991). Until the prepupal molt, stem cells differentiate into gut epithelial cells for the next instar. At the prepupal and adult molts the stem cells differentiate to phenotypes that characterize pupal and adult midguts (Engelhard et al. 1991, Loeb and Hakim 1996, Loeb et al. 2001, 2003).

Histopathological studies have indicated dose-dependent destruction of cultured midgut cells from *H. virescens* larvae in the presence of Bt toxins (Loeb et al. 2001). After 2 days of exposure to 0.8 pg/ μl AA 1-9 or 0.06 pg/ μl HD-73, columnar and goblet cell numbers declined to ca 20% of controls. In contrast, stem cell numbers increased 140-200% greater than the controls. The dynamics of depletion and replacement depended on toxin type and concentration. Two days after toxin removal, cell type ratios returned to approximate pre-toxin levels. The response of cultured midgut cells to Bt toxin injury was similar to injured vertebrate tissues dependent on stem cells for replacement and healing (Loeb et al. 2001). Similar observations were also made in dipteran insects when they were challenged with Bt *israeliensis* (Bti) proteins. Investigations using *Simulium vittatum* larvae infected with Btk HD 255 (Lacey and

Federici 1979) and *S. variegatum* infected with Bti (Rey et al. 1998) demonstrated morphological lesions in the intestinal epithelium which exhibited swollen cells, degenerated brush borders, disorganized nuclei, enlargement of intercellular spaces and cell lysis. Light and electron microscope observations in *Simulium pertinax,* a common black fly, revealed by time and endotoxin concentration, increasing damage of the larva midgut epithelium. The most characteristic effects were midgut columnar cell vacuolization, microvilli damages, epithelium cell contents passing into the midgut lumen and finally, cell death (Smouse and Nishiura 1997, Cavados et al. 2004).

1.3.4 Transgenic crops

The use of *Bt* to control insect pests is not new. Commercial insecticides containing *Bt* and its toxins (e.g., Dipel®, Thuricide®, Vectobac®) have been in the market for over 40 years. *Bt*-based insecticides are considered safe for mammals and birds, and almost all non-target insects (and typically safer than most conventional insecticides). What is relatively new is that *Bt* crops contain a modified version of the Cry gene that has been incorporated into the plant's DNA, so that the plant's cellular machinery produces the toxin (Li et al. 2003). When a susceptible insect feeds on a leaf, bores into a stem, or feeds on essentially any other tissue of a *Bt*-containing plant, it ingests toxin and will either severely stunt or die within a few days.

Numerous crop plants have been transformed to express Bt Cry proteins under the influence of constitutive promoters such as CaMV35S (Perlak et al. 1990, 2001). Initially, Bt crops were not very effective due to relatively low expression of insecticidal proteins, however, a combination of methods including the use of plant stable *Bt* Cry

proteins, plant-specific promoters and different expression methods (e.g. expression in chloroplast DNA as compared to nuclear DNA) (Kota et al. 1999) have changed the scenario.

The US Environmental Protection Agency (EPA) classifies Bt crop plants under the Plant Incorporated Protectants (PIP) category. PIPs are pesticidal substances produced by plants and the genetic material necessary for the plant to produce the substance. The current list of Bt crop plants registered for commercial cultivation can be found on the EPA's webpage at http://www.epa.gov/pesticides/biopesticides/pips/pip_list.htm and the currently registered PIPs for experimental use can be found at http://www.epa.gov/pesticides/biopesticides/pips/current_pip_eups.htm

1.3.4.1 Bt cotton and its commercialization

The first generation of *Bt* cotton, Bollgard® produces Cry1Ac that has been rapidly adopted by growers since its commercial introduction into the US in 1996. Bollgard® provides effective protection from feeding damage by lepidopteran insect pests such as *H. virescens*, *P. gossypiella* and *H. zea* (Gould 1998). Bollgard® has been approved for commercial cultivation in nine countries (James 2005) including two developed countries such as USA and Australia and seven developing countries namely, Argentina, China, Colombia, India, Indonesia, Mexico and South Africa (Sivasupramaniam et al. 2007). First generation *Bt* cotton is referred to as Ingard® in Australia whereas in all other countries it is called as Bollgard® (James 2005). The global adoption of Bollgard® has increased dramatically from 800,000 ha in its first year of introduction (1996) to 5.7 m ha

in 2003. *Bt* cotton acreage has increased tremendously in the USA from 0.8 to 2.8 m ha in 2006 accounting for 52% of total cotton acreage (Brookes and Barfoot 2006). Its adoption has helped US farmers manage cotton insect pests effectively and increase their net income by $50/Ha, thereby increasing the total net value of US cotton production by $103 million in 2001. In addition, it is also safer to the environment including a 841.5 metric tonnes reduction in insecticide active ingredients per year (Perlak et al. 2001, James 2002, Chitkowski et al. 2003, Mendelsohn et al. 2003, Head et al. 2005).

Bollgard® II, Event 15985 was developed by inserting the Cry2Ab2 gene to Bollgard® (DP50B) (Greenplate et al. 2003). Bollgard® II was introduced into Australia in 2002 and the USA in 2003. This dual gene cultivar has expanded the range of benefit to growers and the environment. Bollgard II® provides equivalent or increased control of major target pests of cotton compared to Bollgard®, with additional control of secondary lepidopteran insect pests such as beet armyworm *Spodoptera exigua* (Hübner) and fall armyworm *Spodopeta frugiperda* (Smith) (Sivasupramaniam et al. 2008). Pyramided *Bt* crops also provides an added dimension to effective resistance management (Roush 1998, Zhao et al. 2003). *Bt* cotton varieties expressing Cry1F and Cry1Ac have been commercialized and marketed by Dow AgroSciences, and additional varities expressing VIP3A and Cry1Ab are being developed by Syngenta, respectively and should be available commercially within the next 1-3 years. VIP3A is a vegetative insecticidal protein produced by *B. thuringiensis* during vegetative growth, and represents a new family of insecticidal proteins (Lee et al. 2003). One of the interesting features of Vip3A is that it shares no sequence homology with known δ-endotoxins (Estruch et al. 1996).

The mode of action of Vip3A has been examined and has been shown to target the midgut epithelium, where binding to midgut cells is followed by progressive degeneration of the epithelial layer (Yu et al. 1997). Receptors binding to VIP3A do not bind Cry proteins ensuring values in future resistance management plans.

Both Cry2Ab2 and Cry1F found in Bollgard® II and Wide Strike, respectively, are less toxic (susceptibility values are in the next paragraph) to *H. zea* compared to Cry1Ac (Sivasupramaniam et al. 2008). However, the low toxicity has been overcome by expressing toxin at higher levels (Greenplate et al. 2003).

1.3.4.2 Insecticidal protein expression

Bollgard® expresses Cry1Ac in all above ground tissues and throughout the season. Expression levels vary in different plant tissues (Greenplate 1999, Greenplate et al. 2003, Kranthi et al. 2005, Sivasupramaniam et al. 2008), crop growth stages (Greenplate et al. 2003, Bird and Akhurst 2005) and varieties (Adamczyk and Sumerford 2001, Adamczyk et al. 2001) and/or hybrids (Kranthi et al. 2005). Bollgard® leaves have the highest expression of toxin concentrations followed by squares, bolls and flower parts (Greenplate 1999, Kranthi et al. 2005).

1.3.4.3 Susceptibility of *H. zea* to different Bt proteins found in Bt cotton

H. zea has a naturally higher tolerance to Cry1Ac than the other two target species, *P. gossypiella* and *H. virescens*, with wide variation in susceptibility among field populations (10-40-fold) (Stone and Sims 1993, Luttrell et al. 1999, Ali et al. 2006). *H. armigera* (Liao et al. 2002, Akhurst et al. 2003) is known to have similar susceptibility (LC_{50}=10µg/g) to Cry1Ac as *H. zea* (MacIntosh et al. 1990). Wide variation (71-fold)

(LC_{50}: 0.01 to 0.71 µg/ml) in susceptibility of *H. armigera* has been observed in India (Kranti et al. 2001, Jalali et al. 2004, Krishnappa et al. 2005).

Cry2A (LC_{50}=375.78ng/larvae) is less toxic to *H. zea* compared to Cry1Ac (LC_{50}=63.60ng/larvae) (Karim et al. 2000). Similar observations were also reported by Sivasupramaniam et al. (2008) reported that Cry2Ab2 (LC_{50}=17.476 µg/ml) is less toxic to H. zea than Cry1Ac (LC_{50}=0.87 µg/ml).Among the Cry2A toxins, Cry2Aa is less toxic (LC_{50}=681 ng/diet cup) compared to Cry2Ab2 (LC_{50}=364 ng/diet cup) (Dankocsik et al. 1990), which is present in the Bollgard® II.

Cry1F is another crystal protein found in new varieties of *Bt* cotton from Dow Agrosciences. Cry1F has relatively low toxicity ($LC_{50} \geq 57.0$ ng/mm^2 of diet surface) to *H. zea* however it is highly effective against *O. nubilalis* (LC_{50}=0.27) and *S. exigua* (LC_{50}=25.6) (Chambers et al. 1991).

VIP3A is vegetative insecticidal protein in VIPCOT®, a new *Bt* cotton event being developed by Syngenta has an LC_{50} between 112.5 and 420 ng/ cm^2 against *H. zea* (Estruch et al. 1996, Lee et al. 2003).

1.3.5 Selection for Bt resistance

Insect strains resistant to *Bt* proteins have been selected from laboratory or field-collected insects (Table 1). Different selecting agents such as laboratory-produced or commercially available *Bt* proteins were used as selection agents. For laboratory produced *Bt* proteins: In some instances, genetically transformed bacteria such as *E. coli*, *Pseudomonas fluorescens*, and *Bacillus* etc. were used for generating *Bt* protein inclusion bodies. In

some cases these inclusion bodies were soluble at alkaline pH (10.5) releasing protoxins which were, sometimes, activated using either trypsin or chymotrypsin to form activated toxin. All of these different forms such as inclusion bodies, protoxin and toxins have been used in various insect selection experiments.

Commercial *Bt* formulations such as MVP II (containing Cry1Ac protoxin inclusion bodies encapsulated in *Pseudomonas fluorescens* cells), and Dipel® (containing the HD-1 strain of Btk) Xentari® (containing *Bt aizawai*) have been used as selection agents for developing resistant insects. As will be discussed later, the use of formulations may add at least another variable into resistance selection due to the various non-described ingredients found in formulations.

First instars were selected either individually or *en masse* either by incorporating *Bt* proteins into the diet (diet incorporation), overlaying Bt proteins onto the diet surface (diet overlay) or using different tissues from Bt-crops. Bioassay exposure times (7-21 D) varied across insect species depending on their biology and susceptibility to Cry toxins. Different selection criteria such as survival and growth inhibition were followed, and selected insects were reared on diet containing no Bt-protein until pupation. Resistance was determined by conducting bioassays and comparing these results with bioassays conducted against a parent susceptible population and thereby developing resistance ratios (RR).

1.3.6 Frequency of resistant alleles

The risk of rapid pest adaptation to an insecticide is highly dependent on the initial frequency of resistance alleles in field populations and strategies for delaying pest resistance are based primarily on theoretical models using these frequencies. One key assumption of such models is that genes conferring resistance are rare (less than 10^{-6}) in the field. The frequency of resistance alleles has been measured through field sampling of *P. gossypiella* larvae, genetic crossing of field collected males with laboratory selected Cry1Ac-resitant females in *H. virescens*, and female *H. zea* moths collected from light traps in the field. Theoretical models using this data predict that these insects can develop resistance to Cry1Ac very quickly in a span of three-four years (Storer et al. 2003). However, Bt resistance has not been observed even after a decade of Bt cotton cultivation.

The estimated resistance allele frequency in *H. virescens* through individual mating of over 2,000 male moths collected in four states to females of a Bt toxin-resistant laboratory strain, was 1.5 X 10^{-3}(Gould et al. 1997). The estimated frequency of a recessive allele conferring resistance to Cry1Ac was 0.16 (95% confidence interval [CI] = 0.05-0.26) in strains of PBW derived from 10 Arizona cotton fields during 1997. However, this frequency of resistance allele in *P. gossypiella*, has not increased since 1997 even after higher adoption of Bt cotton in Arizona, and the frequency has remained low; between 0.05 and 0.11 during 1998 and 1999, respectively (Tabashnik et al. 2000). Although some variation occurred from 1999 to 2003, the mean resistance allele frequency has not differed significantly between 1998 and 2004 (0.004, 95% CI = 0-0.01) (Tabashnik et al. 2005b). The estimated non-recessive allele frequency of *H. zea* during

2000 was 4.3 X 10^{-4} and 3.9 X 10^{-4} to Cry1Ac and Cry2Aa, respectively (Burd et al. 2003). Initial frequencies of alleles conferring resistance to transgenic Bt poplars producing Cry3A in a natural population of the poplar pest *Chrysomela tremulae* F. was estimated to be 0.0037 (average over three years) for the period 1999-2001 (95% CI = 0.00045-0.0080) (Genissel et al. 2003). To date, extensive screening of European corn borer, *O. nulialis* the major pest targeted by first generation of Bt corn, has not identified any individuals with alleles conferring resistance to Bt corn (Andow et al. 1998, 2000, Bourguet et al. 2003).

1.3.7 Resistance mechanisms

The mode of action of Bt toxins suggests at least three possible physiological or biochemical mechanism(s) of resistance (Ferre et al. 1995, Ferre and van Rie 2002, Griffits and Aroian 2005). The first involves pH- and protease-mediated dissolution and activation of the crystal. Enzymatic changes in the resistant insect gut may have resulted in the detoxification of Bt proteins, or the inability to activate them. Secondly, changes may have occurred in the gut cell membrane, interfering with binding of the toxic moiety. Thirdly, cellular changes may have occurred that influence the sensitivity of the cell to pore formation or their capacity to recover from toxin effects.

1.3.7.1 Altered proteolytic processing

Serine proteases, such as trypsin, chymotrypsin and elastase are important in both solubilization and activation of Bt protoxins (Oppert 1999). In some insects the altered activities of these proteases have resulted in resistance development. Gut protease activity has been altered in Bt subspecies *entomocidus* (Bte HD-198) resistant strain 198^{r}

of Indian meal moth *Plodia interpunctella* (Hübner) (Oppert et al. 1996). Western blot analysis showed that 198^r enzymes were much less effective than Bt susceptible (688^s) or *Bt* subsp *kurstaki* (strain HD-1, Dipel®) resistant strain (Dpl^r) in hydrolyzing protoxin. In addition, protoxin hydrolysis produced many non-toxic intermediates with a different proteolytic pattern, and proteolysis was incomplete even after 4 h incubation as compared to complete hydrolysis in the other two strains. The proteolytic enzymes were shown to be trypsin-like enzymes (Oppert et al. 1997). Assays conducted using the trypsin diagnostic substrate, BApNA (N-α-benzoyl–L-arginine p-nitroanilide), revealed reduced activities of enzymes from Bt subsp *aizawai* (133^r) and *entomocidus* (198^r) resistant strains. The specific activity of gut proteases from these strains was less than one-half of those in the *kurstaki*-resistant and susceptible strains. Activity from the *kurstaki* resistant strain was approximately 30% higher than the activity from the parent susceptible strain, suggesting adoption of different resistant mechanisms by this strain.

Two (~45 and ~25 k Da proteins) major BApNA hydrolyzing enzymes were identified from gut extracts of susceptible insects, and absent in both resistant strains. A subsequent study demonstrated a genetic linkage between decreased susceptibility to Cry1Ac and the absence of a major gut protease. Moreover, the involvement of changes in midgut proteases in resistance was further affirmed by the observation of 11-fold higher resistance levels for Cry1Ab protoxin than for Cry1Ab toxin in the 198^r strain (Herrero et al. 2001).

Reduced trypsin-like proteinase activity has been also studied in the activation of protoxins by four selected (KS-SC, KS-NE, IA-1, and IA-3) and one susceptible (IA-S)

strains of European corn borer *O. nubilalis*. The hydrolyzing efficiencies were compared using three synthetic substrates, BA*p*NA for trypsin-like, *N*-succinyl-ala-ala-pro-phe *p*-nitroanilide (SAAPF*p*NA) for chymotrypsin-like and *N*-succinyl-ala-ala-pro-leu *p*-nitroanilide (SAAPL*p*NA) for elastase-like proteinase activities (Huang et al. 1999a).

Enzyme kinetic studies of trypsin-like proteases revealed no change in Michealis constants (K_m) among five strains but V_{max} decreased by 35% in the KS-SC resistant strain as compared to the IA-S. However, the detectable reduction was observed in the hydrolysis of protoxin in the KS-SC strain compared with the IA-S strain. At the same time, no significant differences were found in trypsin activity between the IA-S strain and the three other resistant strains (i.e., KS-NE, IA-1, and IA-3). Similarly there were no detectable differences in the activity of chymotrypsin among all strains examined suggesting different resistance mechanisms in the other three strains (Huang et al. 1999).

In a subsequent study, two forms (soluble and membrane fractions) of trypsin-like proteases were identified (Li et al. 2004a). Serine proteases from soluble fractions of the susceptible strain were more active than those of the resistant strain (KS-SC). When casein was used as a substrate for analysis no significant differences were observed between different fractions of proteases. However when Cry1Ab protoxin was used as a substrate, zymogram analysis indicated that approximately 20% less protoxin was hydrolyzed with soluble extracts from the resistant strain when compared with similar extracts from the susceptible strain.

Enzymes from the CP73-3 strain of *H. virescens* resistant to Bt subsp kurstaki (HD-73) were reported to process more slowly, and to degrade toxin faster than enzymes

from the susceptible strain (Forcada et al. 1996). In the NO-95C colony of *P. xylostella*, resistant levels for crystalline Cry1Ca protoxin were about 2.5-fold higher than for Cry1Ca toxin. Thus, reduced conversion to toxin is a minor mechanism of resistance in this strain (Liu et al. 2000).

1.3.7.2 Receptor binding

Four different receptors have been identified as binding sites of Bt Cry toxins in insects. Among the four, cadherin-like proteins with molecular masses between 175 & 220 k Da (Vadlamudi et al. 1993, 1995; Nagamatsu et al. 1998a, 1998b; Dorsch et al. 2002, Flannagan et al. 2005) and aminopeptidase N (APN) with molecular masses in the range of 108 to 252 k Da (Sangadala et al. 1994, Knight et al. 1995, Cheng and Nickerson 1996, Luo et al. 1996, 1997; Cooper et al. 1998, Yaoi et al. 1999, Banks et al. 2001, Agrawal et al. 2002, Hossain et al. 2004, Liu et al. 2004) are the most studied receptors. These receptors are known to preferentially partition into lipid rafts (Zhuang et al. 2002). Recently anionic glyconjugate (Valaitis et al. 2001), actin & alkaline phosphatases (McNall and Adang 2003, Jurat-Fuentes and Adang 2004), 252 kDa (P252) protein (Hossain et al. 2004), and glycolipids (Griffits et al. 2005, W. Moar, Auburn University, personal communication) are added to the possible candidates for binding sites. The binding site modification results either through a reduction in the quantity of binding sites or through an alteration in the receptor itself, affecting the sensitivity for binding (Herrero et al. 2005).

The reduction in binding affinity was first reported for a Dipel®-resistant *P. interpunctella* strain (343^{r}) (van Rie et al. 1990). Binding studies with brush border

membrane vesicles (BBMV) revealed a 50% reduction in binding affinity (K_d = 36.3 ± 22.7nM) for Cry1Ab in 343^r, however no differences were observed with respect to the number of binding sites (R_t=1.77 ± 0.58 pmol/mg of membrane protein) compared to the susceptible strain. Binding affinity was similar in both strains when Cry1Ca was used but the R_t value (0.38 ± 0.07 and 1.15 ± 0.20 pmol/mg for susceptible and resistant strains, respectively) was significantly higher (three-fold) in the resistant strain. This shows that the observed resistance was primarily due to an alteration in the binding site for Cry1Ab and concurrently, the increased affinity to bind Cry1Ca explains its susceptibility to this toxin.

Differences in binding affinity were also observed in resistant (Dpl^r and 198^r) and susceptible (688^s) strains of *P. interpucntella* to Cry1Ab (Herrero et al. 2001). The Dpl^r (K_d =14.48nM) strain showed a 60-fold reduction in binding affinity compared to 688^s (K_d =0.25nM). Conversely, no significant differences were observed between Dpl^r and 198^r binding of ^{125}I-Cry1Ac. These results suggest that the same change in the Cry1A binding site had different effects on Cry1Ab and Cry1Ac binding. Whereas Cry1Ab affinity would be reduced, the change could only affect post binding steps of the Cry1Ac mode of action, such as membrane insertion or pore formation. In contrast to strains 343^r and Dpl^r, strain 198^r showed only a slight reduction in binding of Cry1Ab (five-fold higher K_d and three-fold lower R_t).

Competitive binding analysis revealed two binding sites for Cry1A toxins in three resistant (NO-QA, PEN & PHI) and one susceptible (LAB-V) strain of *P. xylostella.* The reduction in binding sites for Cry1Ab and Cry1Ac was reported as a cause for resistance

in NO-QA and PEN strains; whereas binding of Cry1Aa was unaltered in all three strains (Tabashnik et al. 1997). Further analysis of Cry1Aa binding with PHI indicated a good fit for the two binding sites model (Ballester et al. 1999). The two K_d values (K_{d1}=0.3 ± 0.1 & K_{d2}= 20.3 ± 4.4 nM) for binding to two sites were essentially the same as the K_d values obtained for the susceptible strain (K_{d1}= 0.1 ± 0.1 & K_{d2}= 17.7 ± 1.0 nM). On the other hand, homologous binding studies with NO-QA and PEN strains fit the two binding sites model. The K_d and R_t values of Cry1Aa for NO-QA (4.8 ± 2.7 nM and 1.9 ± 0.8 pmol/mg of protein, respectively) and PEN (4.0 ± 2.8 nM and 1.9 ± 0.8 pmol/mg of protein, respectively) were similar to each other and were intermediate between the values obtained for the two binding sites of LAB-V and PHI.

A four-fold decrease in binding affinity for Cry1Ac was observed in AZP-R (Cry1Ac-resistant PBW) compared to the APHIS-S (susceptible) strain. Though the concentration of binding sites (R_t) was 23-fold higher in the resistant compared to the susceptible strain, the ratio (R_t/K_d) of binding site concentration to dissociation constant did not differ significantly among the two strains (Gonzalez-Cabrera et al. 2003) suggesting that the reduction in the binding affinity is the primary mechanism of insect resistance to Cry1Ac in PBW.

In *H. armigera* Cry1Aa, Cry1Ab and Cry1Ac share common binding sites. Binding experiments in the presence of concanavalin A showed that Cry1Ac and Cry1Ab bind to different epitopes (Estela et al. 2004).

Reduction in binding site numbers was observed in Cry3Aa-resistant Colorado potato beetle *Leptinotarsa decemlineata*. Saturation binding studies with BBMV and ^{125}I-

Cry3Aa revealed approximately 60% less binding of Cry3Aa toxin in the resistant strain compared to the susceptible strain. Nonetheless competitive binding assays showed no differences, suggesting observed resistance was due to changes in the number of binding sites (Loseva et al. 2002).

1.3.7.2.1 Causes for reduction in binding in resistant strains

A mutation that resulted in the deletion of eight amino acids in three alleles (r1, r2 & r3) of the cadherin-like protein was shown to be associated with Cry1Ac resistance in *P. gossypiella* (Morin et al. 2003). However, a mutation that resulted in the deletion of only one amino acid resulted in reduced binding on the cadherin-like receptor in Cry1Ac-resistant *H. virescens* (Xie et al. 2005). The mutated amino acid sequence described above overlaps the Cry1Ab binding site (1363 to 1464) in tobacco hornworm, *Manduca sexta* (L.). The single amino acid mutation in mutant line L1425R from C**T**G to C**G**G was responsible for the observed reduction in binding.

1.3.7.2.2 Identification of resistance genes

The identification and isolation of *Bt* resistant genes has been met with little success. Genetic mapping experiments with the laboratory-selected YHD-2 resistant strain of *H. virescens* showed a tight linkage between resistance to Cry1Ac and a cadherin encoding gene *Bt-R4* or *HevCaLP*. Insertion of a retrotransposon disrupting Bt-R4 in the YHD-2 strain leads to a high level of resistance >10,000 fold, linking this protein to Bt resistance (Gahan et al. 2001).

1.3.7.3 Other resistance mechanisms

Ingestion of a sublethal dose of Cry1Ac by fourth instar CP73-3 *H. virescens* larvae resulted in similar histopathological changes in columnar gut cells compared with cell damage in susceptible larvae (Martinez-Ramirez et al. 1999). Likewise, larvae from both a susceptible colony and another resistant *H. virescens* colony (KCB) showed comparable midgut epithelium damage following Cry1Ac ingestion (Forcada et al. 1999). Therefore, it is possible that resistance in CP73-3 and KCB is due to a more efficient repair (replacement) of damaged midgut cells.

1.3.8 Inheritance of resistance

Resistance development can be delayed by decreasing the dominance of resistance, provided resistance is inherited as a recessive character (Tabashnik et al. 2003, 2004a, 2004b; 2005b). Inheritance can be determined by four different methods; testing on Bt crops, use of leaf-dip, diet incorporated or overlay bioassays (Tabashnik 1991). Among these, bioassays using *Bt* crops are the most realistic of what happens in the field. Leaf-dip bioassays simulate the situations rather well in the field because insects ingest Bt proteins or spore/crystal mixtures along with the fresh plant material. Finally, bioassays (diet incorporation or diet overlay) using artificial diet are the least similar to the field situation.

The degree of dominance (D), dominance (D_{LC}) and effective dominance (D_{ML}) of resistance have been calculated by following Stone (1968) , Bourguet et al. (2000) or many other methods (Preisler et al. 1990). All of these values are based on LC_{50}'s in which $D = (2X_2 - X_1 - X_3) / (X_1 - X_3)$, where X_1, X_2, and X_3 are the logarithms of the LC_{50}'s for the resistant homozygotes, heterozygotes, and susceptible homozygotes,

respectively. *D* values range from -1 (completely recessive resistance) to 1 (completely dominant resistance). $D_{LC}=(D+1)/2$ and $D_{ML}=(ML_{RS}-ML_{SS})/(ML_{RR}-ML_{SS})$. D_{LC} is the estimate of dominance with 0 for completely recessive, 0.5 for semi-dominant and 1.0 for completely dominant trait. D_{ML} defines the effective dominance of survival where ML_{RR}, ML_{SS}, ML_{RS} are the % mortality levels of the resistant, susceptible and hybrid progeny on *Bt* crops.

Resistance to Cry1Ac was reported to be autosomal and inherited as incompletely recessive in *H. armigera* ($D_{LC}=0.26$) (Akhurst et al. 2003), *P. xylostella* (Tabashnik et al.1997, Tang et al. 1997), *H. virescens* (Gould et al. 1992, 1995), *P. gossypiella* (D=-0.61) (Tabashnik et al. 2002a) and *Trichoplusia ni* (Hübner) (D=-0.402) (Janmaat et al. 2004, Kain et al. 2004). However, recent studies from Australia (Bird and Akhurst 2005) and India (Kranthi et al. 2006) suggest that Cry1Ac resistance is inherited as a semi-dominant trait in *H. armigera*. A similar observation has also been made with *H. zea* (Burd et al. 2003, W. Moar, Auburn University, personnel communication). Cry1C resistance was inherited as a recessive character in a *P. xylostella* (D= 0.26) strain that developed high levels of resistance to Bt subsp. *kurstaki* in the field (Liu and Tabashnik 1997b, Zhao et al. 2000) (D=-0.22) and the realized heritability of resistance was 0.10. However, in some insects Bt resistance is inherited as semi-dominant or dominant character (Tabashnik et al. 2000). Incomplete dominance was observed in *O. nubilalis* to Dipel (Huang et al. 1999b), and *P. xylostella* to Cry1Ac (Sayyed et al. 2000). Dominance increased as the concentration of Bt proteins decreased, suggesting the possibility of a

single resistance gene controlling resistance with 3 or more alleles, which might hasten the rate of resistance development.

1.3.9 Fitness costs

The development of resistance in any insect is often associated with decreased fitness. The fitness of a resistant strain may be affected in terms of incubation period, larval duration, pupal duration, adult longevity, fecundity, fertility hatchability, diapause survivability etc.

Reduced survival (51.5%) of Cry1Ac-resistant PBW was observed on non Bt (NBt) cotton compared to susceptible strains; however, there was no difference in their developmental time. Crosses between resistant and susceptible strains indicated that survival costs could be dominant (Carriere et al. 2001b). Additionally, the emergence of PBW moths from overwintering pupae was greatly (71% reduction) affected in the Cry1Ac-resistant strain compared to the susceptible strain (Carriere et al. 2001a).

Resistant larvae feeding on *Bt* cotton required an average of 5.7 D longer to develop than susceptible larvae on NBt cotton. This developmental asynchrony, therefore, favors assortative mating among resistant moths from *Bt* plants. In the field, the extent of developmental asynchrony and assortative mating would be affected by variation in toxin expression in plant tissues and crop phenology, weather and the overlapping of generations (Liu et al. 1999).

Reduced developmental time and pupal weight was observed in Cry1Ac-resistant *P. gossypiella* when they were tested on increasing concentrations of Cry1Ac in artificial

diet. In addition, tests conducted on NBt cotton using resistant and susceptible strains indicated lower survivability, slower development, lower pupal weight and fecundity in the resistant strain compared to the susceptible strain (Liu et al. 2001c).

Glasshouse experiments using Bt cotton showed that 50 and 62% of BX (Cry1Ac-resistant) strain of *H. armigera* could complete larval development on initial (<15 weeks) and later (>15 weeks) crop stages, respectively. However, no susceptible larvae survived to pupation on Bt cotton plants. However, their developmental rate was faster on NBt cotton compared to the BX strain, which took 7 D more. Though studies on NBt cotton indicated no developmental delay in F1 progeny of reciprocal crosses indicating recessive inheritance of resistance, results from studies on Bt cotton showed that F1 progeny can survive to later instars indicating partially dominant inheritance (Bird and Akhurst 2004).

Fitness costs can vary in resistance strains on different host plants. Dipel®-resistant *T. ni* performed differently on cucumber, tomato and pepper, among which pepper is least preferred host. None of the P_R (Dipel® resistant strain) larvae survived on pepper, contrasting to more than 50% survivors on two other crops (Jaanmat and Myers 2005).

1.3.10 Cross resistance between Cry proteins

Cross resistance is defined as tolerance to a usually toxic substance as a result of exposure to a similarly acting substance. The risk of cross resistance occurring between Cry proteins is substantiated because their mode of action is similar. Though, different Cry proteins can bind to different receptors, there are certain overlapping binding sites which can bind to more than one Cry protein. In one case, one gene was demonstrated to

confer resistance to four different Cry proteins (Tabashnik et al. 1997). Among those four Cry toxins, three (Cry1Aa, Cry1Ab, and Cry1Ac) were closely related and the other (Cry1F) displayed low sequence similarity with others. Available information on cross resistance is tabulated in Table 1.

Cross-resistance patterns vary considerably among different insects and different Cry toxins. Selection for Cry1Ac resistance in *H. virescens* (YHD2) (Gould et al. 1995), and *P. gossypiella* (AZP-R and APHIS-98R) (Tabashnik et al. 2000, 2002) resulted in high levels of cross resistance to Cry1Aa and Cry1Ab and no or little cross resistance to Cry1Ca or Cry2Aa. Different strains of the same insect species have different cross resistance patterns though they were selected using the same Cry protein. Such examples can be observed in Cry1Ac resistant *H. virescens* strains; CP73-3 >50-fold resistant strain, showed 53-fold cross resistance to Cry2Aa (Gould et al. 1992), whereas YHD2 strain with over 10,000-fold resistance to Cry1Ac did not have any cross resistance to Cry2Aa (Gould et al. 1995). Similar cross resistance patterns were observed in *P. gossypiella,* AZP-R with 3100-fold resistance to Cry1Ac had no cross resistance to Cry1Ja (Tabashnik et al. 2000, 2002) on the other hand, APHIS-98R with just over 100-fold resistance to Cry1Ac had little cross resistance to Cry1Ja (Tabashnik et al. 2000, 2003). These two above-mentioned examples suggest that, probably in the initial stages of resistance development one might tend to see little or no cross resistance as the resistant population is not homogeneous compared to highly resistant strains. This observation has tremendous implications in the field as we see increased adoption of Bt crops expressing more than one Bt insecticidal protein.

P. xylostella strain NO-QA, which was selected for resistance to a mixed formulation of Cry1A toxins, also exhibited no cross resistance to Cry1Ca or Cry2Aa (Tabashnik et al. 1996, 1997). This condition of high level (>500-fold) resistance to Cry1A toxins that does not lead to cross resistance to Cry1Ca is defined as 'mode 1' resistance (Tabashnik et al. 1998). A similar mode was observed in *P. interpunctella* strain 198r, which was selected for resistance to a Bt strain expressing Cry1A, Cry1C and Cry1D proteins (McGaughey and Johnson 1994). These observations suggest two basic types of resistance, one exhibiting high-level, narrow-spectrum resistance, and the other featuring moderate-level, broad-spectrum resistance.

References cited:

Abd-Elghafar,S.F., C.O. Knowles, and M.L. Wall. 1993. Pyrethroid resistance in two field strains of *Helicoverpa zea* (Lepidoptera: Noctuidae). J. Econ. Entomol. 86: 1651-1655.

Adamczyk,J.J., Jr., and D.V. Sumerford. 2001. Potential factors impacting season-long expression of Cry1Ac in 13 commercial varieties of Bollgard® cotton. J. Insect Sci.: 1-13.

Adamczyk,J.J., Jr., D.D. Hardee, L.C. Adams, and D.V. Sumerford. 2001. Correlating differences in larval survival and development of bollworm (Lepidoptera: Noctuidae) and fall armyworm (Lepidoptera: Noctuidae) to differential expression of Cry1A(c) delta-endotoxin in various plant parts among commercial cultivars of transgenic *Bacillus thuringiensis* cotton. J. Econ. Entomol. 94: 284-290.

Agrawal,N., P. Malhotra, and R.K. Bhatnagar. 2002. Interaction of Gene-Cloned and Insect Cell-Expressed Aminopeptidase N of *Spodoptera litura* with Insecticidal Crystal Protein Cry1C. Appl. Environ. Microbiol. 68 (9): 4583-92

Akhurst,R. J., W.James, L.J. Bird, and C.Beard. 2003. Resistance to the Cry1Ac delta-endotoxin of *Bacillus thuringiensis* in the cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae). J. Econ. Entomol. 96: 1290-1299.

Ali,A., and S. Y. Young. 1996. Activity of *Bacillus thuringiensis* Berliner against different ages and stages of *Helicoverpa zea* (Lepidoptera: Noctuidae) on cotton. J. Entomol. Sci. 31: 1-8.

Ali,M.I., R.G. Luttrell, and S.Y. Young, III. 2006. Susceptibilities of *Helicoverpa zea* and *Heliothis virescens* (Lepidoptera: Noctuidae) populations to Cry1Ac insecticidal protein. J. Econ. Entomol. 99: 164-175.

Andow,D.A., D.M. Olson, R.L. Hellmich, D.N. Alstad, and W.D. Hutchison. 2000. Frequency of resistance to *Bacillus thuringiensis* toxin Cry1Ab in an Iowa population of European corn borer (Lepidoptera: Crambidae). J. Econ. Entomol. 93: 26-30.

Andow,D.A., D.N. Alstad, Y.H. Pang, P.C. Bolin, and W.D. Hutchison. 1998. Using an F2 screen to search for resistance alleles to *Bacillus thuringiensis* toxin in European corn borer (Lepidoptera: Crambidae). J. Econ. Entomol. 91: 579-584.

Archer,T.L., and E.D. Bynum, Jr. 1994. Corn earworm (Lepidoptera: Noctuidae) biology on food corn on the High Plains. Environ. Entomol. 23: 343-348.

Archer,T.L., and E.D. Bynum. 1998. Corn earworm (Lepidoptera: Noctuidae) damage at various kernel development stages on food maize. Crop Prot.17: 691-695.

Audtho,M., A.P. Valaitis, O. Alzate, and D.H. Dean. 1999. Production of chymotrypsin-resistant *Bacillus thuringiensis* Cry2Aa1 delta -endotoxin by protein engineering. Appl. Environ. Microbiol. 65: 4601-4605.

