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Survival, growth, and behavior of Helicoverpa zea (Boddie) and Heliothis virescens (F.) (Lepidoptera: Noctuidae) on genetically engineered cotton expressing the Vip3A insecticidal protein

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SURVIVAL, GROWTH, AND BEHAVIOR OF *HELICOVERPA ZEA* (BODDIE) AND *HELIOTHIS VIRESCENS* (F.) (LEPIDOPTERA: NOCTUIDAE) ON GENETICALLY ENGINEERED COTTON EXPRESSING THE VIP3A INSECTICIDAL PROTEIN

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ABSTRACT

Larval behavior, survivorship, and injury potential of *Helicoverpa zea* (Boddie), and tobacco budworm, *Heliothis virescens* (F.), was evaluated on cotton plants genetically engineered to express *Bacillus thuringiensis* (Bt) insecticidal proteins, Vip3A and Vip3A +Cry 1Ab (VipCot™). Larvae (2-d-old) of both species were infested on plant terminals of vegetative or flowering stage cotton plants. Regardless of species, more larvae migrated from the plant terminals of Vip3A and VipCot plants compared to larvae infested on a conventional non-Bt cotton line (Coker 312). Larval (2-d-old) survivorship on VipCot™ plant structures was generally lower than that on similar structures of the Vip3A cotton line. However, *H. virescens* survivorship was higher than that for *H. zea* on similar structures of Vip3A plant. Age-specific (2 d, 4 d, 6 d, and 8 d-old larvae) survivorship was further evaluated on flower buds (cotton squares) of Vip3A and VipCot™ plants. Cumulative survivorship levels for both species and all larval ages were significantly lower on Vip3A and VipCot™ squares compared to that for Coker 312. No larvae of either species successfully pupated on VipCot™ squares. A limited number of *H. virescens* larvae completed larval development on Vip3A cotton squares.

Field trials quantified cotton fruiting form injury on these Bt lines from artificial and native infestations of *H. zea* or *H. virescens*. Larvae (L2 stage) of *H. zea* infested in white flowers injured an average of 8.6, 4.6, and 1.0 fruiting forms per larva on Coker 312, Vip3A, and VipCot™ plants, respectively. Similarly, *H. virescens* injured an average of 9.2, 5.9, and 0.9 fruiting forms/larva on Coker 312, Vip3A, and VipCot™ plants, respectively. Native infestations of both species during 2005-2007 injured fewer fruiting forms on the Bt lines compared to that on Coker 312 plants. Seasonal efficacy patterns showed the VipCot™ plants to be more durable with less fruiting form injury than that recorded on Coker 312 and Vip3A, especially during

periods of peak insect infestations. The combination of two proteins in the VipCot™ line generally improved efficacy against these pests compared to that of the single protein in the Vip3A line.

CHAPTER 1

INTRODUCTION

Cotton (*Gossypium hirsutum* L.) is an important crop in the United States with 15,274,000 acres producing a mean yield of 831 lb lint/acre in 2006 (Williams 2007). Louisiana is one of 14 states that produce cotton in United States. During 2006, a total of 635,000 acres of cotton were planted in Louisiana with a mean yield of 993 lb/acre (Williams 2007). Several insect pests such as thrips, cotton aphids, tarnished plant bugs, stink bugs, and lepidopteran pests infest cotton at various stages of development. Among these, *H.zea* and *H. virescens* commonly known as the heliothine complex, are the most economically important pests of cotton across the Southern United States. *H. zea* and *H. virescens* are annual pests in Louisiana cotton fields (Leonard et al. 2001).

Helicoverpa zea and *Heliothis virescens* Biology

The *H.zea* and *H. virescens* are classified in the family Noctuidae and order Lepidoptera. The *H. zea* was previously included in the genus *Heliothis*, but was removed based on characters of the genitalia and placed into a new genus, *Helicoverpa* (Hardwick 1970). The tobacco budworm, *Heliothis virescens* (F.), and bollworm, *Helicoverpa zea*, are serious pests of cotton throughout much of the Southeastern and Mid-Southern United States (Gore and Adamczyk 2004).

The *H. zea* and *H. virescens* are multivoltine species with several generations occurring each year (Oliver and Chapin 1981). They develop through four development stages–egg, larva, pupa and adult in approximately 35-50 days. The egg when freshly laid is cream colored with distinct longitudinal ridges from top to bottom (Bohmfalk et al. 1982). The newly hatched larvae are creamy-white with a distinct black head (Oliver and Chapin 1981). The larval color varies

from light green to dark reddish brown with stripes running across the body (Bohmfalk et al. 1982). Both species of heliothine larvae look similar but can be distinguished by the absence of a molar region on the mandible and the absence of spinules on chalazas one and two on abdominal segments one, two, and eight in *H. zea* (Oliver and Chapin 1981). The larva develops through four to five stadia for a total of five to six instars. The final instar emigrates from the plant, burrows into the soil, and pupates beneath the surface (Bohmfalk et al. 1982). The *H. zea* and *H. virescens* over winter as pupae in the soil. The adult *H. zea* is approximately 1.9 cm long with a 3.8 cm wingspan and is light brown in color with shades of olive green, orange, or brown (Oliver and Chapin 1981). The orbicular spot on the hind wing is faint with a dark spot in the center. *H. virescens* adults are about 1.9 cm long with a wingspan of 2.5 to 3.7cm. The forewing is light to olive green with three or four dark colored oblique bands and hind wing white with a reddish-brown border (Oliver and Chapin 1981).