Bailey,W.D., C. Brownie, J.S. Bacheler, F. Gould, G.G. Kennedy, C.E. Sorenson, and R.M. Roe. 2001. Species diagnosis and *Bacillus thuringiensis* resistance monitoring of *Heliothis virescens* and *Helicoverpa zea* (Lepidoptera: Noctuidae) field strains from the Southern United States using feeding disruption bioassays. J. Econ. Entomol. 94: 76-85.

Baldwin,K.M., and R.S. Hakim. 1991. Growth and differentiation of the larval midgut epithelium during molting in the moth, *Manduca sexta*. Tissue and Cell23:411-22.

Ballester,V., B. Escriche, J.L. Mensua, G.W. Riethmacher, and J. Ferre. 1994. Lack of cross-resistance to other *Bacillus thuringiensis* crystal proteins in a population of *Plutella xylostella* highly resistant to CryIA(b). Biocontrol Sci. Technol.4:437-43.

Ballester,V., F. Granero, B.E. Tabashnik, T.Malvar, and J. Ferre. 1999. Integrative model for binding of *Bacillus thuringiensis* toxins in susceptible and resistant larvae of the diamondback moth (*Plutella xylostella*). Appl. Environ.Microbiol.65:1413-19.

Banks,D.J., J.L. Jurat-Fuentes, D.H. Dean and M.J. Adang. 2001. *Bacillus thuringiensis* Cry1Ac and Cry1Fa ™-endotoxin binding to a novel 110 kDa aminopeptidase in *Heliothis virescens* is not *N*-acetylgalactosamine mediated. Insect Biochem. Molec. Biol. 31: 909-18

Bates, S.L., J. Zhao, R.T. Roush, and A.M. Shelton. 2005. Insect resistance management in GM crops: past, present and future. Nature Biotech.23:57-62.

Bauer, L.S. 1995. Resistance: a threat to the insecticidal crystal proteins of *Bacillus thuringiensis*. Fla. Entomol. 78: 414-443.

Baxter,S.W., J.Z. Zhao, L.J. Gahan, A.M. Shelton, B.E. Tabashnik, and D.G. Heckel. 2005. Novel genetic basis of field-evolved resistance to Bt toxins in *Plutella xylostella*. Insect Mol. Biol. 14:327-334

Beegle,C.C., and T. Yamamoto. 1992. Invitation paper (C.P. Alexander Fund): History of *Bacillus thuringiensis* Berliner research and development. Can. Entomol. 124: 587-616.

Bell,M.R., and J.L. Hayes. 1994. Areawide management of cotton bollworm and tobacco budworm (Lepidoptera: Noctuidae) through application of a nuclear polyhedrosis virus on early-season alternate hosts. J. Econ. Entomol. 87: 53-57.

Bergvinson,D.J. 2005. *Heliothis/Helicoverpa* problem in the Americas: biology and management, pp. 7-37. In H. C. Sharma [ed.], *Heliothis*/*Helicoverpa* management: emerging trends and strategies for future research. Ltd, New Delhi India.

Berliner,E. 1915. Uber die schlafsucht der Mehlottenraupe (*Ephestia kuhniella*, Zell.) und ihren erreger *Bacillus thuringiensis* n. sp. Z. angew. Entomol. 2: 29-56.

Bird,L.J., and R.J. Akhurst. 2004. Relative fitness of Cry1A-resistant and -susceptible *Helicoverpa armigera* (Lepidoptera: Noctuidae) on conventional and transgenic cotton. J. Econ. Entomol. 97: 1699-709.

Bird,L.J., and R.J. Akhurst. 2005. Fitness of Cry1A-resistant and -susceptible *Helicoverpa armigera* (Lepidoptera: Noctuidae) on transgenic cotton with reduced levels of Cry1Ac. J. Econ. Entomol. 98: 1311-1319.

Bolin,P.C., W.D. Hutchison, and D.A. Andow. 1999. Long-term selection for resistance to *Bacillus thuringiensis* Cry1Ac endotoxin in a Minnesota population of European corn borer (Lepidoptera: Crambidae). J. Econ. Entomol. 92: 1021-1030.

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

Bourguet,D., J. Chaufaux, M. Seguin, C. Buisson, J.L. Hinton, T.J. Stodola, P. Porter, G. Cronholm, L.L. Buschman, and D.A. Andow. 2003. Frequency of alleles conferring resistance to Bt maize in French and US corn belt populations of the European corn borer, *Ostrinia nubilalis*. Theor. Appl. Genet. 106: 1225-1233.

Bravo,A. 1997. Phylogenetic relationships of *Bacillus thuringiensis* delta -endotoxin family proteins and their functional domains. J. Bacteriol. 179: 2793-2801.

Bravo,A., J. Sanchez, T. Kouskoura, and N. Crickmore. 2002. N-terminal activation is an essential early step in the mechanism of action of the *Bacillus thuringiensis* Cry1Ac insecticidal toxin. J. Biol. Chem.277: 23985-23987.

Brookes, G. and P. Barfoot. 2006. GM crops: First ten years-Global socio-economic and environmental impacts. ISAAA Brief No. 36.ISAAA Ithaca, NY.

Brown,T.M., P.K. Bryson, D.S. Brickle, J.T. Walker, and M.J. Sullivan. 1997. Pyrethroid-resistant *Helicoverpa zea* in cotton in South Carolina. Resistant Pest Management 9: 26-27.

Brown,T.M., P.K. Bryson, D.S. Brickle, S .Pimprale, F. Arnette, M.E. Roof, J.T. Walker, and M.J. Sullivan. 1998. Pyrethroid-resistant *Helicoverpa zea* and transgenic cotton in South Carolina. Crop Prot.17: 441-445.

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: 137-142.

Butler,G.D., Jr. 1976. Bollworm: development in relation to temperature and larval food. Environ. Entomol. 5: 520-522.

Cannon,R.J.C. 2000. Bt transgenic crops: risks and benefits. Integrated Pest Management Reviews 5: 151-173.

Cao,J., J.D. Tang, N. Strizhov, A.M. Shelton, and E.D. Earle. 1999. Transgenic broccoli with high levels of *Bacillus thuringiensis* Cry1C protein control diamondback moth larvae resistant to Cry1A or Cry1C. Mol. Breed. 5: 131-141.

Carriere,Y., C. Ellers-Kirk, A.L. Patin, M.A. Sims, S. Meyer, Y.B. Liu, T.J. Dennehy, and B.E. Tabashnik. 2001a. Overwintering cost associated with resistance to transgenic cotton in the pink bollworm (Lepidoptera: Gelechiidae). J. Econ. Entomol. 94: 935-941.

Carriere,Y., C. Ellers-Kirk, Y.B. Liu, M.A. Sims, A.L. Patin, T.J. Dennehy, and B.E. Tabashnik. 2001b. Fitness costs and maternal effects associated with resistance to transgenic cotton in the pink bollworm (Lepidoptera: Gelechiidae). J. Econ. Entomol. 94: 1571-1576.

Carroll,J., D. Convents, J.v. Damme, A. Boets, J.van Rie, and D.J. Ellar. 1997. Intramolecular proteolytic cleavage of *Bacillus thuringiensis* Cry3A delta - endotoxin may facilitate its coleopteran toxicity. J. Invertebr. Pathol.70: 41-49.

Casida, J.E., and G.B. Quistad. 1998. Golden age of insecticide research: past, present, or future? Annu. Rev. Entomol. 43: 1-16.

Cavados,C.F.G., S. Majerowicz, J.Q. Chaves, C.J.P.C. Araujo-Coutinho, and L. Rabinovitch. 2004. Histopathological and ultrastructural effects of delta - endotoxins of *Bacillus thuringiensis* serovar israelensis in the midgut of Simulium pertinax larvae (Diptera, Simuliidae). Memorias do Instituto Oswaldo Cruz 99: 493-498.

Chambers,J.A., A. Jelen, M.P. Gilbert, C.S. Jany, T.B. Johnson, and C. Gawron-Burke. 1991. Isolation and characterization of a novel insecticidal crystal protein gene from *Bacillus thuringiensis* subsp. aizawai. J. Bacteriol. 173: 3966-3976.

Chapman,R.F. 1998. The insects: structure and function. The insects: structure and function. Cambridge University Press Cambridge UK: 1998. Ed. 4 xvii + 770.

Cheng,D., and K.W. Nickerson. 1996. The *Bacillus thuringiensis* insecticidal toxin binds biotin-containing proteins. Appl. Environ. Microbiol. 62: 2932-2939.

Chitkowski,R.L., S.G. Turnipseed, M.J. Sullivan, and W.C. Bridges, Jr. 2003. Field and laboratory evaluations of transgenic cottons expressing one or two *Bacillus thuringiensis* var. kurstaki Berliner proteins for management of noctuid (Lepidoptera) pests. J. Econ. Entomol. 96: 755-762.

Choma,C.T., and H. Kaplan. 1990. Folding and unfolding of the protoxin from *Bacillus thuringiensis*: evidence that the toxic moiety is present in an active conformation. Biochemistry (Washington) 29: 10971-10977.

Choma,C.T., and H. Kaplan. 1992. *Bacillus thuringiensis* crystal protein: effect of chemical modification of the cysteine and lysine residues. J. Invertebr. Pathol.59: 75-80.

Choma,C.T., W.K. Surewicz, P.R. Carey, M. Pozsgay, T. Raynor, and H. Kaplan. 1990. Unusual proteolysis of the protoxin and toxin from *Bacillus thuringiensis*. Structural implications. Eur. J. Biochem.189: 523-527.

Convents,D., M. Cherlet, J.v. Damme, I. Lasters, and M. Lauwereys. 1991. Two structural domains as a general fold of the toxic fragment of the *Bacillus thuringiensis* delta -endotoxins. Eur. J. Biochem.195: 631-635.

Cooper,M.A., J. Carroll, E.R. Travis, D.H. Williams, and D.J. Ellar. 1998. *Bacillus thuringiensis* Cry1Ac toxin interaction with *Manduca sexta* aminopeptidase N in a model membrane environment. Biochem. J. (London) 333: 677-683.

Crickmore,N., D.R. Zeigler, J. Feitelson, E. Schnepf, J. v. Rie, D. Lereclus, J. Baum, and D.H. Dean. 1998. Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62: 807-813.

Dai, S., and S.S. Gill. 1993. In vitro and in vivo proteolysis of the *Bacillus thuringiensis* subsp. israelensis CryIVD protein by Culex quinquefasciatus larval midgut proteases. Insect Biochem Mol Biol23: 273-283.

Dankocsik, C., W.P. Donovan, and C.S. Jany. 1990. Activation of a cryptic crystal protein gene of *Bacillus thuringiensis* subspecies kurstaki by gene fusion and determination of the crystal protein insecticidal specificity. Mol. Microbiol. 4: 2087-2094.

de Maagd, R.A., A. Bravo, C. Berry, N. Crickmore, and H.E. Schnepf. 2003. Structure, diversity, and evolution of protein toxins from spore-forming entomopathogenic bacteria. Ann. Rev. Genet. 37: 409-433.

de Maagd,R.A., D. Bosch, and W. Stiekema. 1999a. *Bacillus thuringiensis* toxin-mediated insect resistance in plants. Trends Plant Sci. 4: 9-13.

de Maagd,R.A., P.L. Bakker, L. Masson, M.J. Adang, S. Sangadala, W. Stiekema, and D. Bosch. 1999b. Domain III of the *Bacillus thuringiensis* delta-endotoxin Cry1Ac is involved in binding to *Manduca sexta* brush border membranes and to its purified aminopeptidase N. Mol. Microbiol. 31: 463-471.

Dorsch,J.A., M. Candas, N.B. Griko, W.S.A. Maaty, E.G. Midboe, R.K. Vadlamudi and L.A. Bulla. 2002. Cry1A toxins of *Bacillus thuringiensis* bind specifically to a region adjacent to the membrane-proximal extracellular domain of BT-R1 in *Manduca sexta*: involvement of a cadherin in the entomopathogenicity of *Bacillus thuringiensis*. Insect Biochem. Molec. Biol. 32: 1025-36

Ellsworth,P.C., and J.R. Bradley, Jr. 1992. Comparative damage potential and feeding dynamics of the European corn borer (Lepidoptera: Pyralidae) and cotton bollworm (Lepidoptera: Noctuidae) on cotton bolls. J. Econ. Entomol. 85: 402-410.

Engelhard,E.K., B.A. Keddie, and L.E. Volkman. 1991. Isolation of third, fourth, and fifth instar larval midgut epithelia of the moth, *Trichoplusia ni*. Tissue Cell 23: 917-928.

Ernst,G.H., and V. Dittrich. 1992. Comparative measurements of resistance to insecticides in three closely-related Old and New World bollworm species. Pestic. Science 34: 147-152.

Estada,U., and J. Ferre. 1994. Binding of insecticidal crystal proteins of *Bacillus thuringiensis* to the midgut brush border of the cabbage looper, Trichoplusia ni (Hubner) (Lepidoptera: Noctuidae), and selection for resistance to one of the crystal proteins. Appl. Environ. Microbiol. 60: 3840-3846.

Estela,A., B. Escriche, and J. Ferre. 2004. Interaction of *Bacillus thuringiensis* toxins with larval midgut binding sites of *Helicoverpa armigera* (Lepidoptera: Noctuidae). Appl. Environ. Microbiol. 70: 1378-1384.

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.

Fan,X., Zhao J.Z., FanY., and Shi X. 2000. Inhibition of transgenic Bt plants to the growth of cotton bollworm. Plant Prot. 26(2):3–5.

Federici,B.A. 2005. Insecticidal bacteria: an overwhelming success for invertebrate pathology. J. Invertebr. Pathol. 89: 30-8.

Federici,B.A., and L.S. Bauer. 1998. Cyt1Aa protein of *Bacillus thuringiensis* is toxic to the cottonwood leaf beetle, *Chrysomela scripta*, and suppresses high levels of resistance to Cry3Aa. Appl. Environ. Microbiol. 64: 4368-4371.

Ferre,J., and J. van Rie. 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol. 47: 501-533.

Ferre,J., B. Escriche, Y. Bel, and J. v. Rie. 1995. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis* insecticidal crystal proteins. FEMS Microbiol. Lett. 132: 1-7.

Ferre,J., M.D. Real, J. van Rie, S. Jansens, and M. Peferoen. 1991. Resistance to the *Bacillus thuringiensis* bioinsecticide in a field population of *Plutella xylostella* is due to a change in a midgut membrane receptor. Proc. Natl. Acad. Sci. USA 88: 5119-5123.

Flannagan,R. D., C. G. Yu, J.P. Mathis, T.E. Meyer, X. M. Shi, H.A.A. Siqueira, and B.D. Siegfried. 2005. Identification, cloning and expression of a Cry1Ab cadherin receptor from European corn borer, *Ostrinia nubilalis* (Hubner) (Lepidoptera: Crambidae). Insect Biochem. Mol. Biol. 35: 33-40.

Forcada,C., E. Alcacer, M.D. Garcera, A. Tato, and R. Martinez. 1999. Resistance to *Bacillus thuringiensis* Cry1Ac toxin in three strains of *Heliothis virescens*: proteolytic and SEM study of the larval midgut. Arch. Insect Biochem. Physiol. 42: 51-63.

Forcada,C., E. Alcacer, M.D. Garcera, and R. Martinez. 1996. Differences in the midgut proteolytic activity of two *Heliothis virescens* strains, one susceptible and one resistant to *Bacillus thuringiensis* toxins. Arch. Insect Biochem. Physiol. 31: 257-272.

Gahan,L.J., F. Gould, and D.G. Heckel. 2001. Identification of a gene associated with Bt resistance in *Heliothis virescens*. Science (Washington) 293: 857-860.

Genissel,A., S. Augustin, C. Courtin, G. Pilate, P. Lorme, and D. Bourguet. 2003. Initial frequency of alleles conferring resistance to *Bacillus thuringiensis* poplar in a field population of *Chrysomela tremulae*. Proc. R. Soc. B: 791-797.

Georghiou,G.P., and M.C. Wirth. 1997. Influence of exposure to single versus multiple toxins of *Bacillus thuringiensis* subsp. *israelensis* on development of resistance in the mosquito *Culex quinquefasciatus* (Diptera: Culicidae). Appl. Environ. Microbiol. 63: 1095-1101.

Geraud,F., L. Fernandez, D.T. Chirinos, O. Martinez, and A. Casanova. 1996. Biology of the tomato fruitworm, *Heliothis zea* (Boddie), Lepidoptera: Noctuidae, reared on different substrates under laboratory conditions. [Spanish]. Revista de la Facultad de Agronomia, Universidad del Zulia 13: 35-48.

Gill, S. S., E. A. Cowles, and P. V. Pietrantonio. 1992. The mode of action of *Bacillus thuringiensis* endotoxins. Annu. Rev. Entomol. 37: 615-636.

Gonzalez-Cabrera, J., B. Escriche, B. E. Tabashnik, and J. Ferre. 2003. Binding of *Bacillus thuringiensis* toxins in resistant and susceptible strains of pink bollworm (*Pectinophora gossypiella*). Insect Biochem. Mol. Biol. 33: 929-935.

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: 699-705.

Gould,F. 1991. The evolutionary potential of crop pests. Am. Sci. 79: 496-507.

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

Gould,F. 2000. Testing Bt refuge strategies in the field. Nature Biotechnol. 18: 266-267.

Gould,F., A. Anderson, A. Jones, D. Sumerford, D.G. Heckel, J. Lopez, S. Micinski, R. Leonard, and M. Laster. 1997. Initial frequency of alleles for resistance to *Bacillus thuringiensis* toxins in field populations of *Heliothis virescens*. Proc. Natl. Acad. Sci. USA 94: 3519-3523.

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

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., J.W. Mullins, S.R. Penn, A. Dahm, B.J. Reich, J.A. Osborn, P.R. Rahn, L. Ruschke, and Z.W. Shappley. 2003. Partial characterization of cotton plants expressing two toxin proteins from *Bacillus thuringiensis*: relative toxin contribution, toxin interaction, and resistance management. J. Appl. Entomol. 127: 340-347.

Griffitts,J.S., and R.V. Aroian. 2005. Many roads to resistance: how invertebrates adapt to Bt toxins. Bioessays 27: 614-624.

Griffitts,J.S., S.M. Haslam, T.L. Yang, S.F. Garczynski, B. Mulloy, H. Morris, P.S. Cremer, A. Dell, M.J. Adang, and R.V. Aroian. 2005. Glycolipids as receptors for *Bacillus thuringiensis* crystal toxin. Science (Washington) 307: 922-925.

Haider,M.Z., and D.J. Ellar. 1989. Mechanism of action of *Bacillus thuringiensis* insecticidal delta-endotoxin: interaction with phospholipid vesicles. Biochimica et biophysica acta: International journal of biochemistry and biophysics 978: 216-222.

Hama,H., K. Suzuki, and H. Tanaka. 1992. Inheritance and stability of resistance to *Bacillus thuringiensis* formulations of the diamondback moth, *Plutella xylostella* (Linnaeus) (Lepidoptera: Yponomeutidae). Appl. Entomol Zool. 27: 355-362.

Head,G., W. Moar, M. Eubanks, B. Freeman, J. Ruberson, A. Hagerty, and S. Turnipseed. 2005. A multiyear, large-scale comparison of arthropod populations on commercially managed Bt and non-Bt cotton fields. Environ. Entomol. 34: 1257-1266.

Herrero,S., B. Oppert, and J. Ferre. 2001. Different mechanisms of resistance to *Bacillus thuringiensis* toxins in the Indianmeal moth. Appl. Environ. Microbiol. 67: 1085-1089.

Herrero,S., T. Gechev, P.L. Bakker, W.J. Moar, and R.A. de Maagd. 2005. *Bacillus thuringiensis* Cry1Ca-resistant *Spodoptera exigua* lacks expression of one of four Aminopeptidase N genes. BMC Genomics. 6 JUN 24 2005.

Himeno,M., N. Koyama, T. Funato, and T. Komano. 1985. Mechanism of action of *Bacillus thuringiensis* insecticidal delta-endotoxin on insect cells in vitro. Agric. Biol. Chem. 49: 1461-1468.

Hofmann,C., P. Luthy, R. Hutter, and V. Pliska. 1988. Binding of the delta endotoxin from *Bacillus thuringiensis* to brush-border membrane vesicles of the cabbage butterfly (*Pieris brassicae*). Eur. J. Biochem.173: 85-91.

Hofte,H., and H.R. Whiteley. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. 53: 242-255.

Hossain,D.M., Y. Shitomi, K. Moriyama, M. Higuchi, T. Hayakawa, T. Mitsui, R. Sato, and H. Hori. 2004. Characterization of a novel plasma membrane protein, expressed in the midgut epithelia of *Bombyx mori* that binds to Cry1A toxins. Appl. Environ. Microbiol. 70: 4604-4612.

Hsu,E.L., and S.J. Yu. 1991. Insecticide resistance in the corn earworm, *Heliothis zea* (Boddie). Resistant Pest Management 3: 18.

Huang,F., K. Zhu, L.L. Buschman, R.A. Higgins, and B. Oppert. 1999a. Comparison of midgut proteinases in *Bacillus thuringiensis*-susceptible and -resistant European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae). Pestic. Biochem. Physiol. 65: 132-139.

Huang,F., L.L. Buschman, R.A. Higgins, and W.H. McGaughey. 1999b. Inheritance of resistance to *Bacillus thuringiensis* toxin (Dipel ES) in the European corn borer. Science (Washington) 284: 965-967.

Huang,F., R.A. Higgins, and L.L. Buschman. 1997. Baseline susceptibility and changes in susceptibility to *Bacillus thuringiensis* subsp. kurstaki under selection pressure in European corn borer (Lepidoptera: Pyralidae). J. Econ. Entomol. 90: 1137-1143.

Ishiwata,S. 1901. One of a kind of several flasherne (sotto disease). Dainihan Sanbshi Kaiho 9: 1-5.

Jackson,R.E., J.R. Bradley, Jr., J.W. van Duyn, and F. Gould. 2004. Comparative production of *Helicoverpa zea* (Lepidoptera: Noctuidae) from transgenic cotton expressing either one or two *Bacillus thuringiensis* proteins with and without insecticide oversprays. J. Econ. Entomol. 97: 1719-1725.

Jalali,S.K., K.S. Mohan, S.P. Singh, T.M. Manjunath, and Y. Lalitha. 2004. Baseline-susceptibility of the old-world bollworm, *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) populations from India to *Bacillus thuringiensis* Cry1Ac insecticidal protein. Crop Prot.23: 53-59.

James,C. 2002. Global review of commercialized transgenic crops: 2001. Feature: Bt cotton. ISAAA Briefs. ISAAA: Ithaca, NY

James,C. 2005. Preview - global status of commercialized biotech/GM crops: 2005. ISAAA Briefs. ISAAA: Ithaca, NY

Janmaat,A.F., and J. Myers. 2003. Rapid evolution and the cost of resistance to *Bacillus thuringiensis* in greenhouse populations of cabbage loopers, *Trichoplusia ni.* Proc. R. Soc. B: 2263-2270.

Janmaat,A.F., and J.H. Myers. 2005. The cost of resistance to *Bacillus thuringiensis* varies with the host plant of *Trichoplusia ni*. Proc.R.Soc. B: 1031-1038.

Janmaat,A.F., P. Wang, W. Kain, J.Z. Zhao, and J. Myers. 2004. Inheritance of resistance to *Bacillus thuringiensis* subsp. kurstaki in *Trichoplusia ni*. Appl. Environ. Microbiol. 70: 5859-5867.

Jenkins,J.L., and D.H. Dean. 2000. Exploring the mechanism of action of insecticidal proteins by genetic engineering methods. Genetic Engineering; Principles & Methods 22: 33-54.

Johnson,D.E., G.L. Brookhart, K.J. Kramer, B.D. Barnett, and W.H. McGaughey. 1990. Resistance to *Bacillus thuringiensis* by the Indian meal moth, *Plodia interpunctella*: comparison of midgut proteinases from susceptible and resistant larvae. J. Invertebr. Pathol.55: 235-244.

Jurat-Fuentes,J.L., and M.J. Adang. 2004. Characterization of a Cry1Ac-receptor alkaline phosphatase in susceptible and resistant *Heliothis virescens* larvae. Eur. J. Biochem.271: 3127-3135.

Kain,W.C., J.Z. Zhao, A.F. Janmaat, J. Myers, A.M. Shelton, and P. Wang. 2004. Inheritance of resistance to *Bacillus thuringiensis* Cry1Ac toxin in a greenhouse-derived strain of cabbage looper (Lepidoptera: Noctuidae). J. Econ. Entomol. 97: 2073-2078.

Karim,S., S. Riazuddin, F. Gould, and D.H. Dean. 2000. Determination of receptor binding properties of *Bacillus thuringiensis* delta-endotoxins to cotton bollworm (*Helicoverpa zea)* and pink bollworm (*Pectinophora gossypiella*) midgut brush border membrane vesicles. Pestic. Biochem. Physiol. 67: 198-216.

Knight,P.J.K., B.H. Knowels and D.J. Ellar. 1995. Molecular cloning of an insect aminopeptidase N that serves as a receptor for *Bacillus thuringiensis* CryIA(c) toxin. J. Biol. Chem. 270:17765-70

Knowles,B.H. 1994. Mechanism of action of *Bacillus thuringiensis* insecticidal delta - endotoxins. Adv. Insect Physiol. 24: 275-308.

Knowles,B.H., P.J.K. Knight, and D.J. Ellar. 1991. N-acetyl galactosamine is part of the receptor in insect gut epithelia that recognizes an insecticidal protein from *Bacillus thuringiensis*. Proc. R. Soc. Lond. 1991. 245: 31-35.

Kolodny-Hirsch,D.M., and F.P. Harrison. 1982. Comparative damage and leaf area consumption by the tobacco budworm and corn earworm on Maryland tobacco. J. Econ. Entomol. 75: 168-172.

Kota,M., H. Daniell, S. Varma, S.F. Garczynski, F. Gould, and W.J. Moar. 1999. Overexpression of the *Bacillus thuringiensis* (Bt) Cry2Aa2 protein in chloroplasts confers resistance to plants against susceptible and Bt-resistant insects. Proc Natl Acad Sci USA 96: 1840-1845.

Kranthi,K.R., C.S. Dhawad, S.R. Naidu, K. Mate, G.T. Behere, R.M. Wadaskar, and S. Kranthi. 2006. Inheritance of resistance in Indian *Helicoverpa armigera* (Hübner) to Cry1Ac toxin of *Bacillus thuringiensis*. Crop Prot. 25: 119.

Kranthi,K.R., S. Kranthi, and R.R. Wanjari. 2001. Baseline toxicity of Cry1A toxins to *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) in India. Int. J. Pest Manag. 47: 141-145.

Kranthi,K.R., S. Kranthi, S. Ali, and S.K. Banerjee. 2000. Resistance to 'CryIAc delta - endotoxin of *Bacillus thuringiensis*' in a laboratory selected strain of *Helicoverpa armigera* (Hubner). Curr. Sci. 78: 1001-1004.

Kranthi,K.R., S. Naidu, C.S. Dhawad, A. Tatwawadi, K. Mate, E. Patil, A.A. Bharose, G.T. Behere, R.M. Wadaskar, and S. Kranthi. 2005. Temporal and intra-plant variability of Cry1Ac expression in Bt-cotton and its influence on the survival of

the cotton bollworm, *Helicoverpa armigera* (Hubner) (Noctuidae: Lepidoptera). Curr. Sci. 89: 291-298.

Krishnappa,C., K. Archana, K. Vinay, and G.T. Gujar. 2005. Baseline susceptibility of the American bollworm, *Helicoverpa armigera* (Hubner) to *Bacillus thuringiensis* Berl var. kurstaki and its endotoxins in India. Curr. Sci. 88: 167-175.

Lacey,L.A., and B.A. Federici. 1979. Pathogenesis and midgut histopathology of *Bacillus thuringiensis* in *Simulium vittatum* (Diptera: Simuliidae). J. Invertebr. Pathol.33: 171-182.

Lambert,B., and M. Peferoen. 1992. Insecticidal promise of *Bacillus thuringiensis*. BioScience 42: 112-122.

Lambert,B., L. Buysse, C. Decock, S. Jansens, C. Piens, B. Saey, J. Seurinck, K. v. Audenhove, J. van Rie, A. v. Vliet, and M. Peferoen. 1996. A *Bacillus thuringiensis* insecticidal crystal protein with a high activity against members of the family Noctuidae. Appl. Environ. Microbiol. 62: 80-86.

Lecadet,M.M., E. Frachon, V.C. Dumanoir, H. Ripouteau, S. Hamon, P. Laurent, and I. Thiery. 1999. Updating the H-antigen classification of *Bacillus thuringiensis*. J. Appl. Microbiol. 86: 660-672.

Lee,M., F. Rajamohan, F. Gould, and D.H. Dean. 1995. Resistance to *Bacillus thuringiensis* CryIA delta -endotoxins in a laboratory-selected *Heliothis virescens* strain is related to receptor alteration. Appl. Environ. Microbiol. 61: 3836-3842.

Lee,M.K., F.S. Walters, H. Hart, N. Palekar, and J.S. Chen. 2003. The mode of action of the *Bacillus thuringiensis* vegetative insecticidal protein Vip3A differs from that of Cry1Ab delta -endotoxin. Appl. Environ. Microbiol. 69: 4648-4657.

Leeper,J.R., and K.F. Raffa. 1986. Baseline data for evaluating resistance development: doseage/mortality studies using two test methods with chlorpyrifos, methomyl and permethrin in *Heliothis virescens* (F.) and *Heliothis zea* (Boddie) (Lepidoptera: Noctuidae). Trop. Pest Manag. 32: 137-145

Leonard,B.R., J.B. Graves, T.C. Sparks, and A.M. Pavloff. 1988. Variation in resistance of field populations of tobacco budworm and bollworm (Lepidoptera: Noctuidae) to selected insecticides. J. Econ. Entomol. 81: 1521-1528.

Li,H.R., B. Oppert, K.Y. Zhu, R.A. Higgins, F.N. Huang, and L.L. Buschman. 2003. Transgenic plants expressing *Bacillus thuringiensis* delta-endotoxins. Entomologia Sinica 10: 155-166.

Li,H.R., B. Oppert, R.A. Higgins, F.N. Huang, K.Y. Zhu, and L.L. Buschman. 2004a. Comparative analysis of proteinase activities of *Bacillus thuringiensis*-resistant and -susceptible *Ostrinia nubilalis* (Lepidoptera: Crambidae). Insect Biochem. Mol. Biol. 34: 753-762.

Li,H.R., J. Gonzalez-Cabrera, B. Oppert, J. Ferre, R.A. Higgins, L.L. Buschman, G.A. Radke, K.Y. Zhu, and F.N. Huang. 2004. Binding analyses of Cry1Ab and Cry1Ac with membrane vesicles from *Bacillus thuringiensis*-resistant and -susceptible *Ostrinia nubilalis*. Biochem. Biophys. Res. Commun. 323: 52-57.

Li,J., J. Carroll, and D.J. Ellar. 1991. Crystal structure of insecticidal delta -endotoxin from *Bacillus thuringiensis* at 2.5 A resolution. Nature (London) 353: 815-821.

Liao,C., D.G. 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,K., H. Yao, H. Yang, and H. Hong. 2004. Progress of studies on insect midgut aminopeptidase N serving as *Bacillus thuringiensis* insecticidal crystal protein toxin receptor. [Chinese]. Entomological Knowledge Institute of Zoology, Chinese Academy of Sciences, Beijing, China: 203-207.

Liu,Y., and B.E. Tabashnik. 1997. Experimental evidence that refuges delay insect adaptation to *Bacillus thuringiensis*. Proc.R.Soc. B: 605-610.

Liu,Y., and B.E. Tabashnik. 1997. Inheritance of resistance to the *Bacillus thuringiensis* toxin Cry1C in the diamondback moth. Appl. Environ. Microbiol. 63: 2218-2223.

Liu,Y., and B.E. Tabashnik. 1997b. Synergism of *Bacillus thuringiensis* by ethylenediamine tetraacetate in susceptible and resistant larvae of diamondback moth (Lepidoptera: Plutellidae). J. Econ. Entomol. 90: 287-292.

Liu,Y., B.E. Tabashnik, and M. Pusztai-Carey. 1996. Field-evolved resistance to *Bacillus thuringiensis* toxin CryIC in diamondback moth (Lepidoptera: Plutellidae). J. Econ. Entomol. 89: 798-804.

Liu,Y., B.E. Tabashnik, L. Masson, B. Escriche, and J. Ferre. 2000. Binding and toxicity of *Bacillus thuringiensis* protein Cry1C to susceptible and resistant diamondback moth (Lepidoptera: Plutellidae). J. Econ. Entomol. 93: 1-6.

Liu,Y., B.E. Tabashnik, T.J. Dennehy, A.L. Patin, and A.C. Bartlett. 1999. Development time and resistance to Bt crops. Nature (London) 400: 519.

Liu,Y.B., and B.E. Tabashnik. 1997a. Synergism of *Bacillus thuringiensis* by ethylenediamine tetraacetate in susceptible and resistant larvae of diamondback moth (Lepidoptera: Plutellidae). J. Econ. Entomol. 90: 287-292.

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. Applied & Environmental Microbiology 67: 3216-3219.

Liu,Y.B., B.E. Tabashnik, S.K. Meyer, Y. Carriere, and A.C. Bartlett. 2001. Genetics of pink bollworm resistance to *Bacillus thuringiensis* toxin Cry1Ac. J. Econ. Entomol. 94: 248-252.

Liu,Y.B., B.E. Tabashnik, T.J. Dennehy, A.L. Patin, M.A. Sims, S.K. Meyer, and Y. Carriere. 2001c. Effects of Bt cotton and Cry1Ac toxin on survival and development of pink bollworm (Lepidoptera: Gelechiidae). J. Econ. Entomol. 94: 1237-1242.

Loeb,M.J., and R.S. Hakim. 1996. Insect midgut epithelium in vitro: an insect stem cell system. J. Insect Physiol. 42: 1103-1111.

Loeb,M.J., E.A. Clark, M. Blackburn, R.S. Hakim, K. Elsen, and G. Smagghe. 2003. Stem cells from midguts of lepidopteran larvae: clues to the regulation of stem cell fate. Arch. Insect Biochem. Physiol. 53: 186-198.

Loeb,M.J., P.A. W. Martin, R.S. Hakim, S. Goto, and M. Takeda. 2001. Regeneration of cultured midgut cells after exposure to sublethal doses of toxin from two strains of *Bacillus thuringiensis*. J. Insect. Physiol. 47: 599-606.

Lord,J.C. 2005. From Metchnikoff to Monsanto and beyond: the path of microbial control. J. Invertebr. Pathol.89: 19-29.

Lorence,A., A. Darszon, C. Diz, A. Lievano, R. Quintero, and A. Bravo. 1995. Delta - Endotoxins induce cation channels in *Spodoptera frugiperda* brush border membranes in suspension and in planar lipid bilayers. FEBS Letters 360:217-222.

Loseva,O., M. Ibrahim, M. Candas, C.N.Koller, L.S. Bauer and L.A. Bulla Jr. 2002. Changes in protease activity and Cry3Aa toxin binding in the Colorado potato beetle: implications for insect resistance to *Bacillus thuringiensis*. Insect Biochem. Molec. Biol. 32: 567-77

Lu, MG., C.H. Rui, J.Z. Zhao, G.L. Jian, X.L. Fan, and X.W. Gao. 2004. Selection and heritability of resistance to *Bacillus thuringiensis* subsp kurstaki and transgenic cotton in *Helicoverpa armigera* (Lepidoptera: Noctuidae). SO - Pest Manag sci. 60(9). September 2004. 887-893.

Luo,K., B.E. Tabashnik, and M.J. Adang. 1997. Binding of *Bacillus thuringiensis* Cry1Ac toxin to aminopeptidase in susceptible and resistant diamondback moths (*Plutella xylostella*). *Appl. Environ. Microbiol.* 63:1024–27

Luttrell,R.G., G.P. Fitt, F.S. Ramalho, and E.S. Sugonyaev. 1994. Cotton pest management: part 1. A worldwide perspective. Annu. Rev. Entomol. 39: 517-526.

Luttrell,R.G., L. Wan, and K. Knighten. 1999. Variation in susceptibility of noctuid (Lepidoptera) larvae attacking cotton and soybean to purified endotoxin proteins and commercial formulations of *Bacillus thuringiensis*. J. Econ. Entomol.92:21-32.

MacIntosh,S.C., T.B. Stone, R.S. Jokerst, and R.L. Fuchs. 1991. Binding of *Bacillus thuringiensis* proteins to a laboratory-selected line of *Heliothis virescens*. Proc. Natl. Acad. Sci. USA 88: 8930-3.

MacIntosh,S.C., T.B. Stone, S.R. Sims, P.L. Hunst, J.T. Greenplate, P.G. Marrone, F.J. Perlak, D.A. Fischhoff, and R.L. Fuchs. 1990. Specificity and efficacy of purified

Bacillus thuringiensis proteins against agronomically important insects. J. Invertebr. Pathol.56: 258-66.

Martinez-Ramirez,A.C., F. Gould, and J. Ferre. 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 Sci. Technol. 9: 239-246.

McCutchen,B.F., F.W. Plapp, Jr., S.J. Nemec, and C. Campanhola. 1989. Development of diagnostic monitoring techniques for larval pyrethroid resistance in *Heliothis* spp. (Lepidoptera: Noctuidae) in cotton. J. Econ. Entomol. 82: 1502-1507.

McGaughey,W.H., and D.E. Johnson. 1987. Toxicity of different serotypes and toxins of *Bacillus thuringiensis* to resistant and susceptible Indianmeal moths (Lepidoptera: Pyralidae). J. Econ. Entomol. 80: 1122-1126.

McGaughey,W.H., and D.E. Johnson. 1992. Indianmeal moth (Lepidoptera: Pyralidae) resistance to different strains and mixtures of *Bacillus thuringiensis*. J. Econ. Entomol. 85: 1594-1600.

McGaughey,W.H., and D.E. Johnson. 1994. Influence of crystal protein composition of *Bacillus thuringiensis* strains on cross-resistance in Indianmeal moths (Lepidoptera: Pyralidae). J. Econ. Entomol. 87: 535-540.

McGaughey,W.H., and R.W. Beeman. 1988. Resistance to *Bacillus thuringiensis* in colonies of Indianmeal moth and almond moth (Lepidoptera: Pyralidae). J. Econ. Entomol. 81: 28-33.

McNall,R.J. and M.J. Adang. 2003. Identification of novel *Bacillus thuringiensis* Cry1Ac binding proteins in *Manduca sexta* midgut through proteomic analysis. Insect Biochem. Molec. Biol. 33: 999-1010

Mendelsohn,M., J. Kough, Z. Vaituzis, and K. Matthews. 2003. Are Bt crops safe? Nature Biotechnology 21: 1003-1009.

Meng, F., J. Shen, W. Zhou, and H. Cen. 2004. Long-term selection for resistance to transgenic cotton expressing *Bacillus thuringiensis* toxin in *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae). (Special issue: Current research at the Scottish Agricultural College). Pest Manag. Sci. 60: 167-172.

Miranda, R., F.Z. Zamudio, and A. Bravo. 2001. Processing of Cry1Ab delta -endotoxin from *Bacillus thuringiensis* by *Manduca sexta* and Spodoptera frugiperda midgut proteases: role in protoxin activation and toxin inactivation. Insect Biochem. Mol. Biol. 31: 1155-1163.

Moar,W.J., M. Pusztai-Carey, H. v. Faassen, D. Bosch, R. Frutos, C. Rang, K. Luo, and M.J. Adang. 1995. Development of *Bacillus thuringiensis* CryIC resistance by *Spodoptera exigua* (Hubner) (Lepidoptera: Noctuidae). Appl. Environ. Microbiol. 61: 2086-2092.

Mohan,M and G.T. Gujar. 2003. Characterization and comparison of midgut proteases of *Bacillus thuringiensis* susceptible and resistant diamondback moth. J. Invertebr. Pathol. 82:1-11

Morin,S., R.W. Biggs, M.S. Sisterson, L. Shriver, C. Ellers-Kirk, D. Higginson, D. Holley, L.J. Gahan, D.G. Heckel, Y. Carriere, T.J. Dennehy, J.K. Brown, and

B.E. Tabashnik. 2003. Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm. Proc Natl Acad Sci USA: 5004-5009.

Muller-Cohn,J., J. Chaufaux, C. Buisson, N. Gilois, V. Sanchis, and D. Lereclus. 1996. *Spodoptera littoralis* (Lepidoptera: Noctuidae) resistance to CryIC and cross-resistance to other *Bacillus thuringiensis* crystal toxins. J. Econ. Entomol. 89: 791-797.

Nagamatsu, Y., S. Toda, T. Koike, Y. Miyoshi, S. Shigematsu, and M. Kogure. 1998b. Clonning, sequencing, and expression of the *Bombyx mori* receptor for *Bacillus thuringiensis* Insecticidal CryIA(a) toxin. Biosci. Biotechnol. Biochem. 62(4): 727-34

Nagamatsu,Y., S. Toda, F. Yamaguchi, M. Ogo, M. Kogure, M. Nakamura, Y. Shibata, and T. Katsumoto. 1998a. Identification of *Bombyx mori* midgut receptor for *Bacillus thuringiensis* insecticidal CryIA(a) toxin. Biosci. Biotechnol. Biochem. 62(4): 718-26

Oppert,B. 1999. Protease interactions with *Bacillus thuringiensis* insecticidal toxins. Arch. Insect Biochem. Physiol. 42: 1-12.

Oppert,B., K.J. Kramer, D. Johnson, S.J. Upton, and W.H. McGaughey. 1996. Luminal proteinases from *Plodia interpunctella* and the hydrolysis of *Bacillus thuringiensis* CryIA(c) protoxin. Insect Biochem. Mol. Biol. 26: 571-583.

Oppert,B., K.J. Kramer, D.E. Johnson, S.C. MacIntosh, and W.H. McGaughey. 1994. Altered protoxin activation by midgut enzymes from a *Bacillus thuringiensis* resistant strain of *Plodia interpunctella*. Biochem. Biophys. Res. Commun. 198: 940-947.

Oppert,B., K.J. Kramer, R.W. Beeman, D. Johnson, and W.H. McGaughey. 1997. Proteinase-mediated insect resistance to *Bacillus thuringiensis* toxins. J. Biol. Chem.272: 23473-23476.

Pang,A.S.D., J.L. Gringorten, and C. Bai. 1999. Activation and fragmentation of *Bacillus thuringiensis* delta -endotoxin by high concentrations of proteolytic enzymes. Can. J. Microbiol. 45: 816-825.

Perlak,F.J., M. Oppenhuizen, K. Gustafson, R. Voth, S. Sivasupramaniam, D. Heering, B. Carey, R.A. Ihrig, and J.K. Roberts. 2001. Development and commercial use of Bollgard(R) cotton in the USA: Early promises versus today's reality. Plant J. 27: 489-501.

Perlak,F.J., W.R. Deaton, T.A. Armstrong, R.I. Fuchs, S.R. Sims, J.T. Greenplate, and D.A. Fischhoff. 1990. Insect resistant cotton plants. Bio/Technology 8: 939-943.

Preisler,H.K., M.A. Hoy, and J.L. Robertson. 1990. Statistical analysis of modes of inheritance for pesticide resistance. J. Econ. Entomol. 83: 1649-1655.

Rajamohan,F., S.R.A. Hussain, J.A. Cotrill, F. Gould, and D.H. Dean. 1996. Mutations at domain II, loop 3, of *Bacillus thuringiensis* CryIAa and CryIAb delta -endotoxins suggest loop 3 is involved in initial binding to lepidopteran midguts. J. Biol. Chem.271: 25220-25226.

Rey,D., A. Long, M.P. Pautou, and J.C. Meyran. 1998. Comparative histopathology of some Diptera and Crustacea of aquatic alpine ecosystems, after treatment with *Bacillus thuringiensis* var. israelensis. Entomologia Experimentalis et Applicata 88: 255-263.

Reyes-Ramirez,A., and J.E. Ibarra. 2005. Fingerprinting of *Bacillus thuringiensis* type strains and isolates by using Bacillus cereus group-specific repetitive extragenic palindromic sequence-based PCR analysis. Appl. Environ. Microbiol. 71: 1346-1355.

Roush,R.T. 1998. Two-toxin strategies for management of insecticidal transgenic crops: can pyramiding succeed where pesticide mixtures have not? Philos. Trans. R. Soc. Lond. 1998. 353: 1777-1786.

Sangadala,S., F.S. Walters, L.H. English, and M.J. Adang. 1994. A mixture of *Manduca sexta* aminopeptidase and phosphatase enhances *Bacillus thuringiensis* insecticidal CryIA(c) toxin binding and 86Rb+-K+ efflux in vitro. J. Biol. Chem.269: 10088-10092.

Sayyed,A.H., R. Haward, S. Herrero, J. Ferre, and D.J. Wright. 2000. Genetic and biochemical approach for characterization of resistance to *Bacillus thuringiensis* toxin Cry1Ac in a field population of the diamondback moth, *Plutella xylostella*. Appl. Environ. Microbiol. 66: 1509-1516.

Schnepf,E., N. Crickmore, J. v. Rie, D. Lereclus, J. Baum, J. Feitelson, D.R. Zeigler, and D. H. Dean. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62: 775-806.

Schnepf,H.E. 1995. *Bacillus thuringiensis* toxins: regulation, activities and structural diversity. Curr. Opin. Biotechnol. 6: 305-312.

Shelton,A.M., J.Z. Zhao, and R.T. Roush. 2002. Economic, ecological, food safety, and social consequences of the deployment of Bt transgenic plants. Annu. Rev. Entomol. 47: 845-881.

Siqueira,H.A.A., D. Moellenbeck, T. Spencer, and B.D. Siegfried. 2004. Cross-resistance of Cry1Ab-selected *Ostrinia nubilalis* (Lepidoptera: Crambidae) to *Bacillus thuringiensis* delta-endotoxins. J. Econ. Entomol. 97.

Sivasupramaniam, S., G.P. Head, L. English, Y. J. Li and T.T. Vaughn.2007. A global approach to resistance monitoring. J. Invertebr. Pathol. 95: 224-226.

Sivasupramaniam, S., W. J. Moar, L.G. Ruschke, J. A. Osborn, C. Jiang, J. L. Sebaugh, G.R. Brown, Z.W. Shappley, M.E. Oppenhuizen, J.W. Mullins, and J. T. Greenplate 2008. Toxicity and characterization of cotton expressing *Bacillus thuringiensis* Cry1Ac and Cry2Ab2 proteins for control of lepidopteran pests. J. Econ. Entomol. 101: 546-554

Smouse,D., and J. Nishiura. 1997. A *Bacillus thuringiensis* delta -endotoxin induces programmed cell death in mosquito larvae. Cell Death Differ. 4: 560-569.

Stone,T.B., and S.R.Sims SR. 1993. Geographic susceptibility of *Heliothis virescens* and *Helicoverpa zea* (Lepidoptera: Noctuidae) to *Bacillus thuringiensis*. *J. Econ. Entomol*. 86(4):989-994.

Storer,N.P., S.L. Peck, F. Gould, J.W. van Duyn, and G.G. Kennedy. 2003. Sensitivity analysis of a spatially-explicit stochastic simulation model of the evolution of resistance in *Helicoverpa zea* (Lepidoptera: Noctuidae) to Bt transgenic corn and cotton. J. Econ. Entomol. 96: 173-187.

Suresh,R., G.D. Buntin, J.N. All, B.E. Tabashnik, P.L. Raymer, M.J. Adang, D.A. Pulliam, and C.N. Stewart, Jr. 1998. Survival, development, and oviposition of resistant diamondback moth (Lepidoptera: Plutellidae) on transgenic canola producing a *Bacillus thuringiensis* toxin. J. Econ. Entomol. 91: 1239-1244.

Tabashnik,B.E. 1991. Determining the mode of inheritance of pesticide resistance with backcross experiments. J. Econ. Entomol. 84: 703-712.

Tabashnik,B.E. 1994. Evolution of Resistance To Bacillus-Thuringiensis. Annu. Rev. Entomol. 39: 47-79.

Tabashnik,B.E., A.L. Patin, T.J. Dennehy, Y. Liu, Y. Carriere, M.A. Sims, and L. Antilla. 2000. Frequency of resistance to *Bacillus thuringiensis* in field populations of pink bollworm. Proc. Natl. Acad. Sci. USA 97: 12980-12984.

Tabashnik,B.E., F. Gould, and Y. Carriere. 2004. Delaying evolution of insect resistance to transgenic crops by decreasing dominance and heritability. J. Evol. Biol. 17: 904-12.

Tabashnik,B.E., F.R. Groeters, N. Finson, and M.W. Johnson. 1994. Instability of resistance to *Bacillus thuringiensis*. Biocontrol Sci. Technol. 4: 419-426.

Tabashnik,B.E., J.M. Schwartz, N. Finson, and M.W. Johnson. 1992. Inheritance of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). J. Econ. Entomol. 85: 1046-1055.

Tabashnik,B.E., K.W. Johnson, J.T. Engleman, and J.A. Baum. 2000. Cross-resistance to *Bacillus thuringiensis* toxin Cry1Ja in a strain of diamondback moth [*Plutella xylostella*] adapted to artificial diet. J. Invertebr. Pathol.76: 81-83.

Tabashnik,B.E., N. Finson, and M.W. Johnson. 1991. Managing resistance to *Bacillus thuringiensis*: lessons from the diamondback moth (Lepidoptera: Plutellidae). J. Econ. Entomol. 84: 49-55.

Tabashnik,B.E., N. Finson, and M.W. Johnson. 1992. Two protease inhibitors fail to synergize *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). J. Econ. Entomol. 85: 2082-2087.

Tabashnik,B.E., N. Finson, C.F. Chilcutt, N.L. Cushing, and M.W. Johnson. 1993. Increasing efficiency of bioassays: evaluating resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). J. Econ. Entomol. 86: 635-644.

Tabashnik,B.E., N. Finson, F.R. Groeters, W.J. Moar, M.W. Johnson, K. Luo, and M.J. Adang. 1994. Reversal of resistance to *Bacillus thuringiensis* in *Plutella xylostella*. Proc. Natl. Acad. Sci. USA 91: 4120-4124.

Tabashnik,B.E., N. Finson, M.W. Johnson, and D.G. Heckel. 1994. Cross-resistance to *Bacillus thuringiensis* toxin CryIF in the diamondback moth (*Plutella xylostella*). Appl. Environ. Microbiol. 60: 4627-4629.

Tabashnik,B.E., N. Finson, M.W. Johnson, and D.G. Heckel. 1995. Prolonged selection affects stability of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). J. Econ. Entomol. 88: 219-224.

Tabashnik,B.E., N. Finson, M.W. Johnson, and W.J. Moar. 1993. Resistance to toxins from *Bacillus thuringiensis* subsp. kurstaki causes minimal cross-resistance to B. thuringiensis subsp. aizawai in the diamondback moth (Lepidoptera: Plutellidae). Appl Environ Microbiol 59: 1332-1335.

Tabashnik,B.E., N.L. Cushing, N. Finson, and M.W. Johnson. 1990. Field development of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). J. Econ. Entomol. 83: 1671-1676.

Tabashnik,B.E., R.W. Biggs, D.M. Higginson, S. Henderson, D.C. Unnithan, G.C. Unnithan, C. Ellers-Kirk, M.S. Sisterson, T.J. Dennehy, and Y. Carriere. 2005a. Association between resistance to Bt cotton and cadherin genotype in pink bollworm. J. Econ. Entomol. 98: 635-44.

Tabashnik,B.E., T. Malvar, Y. Liu, N. Finson, D. Borthakur, B. Shin, S. Park, L. Masson, R.A. de Maagd, and D. Bosch. 1996. Cross-resistance of the diamondback moth indicates altered interactions with domain II of *Bacillus thuringiensis* toxins. Appl. Environ. Microbiol. 62: 2839-2844.

Tabashnik,B.E., T.J. Dennehy, and Y. Carriere. 2005b. Delayed resistance to transgenic cotton in pink bollworm. Proc. Natl. Acad. Sci. USA 102: 15389-15393.

Tabashnik,B.E., T.J. Dennehy, M.A. Sims, K. Larkin, G.P. Head, W.J. Moar, and Y. Carriere. 2002. Control of resistant pink bollworm (*Pectinophora gossypiella*) by transgenic cotton that produces *Bacillus thuringiensis* toxin Cry2Ab. Appl. Environ. Microbiol. 68: 3790-3794.

Tabashnik,B.E., Y. Carriere, T.J. Dennehy, S. Morin, M.S. Sisterson, R.T. Roush, A.M. Shelton, and J.Z. Zhao. 2003b. Insect resistance to transgenic Bt crops: lessons from the laboratory and field. J. Econ. Entomol. 96: 1031-8.

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

Tabashnik,B.E., Y. Liu, T. Malvar, D.G. Heckel, L. Masson, and J. Ferre. 1998. Insect resistance to *Bacillus thuringiensis*: uniform or diverse? Philos. Trans. R. Soc. Lond. 1998. 353: 1751-1756.

Tabashnik,B.E., Y.B. Liu, D.C. Unnithan, Y. Carriere, T.J. Dennehy, and S. Morin. 2004b. Shared genetic basis of resistance to Bt toxin Cry1ac in independent strains of pink bollworm. J. Econ. Entomol. 97: 721-726.

Tabashnik,B.E., Y.B. Liu, N. Finson, L. Masson, and D.G. Heckel. 1997. One gene in diamondback moth confers resistance to four *Bacillus thuringiensis* toxins. Proc. Natl. Acad. Sci. USA 94: 1640-1644.

Tabashnik,B.E., Y.B. Liu, T. Malvar, D.G. Heckel, L. Masson, V. Ballester, F. Granero, J.L. Mensua, and J. Ferre. 1997. Global variation in the genetic and biochemical basis of diamondback moth resistance to *Bacillus thuringiensis*. Proc. Natl. Acad. Sci. USA 94: 12780-12785.

Tabashnik,B.E., Y.B. Liu, T.J. Dennehy, M.A. Sims, M.S. Sisterson, R.W. Biggs, and Y. Carriere. 2002a. Inheritance of resistance to Bt toxin Cry1Ac in a field-derived strain of pink bollworm (Lepidoptera: Gelechiidae).J.Econ.Entomol.95:1018-26.

Tang,J.D., A.M. Shelton, J. van Rie, S. d. Roeck, W.J. Moar, R.T. Roush, and M. Peferoen. 1996. Toxicity of *Bacillus thuringiensis* spore and crystal protein to resistant diamondback moth (*Plutella xylostella*).Appl.Environ.Microbiol.62: 564-569.

Tang,J.D., H.L. Collins, R.T. Roush, T.D. Metz, E.D. Earle, and A.M. Shelton. 1999. Survival, weight gain, and oviposition of resistant and susceptible *Plutella xylostella* (Lepidoptera: Plutellidae) on broccoli expressing crylAc toxin of *Bacillus thuringiensis*. J. Econ. Entomol.92: 47-55.

Tang,J.D., S. Gilboa, R.T. Roush, and A.M. Shelton. 1997. Inheritance, stability, and lack-of-fitness costs of field-selected resistance to *Bacillus thuringiensis* in

diamondback moth (Lepidoptera: Plutellidae) from Florida.J. Econ. Entomol.90: 732-741.

Utami,R., and M.E. Whalon. 1995. Inheritance of resistance to *Bacillus thuringiensis* subsp. tenebrionis CryIIIA delta -endotoxin in Colorado potato beetle, (Coleoptera: Chrysomelidae). J. Econ. Entomol. 88: 21-26.

Vadlamudi, R.K., E. Weber, I. Ji, T.H. Ji, and L.A. Bulla. 1995. Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis*. J. Biol. Chem. 270: 5490-94

Vadlamudi,R.K., T.H. Ji, and L.A. Bulla. 1993. A specific binding protein from *Manduca sexta* for the insecticidal toxin of *Bacillus thuringiensis* subsp. Berliner. J. Biol. Chem. 268(17):12334-340

Valaitis,A.P., J.L.Jenkins, M.K. Lee, D.H. Dean and K.J. Garner. 2001. Isolation and partial characterization of gypsy moth BTR-270, an anionic brush border membrane glycoconjugate that binds *Bacillus thuringiensis* Cry1A toxin with high affinity. Arch. Insect Biochem. Physiol.46: 186-200

van Rie,J., S. Jansens, H. Hofte, D. Degheele, and H.v. Mellaert. 1990a. Receptors on the brush border membrane of the insect midgut as determinants of the specificity of *Bacillus thuringiensis* delta-endotoxins. Appl. Environ. Microbiol. 56: 1378-1385.

van Rie,J., W.H. McGaughey, D.E. Johnson, B.D. Barnett, and H.v. Mellaert. 1990b. Mechanism of insect resistance to the microbial insecticide *Bacillus thuringiensis*. Science (Washington) 247: 72-74.

Whalon,M.E., and B.A. Wingerd. 2003. Bt: mode of action and use. Arch. Insect Biochem. Physiol. 54: 200-211.

Whalon,M.E., D.L. Miller, R.M. Hollingworth, E.J. Grafius, and J.R. Miller. 1993. Selection of a Colorado potato beetle (Coleoptera: Chrysomelidae) strain resistant to *Bacillus thuringiensis*. J. Econ. Entomol. 86: 226-233.

Wirth,M.C., A. Delecluse, B.A. Federici, and W.E. Walton. 1998. Variable cross-resistance to Cry11B from *Bacillus thuringiensis* subsp. jegathesan in *Culex quinquefasciatus* (Diptera: Culicidae) resistant to single or multiple toxins of *Bacillus thuringiensis* subsp. israelensis. Appl. Environ. Microbiol.64:4174-4179.

Wirth,M.C., and G.P. Georghiou. 1997. Cross-resistance among CryIV toxins of *Bacillus thuringiensis* subsp. israelensis in *Culex quinquefasciatus* (Diptera: Culicidae). J. Econ. Entomol. 90: 1471-1477.

Wirth,M.C., G.P. Georghiou, and B.A. Federici. 1997. CytA enables CryIV endotoxins of *Bacillus thuringiensis* to overcome high levels of CryIV resistance in the mosquito, *Culex quinquefasciatus*. Proc. Natl. Acad. Sci. USA 94: 10536-10540.

Wright,D.J., M. Iqbal, F. Granero, and J. Ferre. 1997. A change in a single midgut receptor in the diamondback moth (*Plutella xylostella*) is only in part responsible for field resistance to *Bacillus thuringiensis* subsp. kurstaki and B. thuringiensis subsp. aizawai. Appl. Environ. Microbiol. 63: 1814-1819.

Xie,R.Y., M.B. Zhuang, L.S. Ross, I. Gomez, D.I. Oltean, A. Bravo, M. Soberon, and S.S. Gill. 2005. Single amino acid mutations in the cadherin receptor from *Heliothis virescens* affect its toxin binding ability to Cry1A toxins. J. Biol. Chem.280: 8416-8425.

Yamagiwa,M., M. Esaki, K. Otake, M. Inagaki, T. Komano, T. Amachi, and H. Sakai. 1999. Activation process of dipteran-specific insecticidal protein produced by *Bacillus thuringiensis* subsp. israelensis. Appl. Environ. Microbiol.65:3464-3469.

Yaoi,K., K. Nakanishi, T. Kadotani, M. Imamura, N. Koizumi, H. Iwahana, and R. Sato. 1999. *Bacillus thuringiensis* Cry1Aa toxin-binding region of *Bombyx mori* aminopeptidase N. FEBS Letters 463: 221-224.

Yu,C., M.A. Mullins, G.W. Warren, M.G. Koziel, and J.J. Estruch. 1997. The *Bacillus thuringiensis* vegetative insecticidal protein Vip3A lyses midgut epithelium cells of susceptible insects. Appl. Environ. Microbiol. 63: 532-536.

Zalunin,I.A., L.P. Revina, L.I. Kostina, G.G. Chestukhina, and V.M. Stepanov. 1998. Limited proteolysis of *Bacillus thuringiensis* CryIG and CryIVB delta - endotoxins leads to formation of active fragments that do not coincide with the structural domains. J. Protein Chem. 17: 463-471.

Zhao,J., H.L. Collins, J.D. Tang, J. Cao, E.D. Earle, R.T. Roush, S. Herrero, B. Escriche, J. Ferre, and A.M. Shelton. 2000. Development and characterization of diamondback moth resistance to transgenic broccoli expressing high levels of Cry1C. Appl. Environ. Microbiol. 66: 3784-3789.

Zhao,J.Z., J. Cao, Y. Li, H.L. Collins, R.T. Roush, E.D. Earle, and A.M. Shelton. 2003. Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance evolution. Nat. Biotechnol. 21: 1493-1497.

Zhuang,M.B., D.I. Oltean, I. Gomez, A.K. Pullikuth, M. Soberon, A. Bravo, and S. S. Gill. 2002. *Heliothis virescens* and *Manduca sexta* lipid rafts are involved in

Cry1A toxin binding to the midgut epithelium and subsequent pore formation. J. Biol. Chem.277: 13863-13872.

Table 1. Selected insect species and strains that have developed resistance to Bt proteins*

Species	Selecting agent		Name[c]	Details on Resistance			Resistance mechanism[f]	References
	Type[a]	Form[b]		ES[d]	Formulation /Toxin	RR[e]		
P. interpunctella	Dipel	FSC	343-R	13	Dipel	100		McGauhey 1985
					Btk HD-337	>100		McGaughey and Johnson 1987
					Bte HD-198	1.1		
					Bta HD-133	1.4		
				36	Dipel	>250	≡ processing	McGaughet and Beeman 1988, Johnson et al.1990
					Cry1Ab	(877)	↓ binding	van Rie et al. 1990b
					Cry1Ca	0.3		
P. interpunctella	*Bte* HD-198	SC	198r	19	HD-198	21		McGaughey and Johnson 1992
					HD-198	32		McGaughey and Johnson 1994
					Cry1Aa	10		
					Cry1Ab	27		
					Cry1Ac	150		
					Cry1Ba	9		
					Cry1Ca	5		
					Cry2A	20		
					Cry1Ac-PT	128	↓ activation	Oppert et al.1994
					Cry1Ab-PT	264		Herrero et al. 2001
					Cry1Ab	25	Δ binding	
					Cry1Ac	n.d	≡ binding	

Species	Selecting agent		Name[c]	Details on Resistance			Resistance mechanism[f]	References
	Type[a]	Form[b]		ES[d]	Formulation /Toxin	RR[e]		
P interpunctella	*Bta* HD-133	SC	133r	23	HD-133	61.5		McGaughey and Johnson 1992
					HD-133	94		McGaughey and Johnson 1994
					HD-1	45		
					Cry1Aa	17		
					Cry1Ab	226		
					Cry1Ac	789		
					Cry1Ba	44		
					Cry1Ca	19		
					Cry2A	24		
P. interpunctella	Dipel	FSC	Dipel[r]	24	Dipel	70		McGaughey and Johnson 1992
					Cry1Ac	2816		McGaughey and Johnson 1994
					Cry1Ab-PT	1049		Herrero et al. 2001
					Cry1Ab	290	↓ binding	
P. xylostella	*Btk*	FSC	BL§	Field	Dipel	1		Ferre et al. 1991
					Cry1Ab	>200	↓ binding	
					Cry1Ba	2		
					Cry1Ca	0.5		
P. xylostella	*Btk*	FSC			Dipel	0.4		Ballester et al. 1994
					Cry1Aa	1.3		
					Cry1Ab	236		
					Cry1Ac	1		
					Cry1Ba	1		

Species	Selecting agent		Name[c]	Details on Resistance			Resistance mechanism[f]	References
	Type[a]	Form[b]		ES[d]	Formulation /Toxin	RR[e]		
	Cry1Ab/ Cry1Ac-1Ab	T/CC	PHI		Cry1Aa	>1	≡ binding	Ballester et al. 1999, Tabashnik et al. 1997
					Cry1Ab	>1	↓ binding	Tabashnik et al. 1997
					Cry1Ac	>1	≡ binding	Ballester et al. 1999, Tabashnik et al. 1997
					Cry1Ca	(1)		Tabashnik et al. 1997
					Cry1Fa	(1)		
					Cry1Ja	(1)		
P. xylostella	*Btk*	FSC	NO	Field	Dipel	26.5		Tabashnik et al.1990
	Dipel	FSC	NO-Q	9	Dipel	820		Tabashnik et al.1991
			NO-Q	15	Dipel	low		Tabashnik et al.1994
	Dipel	FSC	NO-QA		Dipel	3300	≡ processing	Liu and Tabashnik 1997, Tabashnik et al. 1992, 1993
					Cry1Ac	>59	↓ binding	Tabashnik et al.1994
					Cry1Aa	>100	Δ binding	Ballester et al. 1999, Tabashnik et al. 1996, 1997
					Cry1Ab	>100	↓ binding	Tabashnik et al. 1996, 1997
					Cry1Ac	>100	↓ binding	
					Cry1Ba	3		Tabashnik et al. 1996
					Cry1Ca	2		
					Cry1Da	3		
					Cry1Fa	>100		
					Cry1Ia	3		
					Cry1Ja	>140		

Species	Selecting agent		Name[c]	Details on Resistance			Resistance mechanism[f]	References
	Type[a]	Form[b]		ES[d]	Formulation /Toxin	RR[e]		
					Cry1Ac-canola	98/0		Suresh et al. 1998
	Cry1Ac	NO-QAGE			Cry1Aa	>20000		Tabashnik et al. 2000
					Cry1Ab	>10000		
					Cry1Ac	>40000		
					Cry1Bb	2		
					Cry1Ca	1		
					Cry1Da	1		
					Cry1Fa	>10000		
					Cry1Ja	>2000		
					Cry2Aa	5		
					Cry9Ca	2		
P. xylostella	*Btk /Bta*	FSC	NO-95	Field	Cry1Ca	22		Liu and Tabashnik 1997
					Dipel	134		Liu et al. 1996
					Xentari	3		
	Cry1Ca	CC	NO-95C	6	Cry1Ca	62		Liu and Tabashnik 1997
	Cry1Ca	T	NO-95C	5	Cry1Ca	19	≡ binding	Liu et al. 2000
				5	Cry1Ca crystal	48		

Species	Selecting agent		Name[c]	Details on Resistance			Resistance mechanism[f]	References
	Type[a]	Form[b]		ES[d]	Formulation/ Toxin	RR[e]		
P. xylostella	*Btk*	FSC	Loxa A	Field	Javelin	300		Tang et al. 1996
					Dipel	22		
					Cry1Aa	>200		
					Cry1Ab	>200	↓ binding	
					Cry1Ac	>200		
					Cry1Ba	2.5		
					Cry1Ca	3.4		
					Cry1Da	1		
					Cry9Ca	1		Lambert et al. 1996
					Cry1Ac-broccoli	95/20		Tang et al. 1999
P. xylostella	*Btk /Bta*	FSC	Cry1C-Sel	Field	Cry1Ca	31		Zhao et al. 2000
	Cry1Ca	PT	Cry1C-Sel	6	Cry1Ca	120		Cao et al. 1999
	Cry1Ca	PT/Cry1C broccoli	Cry1C-Sel		Cry1Ca	12400	Δ binding	Zhao et al. 2000
P. xylostella	*Btk*	FSC		Field				Tabashnik et al. 1997
	Cry1Ac/ Cry1Ca	SC	PEN	6	Cry1Aa	High	Δ binding	
				6	Cry1Ab	High	↓ binding	
				6	Cry1Ac	High	↓ binding	
				6	Cry1Ca	No		
				6	Cry1Fa	High		
				6	Cry1Ja	>1000		
P. xylostella	*Btk /Bta*	FSC	SERD3	Field	Dipel	330		Wright et al. 1997
					Florbac	160		
					Cry1Aa	n.d.	≡ binding	

Species	Selecting agent		Name[c]	Details on Resistance			Resistance mechanism[f]	References
	Type[a]	Form[b]		ES[d]	Formulation/ Toxin	RR[e]		
					Cry1Ab	n.d.	↓ binding	
					Cry1Ac	n.d.	≡ binding	
	Dipel	FSC	Btk-Sel	3	Dipel	600		
				3	Florbac	60		
	Florbac	FSC	Bta-Sel	3	Florbac	300		
				3	Dipel	80		
P. xylostella	*Btk*	FSC	UNSEL-MEL	Field	Cry1Ab	121		Sayyed et al. 2000
					Cry1Ac	300		
					Dipel	40		
					Xentari	13		
	Cry1Ac	Toxin	1AcSEL-MEL§	5	Cry1Ac	10500	↓ binding	
				5	Cry1Ab	264	↓ binding	
				5	Dipel	59		
				5	Xentari	10		
	Cry1Ab	toxin	1AbSEL-MEL§	5	Cry1Ab	500		Sayyed et al. 2000
				5	Cry1Ac	7000		
				5	Dipel	81		
				5	Xentari	16		
	Dipel	FSC	*Btk*SEL-MEL§	5	Dipel	112		
				5	Cry1Ac	10700		
				5	Cry1Ab	900		

Species	Selecting agent		Name[c]	Details on Resistance			Resistance mechanism[f]	References
	Type[a]	Form[b]		ES[d]	Formulation/ Toxin	RR[e]		
				5	Xentari	8		
	Xentari	FSC	*Bta*SEL-MEL§	5	Xentari	30		
				5	Cry1Ac	7260		
				5	Cry1Ab	420		
				5	Dipel	40		
P. xylostella	*Btk*	FSC	ROO	Greenhouse	Toarow CT	704		Hama et al. 1992
					Thuricide	160		
					Dipel	23		
					Bacilex	4		
H. virescens	Cry1Ac/ Cry1Ac	T/ CC	YHD2		Cry1Aa	32*	↓ binding	Gould et al. 1995, Lee et al. 1995
					Cry1Ab	>2300	≅ binding	
				19	Cry1Ac	>10000	≅ binding	
					Cry1Fa CellCap	3700		Gould et al. 1995
					Cry1Ca CellCap	2.5		
					Cry2Aa	25		
	Cry1Ac	CC	YHD21000MVP		Cry1Aa	>20	↓ binding	
					Cry1Ab	2000	↓ binding	
					Cry1Ac CellCap	23,0000	↓ binding	
					Cry1Fa	130	↓ binding	
					Cry2Aa	9.5		Kota et al. 1999

Species	Selecting agent		Name[c]	Details on Resistance			Resistance mechanism[f]	References
	Type[a]	Form[b]		ES[d]	Formulation/ Toxin	RR[e]		
H. virescens	Cry1Ab	CC	SEL	22	Cry1Ab Cell Cap	71	Δ binding, ≡ processing	MacIntosh et al. 1991
	Dipel	FSC		22	Cry1Ac	16	Δ binding, ≡ processing	
				22	Dipel	57		
H. virescens	Cry1Ac	T	CP73-3	17	Cry1Ab	13	≅ binding ↓ activation ↑ degradation ↑ cell repair	Forcada et al. 1996, Gould et al. 1992, Martinez-ramirez et al. 1999
				17	Cry1Ac	50	≅ binding ↑ cell repair	Gould et al. 1992, Martinez-ramirez et al. 1999
				17	Cry2Aa	53		Gould et al. 1992
	Cry2Aa	PT	CxC1000IIA	24	Cry1Ac	>100		Kota et al. 1999
				24	Cry2Aa	>330		
				24	Cry2Aa-cotton	0/n.d.		
H. armigera	Cry1Ac	PT	BX	24	Cry1Ac	111	↓B	Akhurst et al. 2003
					Cry1Ab	157		
					Cry2Aa	1		
					Cry2Ab	1.4		
					Dipel	5		
					Xentari	7		
					MVP	69		
					HD73 spore/crystal	188		

Species	Selecting agent		Name[c]	Details on Resistance			Resistance mechanism[f]	References
	Type[a]	Form[b]		ES[d]	Formulation/ Toxin	RR[e]		
	Cry1Ac	Cotton leaves		42	HD73 Cry1Ac -PT	1683.8		Meng et al. 2004
				42	Dipel	15.7		
				43	21% MVP II WP	1779.8		
				43	21% MVP II liquid	1233.4		
		Cotton leaves	RC	22	Btk	3.1		Lu et al. 2004
				22	Cry1Ac	11		
		Btk	RB	22	Btk	5.2		
				22	Cry1Ac	4.9		
O. nubilalis	Dipel	FSC	KS-SC-R	7	Dipel	73		Huang et al. 1997
	Cry1Ac	CC	S-I	8	Cry1Ac CellCap	162		Bolin et al. 1999
O. nubilalis	Cry1Ab	PT	Europe-R	69	Cry1Ab	9.8		Siqueira et al. 2004
					Cry1Ac	35.4		
	Cry1Ab		RSST-R	41	Cry1Ab	9.0		
					Cry1Ac	52.6		
	Cry1Ab		Nebraska-R	50	Cry1Ab	1.9		
					Cry1Ac	7.2		
	Cry1Ab		Iowa-R	61	Cry1Ab	1.2		
					Cry1Ac	5.4		
S. exigua	*Btk* HD-1	SC		20	*Btk* HD-1	1		Moar et al. 1995
S. exigua	Cry1Ca	IB/T		25	Cry1Ca	850	Δ binding ↑ NS binding	
				22	Cry1Ab	93		
				34	Cry2Aa	73		
				34	Cry9Ca	12		

Species	Selecting agent		Name[c]	Details on Resistance			Resistance mechanism[f]	References
	Type[a]	Form[b]		ES[d]	Formulation/ Toxin	RR[e]		
S. littoralis	Cry1Ca	SC		14	Cry1Ca spcry	>500		Muller-Cohn et al. 1996
				14	Cry1Da spcry	7		
				14	Cry1Ea spcry	34		
				14	Cry1Fa spcry	1		
				14	Cry1Ab protoxin	3		
				14	*Bta* 7.29 spcry	7		
P. gossypiella	Cry1Ac	CC	APHIS-98R		Cry1Ac CellCap	>100		Liu et al. 2001
					Cry1Aa protoxin	high		Tabashnik et al. 2000
					Cry1Ab protoxin	high		
					Cry1Ac protoxin	high		
					Cry1Bb protoxin	weak		
					Cry1Ca protoxin	no		
					Cry1Da protoxin	no		
					Cry1Fa protoxin	no		
					Cry1Ja protoxin	weak		
					Cry2Aa protoxin	no		
					Cry9Ca	no		
P. gossypiella	Cry1Ac	CC	AZP-R		Cry1Ac Cell Cap	300		Tabashnik et al. 2000
					Cry1Ac-cotton	40/1.6		
					Cry1Aa protoxin	Yes		
					Cry1Ab protoxin	Yes		
					Cry1Ac protoxin	Yes		
					Cry1Bb protoxin	Weak		
					Cry1Ca protoxin	No		
					Cry1Da protoxin	No		
					Cry1Fa protoxin	No		

Species	Selecting agent		Name[c]	Details on Resistance			Resistance mechanism[f]	References
	Type[a]	Form[b]		ES[d]	Formulation/ Toxin	RR[e]		
					Cry1Ja protoxin	No		
					Cry2Aa protoxin	No		
T. ni	Cry1Ab	T		7	Cry1Ab	31		Estada and Ferre 1994
					Cry1Aa	1		
					Cry1Ac	0.5		
C. scripta	Cry3Aa	CC		35	Cry3Aa CellCap	>3000		Bauer 1995
					Cry3Aa crystal	>5000		Federici and Bauer 1998
					Cry1Ba crystal	400		
					Cry1Ba protoxin	100		
					Cyt1Aa	1.2		
L. decemlineata	Cry3Aa	CC		12	Cry3Aa CellCap	59		Whalon et al. 1993
				29	Cry3Aa CellCap	293		Utami and Whalon 1995
C. quinquefasciatus	C+O+L+T	Cry11A	Cq4D	28	11A	42.9	>913**	Georghiou and Wirth, 1997
					4A/4B	9.3	41.6**	Wirth and Georghiou
					4A/4B/11A	4.7	13.5**	1997
					4A/4B/11A/Cyt1	2.1	1.1**	
					11A	>1000		Wirth et al. 1997
					11A/Cyt1	7.1		
					11B	9.2	53.1**	Wirth et al. 1998
					11B/Cyt1	7.1	17.5**	
		Cry4A/ Cry4B	Cq4AB		4A/4B	16.3	>122**	Georghiou and Wirth 1997
					11A	4.3	>350**	Wirth and Georghiou
					4A/4B	2.5	11**	1997

Species	Selecting agent		Name[c]	Details on Resistance			Resistance mechanism[f]	References
	Type[a]	Form[b]		ES[d]	Formulation/ Toxin	RR[e]		
					4A/4B/11A	10.4	16.2**	
					4A/4B/11A/Cyt1	2.1	3.2**	
					4A/4B	51.1		Wirth et al. 1997
					4A/4B /Cyt1	0.8		
					11B	9.7	80.7**	Wirth et al. 1998
					11B/Cyt1	1.6	1.6**	
		Cry4A/ Cry4B/ Cry11A	Cq4A BD		4A/4B/11A	13.3	91**	Georghiou and Wirth 1997, Wirth and Georghiou 1997
					11A	18.6	185**	Georghiou and Wirth 1997
					4A/4B	5	12.9**	
					4A/4B/11A/Cyt1	1.8	1.2**	
					4A/4B/11A	35.4		Wirth et al. 1997
					4A/4B/11A/Cyt1	1.3		
					11B	56.2	347**	Wirth et al. 1998
					11B/Cyt1	3.7	3.7**	
		Cry4A/ Cry4B/ Cry11A/ Cyt1A	Cq4A BD Cyt		4A/4B/11A/Cyt1	2;3	2**	Georghiou and Wirth 1997 Wirth and Georghiou 1997
					11A	10.4	30.1**	Wirth and Georghiou 1997
					4A/4B	5	10.2**	
					4A/4B/11A	5.1	8.1**	

*This table is adopted from Ferre and van Rie 2002 and literature in this table is updated with research publications after 2002 till 2006; Note: Foot note for the table is in next page

[a]*Bta* (*B. thuringiensis* var. *aizawai*) and *Btk* (*B. thuringiensis* var. *kurstaki*) refer to commercial formulations of *B. thuringiensis*. Dipel, Javelin,Toarow CT and Thuricide are tradenames of formulations of *Btk*. Florbac and Xentari are tradenames for commercial formulations of *Bta*; Bacilex is a tradename for a commercial formulation of a mixture of *Btk* and *Bta* respectively. *Bte* = *B. thuringiensis* var. *entomocidus*.

[b]Different forms of selecting agent have been used: formulated spore-crystal preparations (FSC), spore-crystal preparations (SC), micro encapsulated recombinant *Pseudomonas fluorescens* cells expressing a *cry* gene (CC), inclusion bodies from recombinant *Escherichia coli* cells expressing a *cry* gene (IB), protoxin (PT) and activated toxin (T).

[c]Name of the resistant strain as given in the reference paper, except when followed by '§ ': these names are arbitrary (or adapted) names given by the authors of this paper. *P. interpunctella* strain 343-R has also been referred to as strain 343.

[d]When available, the number of episodes of selection after which the insects were tested, is given.

[e]RR= resistance ratio; for Bt strains or (pro) toxins this is defined as the LC_{50} (or LD_{50}) of resistant strain divided by the LC_{50} (or LD_{50}) of susceptible control strain, except when the value is followed by '*': this values refers to a difference in larval weight between resistant and susceptible larvae when larvae were reared on artificial diet with 100 µg toxin/ml. Values in parentheses are estimates. For Bt-plants two values, separated by '/', are given for the adapted survival on Bt-plants (= % survival on the Bt-plant divided by % survival on non-Bt-plants) of the resistant and susceptible strain, respectively. -: not determined. All values for Cry1 and Cry9 proteins refer to activated toxins unless otherwise indicated.

[f] When available, the mechanism of resistance to the particular toxin is given: reduced binding (↓ binding), slightly altered binding (Δ binding), unaltered binding (≡ binding), apparently unaltered binding (≅ binding), increased non-specific binding (↑ NS binding), reduced protoxin activation (↓ activation), increased toxin degradation (↑ degradation), unaltered proteolytic processing (≡ processing), increased cell repair or cell replacement (↑ cell repair). In case of binding, only results of binding experiments to native BBMVs are given.

CHAPTER 2:

PRODUCTION AND CHARACTERIZATION OF *BACILLUS THURINGIENSIS* CRY1AC-RESISTANT COTTON BOLLWORM, *HELICOVERPA ZEA* (BODDIE)

Laboratory-selected *Bt*-resistant colonies are important tools for elucidating *Bt* resistance mechanisms and helping to determine appropriate resistance management strategies for *Bt* crops. However, some important pest insects such as the cotton bollworm, *Helicoverpa zea*, have proven difficult to select for stable resistance, especially when this insect is a pest of both *Bt* cotton and *Bt* corn. Here, two laboratory populations of *H. zea* (AR and MR), resistant to the *Bt* protein found in all commercial *Bt* cotton varieties in the US (Cry1Ac), were established by selection with either Cry1Ac activated toxin (AR) or MVP II (MR). Cry1Ac toxin reflects the form ingested by *H. zea* when feeding on *Bt* cotton, whereas MVP II is a Cry1Ac formulation used for resistance selection and monitoring. The resistance ratio (RR) for AR reached >100-fold after 11 generations and has been maintained at this level for 9 generations. This is the first report of stable Cry1Ac resistance in *H. zea*. MR crashed after 11 generations (similar to previous observations), reaching only a RR of 12 after 7 generations. AR was only partially cross-resistant to MVP II (10% of expected cross resistance) suggesting that MVP II does not have the same Cry1Ac selection pressure as Cry1Ac toxin against *H.*

zea and that proteases may be involved with resistance. AR was highly cross-resistant to Cry1Ab toxin, but only slightly cross-resistant to Cry1Ab-expressing corn leaf powder.

AR was not cross-resistant to Cry2Aa2, Cry2Ab2-expressing corn leaf powder, Vip3A and cypermethrin. Toxin binding assays showed no significant differences, indicating that resistance was not linked to a reduction in binding. These results aid in understanding why this major pest of cotton and corn has not yet evolved *Bt* resistance, and highlight the need to choose carefully the form of *Bt* protein used in experimental studies.

Introduction

Transgenic cotton expressing *Bacillus thuringiensis* (*Bt*) Cry1Ac has been used commercially in the US since 1995 (10) and the area under *Bt* cotton production has steadily increased over that period (22). *Bt* cotton provides excellent control of many lepidopteran pests of cotton, and thereby exerts tremendous selection pressure for resistance. Concerns regarding resistance to *Bt* cotton and *Bt* corn have led the U.S. Environmental Protection Agency (EPA) to mandate Insect Resistance Management (IRM) strategies for all target pests of *Bt* crops (11). Perhaps partly because of these IRM strategies, there has yet to be a case of field resistance to *Bt* cotton after 10 years of intense cultivation (4).

In the US, tobacco budworm, *Heliothis virescens* F., pink bollworm, *Pectinophora gossypiella* (Saunders), and cotton bollworm, *Helicoverpa* zea (Boddie), are the three major target pests of *Bt* cotton. Although current *Bt* varieties express a high dose of Cry1Ac against *H. virescens* and *P. gossypiella*, it is still not sufficient to kill all

H. zea (21). In particular, high *H. zea* population pressure and varied expression of Cry1Ac in different cotton tissues associated with plant age and stress can result in increased *H. zea* larval survival (1, 19,21). *H. zea* is highly polyphagous and can be a major pest in field corn, and is the key pest in sweet corn in many areas. Therefore, *H. zea* also is exposed to Cry1Ab in Cry1Ab-expressing *Bt* corn, which is similar in structure (>90% amino acid similarity) (8) and mode of action to Cry1Ac. Cross-resistance to Cry1Ab has been reported in populations of *Helicoverpa armigera* (2), *H. virescens* (17), *H. zea* (33), *P. gossypiella* (45,46,48), and *Trichoplusia ni* (51). These factors increase the likelihood of resistance development to *Bt* cotton by *H. zea* (6, 33).

Cry1Ac resistance in *Bt* cotton pests such as *H. virescens* (17,18) and *P. gossypiella*(45,46,48) is relatively well-studied, using populations selected in the laboratory with MVP II (a commercial formulation containing Cry1Ac protoxin inclusion bodies encapsulated in *Pseudomonas fluorescens* cells), and these results have helped formulate nationwide IRM strategies. However, these IRM strategies may not be optimal for *H. zea* because *Bt* resistance mechanisms and other factors can be different in different insect species (14, 20). Therefore it is of great interest to establish a Cry1Ac-resistant *H. zea* population to examine mechanisms of *Bt* resistance, patterns of cross-resistance and other parameters in this insect.

Several attempts at selecting for Cry1Ac-resistance in *H. zea* using MVP II have been met with limited success (R.E. Jackson, USDA ARS, Stoneville, MS., personal communication, WJM unpublished data). Possible reasons for this limited success include: fitness costs involved with resistance to the Cry1Ac protoxin or other

compounds in MVP II, or the allele frequency for MVP II resistance being very low (6). Furthermore, resistance selection using MVP II may not adequately reflect Cry1Ac resistance selection to *H. zea in planta* because although *Bt* cotton expresses full-length solubilized Cry1Ac protoxin (39), it is at least partially activated to toxin by plant proteases immediately upon plant cell disruption. This observation is similar to Cry1Fa in cotton, where the full-length protoxin is expressed, but only activated toxin is recovered from plant tissue (16). Besides the use of MVP II, other Cry1Ac forms or preparations have also been used for resistance selection including *E. coli* containing Cry1Ac inclusion bodies, *Bt* Cry1Ac protoxin crystals with spores, and Cry1Ac activated toxin (2,28,33,53). However, it is not known whether there are differences between these different Cry1Ac forms in terms of resistance selection as it pertains to *Bt* cotton. Insect susceptibility to *Bt* proteins may vary with the form of *Bt* protein ingested, especially in *Bt*-tolerant species, and the form of the *Bt* protein used may have a dramatic impact on the resulting *Bt* resistance mechanism(s) (26,34,38).

In this paper, we report for the first time that moderately high and stable resistance to Cry1Ac toxin has been attained in *H. zea*, and this resistance has at least been partly characterized. This strain has differential susceptibilities to various forms of Cry1Ac and Cry1Ab, is still susceptible to cypermethrin, and, unlike in most Cry1Ac-resistant insects, resistance does not appear to be due to alterations in receptor binding (14, 20).

MATERIALS AND METHODS

Insect strains: A laboratory susceptible colony of *H. zea* (SC) was established in

September 2004 from a laboratory colony from Monsanto (Union City, TN). The culture at Monsanto is annually infused with insects collected from corn. Insects were reared on pinto bean-based artificial diet at 27 ± 1 ^{0}C with a photoperiod of 14:10h (L:D) (35).

***Bt* proteins and Pyrethroids**: An *E. coli* strain expressing Cry1Ac protoxin from *B. thuringiensis* subsp. *kurstaki* strain HD-1 (provided by L. Masson, Biotechnology Research Institute, National Research Council, Montreal, Canada) was cultured, and the activated toxin prepared as indicated elsewhere (34,40). Cry2Aa2 protoxin was prepared as described by Moar et al. (34). Cry1Aa (*B. thuringiensis* EG1273), Cry1Ab (*B. thuringiensis* EG7077) and Cry1Ac (EG11070) clones were provided by Ecogen Inc. (Langhorne, Pennsylvania) and were used to prepare trypsin-activated toxins as described by Estela et al.(12). MVP II and lyophilized corn leaf powder containing Cry1Ab (229.55µg/g) and Cry2Ab2 (6mg/g) were supplied by Monsanto (St. Louis, MO). MVP II is a formulated, freeze dried powder containing 19.1% Cry1Ac protoxin inclusion bodies encapsulated in *Pseudomonas fluorescens*. 100% active salt-free Vip3A was supplied by Syngenta (Greensboro, NC). A representative pyrethroid, cypermethrin, cyano (3-phenoxyphenyl) methyl 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate (92% a.i. PL86-172) was supplied by FMC Corporation (Philadelphia, PA).

Selection experiments: Selection experiments were initiated after four generations of rearing SC and generating baseline susceptibility values for Cry1Ac toxin and MVP II.

Two strains of *H. zea* were selected for Cry1Ac resistance on artificial diet (MR and AR, discussed below). *Bt* protein concentrations were prepared in distilled water and mixed thoroughly 20:80 w:w with artificial diet when the diet temperature was <60 ^{0}C

and poured onto selection trays. 96 and 384 well micro-titer plates were used as selection arenas. Typically at least 2,000 neonates were used for selection for each of the first three generations followed by at least 1,000 neonates for most subsequent selections. Individual neonates were exposed to MVP II (MR) or Cry1Ac toxin (AR) for 7d; only those larvae that molted (based on larval head capsule size) were selected and reared to pupation on diet containing no *Bt* protein.

AR was selected at 50 (generation 1), 80 (generation 2-3) and 200 (generation 4-5) µg Cry1Ac activated toxin/g. After five generations, selection concentration was increased to 500 µg/g and was not increased further due to limited supply of toxin; for every 60 grams of diet, 30 mg of activated toxin was required. AR was selected for resistance every generation; currently this strain is under its 25^{th} generation of selection. Preliminary experiments showed that MVP II was 2-3 fold less toxic than Cry1Ac toxin. Therefore, MR was selected at 100 (generation 1), 200 (generation 2), 500 (generation 3-5) and 1000 (generation 6-8) µg of Cry1Ac in MVP II/g diet. Selection of MR could not be continued beyond 8 generations due to suboptimal larval number (reduced hatching percent), ultimately leading to loss of the strain after generation 11.

Testing resistance: At selected generations, diet incorporation bioassays were conducted concurrently for SC and resistant strains to determine resistance levels. Five to seven concentrations of *Bt* compounds were incorporated into artificial diet as described above and assayed against neonates (0 - 16 h old). Each *Bt* compound-diet concentration was poured into 16-32 wells of a 128 well CD International bioassay tray (CD International, Pitman, NJ). One “active” neonate was loaded per well, covered with ventilated covers

(CD International, Pitman, NJ) and the bioassay trays were incubated at 27 ± 1 ^{0}C and 60% RH with a photoperiod of 14:10h L: D. Assays were rated after 7d; dead and first instar larvae were considered as dead (3). Bioassays were replicated at least three times.

Cross-resistance to MVP II: Initial LC_{50} values generated for SC against Cry1Ac activated toxin and MVP II at generation 0 indicated a 2.9-fold increase for MVP II (Table 1). Based on these observations, similarly higher LC_{50} values for AR were expected when tested against MVP II compared to 1Ac toxin. To test this assumption, bioassays were conducted with AR using MVP II after 7, 11 and 16 generation of selection and concurrently with Cry1Ac toxin. After calculating LC_{50} values from probit analysis, the ratios of LC_{50} values for MVP II and activated Cry1Ac toxin were generated for AR and SC (Table 2).

Cross-resistance to other Bt proteins and cypermethrin: Tests for cross-resistance to other Cry proteins (Cry1Ab toxin, Cry1Ab-corn powder, Cry2Aa2, & Cry2Ab2-corn powder), Vip3A and cypermethrin were conducted between generations 15 and 20 of selection (RR about 100-fold). The expression level of Cry1Ab and Cry2Ab2 in corn tissue was too low to obtain sufficient mortality; therefore, growth rates on diets containing a range of concentrations were used. Mean larval weight was recorded and percent weight loss (compared to the untreated control) in different concentrations of *Bt* proteins was calculated considering mean larval weight in untreated control as 100%.

AR and SC larvae were reared to third instar (8.32 ± 1.29 mg) on untreated diet diluted with 20% water and treated topically on the thoracic terga with 0.5 µl of acetone only (control) or 0.5 µl acetone with a range of cypermethrin concentrations (49). Twelve

larvae were tested per concentration; treated larvae were transferred to 24 well bioassay trays containing diet. Additionally, 10 AR larvae (weight: 7.83 ± 1.46 mg) from Cry1Ac selection (500 μg/g of diet) were treated at 1.99 ng/mg body weight. Mortality was assessed after 24 h. All treatments were replicated three times and each replication consisted of a total of seven concentrations and a control except as described above. Lethal doses were calculated using probit analysis (Polo Plus®) and adjusted for body weight.

Labeling of Cry1Ac and Cry1Aa toxins: Cry1Aa and Cry1Ac toxins used for binding experiments were obtained from recombinant *Bt* strains EG1273, and EG11070, respectively. Both toxins were trypsin-activated, dialyzed overnight and purified by anion-exchange chromatography in a Mono Q HR 5/50 column using an ÄKTA explorer 100x explorer system (GE Healthcare, Uppsala, Sweden) using a 30 ml gradient of 20 mM Tris-HCl (pH 8.6) to 20 mM Tris-HCl (pH 8.6), 1 M NaCl, as described by Estela *et al*.(12). Sample purity was determined using SDS-PAGE, and protein concentration was determined by densitometric analysis using bovine serum albumin as a standard.

Labeling of Cry1Aa and Cry1Ac was performed by incubating 20 μg of toxin with 0.30 mCi of [^{125}I]NaI (Nucliber, Madrid, Spain) using chloramine-T (50). Toxins were labeled twice to have relatively fresh labeled-toxins throughout the study. The specific activities obtained for Cry1Aa and Cry1Ac were, respectively, 2.3 and 47 mCi/mg (first labeling) and 0.6 and 1.4 mCi/mg (second labeling).

BBMV preparation and binding assays: Fifth instar AR and SC larvae were dissected in MET buffer (250 mM mannitol, 17 mM Tris-HCl, 5 mM EGTA, pH 7.5) and midguts

were removed and frozen at -80 ºC. Frozen midguts were shipped on dry ice to the University of Valencia. Brush border membrane vesicles (BBMV) were prepared by the differential magnesium precipitation method (52), frozen in liquid nitrogen and stored at -80 ºC until used. BBMV protein concentrations were determined by Bradford (5).

Binding experiments were performed as previously described (12). A fixed amount of ^{125}I-labeled-toxins and of BBMV (0.05 mg/ml) was incubated for 1 h at room temperature with increasing concentrations of unlabeled homologous toxin in 0.1 ml final volume of binding buffer (PBS-0.1% BSA: 1 mM KH_2PO_4; 10 mM Na_2HPO_4; 137 mM NaCl; 2.7 mM KCl, pH 7.4, 0.1% BSA). After 10 min 16000 x*g* centrifugation, pellets were washed twice in binding buffer. The final radioactivity remaining in the BBMV pellets was measured in a 1282 Compugamma CS gamma counter (LKB, Pharmacia). Experiments were replicated twice. N-acetylgalactosamine (GalNAc) was obtained from Sigma (St. Louis, Missouri). Cry1Ac binding in the presence of the GalNac inhibitor was performed as described above, but with a pre-incubation of ^{125}I-Cry1Ac with GalNAc for 45 min at room temp., prior to the start of the assay with the addition of the BBMV. Experiments were replicated three times.

Data analysis: Bioassay data were analyzed by probit analysis (15) using POLO-plus (LeOra Software, Berkeley, CA, U.S.A.). LC_{50} values with non-overlapping 95% fiducial limits were considered as significantly different. Resistance ratios (RR) were calculated by dividing the LC_{50} values for AR with that of SC. The percent weight loss data for AR and SC in Cry1Ab and Cry2Ab cross-resistance studies was subjected to paired t-tests using SPSS (44). A chi square test was conducted to test for significant differences

between ratios of MVP II and Cry1Ac activated toxin LC_{50} values for both AR and SC. Binding results were analyzed with the LIGAND computer program (37).

RESULTS

Selection response in AR and MR: There was a significant increase in resistance after four generations of selection using Cry1Ac activated toxin (AR) and MVP II (MR) compared to the susceptible colony (SC) (Table 1). During the first seven generations, the rate of resistance evolution was 3 times faster in AR than in MR. The rate of resistance evolution in AR increased with an increase in selection pressure and 12, 36 and 123-fold resistance were observed after 4, 7 and 11 generations of selection, respectively (Table 1). Resistance (based on mortality) in AR did not increase further, as the selection concentration was not increased above 500 μg/g diet due to limited toxin availability, however, there has been an increase in the number of large (3^{rd} instar) larvae in subsequent generations (KJA, unpublished data). Resistance in MR did not increase above 17-fold, even after selecting at higher concentrations for 3 additional generations (Table 1). Selection in MR could not be continued beyond 8 generations due to reduced larval numbers (lower percentage egg hatch), ultimately leading to loss of the strain after 11 generations.

Cross-resistance of AR to MVP II: The ratio of LC_{50} values for MVP II to that of Cry1Ac activated toxin for both strains indicated significant ($\chi^2 = 6.16$, p=0.01, df = 1) differences (Table 2). Based on these ratios MVP II was more toxic to AR than expected

(should be less toxic, as observed in SC) resulting in only partial cross-resistance (Table 2).

Cross-resistance of AR to other Bt proteins and cypermethrin: There was significant cross-resistance to Cry1Ab activated toxin (Table 3). AR larvae lost significantly ($t = 14.70$, $p = 0.045$) less (11%) weight at the highest concentration of Cry1Ab-expressing corn powder (3.84 μg/g) able to be diet-incorporated (Fig. 1a). There was no cross-resistance to Cry2Aa2 protoxin inclusion bodies, Cry2Ab2-expressing corn powder (Fig 1b. $t = -0.385$, $p = 0.72$), Vip3A and cypermethrin (Table 3). AR was also tested with cypermethrin while being reared on diet containing 500 μg/g of Cry1Ac activated toxin. Results (45.8 ± 5.9% mortality at 1.99 ng/mg body weight) were not different when AR was reared on regular diet (containing no Cry1Ac).

Binding of ^{125}I-labeled Cry1A toxins to BBMV: Binding of ^{125}I-Cry1Ac to brush border membrane vesicles **(**BBMV) from AR and SC did not show significant differences even when AR was at its highest resistance ratio. As shown in Fig 2a, homologous competition curves followed a similar pattern with BBMV from both strains. Binding parameters (dissociation constant, K_d, and concentration of binding sites, R_t) obtained from the competition experiments were not significantly different (t tests, $p > 0.05$; Table 4). Binding of Cry1Aa was tested because this toxin shares binding sites with Cry1Ac (24) and it has been shown that, in some resistant strains, alteration of a Cry1A common binding site may be observed when no differences with Cry1Ac are detected due to contribution of other binding sites (29,43). In our case, binding of ^{125}I-Cry1Aa did not show significant differences between SC and AR (Fig. 2b, Table 4)

To differentiate between binding of Cry1Ac which takes place solely through domain II from binding that requires domain III, N-acetylgalactosamine (GalNac) was used as a diagnostic tool, as this sugar inhibits binding of Cry1Ac through domain III to GalNac residues in the membrane. Preincubation of ^{125}I-Cry1Ac with GalNac prior to BBMV resulted in partial inhibition of binding (~34%), however, this inhibition was similar in both strains (Fig. 3).

DISCUSSION

Since the advent of transgenic *Bt* crops, determining the most appropriate form of a *Bt* protein for resistance selection has been an issue for debate. There is a fine balance between what forms of the protein(s) are 1) expressed in plants, 2) present in insects upon ingestion, 3) available for testing, and 4) the susceptibility of target insects to these various protein forms (requiring large quantities of protein if susceptibility is low). Historically, and in some cases currently, truncated *Bt* proteins are expressed within transgenic plants, in other cases, full-length protoxins are expressed. However, recent reports by Gao et al. (16) and Li et al. (31) demonstrate that what the insect actually ingests may be different than what is originally expressed in the plant. As a result, researchers are faced with a dilemma of choosing the most appropriate form of the protein while facing potential logistical constraints. Choosing is not an easy task because insects can vary in their susceptibility to the various forms of *Bt* proteins (26).

Results in this study demonstrate that Cry1Ac-resistant *H. zea* can be selected and maintained using Cry1Ac activated toxin in the laboratory. The laboratory strain originating from Monsanto has had annual infusions of *H. zea* collected from corn and

therefore should have higher genetic variability (and therefore higher *Bt* resistant allele frequency) than laboratory colonies with no infusion of field derived insects. Our initial LC_{50} values for SC of 9 μg/g diet (Cry1Ac toxin) and 26 μg/g diet (MVP II) are significantly higher than those reported by Luttrell et al.(33) for Cry1Ac toxin (0.02 μg/g diet for colony 9103Z) and Ali et al. (3) for MVP II (2.08 μg/g diet), respectively, for their laboratory *H. zea* colony that has had no infusion of field insects for at least 10 years. If we compare the LC_{50} of AR at generation 19 to 9103Z, we would observe a RR of ~ 69,500. Although other variables such as bioassay methodology and host strain need to be considered, these results suggest that *H. zea* can be selected to have tremendous differences in Cry1Ac susceptibility relative to a highly homogeneous laboratory colony. Higher levels of resistance in AR were not sought due to the naturally high tolerance to Cry1Ac and cannibalistic nature of *H. zea*, both resulting in the need for relatively large quantities of Cry1Ac activated toxin to rear these insects individually. The availability of appropriate selection materials, especially purified protein and plant material, is still a major constraint for producing resistant colonies, especially for those insects that have a relatively high tolerance to *Bt* proteins such as *H. zea*.

Resistance development in AR was relatively quick compared to reports for other insects (2, 18). Possible reasons for this relatively rapid rate of resistance evolution include: selecting only larvae that had molted thereby eliminating a higher percentage of susceptible insects in each generation; the use of Cry1Ac activated toxin; and a relatively high initial Cry1Ac toxin resistance allele frequency (6). A relatively rapid rate of

resistance evolution was also observed in *S. exigua* and another strain of *H. zea* selected using Cry1C and Cry1Ac activated toxin, respectively (33,35).

The loss of MR after achieving only 17-fold resistance is contrary to reports for *H. virescens* (17,18) and *P. gossypiella* (46,48). However, our current results with *H. zea* agree with previous unpublished observations by at least two different laboratories. Furthermore, concurrent selection with the same parental colony (SC) resulting in moderately high and stable resistance to Cry1Ac toxin but not to MVP II further validates prior reports. Only partial cross resistance in AR to MVP II suggests further that MVP II may not be the most effective Cry1Ac selection agent against *H. zea* considering that the Cry1Ac toxin fragment in MVP II is identical to the Cry1Ac toxin used in selection (8). The above statement is based on the following assumptions: Cry1Ac protoxin (e.g. MVP II) is not the only or primary form of Cry1Ac ingested by *H. zea* when feeding on *Bt* cotton; and the genes necessary to develop resistance to the Cry1Ac protoxin inclusion bodies (as in MVP II) were as high in the population as that for Cry1Ac activated toxin (6).

There was a 2.1 to 2.8-fold difference in toxicity between Cry1Ac activated toxin and MVP II for SC, and ~2-fold difference would be expected after cleavage of Cry1Ac from ~130 kDa to ~65 kDa (35). Therefore, *H. zea* (SC) does not appear to have difficulty converting protoxin to toxin, although potential difficulties could have been masked by increased toxicity of other compounds in MVP II (sublethal toxicity to heat-treated MVP II was observed at the highest rate of MVP II tested in MR selection studies, WJM, unpublished data). MVP II is used to determine *H. zea* susceptibility in field

populations as part of the EPA-mandated *Bt* resistance monitoring program. Although the precise form and ratio of Cry1Ac toxin and protoxin in *Bt* cotton is uncertain, results presented here would suggest that the specific methodologies used for determining *H. zea* susceptibility to Cry1Ac in the monitoring program should carefully consider the form of Cry1Ac protein used. Our results would suggest that, if relatively low levels of field resistance were to evolve comparable to that which developed in AR, monitoring bioassays using MVP II might not be able to identify these resistant individuals.

Cross resistance studies are invaluable for determining suitable insecticidal compounds for pyramiding with Cry1Ac, as well as to help determine possible resistance mechanisms. Current resistance management theory promotes the sequential or simultaneous use of different insecticidal compounds provided that cross-resistance does not occur among these different toxins (41). As also reported for other Cry1Ac-resistant insects (2,17,33,45,46,48,51), AR was cross-resistant to Cry1Ab. This is not unexpected because Cry1Ab and Cry1Ac toxins share >90% aa homology (8). However, this cross-resistance is unlikely to be related to changes in binding affinity of Cry1A toxins because no binding differences were observed between SC and AR (24). Only slight cross resistance to Cry1Ab corn leaf powder indicates either a possible interaction of Cry protein with leaf secondary metabolites or that most/all of Cry1Ab was only partially activated and that AR may have difficulty in proteolytically cleaving the protoxin. The fact that corn leaf material was immediately freeze-dried after harvesting, and then ground into powder, would suggest that plant proteases might have been unable to degrade/activate Cry1Ab until after ingestion, indirectly implicating proteolysis as a

potential resistance mechanism. Cry1Ab protoxin activation in corn is further supported from a recent study that showed that corn extract partially activated Cry1Ab protoxin, suggesting that Cry1Ab protoxin is partially activated by proteases in *Bt* corn (31)

Lack of cross resistance to Cry2Aa2 and Cry2Ab2 was probably due to differences in aa sequence and mode of action between Cry1Ac and Cry2A (8,9). Cry1Ac-resistant *H. virescens* (strain YHD2), *P. gossypiella* and *H. armigera* have shown no detectable cross resistance to Cry2A proteins (2,17,46,48). Therefore, our results also confirm that the use of Cry2Ab2 pyramided with Cry1Ac (as occurs in Bollgard II®) should be a viable approach for managing potential resistance to Cry1Ac. There was also no cross resistance to Vip3A in AR. This would be expected because this protein does not share any sequence homology with Cry1Ac and is known to bind to separate receptors (13,30,54). Therefore, these results suggest that Vip3A would also be a valuable asset in pyramiding *Bt* proteins for delaying *Bt* resistance development in *H. zea* (as occurs in VipCot®).

Cypermethrin was tested for cross resistance in AR because growers often spray *Bt* cotton with pyrethroids when high *H. zea* populations exist, and pyrethroid oversprays are currently recommended to mitigate *H. zea* resistance to *Bt* cotton (21). AR was tested with cypermethrin both on untreated and Cry1Ac-treated diet. The primary reasoning behind the use of Cry1Ac-treated diet was to more realistically simulate pyrethroid exposure to a potentially *Bt*-resistant *H. zea* larva feeding on *Bt* cotton. Because no cross-resistance to cypermethrin was observed for larvae feeding either on untreated or Cry1Ac-treated diet, these results would suggest that pyrethroids can continue to be used

when necessary, and probably have been a valuable *Bt* cotton IRM practice since the introduction of *Bt* cotton in 1996.

The narrow spectrum of *Bt* resistance suggests an alteration in the binding site of Cry1Ac (14). However, in contrast to other Cry1Ac resistant insects, we did not detect any significant reduction in binding. Lack of Cry1Ac binding has been reported in some Cry1Ac resistant populations of *H. virescens* (23), *P. gossypiella* (36), *H. armigera* (2), *P. xylostella* (42, 47) and *T. ni* (51). Because Cry1Ac is known to bind to GalNac residues of glycosylated membrane proteins (27), we tried to dissect Cry1Ac binding using GalNac as an inhibitor, thus discriminating between GalNac-dependent and GalNac-independent binding (12). Again, we could not find any binding difference between both strains. Another way to look for binding alterations is to use different Cry1A toxins known to bind to a common receptor. Cry1Aa, Cry1Ab and Cry1Ac share binding sites in *H. zea* (24). Although Cry1Aa has low toxicity to *H. zea*, this toxin was used in binding analyses as a diagnostic tool because it has been shown that in *H. virescens* and *Ostrinia nubilalis*, resistant insects that showed reduced or no binding of Cry1Aa to the Cry1A common receptor still could bind Cry1Ac (29,43). Similar to Cry1Ac, there were no significant differences in Cry1Aa binding in terms of either dissociation constants (K_d) or concentration of binding sites (R_t) for Cry1Aa among the samples. Therefore, reduction in binding does not seem to be the mechanism of resistance in AR, in spite of the narrow spectrum of cross-resistance observed. The fact that total cross-resistance does not even extend to protoxin forms of Cry1Ac (MVP II) and Cry1Ab (*Bt*-corn powder), might be indicative of a differential activation of protoxin in the insect

midgut, as opposed to *in vitro* bovine-trypsin activation (25, 38). Alternatively, the C-terminal end of the protoxin may protect the active toxin from the degradative action of midgut proteases, resulting in a higher yield of the fully active toxin (7, 32).

Results from this study demonstrate that broad assumptions cannot be made that all target pests will respond in the same manner to a particular *Bt* (protein or formulation). Because AR represents just a single strain, additional selections against geographically distinct *H. zea* populations are recommended to determine potential different resistance characteristics, as has been demonstrated for *H. virescens* (17,18). Although AR is currently only about 100-fold resistant to Cry1Ac, we feel that this level of resistance is appropriate for characterization because: *H. zea* is 10-40 fold less susceptible to Cry1Ac than *H. virescens* or *P. gossypiella*; a lower level of resistance necessary to survive on *Bt* cotton might be expected and lower levels of *Bt* resistance not resulting in total survivorship on *Bt* cotton might be appropriate for initiating alternative control strategies, and higher levels of resistance are difficult to achieve due to logistical constraints. We have shown that *H. zea* does react differently to Cry1Ac activated toxin and MVP II than other cotton pests, and therefore this information can be used to more adequately adopt cotton IRM strategies for all target pests. Possible implications could include: If *H. zea* has difficulty evolving resistance to full-length or mature forms of *Bt* proteins (as suggested for MVP II), proteins could be designed appropriately; if resistance is not primarily due to binding differences, other potential resistance mechanisms should be explored. Our results also show that Cry1Ac-resistant *H. zea* is susceptible to Cry2Ab2 (found in Bollgard II®), Vip3A (found in VipCot®), and

pyrethroids such as cypermethrin. These results show that the cotton growing community has many alternative control methods to help delay the evolution of Cry1Ac (and other *Bt* proteins) resistance for the future.

Acknowledgements

The authors thank Nancy Adams, Monsanto Co., Union City, TN for providing *H. zea*; Monsanto Co. for providing Cry1Ab, Cry2Ab2 and Syngenta for providing Vip3A; and G. Head, Monsanto Co., Saint Louis, MO for review of this manuscript.

This research was supported by USDA, Cotton Incorporated and by the Spanish Ministry of Education and Science (projects No. AGL2003-09282-C03-01 and AGL2006-11914).

References

1. Adamczyk, J.J.Jr., D. D.Hardee, L. C. Adams, and D. V. Sumerford. 2001. Correlating differences in larval survival and development of bollworm (Lepidoptera: Noctuidae) and fall armyworm (Lepidoptera: Noctuidae) to differential expression of Cry1A(c) delta-endotoxin in various plant parts among commercial cultivars of transgenic *Bacillus thuringiensis* cotton. J. Econ. Entomol. **94:** 284-290.
2. Akhurst, R.J., James, W., Bird, L.J. and Beard, C. 2003. Resistance to the Cry1Ac delta-endotoxin of *Bacillus thuringiensis* in the cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae). J. Econ. Entomol. **96:** 1290-1299.

3. Ali, M.I., R. G. Luttrell, and S.Y. III. Young. 2006 Susceptibilities of *Helicoverpa zea* and *Heliothis virescens* (Lepidoptera: Noctuidae) populations to Cry1Ac insecticidal protein. J. Econ. Entomol. **99:** 164-175.

4. Bates, S.L., J. Z. Zhao, R. T. Roush, and A. M. Shelton. 2005. Insect resistance management in GM crops: past, present and future. Nat. Biotechnol. **23:** 57-62.

5. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:** 248-254.

6. 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:**137-142.

7. Choma, C. T., W. K. Surewicz, P. R. carey, M. Pozsgay, T. Raynor, and H. Kaplan. 1990. Unusual proteolysis of the protoxin and toxin from *Bacillus thuringiensis*. Eur. J. Biochem. **189**:523-527

8. Crickmore, N., D.R. Zeigler, J. Feitelson, E. Schnepf, J.V. Rie, D. Lereclus, J. Baum and D.H. Dean. 1998 Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. **62:** 807-813.

9. English, L, H.L. Robbins, M. A. Von Tersch, C. A. Kulesza, D. Ave, D. Coyle, C. S. Jany and S. L. Slatin. 1994. Mode of action of CryIIA: a *Bacillus thuringiensis* delta-endotoxin. Insect Biochem. Mol. Biol. **24:** 1025-1035.

10. EPA, U.S. 1998. The Environmental Protection Agency's White paper on Bt Plant-Pesticide Resistance Management. *U.S. EPA,* Biopesticides and Pollution Prevention Division (7511W), 401 M Street, SW, Washington, D.C. 14 January 1998. [EPA Publication 739-S-98-001].

11. EPA, U.S. 2001. Environmental Protection Agency. Biopesticides registration action document - *Bacillus thuringiensis* plant incorporated protectants (EPA, Washington, DC, USA 2001). *http://www.epa.gov/pesticides/biopesticides/pips/bt_brad.htm*.

12. Estela, A., B. Escriche, and J. Ferre. 2004 Interaction of *Bacillus thuringiensis* toxins with larval midgut binding sites of *Helicoverpa armigera* (Lepidoptera: Noctuidae). Appl. Environ. Microbiol. **70:** 1378-1384.

13. Estruch, J.J, G. W. Warren, M. A. Mullins, G. J. Nye, J. A. Craig and M. C. 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.

14. Ferre, J. and Van Rie, J. 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. Ann. Rev. Entomol. **47:** 501-533.

15. Finney, D. J.1971. Probit analysis. Cambridge University Press London, England.

16. Gao, Y, K. J. Fencil, X. Xu, D. A. Schwedler, J. R. Gilbert and R. A. Herman. 2006. Purification and characterization of a chimeric Cry1F delta-endotoxin expressed in transgenic cotton plants. J. Agric. Food Chem. **54:** 829-835.

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

18. 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.

19. 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.

20. Griffitts, J. S., and R. V. Aroian**.** 2005. Many roads to resistance: how invertebrates adapt to Bt toxins. Bioessays **27:**614–624.

21. Jackson, R.E., J.R. Jr. Bradley, J.W. Van Duyn, and F. Gould. 2004. Comparative production of *Helicoverpa zea* (Lepidoptera: Noctuidae) from transgenic cotton expressing either one or two *Bacillus thuringiensis* proteins with and without insecticide oversprays. J. Econ. Entomol. **97:**1719-1725.

22. James, C. 2006. Global Status of Commercialized Biotech/GM Crops: 2006. ISAAA Brief No. 35. ISAAA: Ithaca, NY.

23. Jurat-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:**5711-5717.

24. Karim, S., S. Riazuddin, F. Gould, and D. H. Dean. 2000. Determination of receptor binding properties of *Bacillus thuringiensis* delta-endotoxins to cotton bollworm (*Helicoverpa zea)* and pink bollworm (*Pectinophora gossypiella*) midgut brush border membrane vesicles. Pestic. Biochem. Physiol. **67:**198-216.

25. Karumbaiah, L., B. S. Oppert, J. L. Jurat-Fuentes, and M. J. Adang. 2007. Analysis of midgut proteinases from *Bacillus thuringiensis*-susceptible and -resistant *Heliothis virescens* (Lepidoptera: Noctuidae). Comp. Biochem. Physiol. **146:**139-146.

26. Keller, M, B. Sneh, N. Strizhov, E. Prudovsky, A. Regev, C. Koncz, J. Schell and A. Zilberstein. 1996. Digestion of delta-endotoxin by gut proteases may explain reduced sensitivity of advanced instar larvae of *Spodoptera littoralis* to CryIC. Insect Biochem. Mol. Biol. **26:**365-373.

27. Knowles, B.H., P. J. K. Knight and D. J. Ellar. 1991. N-acetyl galactosamine is part of the receptor in insect gut epithelia that recognizes an insecticidal protein from *Bacillus thuringiensis*. *Proc. R. Soc. London* **245:**31-35.

28. Kranthi, K.R., S. Kranthi, S. Ali, and S. K. Banerjee. 2000. Resistance to 'CryIAc delta -endotoxin of *Bacillus thuringiensis*' in a laboratory selected strain of *Helicoverpa armigera* (Hubner). *Curr. Sci.* **78:**1001-1004.

29. Lee, M., F. Rajamohan, F. Gould, and D. H. Dean. 1995. Resistance to *Bacillus thuringiensis* CryIA delta-endotoxins in a laboratory-selected *Heliothis virescens* strain is related to receptor alteration. Appl. Environ. Microbiol. **61:**3836-3842.

30. Lee, M.K., P. Miles, and J. S. Chen. 2006. Brush border membrane binding properties of *Bacillus thuringiensis* Vip3A toxin to *Heliothis virescens* and *Helicoverpa zea* midguts. Biochem. Biophys. Res. Commun. **339:**1043-1047.

31. Li, H., L. L. Buschman, F. Huang, K. Y. Zhu, B. Bonning, and B. Oppert. 2007. Dipel-selected *Ostrinia nubilalis* larvae are not resistant transgenic corn expressing *Bacillus thuringiensis* Cry1Ab. J. Econ. Entomol. *In Press*

32. Lightwood, D. J., D. J. Ellar, and P. Jarrett. 2000. Role of proteolysis in determining potency of *Bacillus thuringiensis* Cry1Ac δ-endotoxin. Appl. Environ. Microbiol. **66:**5174-5181.

33. Luttrell, R.G., L. Wan, and K. Knighten. 1999. Variation in susceptibility of noctuid (Lepidoptera) larvae attacking cotton and soybean to purified endotoxin proteins and commercial formulations of *Bacillus thuringiensis*. J. Econ. Entomol. **92:**21-32.

34. Moar, W.J., J. T. Trumble, R. H. Hice, and P. A. Backman. 1994. Insecticidal activity of the CryIIA protein from the NRD-12 isolate of *Bacillus thuringiensis* subsp. *kurstaki* expressed in *Escherichia coli* and *Bacillus thuringiensis* and in a leaf-colonizing strain of *Bacillus cereus*. Appl. Environ. Microbiol. **60:**896-902.

35. Moar, W.J., M. Pusztai-Carey, H.V. Faassen, D. Bosch, R. Frutos, C. Rang, K. Luo and M. J. Adang. 1995. Development of *Bacillus thuringiensis* CryIC resistance by *Spodoptera exigua* (Hubner) (Lepidoptera: Noctuidae). Appl. Environ. Microbiol. **61:** 2086-2092.

36. Morin, S., R. W. Biggs, M. S. Sisterson, L. Shriver, C. Ellers-Kirk, D. Higginson, D. Holley, L. J. Gahan, D. G. Heckel, Y. Carriere, T. J. Dennehy, J. K. Brown and B. E. Tabashnik. 2003. Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm. Proc. Natl. Acad. Sci. USA. **100:** 5004-5009.

37. Munson, P.J. and D. Rodbard. 1980. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. Anal. Biochem. **107:** 220-239.

38. Oppert, B., K. J. Kramer, D. E. Johnson, S. C. MacIntosh and W. H. McGaughey. 1994. Altered protoxin activation by midgut enzymes from a *Bacillus thuringiensis* resistant strain of *Plodia interpunctella.* Biochem. Biophys. Res. Commun. **198:** 940-947.

39. Perlak, F. J., W. R. Deaton, T. A. Armstrong, R. I. Fuchs, S. R. Sims, J. T. Greenplate and D. A. Fishhoff. 1990. Insect resistant cotton plants. Bio/Technology **8:**939-943.

40. Pusztai-Carey, M., P. Carey, T. Lessard and M. Yaguchi. 1994. US patent # 5356788.

41. Roush, R.T. 1998. Two-toxin strategies for management of insecticidal transgenic crops: can pyramiding succeed where pesticide mixtures have not? Philos. Trans. R. Soc. London **353:**1777-1786.

42. Sayyed, A.H., R. Haward, S. Herrero, J. Ferre and D. J. Wright. 2000. Genetic and biochemical approach for characterization of resistance to *Bacillus thuringiensis* toxin Cry1Ac in a field population of the diamondback moth, *Plutella xylostella*. Appl. Environ. Microbiol. **66:**1509-1516.

43. Siqueira, H.A.A., J. Gonzalez-Cabrera, J. Ferre, R. Flannagan and B. D. Siegfried. 2006. Analyses of Cry1Ab binding in resistant and susceptible strains of the European Corn Borer, *Ostrinia nubilalis* (Hubner) (Lepidoptera: Crambidae). Appl. Environ. Microbiol. **72:** 5318-5324.

44. SPSS. 2006. SPSS Base 14.0 for Windows User's Guide. SPSS Inc., Chicago IL.

45. Tabashnik, B. E., Y. Carriere, T. J. Dennehy, S. Morin, M. A. Sisterson, R. T. Roush, A. M. Shelton and J. Z. Zhao. 2003 Insect resistance to transgenic Bt crops: lessons from the laboratory and field. J. Econ. Entomol. **96:** 1031-1038.

46. Tabashnik, B.E., T. J. Dennehy, M. A. Sims, K. Larkin, G. P. Head, W. J. Moar and Y. Carriere. 2002. Control of resistant pink bollworm (*Pectinophora gossypiella*) by transgenic cotton that produces *Bacillus thuringiensis* toxin Cry2Ab. Appl. Environ. Microbiol. **68:**3790-3794.

47. Tabashnik, B.E., Y. B. Liu, T. Malvar, D. G. Heckel, L. Masson, V. Ballester, F. Granero, J. L. Mensua and J. Ferre. 1997. Global variation in the genetic and biochemical basis of diamondback moth resistance to *Bacillus thuringiensis*. Proc. Natl. Acad. Sci. USA **94:**12780-12785.

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

49. Usmani, K.A. and C. O. Knowles. 2001. Toxicity of pyrethroids and effect of synergists to larval and adult *Helicoverpa zea*, *Spodoptera frugiperda*, and *Agrotis ipsilon* (Lepidoptera: Noctuidae) J. Econ. Entomol. **94:**868-873

50. Van Rie, J., S. Jansens, H. Hofte, D. Degheele and H. Van Mellaert. 1989. Specificity of *Bacillus thuringiensis* δ-endotoxins. Importance of specific receptors on the brush border membrane of the midgut of target insects. Eur. J. Biochem. **186:**239-247.

51. Wang, P, J. Z. Zhao, A. Rodrigo-Simon, W. Kain, A. F. Janmaat, A. M. Shleton, J. Ferre and J. Myers. 2007. Mechanism of resistance to *Bacillus thuringiensis* toxin Cry1Ac in greenhouse population of cabbage looper, *Trichopusia ni*. Appl. Environ. Microbiol. **73:**1199-1207.

52. Wolfersberger, M.G., P. Luethy, P. Maurer, P. Parenti, V. F. Sacchi, B. Giordana, and G. M. Hanozet. 1987. Preparation and partial characterization of amino acid transporting brush border membrane vesicles from the larval midgut of the cabbage butterfly (*Pieris brassicae*). Comp. Biochem. Physiol. **86A:** 301-308.

53. Xu, X., L.Yu, and Y. Wu. 2005. Disruption of a cadherin gene associated with resistance to Cry1Ac delta -endotoxin of *Bacillus thuringiensis* in *Helicoverpa armigera*. Appl. Environ. Microbiol. **71:**948-954.

54. Yu, C., M. A. Mullins, G. W. Warren, M. G. Koziel and J. J. Estruch. 1997. The *Bacillus thuringiensis* vegetative insecticidal protein Vip3A lyses midgut epithelium cells of susceptible insects. Appl. Environ. Microbiol. **63:**532-536.

Table 1. Resistance development in *H. zea* when selected using activated toxin and MVP II

Strain	G[a]	N[b]	LC_{50} (95% FL)[c]	Slope (mean ± SE)	RR[d]
Activated toxin-resistant strain (AR)					
SC[e]		800	9.13 (5.83- 12.53)	1.61± 0.41	
AR	4	384	107.64 (75.37 – 155.6)	1.42 ± 0.3	12.12
SC		480	8.89 (5.71-13.72)	1.71± 0.41	
AR	7	222	321.22 (251.27 – 371)	1.89 ± 0.13	35.91
SC		384	8.94 (6.37 – 15.27)	1.93 ± 0.31	
AR	11	175	1,450 (690 – 2,392)	1.42 ± 0.47	122.67
SC		384	11.82 (7.01-19.24)	1.76 ± 0.42	
AR	16	72	47% survivors @ 1.5mg/g		>100
SC		384	13.90 (9.11- 21.44)	2.41 ± 0.51	
AR	19	192	1,390 (743 –12,017)	1.39 ± 0.46	92.69
SC		192	15.00 (9.90 – 22.45)	2.31 ± 0.52	
MVP II resistant strain (MR)					
SC	0	1120	26.13 (16.34 – 35.62)	1.73 ± 0.24	
MR	4	384	384.3 (282.31 – 568.12)	1.79 ± 0.27	16.61
SC		640	23.13 (16.34 – 35.62)	1.73 ± 0.24	
MR	7	672	298.40 (155.16 – 455.5)	1.67 ± 0.41	12.01
SC		1120	24.84 (13.48 – 41.89)	2.47 ± 0.57	
MR	9-11	No selection due to reduced larval number, resistant strain crashed after 11 generations			

[a]Generations of *H. zea* continuously selected with *Bt*

[b]Total number of insects tested (one to five replicates with one to seven concentrations).

[c]LC_{50} values are in micrograms of *Bt* protein per gram of diet. FL, fiducial limits

[d]Resistance ratio: LC_{50} for AR divided by the LC_{50} for SC

[e]SC: Susceptible colony

Table 2. Cross-resistance of AR to MVP II

Strain	G[a]	N[b]	LC_{50} (95% FL)[c]	Slope (mean ± SE)	RR[d]	MVP II – 1Ac Ratio[e]
AR	7 (36)	192	117.79(63.38 – 175.45)	1.81 ± 0.34	4.7	0.37
SC[f]		1120	24.84 (13.48 – 41.89)	2.47 ± 0.57		2.78
AR	11 (123)	576	197.10 (134.1 – 333.51)	1.36 ± 0.29	7.9	0.14
SC		576	24.94 (13.86 – 44.87)	2.57 ± 0.37		2.11
AR	16 (>100)	448	397.93 (245.87 – 749.86)	1.56 ± 0.42	10.3	0.28
SC		448	38.53 (24.87 – 55.56)	2.41 ± 0.53		2.76

[a]Generations of *H. zea* continuously selected with Cry1Ac activated toxin; Values in parenthesis indicate resistance ratio to Cry1Ac activated toxin.

[b]Total number of insects tested (one to five replicates with one to seven concentrations).

[c]LC_{50}s are in micrograms of Cry1Ac in MVP II per gram of diet. FL, fiducial limits

[d]Resistance ratio: LC_{50} for AR divided by the LC_{50}s for SC

[e]MVP II-1Ac Ratio. Ratio of LC_{50} values for MVP II divided by the LC_{50} values for Cry1Ac activated toxin (data from Table 1)

[f]SC: Susceptible colony

Table 3. Cross-resistance of AR to other *B. thuringiensis* proteins and cypermethrin

Strain	G[a]	Compound	N[b]	LC_{50} (95% FL)[c]	Slope (mean ± SE)	RR[d]
AR	19 (93)	Cry1Ab	200	8.44 % mortality @ 400µg/g of diet		ND[e]
SC[f]			200	133.33 (98.42 – 261.41)	1.82 ± 0.55	
AR	15 (>100)	Vip3A	512	22.29 (15.18 – 31.07)	2.59 ± 0.49	0.94
SC			512	23.73 (16.82 – 33.80)	2.24 ± 0.35	
AR	16 (>100)	Cry2Aa2	672	101.83 (72.60 – 167.39)	2.83 ± 0.51	1.55
SC			672	65.70 (46.27 – 109.34)	1.89 ± 0.27	
AR	16 (>100)	Cypermethrin	288	1.70 (1.11 – 2.61)[g]	2.08 ± 0.40	1.85
SC			288	0.92 (0.61 – 1.33)[g]	2.55 ± 0.52	

[a]Generations of *H. zea* continuously selected with *Bt* protein; values in parenthesis indicates resistance ratio when bioassays were conducted

[b]Total number of insects tested (three to five replicates with five to seven concentrations).

[c]LC_{50}s are in micrograms of *Bt* protein per gram of diet. FL, fiducial limits

[d]Resistance ratio: LC_{50} for AR divided by the LC_{50}s for SC

[e]ND: Not determined because LC_{50} for AR could not be obtained

[f]SC: Susceptible colony

[g]Lethal dose (ng/mg body weight)

Table 4. Dissociation constants (K_d) and concentration of binding sites (R_t) for binding of Cry1A proteins to BBMV from *H. zea*[a].

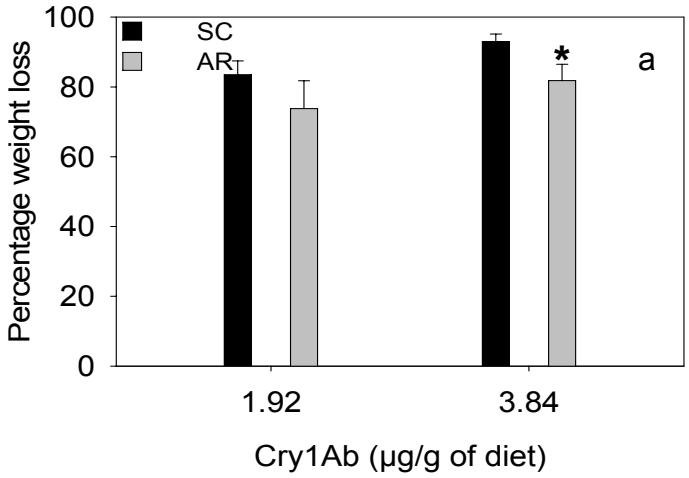
Bt toxin	Sample (gen. selection)	K_d ± SD (nM)	R_t ± SD (pmol/mg) [b]
Cry1Ac	SC[c]	1.1 ± 0.1	17.0 ± 0.9
	AR[c] (4)	0.50 ± 0.3	22.3 ± 4.9
	AR (7)	0.4 ± 0.1	28 ± 15
	AR (11)	2.9 ± 0.1	49 ± 3
Cry1Aa	SC	3.2 ± 0.4	2.7 ± 0.7
	AR (7)	3.8 ± 0.1	4.1 ± 0.7
	AR (11)	3.2 ± 0.3	5.0 ± 1.2

[a]Values are the mean of two replicates for resistant insects and four replicates for the SC strain (using two independently labeled Cry1Ac and Cry1Aa batches)

[b]Expressed as pmol per milligram of total vesicle protein

[c]SC: Susceptible colony; AR: Cry1Ac-resistant colony

Fig 1. Toxicity of Cry1Ab (a) and Cry2Ab2 (b) expressing corn leaf powder to susceptible (SC) and Cry1Ac-resistant (AR) *H. zea*. Values are expressed as percentage weight loss relative to the corresponding controls at different concentrations. Data represent the mean of four replications and error bars are the standard deviation. The asterisk (*) indicates significant differences in t-tests (t =14.70, p=0.045)



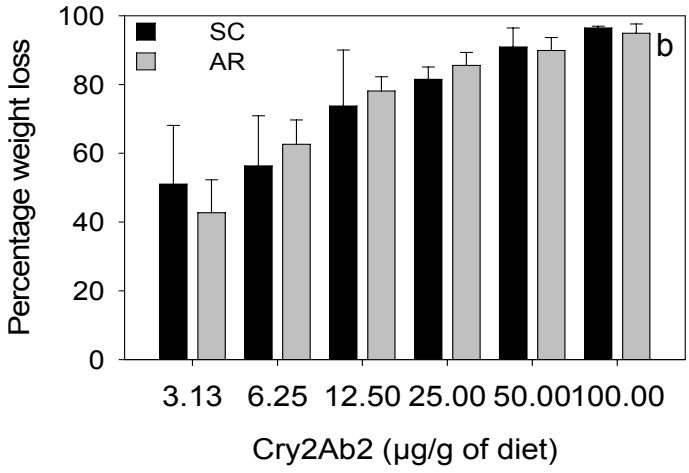


Fig 2. Binding of ^{125}I-Cry1Ac (A) and ^{125}I-Cry1Aa (B) to BBMV from susceptible (SC) (●) and and Cry1Ac-resistant (AR) *H. zea* (▲) at increasing concentrations of unlabeled homologous competitor. Data represent the mean of two experiments and error bars are the SEM.

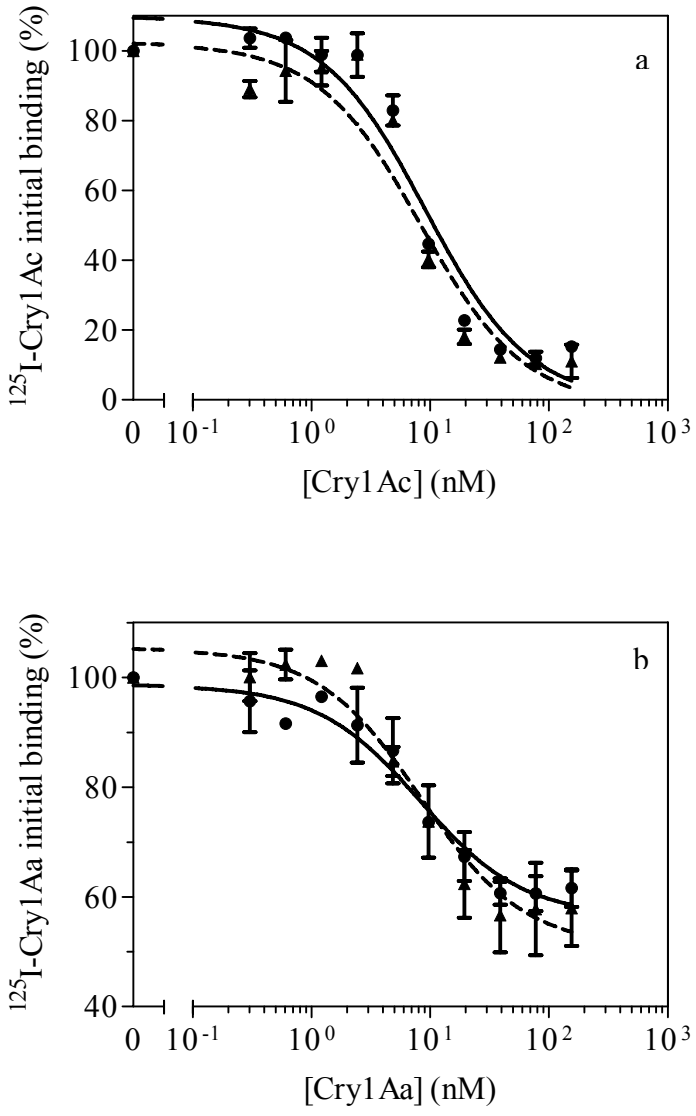
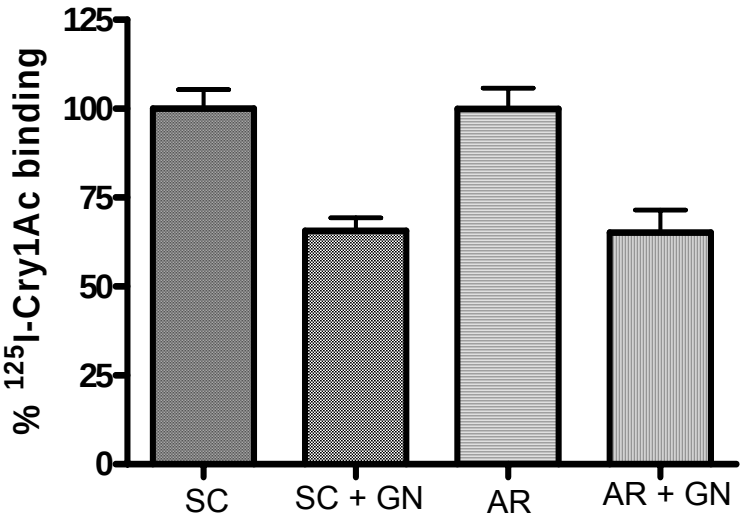


Fig 3. Percent binding of ^{125}I-Cry1Ac to BBMV from susceptible (SC) and Cry1Ac-resistant (AR) *H. zea* in the absence (SC and AR) or the presence (LC+GN and AR+GN) of 25 mM GalNac. Data represent the mean of three experiments and error bars are the SEM.



CHAPTER 3:

FITNESS COSTS ASSOCIATED WITH CRY1AC-RESISTANT *HELICOVERPA ZEA* (LEPIDOPTERA: NOCTUIDAE): A FACTOR COUNTERING SELECTION FOR RESISTANCE TO *BT* COTTON?

ABSTRACT The heritability, stability, and fitness costs in a Cry1Ac-resistant *Helicoverpa zea* colony (AR) were measured in the laboratory. In response to selection, heritability values for AR increased in generations 4 to 7 and decreased in generations 11 to 19. AR had significantly increased pupal mortality, a male-biased sex ratio, and lower mating success compared to the unselected parental strain (SC). AR males had significantly more mating costs compared to females. AR reared on untreated diet had significantly increased fitness costs compared to rearing on Cry1Ac treated diet. AR had significantly higher larval mortality, lower larval weight, longer larval developmental period, lower pupal weight, longer pupal duration, and higher number of morphologically abnormal adults compared to SC. Due to fitness costs after 27 generations of selection as described above, AR was crossed with a new susceptible colony (SC1), resulting in AR1. After just two generations of selection, AR1 exhibited significant fitness costs in larval mortality, pupal weight and morphologically abnormal adults compared to SC1. Cry1Ac-resistance was not stable in AR in the absence of selection. This study

demonstrates that fitness costs are strongly linked with selecting for Cry1Ac resistance in *H. zea* in the laboratory, and fitness costs remain, and in some cases, even increase after selection pressure is removed. These results support the lack of success of selecting, and maintaining Cry1Ac-resistant populations of *H. zea* in the laboratory, and may help explain why field-evolved resistance has yet to be observed in this major pest of *Bt* cotton.

Introduction

The evolution of resistance in target insect populations is the primary concern with the use of crops expressing *Bacillus thuringiensis* (*Bt*) proteins such as Cry1Ac in *Bt* cotton (Bollgard®) in the US and elsewhere. However, even after 12 years of commercial use in the US, there are still no documented cases of field-evolved resistance in Bollgard® to any of the three target pests, especially to bollworm, *Helicoverpa zea* (Boddie) (Moar and Anilkumar 2007). *H. zea* is significantly more tolerant to Cry1Ac present in Bollgard® than other target pests (MacIntosh et al. 1990, Ali et al. 2006, Sivasupramaniam et al. 2008) and can survive on Bollgard® late season (Jackson et al. 2004a). Although resistance management strategies such as "high dose plus refuge" have been used to delay resistance development (Gould 1998) the use of these tactics alone cannot fully explain the total lack of field-evolved resistance. Factors such as fitness costs, stability, and the genetics of resistance may play a significant role in delaying or mitigating resistance evolution (Tabashnik 1994, Gould 1998). Many models have predicted the delay in resistance development due to fitness costs (Caprio 2001, Storer et al. 2003a, 2003b; Gustafson et al. 2006). Studies with laboratory-selected Cry1Ac-resistant insects

such as *Pectinophora gossypiella* (Saunders), *Helicoverpa armigera* (Hubner), and *Plutella xylostella* (L.) support these model predictions by documenting fitness costs and incomplete resistance to *Bt* crops (Liu et al. 1999, Carriére et al. 2001a, 2001b, 2006; Sayyed and Wright 2001, Bird and Akhurst 2004, 2005; Higginson et al. 2005).

Selection for Cry1Ac-resistant *H. zea* in the laboratory has been attempted numerous times over a 10 year period, but all attempts have resulted in colony crashes due to fitness costs (Luttrell et al. 1999, Luttrell R, Univ. of Arkansas, personal communication, Jackson et al. 2004b, Jackson, R., USDA, personal communication., WJM unpublished data, Anilkumar and Moar 2006, Anilkumar et al. 2008). Additionally, although there have been many attempts to rear field-collected *H. zea* populations, often collected from *Bt* crops, with relatively high tolerances to Cry1Ac (MVP II) in the laboratory, they typically cannot be maintained for more than five to seven of generations, with many populations crashing after one to two generations (Luttrell, R, Univ. of Arkansas, personal communication, KJA and WJM, unpublished results). Anilkumar et al. (2008) reported a stable Cry1Ac-resistant *H. zea* strain (AR) after selection with *Bt* Cry1Ac toxin for 11 generations (100-fold resistance) and this colony was maintained at this level for 25 generations under continuous selection. Even though AR was relatively stable, fitness costs were observed during selection and when insects were removed from selection. (Note: This colony was crossed to a susceptible population in Generation 26 to avoid the total collapse of this strain due to fitness costs). Further, higher fitness costs usually affect the stability of resistance in the population, thereby affecting the heritability of resistance. Therefore, a thorough understanding of

the biological traits of *H. zea* affected by fitness costs, heritability and stability of resistance could contribute to the development of more realistic models for predicting the development of resistance and thereby aid in formulating better strategies for effective resistance management. Furthermore, findings in this paper could help explain why field-evolved resistance has yet to be observed in this pest to Bollgard® after 12 years of intense use. Therefore, this study investigated the various biological parameters associated with fitness costs, heritability and stability of resistance exhibited by Cry1Ac toxin-resistant *H. zea* (Anilkumar et al. 2008).

Materials and methods

Insect strains: A laboratory susceptible colony of *H. zea* (SC) was established in September 2004 from a laboratory colony from Monsanto (Union City, TN). The culture at Monsanto was annually infused with field-collected insects; therefore, the population was heterogeneous and contained Cry1Ac-resistant genes (Anilkumar et al. 2008). One strain (AR) was selected from SC for resistance on artificial diet containing *Bt* Cry1Ac toxin for 25 generations by exposing individual neonates for seven days (Anilkumar et al. 2008). Only second and third instars were transferred to 24 well tissue culture plates containing untreated diet and were reared an additional seven days (Ali et al. 2006, Anilkumar et al. 2008, Sivasupramaniam et al. 2008). Late 4^{th} to early 5^{th} instar larvae were transferred to diet cups (30 ml, Bio-serve, Frenchtown, NJ) containing artificial diet and were reared to pupation. Except for selection using Cry1Ac toxin, AR and SC were treated similarly in regards to diet used, number of larvae reared, quality of larvae harvested, and number of adults used for generating subsequent generations.

Resistance heritability and resistance risk assessment: Heritability of resistance (h^2) and resistance risk (G) (number of generations required for 10-fold increase in resistance) were estimated. LC_{50} values for SC and AR conducted simultaneously (Anilkumar et al. 2008) and percent survival in each generation of selection were used for calculating parameters necessary for determining h^2 and G (Tabashnik 1992).

Fitness costs in AR on Cry1Ac treated diet: While conducting selection experiments, a reduction in egg hatch was observed in AR after nine generations of selection (>36-fold resistance, Anilkumar et al. 2008). Further observations indicated no embryo development, confirming egg infertility. Therefore, both resistant (AR) and control (SC) strains were monitored for mating success during resistance selection, and maintenance, respectively, from generations 9-24.

Larvae were selected and reared as discussed above; the resistance ratio of AR (LC_{50} of AR / LC_{50} of SC) exceeded 100-fold. The resulting pupae were sexed and maintained in separate boxes (18x18x7cms) for adult eclosion. Pupal sex ratio was recorded for 15 generations (generations 10 to 24; 6,314 pupae total). Further, pupal mortality (dead pupae and malformed adults) were recorded for 11 generations (generation 15 to generation 25; 4,867 pupae total). The proportions of males were modeled as the number of males in a total population, and were analyzed using a binomial test; the interaction of population and generation were considered as residuals (Hardy 2002, SAS Institute 2003). Pupal mortality was compared between strains using a paired t-test (SPSS 2006).

Initially adults were released into mating cages (34x19x11cms) at a 1:1 sex ratio and thirty moths were maintained per cage. However, additional (maximum of three) moths from either sex were released into mating cages because of premature adult mortality in some of the original 30 moths (within 3 three days). The resultant sex ratio was not significantly different (see results below) from 1:1. Mating cages were covered with white cloth for oviposition and moths were fed a 10% sucrose solution. Egg sheets were replaced daily and incubated at 27 ± 2 ^{0}C until hatching. Adults were maintained in cages until death or when moths quit laying eggs (after 10 days). Dead moths were removed daily from cages, and all surviving moths (after 10 days) were dissected under a stereo microscope to determine mating frequency. Female moths were classified as mated or unmated based on the presence or absence of spermatophore(s) in the spermatheca. Further, females were classified as having mated once, twice, three, four or five times depending on the number of spematophores present in the spermatheca. Mean number of spermatophores produced per male was calculated by taking the total number of spermatophores produced in a generation divided by the number of males released into cages (Bird and Akhurst, 2004). Observations were made for 14 generations (generation 9 [Resistance Ratio {RR} >36-fold], and generations 12 to 24 [RR >100-fold]) from a period spanning nearly two years and from a total of 3,886 moths. Percentage mating, multiple mating and mean number of spermatophores between AR and SC were compared by paired t-tests (SPSS 2006).

Mating propensity observations indicated a reduction in mating success in AR (see results, Table 2; Fig. 2). Therefore, reciprocal crosses between AR and SC (AR[♀]

X SC[♂] and SC[♀] X AR[♂]) were conducted at an equal sex ratio to test if the reduction in mating success was sex-linked. Moths were caged and percent mating was ascertained as described above. Reciprocal crosses were conducted with five male and five female moths spanning three generations, >50 moths (1:1 sex ratio) in two generations (two replicates). Therefore, the total number of moths used in each of the two reciprocal (AR X SC) crosses was 140. Percentage mating, multiple mating and mean number of spermatophores between AR and SC were analyzed by ANOVA (SPSS 2006).

Fitness costs in AR on untreated diet: The relative performances of both susceptible (SC) and >100-fold resistant (AR) strains were measured on untreated artificial diet (referred to hereafter as regular diet) and untreated selection diet (artificial diet diluted with 20% water; used for incorporating *Bt* proteins in selection experiments, referred to hereafter as selection diet) (Anilkumar et al. 2008). A total of 160 larvae (48, 48 and 64 larvae in replication 1, 2 and 3, respectively) for each treatment were tested. Individual neonates were placed on diet in 128 well CD International bioassay trays (CD International, Pitman, NJ) and reared for seven days. Larval weight and instar were recorded after seven days, and larvae were transferred to 30 ml diet cups containing regular diet and reared until pupation. Larval duration and mortality were recorded. All insects were removed from diet on the second day of pupation; weights were recorded and were transferred to a new 30 ml cups (containing no diet). Pupae were sexed and observed daily for adult eclosion. Adults failing to eclose and those with fringed wings were considered as malformed adults. Pupa that did not eclose after 15 days was considered dead.

Growth rate (weight gain per day) was calculated for both strains after the first seven days (on either diet) and at pupation. The growth rate for the first seven days (when insects were exposed to either selection diet or regular diet) was calculated by dividing larval weight by seven. The growth rate after 7 days, (when insects with different exposure background were transferred to regular diet) was calculated by the following equation.

$$\text{Growth rate (weight gain per day)} = \frac{\text{(Pupal weight – larval weight at 7 days)}}{\text{(Larval duration – 7 days)}}$$

Further, the difference in growth rates was calculated by subtracting the growth rate during the first seven days from that determined after seven days. Insects that died prematurely were not included in the analysis.

Thirty adults were released into mating cages and maintained as explained above. Total number of eggs laid was recorded daily and mean number of eggs per female was calculated. Eggs were incubated for 4 days at 27 ± 2 ^{0}C, and hatching percentage was calculated. Each experiment on selection and regular diets was considered as a block, each insect as a replicate and the entire test as a randomized complete block design for analysis. Larval and pupal periods were log transformed to stabilize variance. Larval weight, duration and mortality; pupal weight and duration; and percent malformed adults were analyzed using two-way ANOVA and means were separated using Tukey's least significant differences (SPSS 2006). Growth rates during initial seven days, after seven days, and their difference were analyzed using ANOVA and means were separated using Fisher least significant differences (SPSS 2006).

Crosses with the susceptible strain: AR was crossed with a new susceptible strain (SC1) resulting in AR1, to avoid complete loss of the strain due to fitness costs (see results) associated with Cry1Ac-resistance selection and maintenance. As discussed earlier, the laboratory colony at Monsanto is infused annually with field collected insects; therefore, SC1 is a derivative of SC from the most recent infusion in 2007. SC1 had increased tolerance to Cry1Ac toxin (LC_{50}=31.25µg Cry1Ac/g of diet) compared to SC (LC_{50}=9-15µg Cry1Ac/g of diet, Anilkumar et al. 2008). Even though both reciprocal crosses were attempted, only AR[♀] X SC1[♂] yielded a F_1 population due to mating costs associated with AR males (see results). AR1 was selected at the regular selection concentration of Cry1Ac (500 µg Cry1Ac/g of diet) for two generations. Further, fitness parameters (discussed above) were measured only on regular diet. Three experiments were conducted with 32 larvae each per strain per replication and data were analyzed as discussed above.

Stability of resistance: The desired number of larvae could not be obtained for bioassays when AR was reared on untreated diet for two generations due to extremely high pupal mortality (discussed in Results). Therefore, bioassays were conducted immediately after one generation. Neonates (130 in number) were tested in two replications at 500 µg Cry1Ac/g of diet (concentration used in resistance selection/maintenance experiments) compared with an untreated control. Parallel tests were conducted on AR subjected to continued selection. Paired t-tests were conducted to compare the survivorship of AR on 500 µg Cry1Ac/g of diet when AR was continuously selected at 500 µg Cry1Ac/g of diet, and after AR had been reared one generation on regular diet.

Statistical analysis: All statistical tests were conducted at the 0.05 level of significance using either SPSS or SAS statistical programs, and for those parameters which required transformations for stabilizing the variance, data are presented as non-transformed arithmetic means.

Results

Heritability and resistance risk assessment: The heritability (h^2) of resistance to *Bt* Cry1Ac toxin varied at different generations of selection (Table 1). The h^2 was 0.315 after four generations (12-fold resistance), increased to 0.401 after seven generations (36-fold resistance) and decreased to 0.256 and 0.123 after 11 and 19 generations, (>100-fold resistance) respectively. Resistance risk (G) assessment considering heritability values after 19 generations of selection (0.123) indicated that 9.66 generations are required for a 10-fold increase in resistance.

Fitness costs in AR on Cry1Ac treated diet:

Pupal sex ratio and mortality: There were significant ($F_{1,14}$=9.44, p=0.0083) differences in sex ratios of AR and SC (Fig 1). In 11 of 15 generations AR produced more males than females as compared to only 3 of 15 generations of male bias in SC. Results from 11 generations indicated that mean (± SE) pupal mortality in AR (24.48 ± 2.47%) was significantly (t_{10}=5.244, p<0.001) higher than SC (11.67 ± 1.16%). However, there were no significant differences in mortality between sexes for either AR (t_{10}=-1.138, p=0.284) or SC (t_{10}=-0.881, p=0.401).

Mating studies: Percent mating success during generations 9 and 12 was not significantly (t_1= -1.963, p=0.30) different between AR and SC (Fig. 2). Resistance

ratios for these generations were 36-fold and 122-fold, respectively (Anilkumar et al. 2008). Mating success in AR declined after achieving >100-fold resistance and there was always a 1.5 to 3-fold decrease in mating success for AR compared to SC. Further, significantly (t_{13}=-2.521, p=0.026) more SC females (26%) had multiple mating compared to AR (17%) (Table 2). SC males (1.23 ± 0.10) produced significantly (t_{13}=-5.058, p=0.001) more spermatophores compared to AR (0.58 ± 0.08).

Reciprocal crosses: Mating success between AR and SC was significantly ($F_{3,19}$=14.29, p=0.000) different (Table 3). Reciprocal crosses (AR♀ X SC♂, SC♀ X AR♂) with SC as male had significantly (p=0.046) higher mating compared to AR as male. There were no significant differences in mating between AR and reciprocal cross with AR as male (p=0.35); similarly between SC and SC as male in reciprocal cross (p=0.61). There were no significant differences in multiple mating between either parental strains or their reciprocal crosses ($F_{3,19}$=0.7, p=0.566). The number of spermatophores produced per male was significantly ($F_{3,19}$=3.804, p=0.028) different in AR and SC strains. However, when AR males were used in the reciprocal cross, no significant differences were observed in mean spermatophore/male compared with SC as male (p=0.074) and/or SC strain (p=0.099).

Fitness costs in AR on untreated diet:

Larval weight, duration and mortality: SC gained significantly ($F_{3,574}$=48.178, p=0.000) more weight in seven days compared with AR. Further, selection diet or regular diet did not affect larval weight in SC (Table 4). In contrast, AR on selection diet had significantly lower weight compared to when reared on regular diet. Significant

differences ($F_{3,571}$=124.01, p=0.000) existed between strains for larval duration regardless of diet tested; AR required one additional day to complete larval development compared with SC. Further, rearing larvae either on selection diet or regular diet for one week did not influence the total larval duration in either AR or SC. It is important to note that significantly ($F_{3,11}$=4.623, p=0.037) higher larval mortality was recorded in AR compared to SC, although larval mortality did not differ between selection and regular diet in either AR or SC.

Pupal weight, duration and mortality: Pupal weight of AR on regular diet varied significantly ($F_{3,503}$=25.402, p=0.000) from SC. Interestingly, AR pupal weight on selection diet was not different from SC. SC on regular diet recorded the shortest pupal duration which was significantly ($F_{3,492}$=39.425, p=0.000) different from SC on selection diet and AR on both diets. Production of morphologically abnormal adults in AR was significantly ($F_{3,11}$=14.281, p=0.001) increased (ca 6-fold) when compared with SC, which did not differ between selection diet and regular diet and had the most pronounced effect on fitness in relation to resistance.

Growth rate: During the initial seven days, weight gained per day by SC larvae was significantly ($F_{3,471}$=22.70, p=0.000) higher than AR, but there was no significant (AR: p=0.06, SC: p=0.30) difference between selection diet and regular diet (Table 5). The slowest growth rate (15.82 ± 0.54 mg/day) was observed when AR larvae were exposed to selection diet. After seven days, when both AR and SC were transferred to or continued on regular diet, growth rates were significantly different ($F_{3,471}$=12.34, p=0.000). During this time, the growth rate in SC did not differ significantly (p=0.684)

based on their previous exposure. However, initial exposure influenced the growth rate of AR larvae significantly (p=0.013). The slowest growth rate (28.89 ± 0.62 mg/day) after seven days was observed in AR when they were initially exposed to regular diet.

The difference in growth rate before and after seven days was significantly different ($F_{3,471}$=3.84, p=0.010), and the highest difference (15.44 ± 0.77 mg/day) was observed when AR from selection diet was shifted to regular diet (Table 5). Considering the change in growth rate on regular diet as 100% when SC was moved from selection to regular diet, growth rate increased by 107.35%. However, in a similar comparison, the growth rate increase in AR was 131.18%.

Fecundity and Fertility: Fertility and fecundity in AR on all types of diet could not be determined due to insufficient number of adults (result of high pupal mortality). In SC, the fecundity and fertility were not influenced by the initial seven days exposure to diet of different strengths (Table 4).

Fitness values after crossing AR with SC1: Larval mortality ($F_{1,5}$= 11.148, p=0.029), pupal weight ($F_{1,151}$= 15.426, p=0.000) and percentage malformed adults ($F_{1,5}$= 53.646, p=0.002) differed significantly between AR1 and SC1 (Table 6). However no significant differences were observed in larval weight after seven days ($F_{1,173}$= 1.599, p=0.208), and larval ($F_{1,151}$= 0.003, p=0.957) and pupal periods ($F_{1,104}$= 0.229, p=0.633).

Stability of resistance: Stability of resistance were conducted after only one generation of rearing on regular diet due to extremely high (range=40-80%) pupal mortality leading to the colony crashing. After removing AR from Cry1Ac selection (referred as AR-Unsel in Fig. 4) for one generation, mean (± SE) percent survivors (10.2 ± 1.7) was reduced

significantly (t_1=-7.78, p=0.016) compared to percent survivors (35.4 ± 1.54) when AR was under continuous selection (referred as AR-Sel in Fig. 4).

Discussion

In the present study, heritability (h^2) of resistance, stability of resistance and fitness were assessed in a laboratory selected Cry1Ac-resistant strain of *H. zea* (AR). Heritability (h^2) values initially increased and then decreased over generations, indicating the increase in the genetic homogeneity of the population and hence, resistance factor. At h^2= 0.123, AR could develop 10-fold resistance to Cry1Ac in 10 generations at 30% selection pressure, which is less than the number of generations predicted for tobacco budworm, *Heliothis virescens* (Tabashnik 1992); possible reasons for quicker resistance evolution are discussed in Anilkumar et al. (2008).

Fitness costs and the degree of dominance of fitness costs related to resistance determine the rate of resistance development (Carriére et al. 1994). In most studies, fitness costs were usually measured in the absence of the selection agent, presumably to approximate how long resistance would remain in the absence of field selection (Liu et al. 1999, Carriére et al. 2001a, 2001b; Bird and Akhurst 2004, 2005). This present study shows that under continuous selection AR had significantly higher pupal mortality, a male biased sex ratio, and decreased mating ability of moths compared to SC. Increased pupal mortality for *H. zea* was also reported when larvae originated from Bt-corn (Storer et al. 2001) and Bt-cotton (Jackson et al. 2004a) compared to their non-Bt counterparts. The sex ratio in SC (0.47 ± 0.01) was similar to five batches of larvae (0.48 ± 0.01) collected from non-Bt field corn (during 2006 and 2007) (KJA and WJM, unpublished

data). Further, the sex ratio of AR (0.51 ± 0.01) was similar to 167 larvae collected from a Bt-sweet corn field in 2006 (0.51) that were shown to be highly resistant to Cry1Ac toxin in the F_1 generation (KJA and WJM, unpublished data). Therefore, the male biased sex ratio in AR may be a result of resistance selection, suggesting higher susceptibility of females (De Lame et al. 2001, Shearer and Usmani 2001).

The magnitude of mating costs is expected to be influenced by factors such as mating history, life span, current and past population sizes (bottlenecks, founder effects), environmental conditions, and possibly interactions between these factors (Bird and Akhurst 2004). SC is the parental population of AR (Anilkumar et al. 2008), both colonies were reared in parallel; genetic inbreeding independent from Cry1Ac selection seems an unlikely cause. Even under conditions where there were significantly fewer adult AR compared to SC in a particular generation, the reduction in mating success for AR may not be linked to genetic inbreeding; AR in two generations (Aug-06 and Apr-07) had fewer (61 [30♂:31♀] and 58 [32♂:26♀]) adults but had increased (47 and 25% increase over previous generations) mating success past this potential bottleneck. Percent mating for SC was similar to moths collected from light traps (Hendricks et al. 1970) and lower compared to collections made from sweep net and/or blacklight traps (Latheef et al. 1991). Further, mating increased in AR1 F_1 adults, but was still significantly different from SC1. Additionally, AR1 F_2 adults had reduced mating compared to their parents and the mating success was similar to AR before being crossed to SC1. Therefore, reduced mating in AR may be due to Cry1Ac resistance and not necessarily inbreeding. Further, reciprocal crosses indicate significant mating costs in males as against females. Reduced

mating, mainly because of mating problems in males was also observed in Bt-resistant (selected using Dipel 2X) *P. xylostella* moths (Groeters et al. 1993).

Fitness costs associated with resistance in AR have been demonstrated in many life history traits when reared on untreated diet. Insects adopt different feeding strategies depending on the nutritional quality of the diet or host plants (Woods 1999). Here, AR larvae exposed to selection diet had increased growth rate when shifted to regular diet, and with an additional day they achieved pupal weights similar to SC. The increase in growth rate suggests increased feeding and/or higher assimilation rate, both of which may be due to an increased titer of digestive enzymes (Woods 1999). Interestingly, AR produced a higher percentage of normal adults when exposed to toxin in selection experiments than when reared on untreated diet. This may be due to 1) in the absence of selection, average fitness of individuals may decline due to the accumulation of deleterious mutations (Lynch et al. 1999), 2) elimination of higher percentage of insects with lower fitness (WJM, unpublished data), 3) AR has been selected with Cry1Ac toxin for 26 generations on selection diet containing 20% more water and therefore 20% less nutrients; AR have adapted to these conditions, as would be expected for a highly polyphagous insect (Woods 1999), and 4) exposure to Cry1Ac toxin affects the physiology of the insects such that they obtain higher fitness values from the increased nutrition of *Bt* (Sayyed et al. 2003); or other factors. In the confused flour beetle, *Tribolium confusum*, reduced fitness was observed in a selection-free population compared to population with more intense selection (Lomnicki and Jasienski 2000).

AR required 27 days for adult eclosion on regular diet, compared to 25 days for SC. This resulted in developmental asynchrony (Liu et al. 1999, Bird and Akhurst 2004, 2005) as has been observed in other insects, and may lead to assortative mating (Liu et al. 1999) thereby accelerating the rate of resistance evolution. This should not be relative to *H. zea*, because peak mating occurs on the 4th night after emergence (KJA, unpublished data, Shorey et al. 1968). Assortative mating fitness differences will favor restoration of susceptibility in the absence of insecticide treatments (Groeters et al. 1993). Caprio (2001), using a spatially descriptive model, found that non-random mating along with non-random oviposition could significantly delay resistance evolution.

Long term rearing of insects in the laboratory results in reduced fitness mainly because of the founder effect and/or inbreeding (Roush and Daly, 1990). Therefore, AR was crossed to SC1 to ascertain whether observed reduction in fitness was linked to resistance (Bird and Akhurst 2004) and to save AR from extinction. Even after one generation of crossing with SC1, AR1 had increased fitness costs while feeding on Cry1Ac-treated and untreated diet. These observations strongly suggest that they may be linked to Cry1Ac-resistance as reported in Cry1Ac-resistant *H. armigera* after four crosses with a susceptible strain (Bird and Akhurst 2004). Although AR1 appears similar to AR in terms of survivorship at 500 µg Cry1Ac toxin/g diet, the RR for AR1 is lower than for AR because of increased tolerance of SC1 (LC_{50}=31.25 µg/g diet) compared to SC (LC_{50}=9-15µg/g diet, Anilkumar et al. 2008). This increased tolerance to Cry1Ac in SC1 may also come with fitness costs that were reflected in larval weight, larval and

pupal period not differing between AR1 and SC1; these fitness costs may not be linked to Cry1Ac-resistance.

Resistance in AR was not stable; after one generation of rearing on regular diet AR lost a significant amount of resistance. Similar unstable resistance (from >500-fold to >74-fold) was also reported in Cry1C resistant *Spodoptera littoralis* (Muller-Cohn et al. 1996). However, *Bt* resistance was stable in *Spodoptera exigua* (Moar et al. 1995) and *Plodia interpunctella* (343-R) (McGaughey and Beeman 1988). Both stable and unstable *Bt* resistance was observed in *P. xylostella* (Ferre and Rie 2002). Unstable Cry1Ac resistance in AR may help in understanding observed reductions in the LC_{50} values of field collected populations which had elevated LC_{50} values in $F_{1,}$ but declined rapidly during laboratory colonization (R. Luttrell, Univ. of Arkansas, personal communication). The reduction in resistance may be linked to fitness costs and/or accumulation of deleterious mutations (Lynch et al. 1999).

Contrary to the initial expectations of rapid evolution of *H. zea* resistance to Bt-cotton (Harris 1991, Roush 1997), there are no reports of field control failure(s) after more than a decade of Bollgard® and Bt corn use (Ali et al. 2006, Moar and Anilkumar 2007). This lack of observed field-evolved resistance occurred despite widespread use of Bollgard® and Bt corn during this period. There are a number of mitigating factors which might have contributed to the delay of this pest developing resistance to Bollgard®; 1) the "high dose plus structured refuge", 2) use of pyrethroid insecticide(s) to control bollworms during high infestations *Bt*-cotton (Anilkumar et al. 2008), 3) substantial temporal and spatial bollworm production from non-cotton crop hosts (Gustafson et al.

2006), and 4) fitness costs associated with elevated Cry1Ac resistance or tolerance as shown in these studies and others. The latter has likely played the most important role in delaying resistance development in bollworms. Indeed, Gustafson et al. (2006) incorporated assumed values (none, low and moderate) of fitness costs associated with either recessive or additive inheritance for resistance in modeling the effect of non-Bt crops as effective refuges for IRM. For the Mississippi region, this model predicted a delay in resistance for 6-10, 7-14 and >30 yr with none, low and moderate fitness costs, respectively. We believe that this model has been validated and may indicate even greater delays in resistance development if results from this study (moderate to high fitness costs) are incorporated in their model, assuming laboratory generated results are applicable to the field. Recently, Tabashnik et al. (2008) reported field-evolved Cry1Ac-resistance in *H. zea* based on laboratory assays of different strains collected from the field before (Luttrell et al. 1999) and after (Ali et al. 2006) commercial cultivation of Bt cotton. However, the conclusions of Tabashnik et al. (2008) are directly contradicted by the lack of observed changes in Bt cotton efficacy against *H. zea* and the lack of confirmed Bt resistant *H. zea* populations in the EPA-mandated Bt resistance monitoring program. We believe that the data presented in this present manuscript help to explain why field-evolved resistance has not yet occurred in this pest.

Acknowledgements

Authors thank N. Adams, Monsanto Co., Union City, TN for providing *H. zea*; E. van Santen, Dept. of Agronomy and Soils, and A. Appel, Dept of Entomology, Auburn

University for providing statistical guidance; and G. Head and S. Sivasupramaniam, Monsanto Co., St. Louis, MO, and J. Chandrashekhar for review of this manuscript. This research was supported by the U.S. Department of Agriculture, and Cotton Incorporated.

References cited:

Ali, M.I., R.G. Luttrell, and S. Y. Young. 2006. Susceptibilities of *Helicoverpa zea* and *Heliothis virescens* (Lepidoptera: Noctuidae) populations to Cry1Ac insecticidal protein. J. Econ. Entomol. 99:164–175.

Anilkumar, K. J. and W. J. Moar. 2006. Differential rate of resistance development to *Bt* Cry1Ac in cotton bollworm, *Helicoverpa zea* (Boddie) when selected using MVP II and activated toxin.Pp1478-1483. *In* Proc. Beltwide Cotton Conf. National Cotton Council, Memphis, TN.

Anilkumar, K. J., A. Rodrigo-Simon, J. Ferre, M. Pusztai-Carey, S. Sivasupramaniam, and W. J. Moar. 2008. Production and characterization of *Bacillus thuringiensis* Cry1Ac-resistant cotton bollworm *Helicoverpa zea* (Boddie). Appl. Environ. Microbiol. 74: 462-469.

Bird, L.J., and R. J. Akhurst. 2004. Relative fitness of Cry1A-resistant and -susceptible *Helicoverpa armigera* (Lepidoptera: Noctuidae) on conventional and transgenic cotton. J. Econ. Entomol. 97: 1699-709.

Bird, L.J. and R. J. Akhurst. 2005. Fitness of Cry1A-resistant and -susceptible *Helicoverpa armigera* (Lepidoptera: Noctuidae) on transgenic cotton with reduced levels of Cry1Ac. J. Econ. Entomol. 98:1311–1319.

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. 96:137-142.

Caprio, M. A. 2001. Source-sink dynamics between transgenic and non-transgenic habitats and their role in the evolution of resistance. J. Econ. Entomol. 94:698-705.

Carriére Y, J.-P. Deland, D.A.Roff and C. Vincent. 1994. Life-history costs associated with the evolution of insecticide resistance. Proc. R. Soc. Lond. B. 258:35-40.

Carriére Y, C. Ellers-Kirk, Y.B. Liu, M.A. Sims, A.L. Patin, T. J. Dennehy, and B.E. Tabashnik. 2001a. Fitness Costs and maternal effects associated with resistance to transgenic cotton in the pink bollworm (Lepidoptera: Gelechiidae). J. Econ. Entomol. 94: 1571–1576.

Carrière, Y. Ellers-Kirk, C. Patin, A. L. Sims, M. A. Meyer, S. Liu, Y. B. Dennehy, T. J. and B. E.Tabashnik. 2001b. Overwintering cost associated with resistance to transgenic cotton in the pink bollworm (Lepidoptera: Gelechiidae). J. Econ. Entomol.94: 935-941

Carrière Y, C. Ellers-Kirk, R.W. Biggs, M.E. Nyboer, Unnithan GC, T.J. Dennehy and B.E. Tabashnik. 2006. Cadherin-based resistance to *Bacillus thuringiensis* cotton in hybrid strains of pink bollworm: fitness costs and incomplete resistance. J. Econ. Entomol. 99:1925–1935.

De Lame F. M., J. J Hong, P. W Shearer, and L. B Brattsten. 2001. Sex-related differences in the tolerance of Oriental fruit moth (*Grapholita molesta*) to organophosphate insecticides. Pest Manag. Sci. 57: 827 – 832.

Ferre, J. and J.V. Rie. 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. Ann. Rev. Entomol. 47: 501-533.

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

Groeters, F.R., B. E. Tabashnik, N. Finson, and M. W. Johnson. 1993. Resistance to *Bacillus thuringiensis* affects mating success of the diamondback moth (Lepidoptera: Plutellidae). J. Econ. Entomol. 86: 1035-1039.

Gustafson D.I., G. P. Head, and M. A. Caprio. 2006. Modeling the impact of alternative hosts on *Helicoverpa zea* adaptation to Bollgard cotton. J. Econ. Entomol. 99:2116–2124.

Hardy, C. W. 2002. Sex ratios: Concepts and research methods. Cambridge University Press, Cambridge, United Kingdom. 438pp.

Harris, M.K. 1991. *Bacillus thuringiensis* and pest control. Science. 253:1075.

Hendricks, D.E., H. M. Graham, and A.T. Fernandez. 1970. Mating of female tobacco budworms and bollworms collected from light traps. J. Econ. Entomol. 63: 1228-1321.

Higginson D. M., S. Morin, M. E. Nyboer, R. W. Biggs, B. E. Tabashnik, and Y. Carrière. 2005 Evolutionary trade-offs of insect resistance to *Bacillus thuringiensis* crops: fitness cost affecting paternity. Evolution 59: 915–920.

Jackson, R.E., J.R. Jr. Bradley, J.W. Van Duyn, and F. Gould. 2004a. Comparative production of *Helicoverpa zea* (Lepidoptera: Noctuidae) from transgenic cotton

expressing either one or two *Bacillus thuringiensis* proteins with and without insecticide oversprays. J. Econ. Entomol. 97:1719-1725.

Jackson, R.E., J.R. Bradley, Jr and J.W. van Duyn. 2004b. Performance of feral and Cry1Ac-selected *Helicoverpa zea* (Lepidoptera: Noctuidae) strains on transgenic cottons expressing one or two *Bacillus thuringiensis* spp. *kurstaki* proteins under greenhouse conditions. J. Entomol. Sci. 39: 46-55.

Latheef, M.A., J.D. Lopez, Jr. and J.A. Witz. 1991. Reproductive condition of female corn ear worm (Lepidoptera: Noctuidae) moths from sweep net and blacklight trap collections in corn. Environ. Entomol. 20:736-741.

Liu, Y.B., B. E. Tabashnik, T. J. Dennehy, A. L. Patin, and A. C. Bartlett. 1999. Development time and resistance to Bt crops. Nature 400: 519.

Lomnicki, A., and M. Jasienski. 2000. Does fitness erode in the absence of selection? An experimental test with *Tribolium*. J. Hered. 91:407–411.

Luttrell, R.G., L. Wan, and K. Knighten. 1999. Variation in susceptibility of noctuid (Lepidoptera) larvae attacking cotton and soybean to purified endotoxin proteins and commercial formulations of *Bacillus thuringiensis*. J. Econ. Entomol. 92:21-32.

Lynch M., J. Blanchard, D. Houle, T. Kibota, S. Schultz, L. Vassilieva, and J. Willis. 1999. Perspective: spontaneous deleterious mutation. Evolution. 53:645–663.

MacIntosh, S. C., T. B. Stone, S. R. Sims, P. L. Hunst, J. T. Greenplate, P. G. Marrone, F. J. Perlak, D. A. Fischhoff, and R. L. Fuchs. 1990. Specificity and efficacy of purified *Bacillus thuringiensis* proteins against agronomically important insects. J. Invertebr. Pathol. 56: 258-66.

McGaughey, W.H., and R. W. Beeman. 1988. Resistance to Bacillus thuringiensis in colonies of Indianmeal moth and almond moth (Lepidoptera: Pyralidae). J. Econ. Entomol. 81:28–33.

Moar, W. J. and K. J. Anilkumar. 2007. The power of the pyramid. Science. 318: 1561-1562.

Moar, W.J., M. Pusztai-Carey, H.V. Faassen, D. Bosch, R. Frutos, C. Rang, K. Luo and M. J. Adang. 1995. Development of *Bacillus thuringiensis* CryIC resistance by *Spodoptera exigua* (Hubner) (Lepidoptera: Noctuidae). Appl. Environ. Microbiol. 61: 2086-2092.

Muller-Cohn, J., J. Chaufaux, C. Buisson, N. gilois, V. Sanchis and D. Lereclus. 1996. *Spodoptera littoralis* (Lepidoptera: Noctuidae) resistance to Cry1C and cross resistance to other *Bacillus thuringiensis* crystal toxins. J. Econ. Ent. 89: 791-797.

Roush, R. T. 1997. Bt-transgenic crops: just another pretty insecticide or a chance for a new start in resistance management? Pestic. Sci. 51:328-334.

Roush, R. T. and Daly, J. C. 1990. The role of population genetics in resistance research and management. In *Pesticide resistance in arthropods* (ed. R. T. Roush & B. E. Tabashnik), pp. 97-152. New York: Chapman & Hall.

SAS Institute. 2003. SAS software, release 9.1. SAS Institute, Cary, NC.

Sayyed, A. H., and D. J. Wright. 2001. Fitness costs and stability of resistance to *Bacillus thuringiensis* in a field population of the diamondback moth *Plutella xylostella* L. Ecol. Entomol. 26: 502–508.

Sayyed, A.H., H. Cerda, and D.J.Wright. 2003. Could Bt transgenic crops have nutritionally favourable effects on resistant insects? Ecol. Lett. 6:167–169.

Shearer, P.W., and K.A. Usmani. 2001. Sex-related response to organophosphorus and carbamate insecticides in adult Oriental fruit moth, *Grapholita molesta*. Pest Manag. Sci. 57(9): 822-826

Shorey, H. H., S. U. McFarland, and L. K. Gaston. 1968. Sex pheromones of noctuid moths. XIII. Changes in pheromone quantity as related to reproductive age and mating history, in females of seven species of Noctuidae (Lepidoptera). Ann. Entomol. Soc. Amer. 61:372-376.

Sivasupramaniam, S., W. J. Moar, L.G. Ruschke, J. A. Osborn, C. Jiang, J. L. Sebaugh, G.R. Brown, Z.W. Shappley, M.E. Oppenhuizen, J.W. Mullins, and J. T. Greenplate 2008. Toxicity and characterization of cotton expressing *Bacillus thuringiensis* Cry1Ac and Cry2Ab2 proteins for control of lepidopteran pests. J. Econ. Entomol. 101: 546-554

SPSS. 2006. SPSS Base 14.0 for Windows User's Guide. SPSS Inc., Chicago IL.

Storer N.P., J.W. Van Duyn, and G. G. Kennedy. 2001. Life history traits of *Helicoverpa zea* (Lepidoptera: Noctuidae) on Non-Bt and Bt transgenic corn hybrids in eastern North Carolina. J. Econ. Entomol. 94: 1268–1279

Storer, N. P., S. L. Peck, F. Gould, J. W. Van Duyn, and G. G Kennedy. 2003a. Spatial processes in the evolution of resistance in *Helicoverpa zea* (Lepidoptera: Noctuidae) to Bt transgenic corn and cotton in a mixed agroecosystem: a biology-rich stochastic simulation model. J. Econ. Entomol. 96:156-172.

Storer, N. P., S. L. Peck, F. Gould, J. W. Van Duyn, and G. G. Kennedy. 2003b. Sensitivity analysis of a spatially explicit stochastic simulation model of the evolution of resistance in *Helicoverpa zea* (Lepidoptera: Noctuidae) to Bt transgenic corn and cotton. J. Econ. Entomol. 96:173-187.

Tabashnik, B. E. 1992. Resistance risk assessment: Realized heritability of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae), tobacco budworm (Lepidoptera: Noctuidae), and Colorado potato beetle (Coleoptera: Chrysomelidae). J. Econ. Entomol. 85:1551–1559.

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

Tabashnik, B.E., A.J. Gassmann, D.W. Crowder and Y. Carriére. 2008. Insect resistance to Bt crops: evidence versus theory. Nat. Biotechnol. 26: 199 – 202.

Woods, H. A. 1999. Patterns and mechanisms of growth of fifth-instar *Manduca sexta* caterpillars following exposure to low-or high-protein food during early instars. Physiol. Biochem. Zool. 72:445-454.

Table 1. Heritability (h^2) and resistance risk assessment for resistance to Cry1Ac in *H. zea*

N[a]	LC_{50} values		RR[b]	R[c]	Slope		S[d]	h^{2e}	G[f]
	Initial[g]	Final[h]			Initial	Final			
4	8.89	107.64	12.11	0.271	1.71	1.42	0.860	0.315	3.69
7	8.94	321.22	35.93	0.222	1.93	1.89	0.554	0.401	4.50
11	11.82	1,450.00	122.7	0.190	1.76	1.76	0.742	0.256	5.27
19	15.00	1,390.00	92.67	0.104	2.31	1.39	0.840	0.123	9.66

LC_{50}, resistance ratio and slope values are from Anilkumar et al. 2008

[a]N: Number of generations of continuous selection using *Bt* Cry1Ac treated diet; [b]RR: Resistance ratio; [c]R: response to selection; [d]S: Selection differential; [e]h^2: heritability; [f]G: Resistance risk = Number of generations required for 10-fold increase in resistance; [g]Initial: LC_{50} for unselected parental strain; [h]Final: LC_{50} for resistant strain measured after number of generations of selection

Table 2. Reproductive propensity (mean ± SE) of Cry1Ac-resistant (AR) and susceptible (SC) strains of *H. zea* during selection and rearing, respectively[a].

Strains	N[b]	% mating	% multiple mating	# of Spermatophore /male
AR	2066	40.27 ± 5.18	16.76 ± 3.40	0.58 ± 0.08
SC	1820	71.85 ± 3.53	26.01 ± 2.34	1.23 ± 0.10
t-Test results		$t_{13,1}$= -6.468, p=0.000	$t_{13,1}$= -2.521, p=0.026	$t_{13,1}$=-5.628, p=0.000

[a]Results are from 14 generations of observations; [b]N: Number of moths

Superscript letters after means within a column indicate significant differences at $p<0.05$ level by Tukey's test

Table 3. The reproductive (mean ± SE) success in a Cry1Ac-resistant (AR), susceptible (SC) and their reciprocal crosses[a]

Strain/cross	N[b]	% mating	% multiple mating	Spermatophore /male
AR	960	29.48 ± 2.98[a]	12.04 ± 1.93	0.41 ± 0.13[a]
SC	815	66.29 ± 3.65[b]	21.60 ± 4.43	1.01 ± 0.39[b]
AR ♀ X SC ♂	140	58.48 ± 3.98[b]	20.19 ± 5.52	1.04 ± 0.32[b]
SC ♀ X AR ♂	143	40.24 ± 7.38[a]	16.10 ± 7.25	0.53 ± 0.30[ab]
F-Test results		$F_{3,19}$=14.29	$F_{3,19}$=0.700	$F_{3,19}$=5.772
		p=0.000	p=0.566	p=0.007

[a]Results are from five generations of experiments, [b]N: Number of moths

Superscript letters after means within a column indicate significant differences at $p<0.05$ level by Tukey's test

Table 4. Fitness parameters (mean ± SE) for Cry1Ac-resistant (AR) and susceptible (SC) strains of *H. zea*[a]

Life-history trait	AR		SC	
	Regular diet	Selection diet[b]	Regular diet	Selection diet
Larval weight in 7D (mg)	109.48 ± 3.22[b]	91.04 ± 3.89[a]	144.58 ± 3.95[c]	131.99 ± 4.48[c]
Larval duration (d)	15.01 ± 0.09[b]	15.20 ± 0.11[b]	13.94 ± 0.11[a]	14.09 ± 0.13[a]
Larval mortality (%)	18.23 ± 2.46[b]	12.85 ± 2.11[ab]	5.04 ± 0.63[a]	10.07 ± 3.91[ab]
Pupal weight (mg)	347.70 ± 4.22[a]	355.41 ± 3.37[ab]	375.32 ± 3.34[bc]	368.4 ± 3.67[bc]
Pupal duration (d)	12.02 ± 0.12[b]	12.19 ± 0.12[b]	11.39 ± 0.1[a]	11.82 ± 0.1[b]
Malformed adults (%)	74.38 ± 13.46[b]	71.10 ± 9.49[b]	13.87 ± 1.88[a]	16.10 ± 6.12[a]
Number of eggs	NA[c]	NA	653.74 ± 51.58	583.26 ± 68.53
Hatching (%)	NA	NA	85.29 ± 2.61	86.60 ± 3.12

Superscript letters after means within a row indicate significant differences at $p<0.05$ level by Tukey's test

[a]Results are from 160 larvae; [b]Selection diet (regular diet + 20% water [used for the purpose of adding *Bt* proteins into regular diet in resistance selection experiments]

[c]NA: Not available, experiments were not continued due to higher percentage of malformed adults

Table 5. Growth rate (mean ± SE) for Cry1Ac-resistant (AR) and susceptible (SC) strains of *H. zea* on different strengths of diet[a]

Strains	Diet[b]	Growth rate (mg/day)		
		During 7D	After 7D	Difference
SC	RD	21.09 ± 0.53^{b}	32.93 ± 0.56^{c}	11.83 ± 0.81^{a}
	SD	20.39 ± 0.60^{b}	33.09 ± 0.61^{c}	12.70 ± 0.85^{a}
AR	RD	17.12 ± 0.44^{a}	28.89 ± 0.62^{a}	11.77 ± 0.78^{a}
	SD	15.82 ± 0.54^{a}	31.07 ± 0.51^{b}	15.44 ± 0.77^{b}
F-test results		$F_{3,471}=22.70$, $p=0.000$	$F_{3,471}=12.34$, $p=0.000$	$F_{3,471}=3.84$, $p=0.010$

[a]Results from 160 larvae: [b]RD: Regular diet, SD: Selection diet (RD + 20% water [used for the purpose of adding Bt proteins into regular diet])

Superscript letters after means within a column indicate significant differences at the $p<0.05$ level by Fisher's least significant differences

Table 6. Fitness parameters (mean ± SE) for Cry1Ac-resistant (AR1) and susceptible (SC1) strains of *H. zea* after crossing AR with SC1[a]

Life-history trait	AR1	SC1
Larval weight in 7D (mg)	95.91 ± 6.38	88.99 ± 6.43
Larval duration (d)	15.14 ± 0.17	15.24 ± 0.18
Larval mortality (%)	22.70 ± 3.55[b]	9.69 ± 1.62[a]
Pupal weight (mg)	359.06 ± 7.12[a]	391.72 ± 5.11[b]
Pupal duration (d)	11.59 ± 0.20	11.72 ± 0.14
Malformed adults (%)	60.81 ± 6.49[b]	10.77 ± 2.14[a]

[a]Results are from 96 larvae

Superscript letters after means within life history traits indicate significant differences at the $p<0.05$ level by Tukey's test

Fig 1. Pupal sex ratio of AR and SC strains over time with selection and rearing, respectively

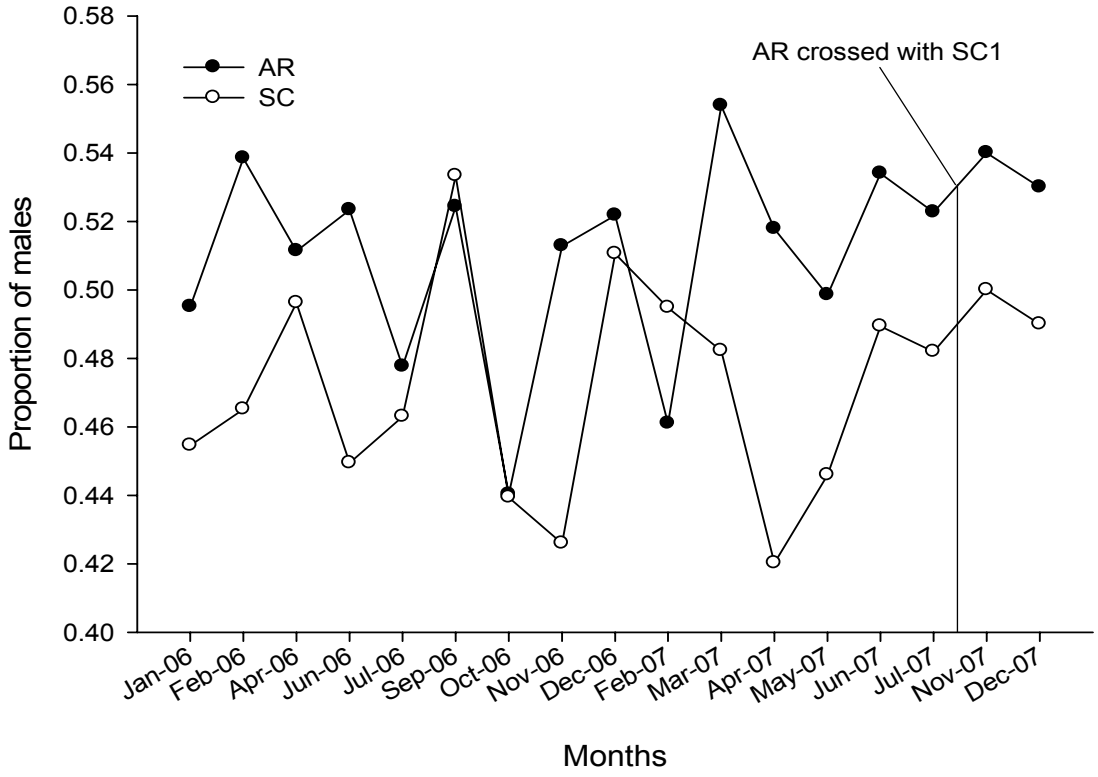


Fig 2. Mating success in AR and SC over time with selection and rearing, respectively

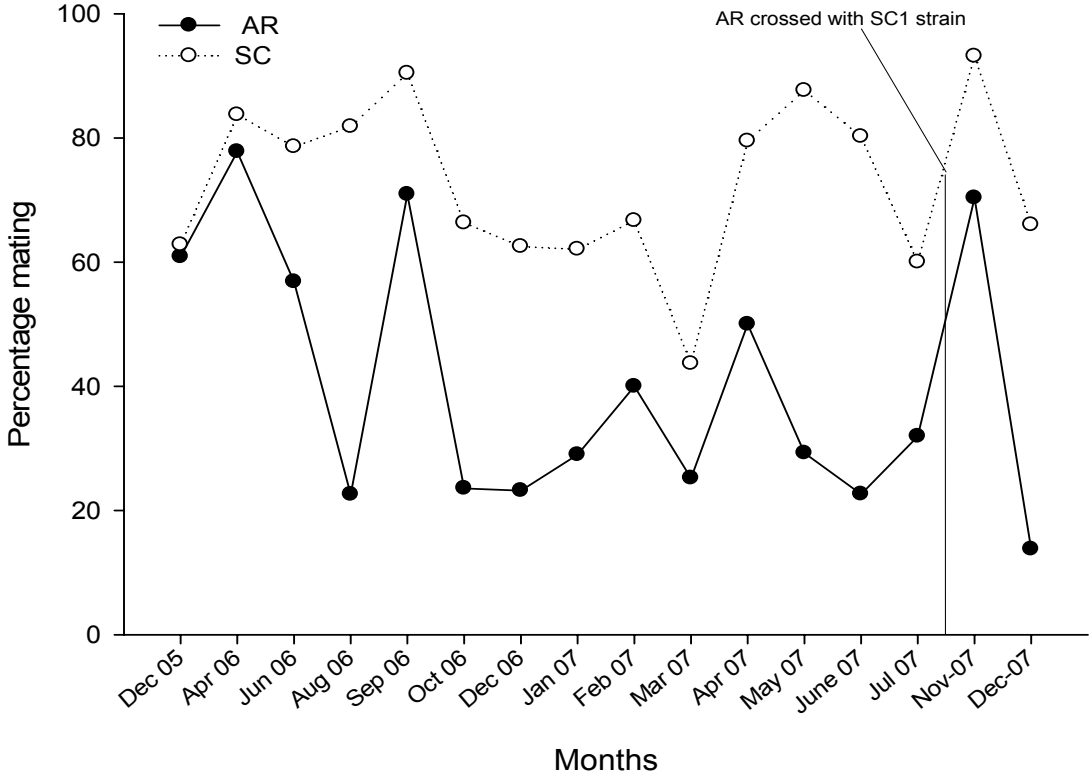


Fig 3. Growth rate differences in AR and SC, when larvae were reared on regular diet after exposing to regular diet and selection diet (20% diluted regular diet) for initial seven days.

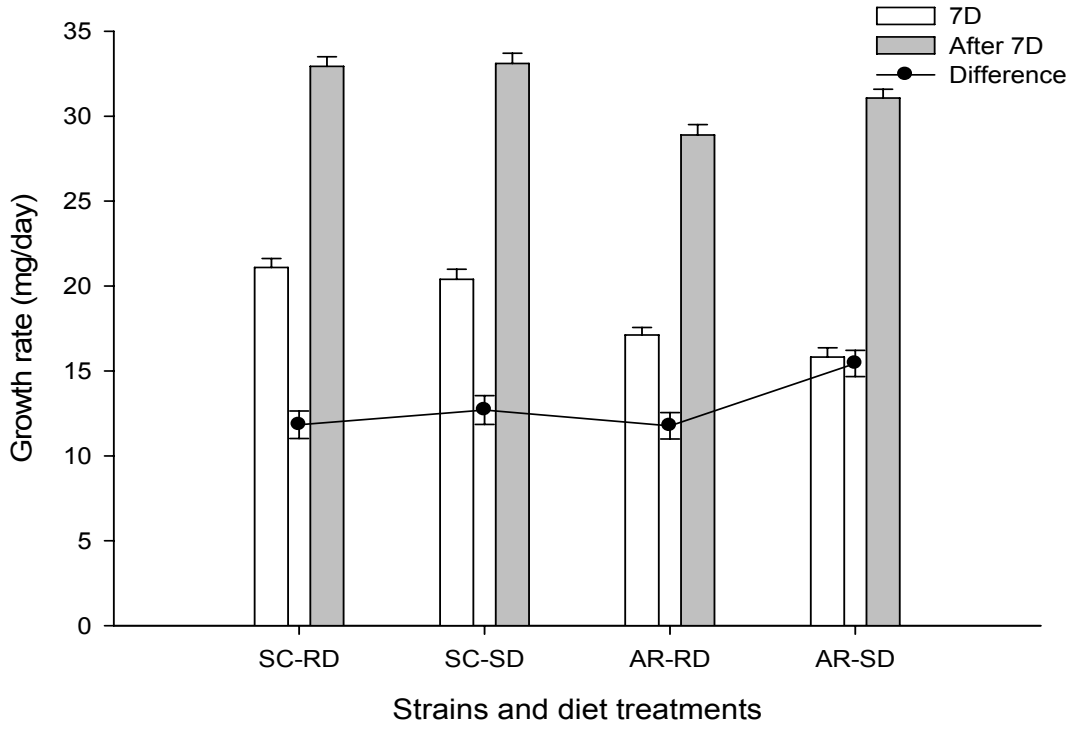
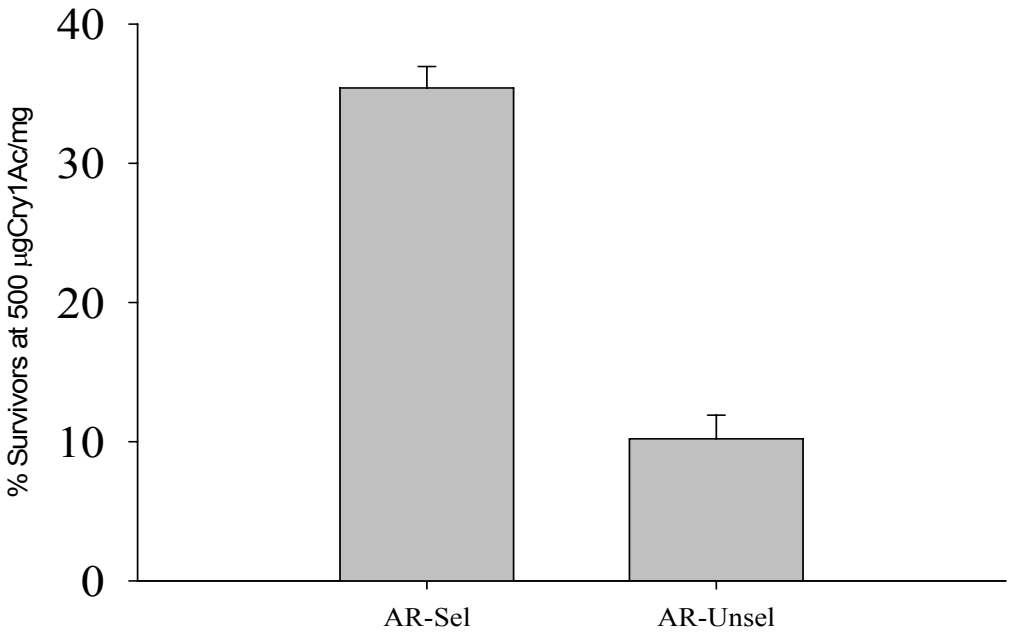


Fig 4. Percent survivors of Cry1Ac-resistant *H. zea* at 500 μg/g Cry1Ac toxin when selected continuously at 500 μg/g Cry1Ac toxin compared to when removed from selection for one generation.



CHAPTER 4:

SYNERGISTIC INTERACTIONS BETWEEN CRY1AC AND GOSSYPOL LIMIT SURVIVAL OF CRY1AC-RESISTANT *HELICOVERPA ZEA* (LEPIDOPTERA: NOCTUIDAE) ON *BT* COTTON

ABSTRACT Results of laboratory experiments with field-cultivated cotton squares demonstrate that Cry1Ac-resistant *Helicoverpa zea* (Boddie) cannot complete larval development on *Bt* cotton, despite being more than 150-fold resistant to Cry1Ac. Diet incorporation bioassays were conducted with Cry1Ac (15 μg/g), gossypol (0.15%), their 2, & 4-fold dilutions; and 4% corn and cotton powders in the presence and absence of Cry1Ac (15 μg/g) to help determine the contribution of these compounds to the results observed using *Bt* cotton. Cry1Ac toxin (15 μg/g) was significantly more lethal to susceptible compared to resistant strain however no differential susceptibility was observed in strains for 0.15% of gossypol. Combinations of Cry1Ac and gossypol, 4% cotton or corn powders were synergistic against resistant, but not to susceptible strain. Gossypol concentration in individual larvae showed no significant differences between strains, or between gossypol alone and gossypol plus Cry1Ac. These results may help understand the inability of resistant strain to complete development on *Bt* cotton, and therefore may help explain the absence of field-evolved resistance to *Bt* cotton by *H. zea*.

Introduction

Bollworm, *Helicoverpa zea* (Boddie) has a naturally high tolerance to *Bacillus thuringiensis* (*Bt*) Cry1Ac compared to other target pests of *Bt* cotton in the US such as tobacco budworm *Heliothis virescens* (F.) and pink bollworm *Pectinophora gossypiella* (Saunders) (MacIntosh et al. 1990b, Sivasupramaniam et al. 2008). This relatively high tolerance coupled with preferential feeding on tissues expressing lower levels of *Bt* protein (Brickle et al. 2001) and toxin attenuation due to abiotic stress and plant phenology can result in complete larval development on Cry1Ac cotton, especially under high insect populations (Jackson et al. 2004a). Additionally, *H. zea* is also exposed to *Bt* Cry1Ab in Cry1Ab-expressing corn, which is similar in structure and function to Cry1Ac (Crickmore et al. 1998). It has been postulated that this high selection pressure increases the likelihood of resistance evolution (Gould 1998) and it is therefore not surprising that models predicted resistance development within 3-7 years (Harris 1991, Roush 1997). Furthermore, the capacity for *H. zea* to develop resistance to Cry1Ac has been demonstrated in laboratory-selected strains (Luttrell et al. 1999, Jackson et al. 2004b, Anilkumar et al. 2008a). However, contrary to these results and predictions, field-evolved resistance has not occurred with *H. zea* even after 12 years of commercial use of *Bt* cotton in the USA (Ali et al. 2008).

Although numerous Cry1Ac-resistant strains of *H. virescens* (Gould et al. 1992, 1995), *P. gossypiella* (Liu et al. 1999, Tabashnik et al. 2000), and *Helicoverpa armigera* (Hubner) (Akhurst et al 2003), have been developed in the laboratory, very few strains have been able to pupate and produce fertile adults on *Bt* cotton (Tabashnik et al. 2003,

Bird and Akhurst 2004, 2005). Of those Lepidopteran strains that could develop to fertile adults on *Bt* cotton, there is little correlation between the relative susceptibility of these strains to Cry1Ac, the level of Cry1Ac resistance in these resistant strains, and the relative survivorship of these resistant strains on *Bt* cotton (Liu et al. 1999, 2001a, 2001b, Akhurst et al. 2003, Tabashnik et al. 2003, Bird and Akhurst et al. 2004, 2005). Possible explanations for this low correlation include interactions of *Bt* proteins with secondary plant metabolites (Carrière et al. 2004), use of a form of *Bt* protein for resistance selection not exclusively found in *Bt* cotton (Liu et al. 1999, 2001a, 2001b, Tabashnik et al. 2000, Akhurst et al. 2003, Henneberry and Jech, 2007, Anilkumar et al. 2008a), increased consumption of plant tissues compared to consumption of artificial diet (Woods, 1999), loss of genes or a reduction in expression of compounds necessary to survive on cotton during laboratory rearing and *Bt* resistance selection, or fitness costs associated with *Bt* resistance (Tabashnik et al. 2003).

Anilkumar et al. (2008a) reported a population of *H. zea* that was selected for stable and moderately high levels of resistance to the Cry1Ac toxin in the laboratory. Because *H. zea* is the most tolerant to Cry1Ac, and arguably the most polyphagous of all target lepidopteran pests of *Bt* cotton expressing Cry1Ac in the US, research was conducted to determine the survivorship of Cry1Ac-resistant and susceptible *H. zea* on field-cultivated *Bt* and non-*Bt* (NBt) cotton squares. Further, this study also explored the interaction of Cry1Ac with gossypol, cotton powder and corn powder in artificial diet to help explain possible reasons for the higher than expected mortality observed for Cry1Ac-resistant *H. zea* in *Bt* cotton.

Materials and Methods

Insect strains: A laboratory susceptible colony of *H. zea* (SC) was established in September 2004 from a laboratory colony from Monsanto (Union City, TN). A resistant strain (AR) was the product of selecting SC for resistance by exposing individual neonates to an artificial diet containing 500 µg *Bt* Cry1Ac toxin/gram diet for 25 generations (Anilkumar et al. 2008a). Seven days after exposure to the Cry1Ac toxin, surviving molted larvae were transferred to an untreated diet and reared until pupation (Ali et al. 2006, Anilkumar et al. 2008a, Sivasupramaniam et al. 2008). Resistance was assessed at selected generations (Anilkumar et al. 2008a) and AR was >150-fold resistant (based on artificial diet bioassays) at the time when survivorship bioassays on *Bt* and non-*Bt* cotton squares were conducted.

Cry1Ac interactions with gossypol, cotton/corn powder: In order to avoid complete loss of AR due to fitness costs associated with Cry1Ac resistance selection and rearing in the laboratory for 26 generations (Anilkumar et al. 2008b), AR was crossed with a new Monsanto susceptible strain (from Union City, TN), SC1, resulting in AR1. SC1 had higher LC_{50} (31.25µg Cry1Ac toxin/g of diet) values compared to SC (8.89 - 15 µg Cry1Ac toxin /g of diet; Anilkumar et al. 2008a). Although both reciprocal crosses were attempted, only AR[♀] X SC1[♂] yielded a viable F_1 population due to mating costs associated with AR males (Anilkumar et al. 2008b). Because Cry1Ac resistance in *H. zea* is inherited as a co-dominant character (Burd et al., 2003, Anilkumar et al. 2008b), high levels of resistance (resistance ratio {RR}>50-fold compared SC1) was observed in F_1 (data not shown) confirming that AR1 was resistant to Cry1Ac toxin. AR1 was selected

at the regular selection concentration of Cry1Ac (500 µg Cry1Ac toxin/g of diet) for two generations (Anilkumar et al. 2008a).

Cry1Ac toxin: An *E. coli* strain expressing Cry1Ac protoxin from *B. thuringiensis* subsp. *kurstaki* strain HD-1 (provided by L. Masson, Biotechnology Research Institute, National Research Council, Montreal, Canada) was cultured, and the activated toxin prepared as indicated elsewhere (Moar et al. 1994, Pusztai-Carey et al. 1994).

Lyophilized leaf tissue powder studies: Lyophilized corn (LH198/LH172) and cotton (C312) leaf tissue powders were supplied by Monsanto.

Plant Studies

Cotton plants: *Bt*-cotton (DPL555) and the near isogenic non-*Bt* cotton (NBt) (DPL491) were planted at the Prattville Agricultural Research Unit, Alabama Agricultural Research Station, Prattville, AL. Planting dates for *Bt*- and NBt-cotton were April 23rd and 24th, 2007, respectively. Cotton plants were cultivated as per typical practices. NBt-cotton plants were treated with Imidacloprid at 1 oz/A on both July 13, and July 24, 2007 for aphid control and tissues were used five days after treatment for bioassays. Cotton plants were >85 days old when squares were harvested. Pin-head to midpoint-stage squares (7-14 D old) were collected, transported to the laboratory, and stored at 4-7 ^{0}C until needed, up to a maximum of 13 d.

Cry1Ac protein quantification: Beginning on the day of field collection, and on days when cotton squares were removed from refrigeration and used in bioassays, a random sample of 10 *Bt* squares was placed at -80 ^{0}C. After all bioassays were completed, all -80 ^{0}C samples were shipped to Monsanto (St. Louis, MO) for Cry1Ac protein quantification.

Square tissues were lyophilized, and Cry1Ac expression was determined using ELISA and compared against a positive *Bt* cotton standard as described in Greenplate (1999) and Sivasupramaniam et al. (2008). Three replicate assays were conducted for each sample.

Survival and development on squares: Individual neonates from both strains were placed on the outside of the square bracts on one square in a petri dish (35x10mm). Moist cotton was placed below squares to reduce desiccation, and squares were changed every three days (replacing larvae on bracts) until experiments were concluded. For both *Bt* and NBt-cotton tests, larvae and squares were transferred to 30 ml cups containing three-five squares when larvae reached 4^{th} instar, and rearing was continued until pupation. Observations on larval mortality and stadia were recorded beginning on the fourth day and subsequently at three day intervals. Larval weights were recorded after seven days and tests were continued until survivors reached pupation. Data on larval mortality, larval weight after seven days, and larval duration were recorded as discussed above. Thirty larvae from each strain were tested in each replication and experiments were repeated three times.

Gossypol studies

Effect of Cry1Ac and gossypol: Growth and development of AR1 and SC1 were evaluated in diet containing 3.75, 7.5, 15 μg Cry1Ac/g diet, or 0.0375, 0.075, 0.15% gossypol (95% in acetic acid crystals, Sigma, St. Louis, MO), and their 1:1 combination (at respective dilutions from the maximum concentration used); and were compared to an untreated control. Because gossypol was dissolved in 1.0 % Dimethylsulfoxide (DMSO), all treatments contained a final concentration of 1.0% DMSO. Gossypol and/or Cry1Ac

toxin were added when diet temperature was <60 ^{0}C, mixed thoroughly and poured into 128 well bioassay trays (CD-International, Pitman, NJ) at one gram per well. 0 - 16 h old neonates were transferred individually into each well and covered with ventilated covers. Bioassay trays were incubated at 27 ± 1 ^{0}C and RH 50% and a photoperiod of 14:10 (L:D) h. Larval mortality, instar and weight were recorded after seven days. Thirty two larvae were tested for each treatment and the experiment was replicated three times.

Gossypol quantification: Five larvae from each replication that survived after 7 days in bioassays above containing gossypol (gossypol alone, gossypol plus Cry1Ac) were weighed and placed individually in 30ml plastic cups containing no diet for 10-12 hours to allow for purging of gut contents. Insects were transferred individually to a microcentrifuge tube and frozen at -80 ^{0}C. Gossypol content per insect was determined using method by Orth et al. (2007)

Lyophilized leaf tissue powder studies

Effect of Cry1Ac and corn/cotton powder: The performances of AR1 and SC1 on 4% cotton powder, 15 μg Cry1Ac/g diet, and their 1:1 combinations were studied in two generations (six replications of 32 larvae/treatment). In one generation (three replicates of 16 larvae/treatment) both strains were evaluated for their susceptibility to 4% corn powder and its interaction with Cry1Ac (15 μg/g diet), and compared to the untreated control. The experimental procedure was similar as explained above except for the absence of DMSO. Further, larvae feeding on cotton powder alone and cotton powder plus Cry1Ac were assayed for gossypol content following the procedure discussed above.

Data analysis:

Plant Studies: Age of the plant at sampling was considered a fixed classification effect and duration of storage as a fixed effects covariate nested with age to analyze the effect of storage on Cry1Ac stability. The sole random effect in the model was replicate(age) and Proc Mixed predicted a separate intercept for each age class with the no intercept option (SAS Institute 2003).

Larval mortality was modeled as logistic regression with SAS Proc NLmixed using the binomial distribution function. The degrees of freedom for t-tests and confidence intervals were calculated as the number of group means minus the number of fitted parameters (Schabenberger and Pierce 2004). We used the CONTRAST statement to evaluate the statistical significance of toxin differences within strains and ESTIMATE to calculate $LT_{50,}$ and LT_{75} values plus associated 95% confidence intervals as well as the contrasts between strains.

The effect of diet on larval development (instars 3 and 4) was modeled with SAS Proc GLIMMIX with a binomial distribution function and the logit link function. Strain, treatment and their interactions were fixed effects and replicate the sole random effect.

Gossypol studies: For larval weight data, replicate x strain interaction means in response to toxin rate were modeled with SAS Proc NLmixed using an exponential decay model with a lower asymptotic limit and a normal distribution function. To improve the ability to detect otherwise small differences between strains and treatments, failure to molt to third instars was considered as mortality and analyzed as discussed above. Due to the relatively small effect of gossypol alone on mortality, lethal concentrations for Cry1Ac

and gossypol mixtures were estimated in terms of concentration of Cry1Ac. Effective concentrations (EC) were calculated similar to the calculation of lethal concentrations. EC_{50} is defined as the effective concentration of toxin which provides 50% weight reduction in a test population (Jalali et al. 2004).

The distribution of gossypol concentration per larva was right skewed; therefore, the data was analyzed using a generalized linear models framework utilizing SAS Proc GLIMMIX. The lognormal distribution function resulted in a symmetrical distribution of residuals. Strain and toxin were fixed effects class variables and toxin rate was treated as a fixed effects covariate. Differences among toxin x strain combinations were then predicted at toxin rates 375, 750, and 1,500 using the AT option of the LSmeans statement with the simulation adjustment to control the Type I error rate.

Interactions of Cry1Ac and gossypol were evaluated as described by Salama et al. (1984). Differences in observed mortality and theoretical mortality for the mixture of Cry1Ac and gossypol were analyzed using χ^2 tests. Interaction was considered 1) synergistic, if observed mortality was more than expected mortality coupled with significant chi square values, 2) additive, if observed mortality was more than expected mortality coupled with non significant chi square values, 3) antagonistic, if observed mortality was less than expected mortality coupled with significant chi square values.

Lyophilized leaf tissue powder studies: Larval mortality was modeled with SAS Proc GLIMMIX with a normal distribution function. The residual variance was modeled using the group option to account for heterogeneous variances among treatments. Larval weight and gossypol concentration/mg larva were modeled using the same procedure but

with lognormal distribution function, which was necessary as residuals under the normal assumption were extremely right-skewed. Treatment, strain and their interaction were treated as fixed effects. However random effects were different for each of data parameters. For larval weight, generation, replicate (generation) and their interactions with fixed effects. For larval mortality, generation and replicate (generation) were considered to be random effects. Least squares diet x strain interaction means were calculated. The slicediff (for larval weight and mortality), pdiff (gossypol concentration/larvae) and simulation options were employed to assess differences among strains and diets while controlling the Type I error rate. Analysis of synergism between Cry1Ac and cotton/corn powders were evaluated as explained above.

Results

Cry1Ac expression in squares: The concentration of Cry1Ac in *Bt* cotton squares quantified using ELISA were not significantly different from levels of Cry1Ac found in the positive control, DP50 Bollgard® squares (Fig 1). Cry1Ac expression was reduced significantly (F_5=248.75, $P < 0.0001$) after 86 days (July 18) after planting; and storage at 4-7 ^{0}C did not affect the stability of the Cry1Ac for all samples except the July 18th samples ($P = 0.0488$).

Survivorship of AR and SC on *Bt*-cotton and NBt-cotton: LT_{50} (lethal time for 50% mortality) values for AR (9.13 d) on *Bt* squares was significantly (P=0.0004) higher compared to SC (LT_{50} = 4.75 d). Similarly, LT_{75} values were significantly (P<0.0001) higher for AR compared to SC (Table 1). Contrastingly, there were no significant differences in LT_{50} values for either SC (23.18 d) or AR (22.33 d), larvae on NBt squares.

Larval weight after seven days was significantly different between Bt and NBt cotton tissues ($P < 0.0001$) but not between strains ($P = 0.4056$) and their interaction with tissues ($P = 0.1248$) (Table 2). Tissue ($P < 0.0001$) and its interaction ($P = 0.0278$) with strain but not the strain alone ($P = 0.0985$) had a significant effect on the number of larvae reaching third instar. The proportion of larvae reaching third instar differed significantly between AR and SC on *Bt* but not on non-Bt tissues and only AR reached fourth instar on *Bt* squares.

Effect of Cry1Ac and gossypol: LC_{50} (µg/g diet) values were significantly different between AR1 (17.56) and SC1 (7.07) for Cry1Ac. In the presence of gossypol, however, the LC_{50} for AR1 (10.04) was decreased substantially and not significantly different ($P = 0.05$) from SC1 on Cry1Ac alone (Table 3). AR1 (10.04) and SC1 (5.41) strains had significantly different LC_{50} values for the Cry1Ac and gossypol mixture. EC_{50} (µg/g diet) values for both AR1 (1,172) and SC1 (1,204) were similar for gossypol but were significantly higher compared to Cry1Ac (AR1=0.99, SC1=0.97) and their mixtures (AR1=1.35, SC1=0.94) (Table 4).

Gossypol concentration/larvae did not differ significantly between strains at any concentration (375, 750 and 1,500µg/g diet) of gossypol in the presence or absence of Cry1Ac (Fig. 3A). However, irrespective of strains, larvae feeding on 0.15% gossypol in the presence of 15 µg Cry1Ac/g of diet had significantly lower gossypol/mg body weight.

Effect of Cry1Ac and cotton/corn powder: Strain ($F_1=49.25$, $P < 0.0001$), treatment ($F_5=672.10$, $P < 0.0001$), and the strain x treatment interaction ($F_5=44.27$, $P < 0.0001$) had a significant effect on larval mortality as measured by failure to molt to third instar

(Table 5). Significant differences between strains were observed in Cry1Ac (P < 0.0001), corn powder (P = 0.0065) and their mixtures (P = 0.0002). However, no significant differences were observed between strains in untreated diet (P = 0.9333), 4% cotton powder in the presence (P = 0.0506), or absence (0.2616) of Cry1Ac treatments. The mortality in mixtures of corn or cotton powders with Cry1Ac were significantly (P < 0.0001) different in AR1 even though AR1 was not (P = 0.6680) differentially susceptible to either cotton or corn powder. However, no such differences were observed in SC1.

Gossypol concentration/larvae did not differ significantly between strains (F_1=0.0, P = 0.9853) and its interaction (F_2=0.70, P = 0.5010) with treatment. However, larvae feeding on different treatments had significantly (F_2=243.84, P < 0.0001) different levels of gossypol (Fig. 3B).

Treatments (F_5=244.01, P < 0.0001), strains (F_1=17.14, P = 0.0005) and their interactions (F_5=16.85, P < 0.0001) influenced larval weight significantly (Fig 4). AR1 and SC1 larval weights differed significantly in Cry1Ac (P < 0.0001), and its mixture with 4% corn powder (P = 0.0027) but not in other treatments.

Synergistic interactions: Considering failure to molt to third instar as mortality, significant synergistic interactions of Cry1Ac with both corn and cotton powders and gossypol were observed only for AR1 compared to SC1 (Table 6). There was only an additive interaction at lower levels of Cry1Ac and gossypol in the diet. Further, the level of synergism varied between corn and cotton powders with Cry1Ac.

Discussion

ELISA results support the conclusion that >150-fold Cry1Ac-resistant *H*. zea (AR) would not be able to complete development on *Bt* cotton squares in commercial fields. ELISA results are particularly important for this study as Prattville, AL, sustained one of its worst droughts (summer 2007) in history, documenting that *Bt* cotton can still produce an efficacious amount of Cry1Ac toxin even under drought conditions. Mean Cry1Ac expression was reduced by 45% from 86 to 124 d after planting which is similar to published results by Greenplate (1999). Refrigeration of harvested squares at 4-7 ^{0}C for up to 13 d did not result in a significant reduction in Cry1Ac levels. These results may help future investigations in which *Bt* cotton squares will need to be refrigerated for extended periods prior to use.

Our results support the use of Cry1Ac toxin for resistance selection, and that this selection can confer partial resistance in *H. zea* to *Bt* cotton that expresses full-length protoxin although some to most may have been processed to active toxin by the time *Bt* protein is ingested (Anilkumar et al. 2008a, Gao et al. 2006, Li et al. 2007). Evaluation of survivorship using squares was justified as young buds (pinhead square) and fruiting structures (squares, flowers, bracts and bolls) are the preferred oviposition and feeding sites for *H. zea* (Torres and Ruberson 2006). Increased survivorship of AR on *Bt* cotton could be attributed to >150-fold level of resistance to Cry1Ac toxin developed in the laboratory (Anilkumar et al. 2008a) and also the increased feeding of AR larvae on bracts compared to SC (KJA and WJM, unpublished data), which express lower levels of toxin compared to other square tissues (Sivasupramaniam et al. 2008).

H. zea is >100-fold less susceptible to Cry1Ac than *P. gossypiella* (LC_{50}=0.1 µg/g) (Anilkumar et al. 2008a, Sivasupramaniam et al. 2008, Liu et al. 2001b). Therefore, AR would be expected to survive on Bollgard®, and at RR's significantly less than those deemed necessary for a Cry1Ac-resistant insect such as *P. gossypiella* (>100-fold and >3,100) (Liu et al. 1999, 2001a, Tabashnik et al. 2003). However, AR could not develop to pupation on *Bt* cotton, even with >150-fold resistance to Cry1Ac toxin (based on artificial diet bioassays). Interestingly, Cry1Ac-resistant *H. armigera* have been shown to survive on *Bt* cotton and produce fertile adults (Bird and Akhurst 2004, 2005). Although *H. zea* (Anilkumar et al. 2008a, MacIntosh et al. 1990b) and *H. armigera* (Akhurst et al. 2003) have similar baseline susceptibility, there are many reports from Australia, China (0.09 – 9.07 µg/ml, Wu et al. 1999), India (0.01– 0.71 µg/ml, Kranthi et al. 2001, Jalali et al. 2004) and Spain (3.5 µg/ml, Avilla et al. 2005) showing variability in significant susceptibility of *H. armigera* to Cry1Ac. These differences may be due to 1) quantity of toxin expressed in the plant, 2) presence of Cry1Ac in both protoxin and toxin form in the plant (Anilkumar et al. 2008a), 3) interaction of toxin and other plant traits (Tabashnik et al. 2003, Carrière et al. 2004), 4) larval age, 5) *Bt* resistance mechanism, 6) insect species and population variation and 7) variation in *Bt* cotton cultivar and growing conditions.

H. armigera (Bird and Akhurst 2004, 2005) and *P. gossypiella* (Liu et al. 1999) could survive to pupation on *Bt* cotton when selected on artificial diets containing Cry1Ac protoxin or MVP II, respectively. However, when *P. gossypiella* was selected for 42 generations using *Bt* cotton bolls (NuCOTN33B), larvae could not complete

development on *Bt* cotton and this strain did not have a reduction in cadherin binding (Henneberry and Jech 2007); similar to reports by Anilkumar et al. (2008a) for Cry1Ac toxin-selected *H. zea*. Therefore, the Cry1Ac form and method of administration (artificial diet vs. plant material) may play an important role when determining RR's needed for surviving on *Bt* cotton.

There were no significant differences in AR and SC survivability and time required for pupation on NBt cotton. However, Cry1Ac-resistant *H. virescens* (Tabashnik et al. 2003), *H. armigera* (Bird and Akhurst 2004, 2005) and *P. gossypiella* (Liu et al. 1999) had significantly slower larval development on NBt cotton and lower adult overwintering survival than susceptible individuals (Carrière et al. 2001, Bird and Akhurst 2004). Additionally, Cry1Ac-resistant *P. gossypiella* had less tolerance to gossypol than susceptible individuals, resulting in fitness being decreased by >50% (Carrière et al. 2001, 2004). In our present study, however, there were no observable fitness costs compared to SC1 when gossypol alone was tested against AR1. Further, gossypol concentration per larvae varied significantly between treatments (gossypol alone and 1:1 mixture of gossypol and Cry1Ac) but not between strains (AR1 and SC1) only at the highest concentrations tested. At the highest concentration of the 1:1 mixture, both strains recorded lower larval weight due to inhibition of feeding induced by pure gossypol (Meisner et al. 1976) and Cry1Ac (Whalon and Wingerd 2003) resulting in significantly less gossypol per body wt. These results suggest that plant secondary metabolites other than gossypol, and/or nutritional factors, may adversely affect AR fitness (Tabashnik et al. 2003).

Although AR1 appears similar to AR in terms of survivorship at 500 μg Cry1Ac toxin/g diet, the RR appears lower for AR1 because of the increased tolerance of SC1 (LC_{50}=31.25 μg/g diet) compared to SC (LC_{50}=9-15μg/g diet, Anilkumar et al. 2008a). Only LC_{50} but not EC_{50} values were different between AR1 and SC1 for Cry1Ac; which is due to 1) larval weight does not change significantly a day before and after molting, 2) EC_{50} values are estimated compared to larval weight in untreated diet treatment. Cry1Ac toxin was tested at 15 μg/g of diet, a concentration which is similar to the amount quantified from *Bt* cotton squares using ELISA. At this concentration 54% of AR1 could develop to pupation (data not shown), suggesting that AR should be able to develop to pupation on *Bt* cotton especially if larvae feed selectively on tissues expressing lower Cry1Ac concentrations compared to squares (Sivasupramaniam et al. 2008). However, none of the AR reached pupation on Bt cotton. Results from Cry1Ac interactions with gossypol, and cotton powder showed synergistic interactions to AR1 but not to SC1 when mortality was measured as failure to molt to third instar; mortality defined as dead larvae, or dead plus larvae in first instar, could not explain the differences observed between treatments. Synergistic interactions of gossypol and Cry1Ac were observed only at the highest concentrations tested. Furthermore, 4% cotton powder was synergistic to AR1 although it contained only 6 μg gossypol/g of diet (much less than the lowest concentration of gossypol tested that showed no differences in activity between SC1 and AR1). In addition to gossypol, cotton plants produce many other insecticidal secondary metabolites such as heliocides H_1 and H_2, hemigossypolone, etc., (Hedin et al. 1991). Therefore, these compounds could potentially interact to reduce AR survivorship on NBt

cotton. Additionally, 4% corn powder was also found to be interacting synergistically with Cry1Ac against AR1 but not to SC1. Although, corn does not have gossypol it has many secondary metabolites such as zeatin, which is found to affect the signaling pathway (jasmonate and salicylate) in plants when damage occurs through insect herbivory resulting in the activation of cytochrome P450 production.

Concentrations of gossypol and Cry1Ac used in this study were based on dry weights, which may not be representative of what an insect might consume while feeding on plants, as nearly 95% of total plant weight is water. However, because the nutritional value of plant material is significantly lower than nutritionally-rich artificial diet, larvae consume 6-8-fold less artificial diet compared to feeding on plant material (Naeem et al. 1992, Woods 1999). Therefore, we believe that the concentrations of gossypol and Cry1Ac used in this study are justifiable.

This is the first report of plant compounds other than protein inhibitors synergizing the activity of *Bt* proteins, especially against Cry1Ac-resistant insects. To date most compounds that are synergistic with *Bt* Cry proteins are other *Bacillus* spp or *Bt* products such as spores or spore crystal mixtures (Tang et al. 1996, Liu et al. 1998, Moar et al. 1989, 1995), zwittermicin A (Broderick et al. 2000), β-exotoxin (Moar et al. 1986) and CytA (Wirth et al. 1997) and a peptide expressed in *E. coli* containing a corresponding *Bt* binding sequence (Chen et al. 2007). However, plant protease inhibitors and several chemical insecticides have also been reported to synergize *Bt* proteins, mixtures (Herfs et al. 1965, MacIntosh et al. 1990a). Gossypol occurs naturally in an enantiomeric mixture of both (+)-gossypol and (-)-gossypol, and their ratio varies among

commercial cultivars. Both of these forms reduced the survivorship of *H. zea*, and a racemic mixture of 1:1 had a synergistic effect at 0.16% (Stipanovic et al. 2006). The gossypol obtained from Sigma used in the current study was extracted from cotton seeds, and the ratio of enantiomeric forms of gossypol was not provided. Therefore, further studies are warranted to quantify the ratios of enantiomers and to evaluate their interactions with Cry1Ac

The synergistic interaction of gossypol, and cotton with Cry1Ac observed in AR1 may help explain the inability of AR to survive and produce fertile adults on *Bt* cotton. Carrière et al. (2004) suggested that increased susceptibility of Cry1Ac-resistant *P. gossypiella* to gossypol was linked to the cadherin mutation resistance mechanism (Morin et al. 2003, Carrière et al. 2006). However, AR has not been shown to have any differences in Cry1Ac binding (essentially eliminating a cadherin mutation as a potential resistance mechanism), and is speculated to have altered proteolysis as a resistance mechanism (Anilkumar et al. 2008a). Additionally, AR1 was not differentially susceptible to gossypol alone compared to SC1. Therefore, future studies are warranted to determine how resistance mechanisms not associated with binding (or fitness costs involved with Cry1Ac selection) are affected by the presence of gossypol and other plant compounds.

Predicting field-evolved *Bt* resistance based on laboratory studies has always been tenuous. Field-evolved resistance has not occurred with *H. zea* even after 12 y of commercial use of *Bt* cotton in the USA (Ali et al. 2006, 2008, Moar and Anilkumar 2007) even though laboratory experiments have shown that *H. zea* does have the

capability to become resistant to the *Bt* protein in *Bt* cotton (Cry1Ac) (Luttrell et al. 1999, Jackson et al. 2004b, Anilkumar et al. 2008a). Results presented in this study help to illustrate that the actual hurdles that *H. zea* must overcome to become resistant to *Bt* cotton in the field are most likely quite complex and help to validate the absence of field-evolved resistance in *H. zea*.

Acknowledgements

The authors thank Nancy Adams, Monsanto Co., Union City, TN for providing *H. zea*; Arthur Appel, Dept of Entomology, Auburn University for providing statistical guidance; and Marianne Carey for providing purified Cry1Ac toxin. This research was partially supported by USDA, and Cotton Incorporated.

References cited:

Akhurst, R.J., W.James, L.J. Bird, and C. Beard. 2003. Resistance to the Cry1Ac delta-endotoxin of *Bacillus thuringiensis* in the cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae). J. Econ. Entomol. 96: 1290-1299.

Ali, I., C. Abel, J.R. Bradley, G. Head, R. Jackson, R. Kurtz, B.R. Leonard, J. Lopez, R.G. Luttrell, W. J. Moar, W. Mullins, J. Ruberson, S. Sivasupramaniam, and N. P. Storer. 2008. Monitoring *Helicoverpa zea* susceptibilities to Bt toxins: Results of 2007 studies. Pp.1020-1034. In Proc. Beltwide Cotton Conf., National Cotton Council, Memphis, TN.

Ali, M.I., R.G. Luttrell, and S. Y. Young. 2006. Susceptibilities of *Helicoverpa zea* and *Heliothis virescens* (Lepidoptera: Noctuidae) populations to Cry1Ac insecticidal protein. J. Econ. Entomol. 99:164–175.

Anilkumar, K. J., A. Rodrigo-Simon, J. Ferre, M. Pusztai-Carey, S. Sivasupramaniam, and W. J. Moar. 2008a. Production and characterization of *Bacillus thuringiensis* Cry1Ac-resistant cotton bollworm *Helicoverpa zea* (Boddie). Appl. Environ. Microbiol. 74: 462-469.

Anilkumar, K.J., M. Pusztai-Carey, and W. J. Moar. 2008b. Fitness associated with Cry1Ac-resistant *Helicoverpa zea* (Lepidoptera: Noctuidae): A factor countering selection for resistance to Bt cotton? J. Econ. Entomol. (In press)

Avilla, C., E. Vargas-Osuna, J. González-Cabrera, J. Ferré and J.E. González-Zamora. 2005. Toxicity of several δ-endotoxins of *Bacillus thuringiensis* against *Helicoverpa armigera* (Lepidoptera: Noctuidae) from Spain. J. Invertebr. Pathol. 90: 51-54.

Bird, L.J. and R.J. Akhurst. 2005. Fitness of Cry1A-resistant and -susceptible *Helicoverpa armigera* (Lepidoptera: Noctuidae) on transgenic cotton with reduced levels of Cry1Ac. J. Econ. Entomol. 98:1311–1319.

Bird, L.J., and R.J. Akhurst. 2004. Relative fitness of Cry1A-resistant and -susceptible *Helicoverpa armigera* (Lepidoptera: Noctuidae) on conventional and transgenic cotton. J. Econ. Entomol. 97: 1699-709.

Brickle, D.S., Turnipseed, S.G. and M.J. Sullivan. 2001. Efficacy of insecticides of different chemistries against *Helicoverpa zea* (Lepidoptera: Noctuidae) in

transgenic *Bacillus thuringiensis* and conventional cotton. J. Econ. Entomol. 94: 86-92.

Broderick, N.A., R.M.Goodman, K.F. Raffa and J. Handelsman. 2000. Synergy between zwittermicin A and *Bacillus thuringiensis* subsp. k*urstaki* against gypsy moth (Lepidoptera: Lymantriidae). Environ. Entomol. 29:101-107.

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. 96:137-142.

Carrière Y, C. Ellers-Kirk, R.W. Biggs, M.E. Nyboer, Unnithan GC, T.J. Dennehy and B.E. Tabashnik. 2006. Cadherin-based resistance to *Bacillus thuringiensis* cotton in hybrid strains of pink bollworm: fitness costs and incomplete resistance. J. Econ. Entomol. 99:1925–1935.

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. 97:1710–1718.

Carrière, Y. Ellers-Kirk, C. Patin, A. L. Sims, M. A. Meyer, S. Liu, Y. B. Dennehy, T. J. and B. E.Tabashnik. 2001. Overwintering cost associated with resistance to transgenic cotton in the pink bollworm (Lepidoptera: Gelechiidae). J. Econ. Entomol.94: 935-941

Chen, J., G. Hua, J.L. Jurat-Fuentes, M.A. Abdullah, and M. J. Adang. 2007. Synergism of *Bacillus thuringiensis* toxins by a fragment of a toxin-binding cadherin. Proc. Natl. Acad. Sci. USA 104: 13901 - 13906.

Crickmore, N., D. R. Zeigler, J. Feitelson, E. Schnepf, J. v. Rie, D. Lereclus, J. Baum, and D. H. Dean. 1998. Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62: 807-813.

Gao, Y, K. J. Fencil, X. Xu, D. A. Schwedler, J. R. Gilbert and R. A. Herman. 2006. Purification and characterization of a chimeric Cry1F delta-endotoxin expressed in transgenic cotton plants. J. Agric. Food Chem. 54**:** 829-835.

Gould, F. 1998. Sustainability of transgenic insecticidal cultivars: Integrating pest genetics and ecology. Ann. 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: 1545-1559.

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

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.

Harris, M.K. 1991. *Bacillus thuringiensis* and pest control. Science. 253:1075.

Hedin, P.A., W. L. Parrott, and J. N. Jenkins. 1991. Effects of cotton plant allelochemicals and nutrients on behavior and development of tobacco budworm. J. Chem. Ecol. 17: 1107-1121.

Henneberry, T.J.and Jech, L.J. 2007. Effects of pink bollworm resistance to transgenic cotton on moth mating, oviposition and larval progeny development. Arthropod Manag. Tests 32, Report No. M3.

Herfs, W. 1965. Die vertraeglichkeit von *Bacillus thuringiensis* Praeparaten mit chemishen Pflanzenschutzmittlen und mit Beistoffen. Z. Pflanzenkra. & Pflanzenschutz. 72:584-599.

Jackson, R.E., J.R. Bradley Jr, J.W. Van Duyn, and F. Gould. 2004a. Comparative production of *Helicoverpa zea* (Lepidoptera: Noctuidae) from transgenic cotton expressing either one or two *Bacillus thuringiensis* proteins with and without insecticide oversprays. J. Econ. Entomol. 97:1719-1725.

Jackson, R.E., J.R. Bradley, Jr and J.W. van Duyn. 2004b. Performance of feral and Cry1Ac-selected *Helicoverpa zea* (Lepidoptera: Noctuidae) strains on transgenic cottons expressing one or two *Bacillus thuringiensis* spp. *kurstaki* proteins under greenhouse conditions. J. Entomol. Sci. 39: 46-55.

Jalali, S. K., K. S. Mohan, S. P. Singh, T. M. Manjunath, and Y. Lalitha. 2004. Baseline-susceptibility of the old-world bollworm, *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) populations from India to *Bacillus thuringiensis* Cry1Ac insecticidal protein. Crop Prot. 23: 53-59.

Kranthi, K.R., S. Kranthi and R.R. Wanjari 2001. Baseline susceptibility of CryI toxins to *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) in India. *Int. J. Pest Manage.* 47 : 141–145.

Li, H., L. L. Buschman, F. Huang, K. Y. Zhu, B. Bonning, and B. Oppert. 2007. DiPel-selected *Ostrinia nubilalis* larvae are not resistant to transgenic corn expressing *Bacillus thuringiensis* Cry1Ab. J. Econ. Entomol. 100: 1862-1870.

Liu, Y.B., B. E. Tabashnik, T. J. Dennehy, A. L. Patin, and A. C. Bartlett. 1999. Development time and resistance to Bt crops. Nature 400: 519.

Liu, Y.B., B. E. Tabashnik, W.J. Moar and R. A. Smith. 1998. Synergism between *Bacillus thuringiensis* spores and toxins against resistant and susceptible diamondback moths (*Plutella xylostella*). Appl. Environ. Microbiol. 64:1385-1389.

Liu, Y.B., B. E. Tabashnik, S. K. Meyer, Y. Carrière and A. C. Bartlett. 2001a. Genetics of pink bollworm resistance to *Bacillus thuringiensis* toxin Cry1Ac. J. Econ. Entomol. 94: 248–252.

Liu, Y.B., B. E. Tabashnik, T. J. Dennehy, A. L. Patin, M. A. Sims, S. K. Meyer and Y. Carrière. 2001b. Effects of Bt cotton and Cry1Ac toxin on survival and development of pink bollworm (Lepidoptera: Gelechiidae). J. Econ. Entomol. 94:1237–1242.

Luttrell, R.G., L. Wan, and K. Knighten. 1999. Variation in susceptibility of noctuid (Lepidoptera) larvae attacking cotton and soybean to purified endotoxin proteins

and commercial formulations of *Bacillus thuringiensis*. J. Econ. Entomol. 92:21-32.

MacIntosh, S. C., G. M. Kishore, F. J. Perlak, P. G. Marrone, T. B., Stone, S. R. Stevens and R.L. Fuchs. 1990a. Potentiation of *Bacillus thuringiensis* insecticidal activity by serine protease inhibitors. J. Agric. Food Chem. 38:1145-1152.

MacIntosh, S. C., T. B. Stone, S. R. Sims, P. L. Hunst, J. T. Greenplate, P. G. Marrone, F. J. Perlak, D. A. Fischhoff, and R. L. Fuchs. 1990b. Specificity and efficacy of purified *Bacillus thuringiensis* proteins against agronomically important insects. J. Invertebr. Pathol. 56: 258-66.

Meisner, J. K.R.S. Ascher, and M. Zur. 1976. Phagodeterrency induced by pure gossypol and leaf extracts of a cotton strain with high gossypol content in the larva of *Spodoptera littoralis*. J. Econ. Entomol. 70: 149-150.

Moar, W. J. and K. J. Anilkumar. 2007. The power of the pyramid. Science. 318: 1561-1562.

Moar, W.J., J. T. Trumble and B. A. Federici. 1989. Comparative toxicity of spores and crystals from the NRD-12 and HD-1 strains of *Bacillus thuringiensis* subsp. *kurstaki* to neonate beet armyworm (Lepidoptera: Noctuidae). J. Econ. Entomol. 82: 1593-1603.

Moar, W.J., Osbrink, W. L. A., and Trumble, J. T. 1986. Potentiation of *Bacillus thuringiensis* var. k*urstaki* with Thuringiensin on beet armyworm (Lepidoptera: Noctuidae). J. Econ. Entomol., 79, 1443-1446.

Moar, W.J., J. T. Trumble, R. H. Hice, and P. A. Backman. 1994. Insecticidal activity of the CryIIA protein from the NRD-12 isolate of *Bacillus thuringiensis* subsp. *kurstaki* expressed in *Escherichia coli* and *Bacillus thuringiensis* and in a leaf-colonizing strain of *Bacillus cereus*. Appl. Environ. Microbiol. 60:896-902.

Moar, W.J., M. Pusztai-Carey, H.v. Faassen, D. Bosch, R. Frutos, C. Rang, K. Luo, and M.J. Adang. 1995. Development of *Bacillus thuringiensis* CryIC resistance by *Spodoptera exigua* (Hubner) (Lepidoptera: Noctuidae). Appl. Environ. Microbiol. 61: 2086-2092.

Morin, S., R. W. Biggs, M. S. Sisterson, L. Shriver, C. Ellers-Kirk, D. Higginson, D. Holley, L. J. Gahan, D. G. Heckel, Y. Carriere, T. J. Dennehy, J. K. Brown, and B. E. Tabashnik. 2003. Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm. Proc. Natl. Acad. Sci. USA 100: 5004-5009.

Naeem, M., G. P. Waldbauer, and S. Friedman. 1992. Heliothis zea larvae respond to diluted diets by increased searching behavior as well as by increased feeding. Entomol. Exp. Appl. 65: 95-98.

Orth, R.G., G. Head, and M. Mierkowski. 2007. Determining larval host plant use by a polyphagous lepidopteran through analysis of adult moths for plant secondary metabolites. J. Chem. Ecol. 33: 1131-1148

Pusztai-Carey, M., P. Carey, T. Lessard and M. Yaguchi. 1994. USA patent # 5356788.

Roush, R.T. 1997. Bt-transgenic crops: just another pretty insecticide or a chance for a new start in resistance management? Pestic. Sci. 51:328-334.

Salama, H.S., M.S. Foda, F.N. Zaki and S. Moawad. 1984. Potency of combinations of *Bacillus thuringiensis* and chemical insecticides on *Spodoptera littoralis* (Lepidoptera: Noctuidae). J. Econ. Entomol. 77:885-890.

Schabenberger, O. and F. J. Pierce. 2004. Contemporary statistical models for the plant and soil sciences. CRC Press, Boca Raton, FL, USA.

Sivasupramaniam S., W. J. Moar, L.G. Ruschke, J. A. Osborn, C. Jiang, J. L. Sebaugh, G.R. Brown, Z.W. Shappley, M.E. Oppenhuizen, J.W. Mullins, and J. T. Greenplate. 2008. Toxicity and characterization of cotton expressing *Bacillus thuringiensis* Cry1Ac and Cry2Ab2 proteins for control of lepidopteran pests. J. Econ. Entomol. 101:546-554.

Stipanovic, R.D., J. Lopez, M. K.Dowd, L.S. Puckhaber,and S. E. Duke. 2006. Effect of racemic and (+)- and (-)-gossypol on the survival and development of *Helicoverpa zea* larvae. J. Chem. Ecol. 32:959-968.

Tabashnik B.E. 1992. Evaluation of synergism among *Bacillus thuringiensis* toxins. Appl. Environ. Microbiol. 58:3343-3346.

Tabashnik B.E., Y. Carrière, T. J. Dennehy, S. Morin, M.S.Sisterson, R. T. Roush, A. M. Shelton and J. Z. Zhao. 2003. Insect Resistance to Transgenic Bt Crops: Lessons from the laboratory and field. J. Econ. Entomol. 96:1031–1038.

Tabashnik, B. E., A. L. Patin, T. J. Dennehy, Y. Liu, Y. Carriere, M. A. Sims, and L. Antilla. 2000. Frequency of resistance to *Bacillus thuringiensis* in field populations of pink bollworm. Proc. Natl. Acad. Sci. USA 97: 12980-12984.

Tang, J.D., A.M. Shelton, J.V. Rie, S.D. Roeck, W. J. Moar, R. T. Roush and M. Peferoen. 1996. Toxicity of *Bacillus thuringiensis* spore and crystal protein to resistant diamondback moth (*Plutella xylostella*). Appl. Environ. Microbiol. 62:564-569.

Torres, J. B. and J. R. Ruberson. 2006. Spatial and temporal dynamics of oviposition behavior of bollworm and three of its predators in Bt and non-Bt cotton fields. Entomol. Exp. et Appl. 120:11–22.

Whalon,M.E., and B.A. Wingerd. 2003. Bt: mode of action and use. Arch. Insect Biochem. Physiol. 54: 200-211.

Wirth, M.C., G. P. Georghiou and B. A. Federici. 1997. CytA enables CryIV endotoxins of *Bacillus thuringiensis* to overcome high levels of CryIV resistance in the mosquito, *culex quinquefaciatus*. Proc. Natl. Acad. Sci. USA 94:10536-10540.

Woods, H. A. 1999. Patterns and mechanisms of growth of fifth-instar *Manduca sexta* caterpillars following exposure to low-or high-protein food during early instars. Physiol. Biochem. Zool. 72:445-454.

Wu K., Guo Y., and Lv N. 1999. Geographic variation in susceptibility of *Helicoverpa armigera* (Lepidoptera: Noctuidae) to *Bacillus thuringiensis* insecticidal protein in China. J. Econ. Entomol. 92:273–278.

Table 1. Lethal time (LT) to mortality for a Cry1Ac-resistant (AR) and a susceptible (SC) *H. zea* on *Bt* (DPL-555) and NBt (DPL-491) cotton squares. The test was conducted in three replicates of 30 insects each; hence the df for *P*-values and confidence intervals was 4.

Squares	Strain	LT_{50}(95% CI) (Days)	LT_{75}(95% CI) (Days)	Slope ± SE
Bt	AR	9.13 (6.99 - 9.13)	13.41 (12.26 – 14.57)	4.50 ± 0.36
	SC	4.75 (3.94 – 5.55)	7.80 (6.95 – 8.65)	4.94 ± 0.51
NBt	AR	22.33 (20.78 - 23.88)	31.33 (28.75 – 33.92)	4.03 ± 0.36
	SC	23.18 (21.56 – 24.80)	32.30 (29.57 – 35.03)	4.11 ± 0.37

Table 2. Performance of a Cry1Ac-resistant (AR) and a susceptible (SC) strains on *Bt* (DPL-555) and NBt (DPL-491) cotton squares. The test was conducted in three replicates of 30 insects each.

Square	Strain	Larval mass at 7d (mg)	Proportions at the end of the experiment (31 d)	
			3^{rd} instars	4^{th} instars
Bt	AR	0.93 (0.69 – 1.25)[a]	0.19(0.07 - 0.42)	0.06 (0.02 – 0.15)
	SC	0.66 (0.45 – 0.96)	0.05 (0.02 - 0.16)	0.00
NBt	AR	4.11 (3.16 – 5.35)	0.76 (0.51 – 0.91)	0.63 (0.51 – 0.74)
	SC	4.57 (3.51 – 5.95)	0.81 (0.58 – 0.93)	0.66 (0.53 – 0.76)
F-Test				
Strain		F_1=0.73 P = 0.4056	F_1=3.49 P = 0.0985	F_1=0.00 P = 0.9537
Tissue		F_1=155.74 P < 0.0001	F_1=108.0 P < 0.0001	F_1=0.00 P = 0.9697
Strain* Tissue		F_1=2.66 P = 0.1248	F_1=7.20 P = 0.0278	F_1=0.00 P = 0.9692

[a]values in the parenthesis are 95% confidence intervals

Table 3. Molt inhibitory concentration (failure to molt to third instar) response of a Cry1Ac-resistant (AR1) and a susceptible (SC1) *H. zea* to Cry1Ac, and its 1:1 mixture with gossypol. The test was conducted in three replicates of 32 insects each.

Treatments	Strain	LC_{50} (µg/g of diet)[a]	Slope	Intercept
Cry1Ac	AR1	17.56 (12.02 – 23.10)[b]	0.10 (0.05 – 0.15)	-1.78 (-2.28 – -1.29)
	SC1	7.07 (5.99 – 8.75)	0.29 (0.22 – 0.35)	-2.87 (-3.51 – -2.24)
Cry1Ac +	AR1	10.04 (8.28 – 11.27)	0.29 (0.22 – 0.36)	-2.02 (-2.54 – -1.50)
Gossypol	SC1	5.41 (4.68 – 6.14)	0.48 (0.36 – 0.60)	-2.60 (-3.29 – -1.91)

[a]LC_{50} values for mixture of Cry1Ac and gossypol are expressed concentrations of Cry1Ac; [b]values in the parenthesis are 95% confidence intervals and are in logit scale.

Table 4. Weight stunting concentration response of a Cry1Ac-resistant (AR1) and a susceptible (SC1) strain of *H. zea* to Cry1Ac, gossypol and their 1:1 mixtures. The test was conducted in three replicates of 32 insects each.

Treatments	Strains	EC_{50} (µg/g of diet)[a]	EC_{90} (µg/g of diet)
Gossypol	AR1	1,171.81 (892.63 – 1,450.98)[b]	2,109. 25 (1,606.73 – 2,611.77)
	SC1	1,204.01 (916.35 – 1,491.66)	2,167.21 (1,649.43 – 2,684.99)
Cry1Ac	AR1	0.99 (0.12-1.87)	3.30 (0.39 - 6.20)
	SC1	0.97 (0.31-1.63)	3.23 (1.04 - 5.42)
Cry1Ac + Gossypol	AR1	1.35 (0.52 - 2.18)	4.48 (1.72 - 7.25)
	SC1	0.94 (0.26 - 1.63)	3.14 (0.86 - 5.41)

[a]EC- Effective concentration (related to stunting - weight related). EC_{50}, EC_{90} – Concentration of Cry1Ac that would stunt the larvae such that they weighed 50 and 10% of that of larvae in the untreated control group; values for Cry1Ac + gossypol are expressed in concentrations of Cry1Ac; [b]values in the parenthesis are 95% confidence intervals.

Table 5. Percent mortality of a Cry1Ac-resistant (AR1) and a susceptible (SC1) *H. zea* in 4% cotton/corn powder in the presence and absence of 15 µg Cry1Ac/g diet.

Treatments	N[a]	Failure to molt to 3rd instar (mean ± SE)		
		AR1	SC1	P value
Untreated diet	192	11.59 ± 2.66[a]	11.98 ± 3.90[a]	0.9333
Cry1Ac	200	20.59 ± 3.04[a]	86.98 ± 3.27[b]	< 0.0001
Cotton powder	222	22.65 ± 3.99[a]	14.23 ± 5.94[a]	0.2616
Cry1Ac + cotton powder	205	98.31 ± 0.69[c]	96.01 ± 1.09[b]	0.0506
Corn powder	48	15.14 ± 1.81[a]	4.65 ± 2.37[a]	0.0002
Cry1Ac + corn powder	48	53.21 ± 6.79[b]	95.83 ± 2.08[b]	0.0065
Strains		F_1=49.25, $P < 0.0001$		
Treatment		F_5=672.10, $P < 0.0001$		
Strain * Treatment		F_5=44.27, $P < 0.0001$		

[a]Number of insects tested; means within a column followed by different superscript letters are significantly different at $P = 0.05$

Table 6. Interactions of Cry1Ac with gossypol, cotton and corn powder as measured by failure to molt into third instars after seven days.

Compounds	Concentration (μg/g)		Strain	N[a]	Mortality (%)		χ^2	Effect
	Cry1Ac	Gossypol			Observed	Expected[b]		
Cry1Ac + Gossypol	15	1500	AR1	96	92.97	37.70	81.02	Synergistic
	7.5	750		96	22.60	15.67	2.13	Additive
	3.75	375		96	6.66	8.32	0.33	Additive
	15	1500	SC1	96	98.92	85.24	2.20	Additive
	7.5	750		96	68.16	61.78	0.66	Additive
	3.75	375		96	36.56	35.17	0.05	Additive
Cry1Ac + Cotton powder (4%)	15	0.006	AR1	222	99.23	22.01	271.04	Synergistic
	15	0.006	SC1	221	95.60	85.15	1.28	Additive
Cry1Ac + Corn powder (4%)	15	NP[c]	AR1	48	47.21	14.75	71.47	Synergistic
	15		SC1	48	95.35	84.80	1.31	Additive

[a]Number of insects tested; [b]Expected mortality is calculated from observed mortalities in different treatments after adjusting for control mortality. [c]gossypol is not present in corn and hence not quantified.

Fig 1. Quantity of Cry1Ac protein (μg Cry1Ac/g lyophilized tissue) expressed in *Bt* cotton squares using ELISA. R1-3 = replicates 1-3. Squares were harvested July 18-Aug 25, and squares were refrigerated at 4-7 ^{0}C until fed to *H. zea* from July 20 – Aug. 29.

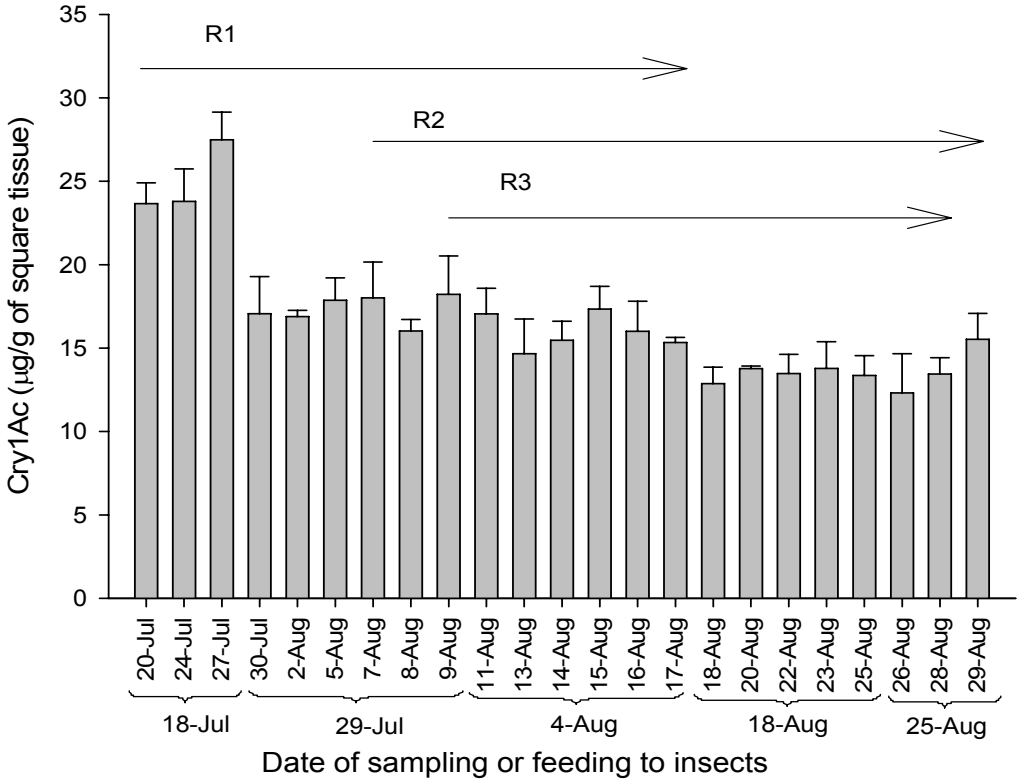


Fig 2. Cumulative % mortality of susceptible (SC) and Cry1Ac-resistant (AR) *H. zea* on *Bt* (DPL-555) and NBt (DPL-491) cotton squares.

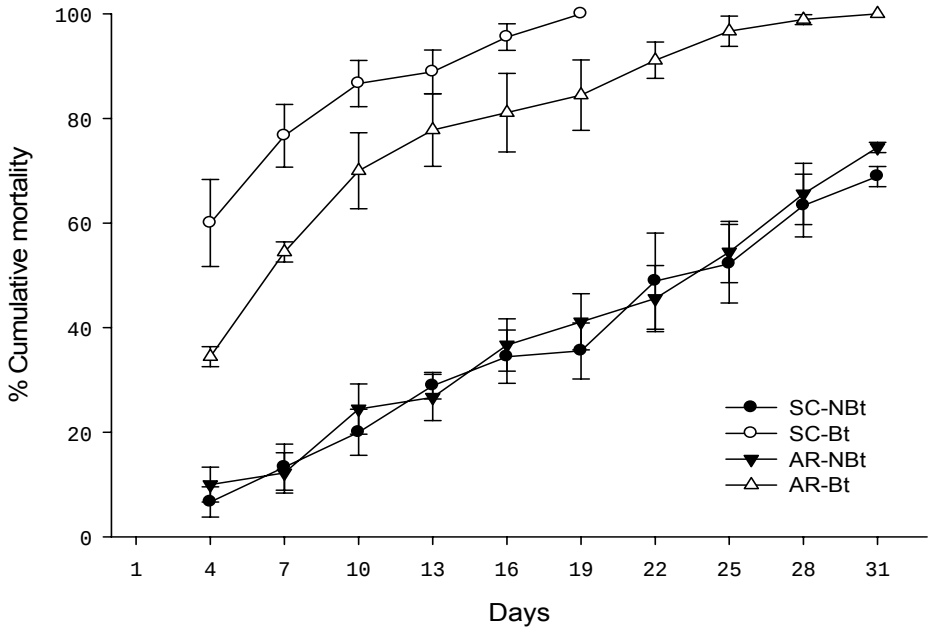


Fig 3. Concentration of gossypol in Cry1Ac-resistant (AR1) and a susceptible (SC1) *H. zea*. A: when fed on different concentrations of gossypol (375, 750 & 1,500 µg/g) in the diet alone and in 1:1combination with Cry1Ac (3.75, 7.5, 15 µg/g). CPG = Cry1Ac + Gossypol; Gos=Gossypol. B: when fed on 4% cotton powder alone or in combination with 15 µg Cry1Ac/g of diet. The data represent the mean of three replications and standard errors are back-transformed values from logarithmic scale.

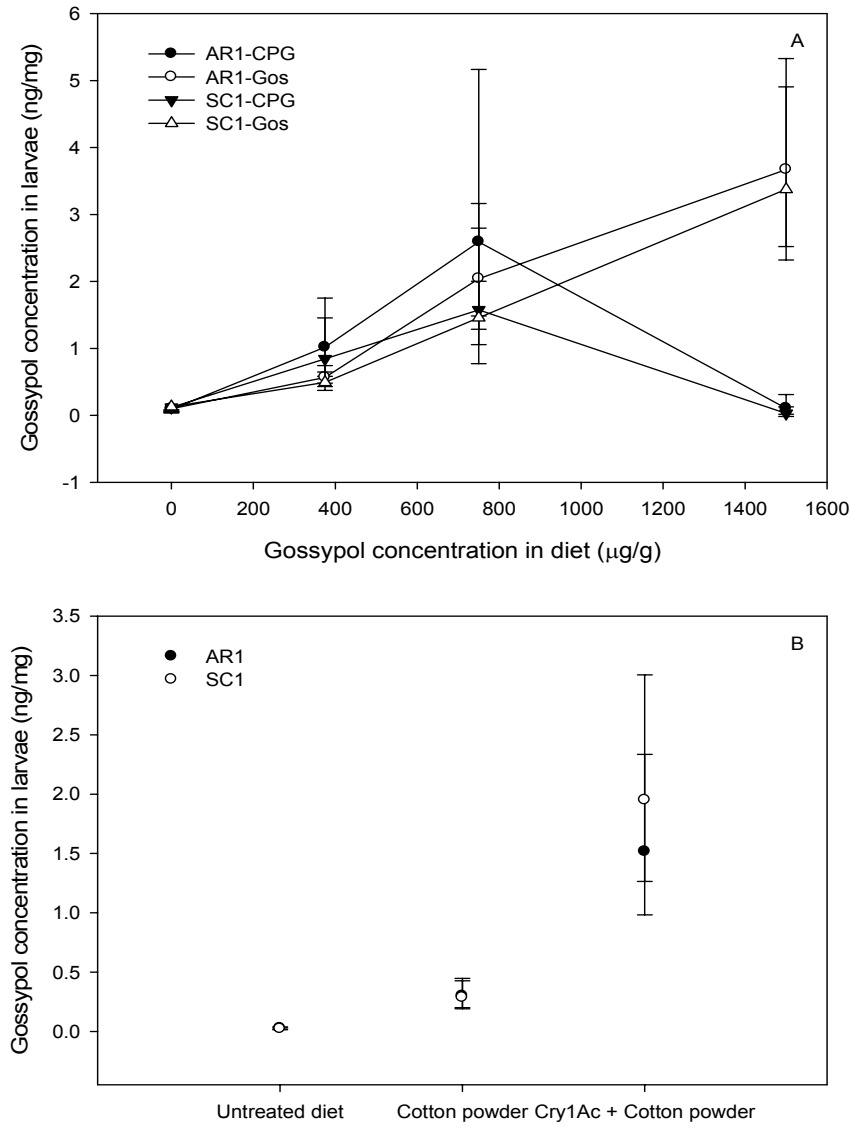


Fig 4. Effect of 4% cotton/corn powder in the presence and absence of 15 µg Cry1Ac/g of diet on larval weight in Cry1Ac-resistant (AR1) and a susceptible (SC1) *H. zea*.