Helicoverpa zea **and** ***Heliothis virescens*** **Damage to Cotton**

The *H. zea* and *H. virescens* are polyphagous species that feed on a variety of cultivated and native plant hosts. *H. zea* larvae have been reported to feed on more than 100 hosts (King and Coleman 1989). Some important hosts include corn; *Zea mays* L., cotton; *Gossypium hirsutum* L., tomato; *Lycopersicon esculentum* L., crimson clover; *trifolium incarnatum* L., soybeans; *Glysine max*, peas; *Pisum sativum*, and peppers; *Piper nigrum* (Oliver and Chapin 1981). The *H. virescens* however has a narrow host range than *H. zea*. The primary host of *H. virescens* is cotton but it also attacks roses, clover, soybean, and other wild hosts (Oliver and Chapin 1981). Oviposition usually occurs on the upper surface of tender foliage and fruiting forms including bracts, blooms, squares, and bolls (Bohmfalk et al. 1982).

Early stage larvae feed primarily on developing squares that are less than two mm in diameter (Reese et al. 1981). First instars of *H. virescens* are generally found feeding on pin-head squares in the plant terminal (Mulrooney et al. 1992). The injured young squares flare outward and abort from plants usually within five to seven days (Bohmfalk et al. 1982). Fye (1972) found that 78 to 100% of damaged fruit at any given time could be found in the upper 0.6 m of the plant. The first three instars of *H. virescens* migrated up to the plant, where as the last two instars moved down (Wilson et al. 1980). Similar behavioral patterns were observed for *H. zea*. The last two instars of *H. zea* feed lower in the plant canopy on older bolls (Farrar and Bradley 1985). Wilson and Gutierrez (1980) found that second instar *H. zea* migrated down the plant feeding on older fruiting forms as larval development progressed. Second and third instars of *H. virescens* were found on squares during early season and on bolls later in the season; whereas fourth instars or older larvae were observed primarily on bolls (Ramalho et al. 1984).

H. virescens larvae damaged more squares than bloom or bolls during the early season than the late season (Kincade et al. 1967). A small number of larvae are capable of causing significant levels of damage. Adkisson et al. (1964) reported that eight to ten *H. zea* larvae per 100 plant terminals are capable of causing significant yield losses, and that control measures are generally recommended when four to five young larvae or eggs per 100 plant terminals are present. Early season plant terminal damage of 40% by *H. virescens* delayed crop maturity and reduced yield (Heilman et al. 1981). Each *H. virescens* is capable of damaging 10 squares, 1.2 blooms, and 2.1 bolls during larval development (Heilman et al. 1981). Heliothine-infested bolls can be susceptible to boll-rotting pathogens (Bohmfalk et al. 1982). In Louisiana, insecticides are usually recommended when squares are at least one third grown and five live larvae per 100 plants plus eggs are present on conventional cotton (Bagwell et al. 2004).

Pest Status

The *H. virescens*/*H. zea* complex remained a primary pest species across the cotton belt during 2006. Heliothines infested 9,428,335 acres of cotton in United States resulting in a yield loss of 0.876% (Williams 2007). During 2006, the number of Louisiana cotton acres infested with *H. virescens* or *H. zea* was 563,400. A statewide yield loss of 25,567 bales of cotton was attributed to these pests (Williams 2007). The heliothine complex ranked second among all arthropod pests in Louisiana (Williams 2007).

Insecticide Resistance

Cotton producers spend $200 million annually on chemical insecticides to control arthropod pests (Jenkins et al. 1991). Until recently, insecticides were the primary strategy used to control Lepidopteran pests in cotton production (Jenkins et al. 1993). Insecticides currently remain a key component of cotton integrated pest management (Graves et al. 1999). In 1991, Louisiana cotton growers averaged 3.5 insecticide applications at a cost of $7.50 per acre to control the heliothine complex (Head 1992). *H. zea* populations were first reported resistant to chlorinated hydrocarbons in Louisiana (Graves et al. 1963) and *H. virescens* populations in Texas (Brazzel 1963). Organophosphorous and carbamate insecticides ultimately replaced chlorinated hydrocarbons. Within a few years, *H. zea* and *H. virescens* developed resistance to these compounds (Wolfenbarger and McGarr 1970, Harris 1972, Wolfenbarger et al. 1973). Pyrethroids were introduced during 1970's and provided excellent control of Heliothines. As a result of continued use of these insecticides, resistance to pyrethroids was documented in *H. virescens* populations from West Texas in 1985 (Luttrell et al. 1987, Plapp and Campanhola 1986). Several instances of pyrethroid resistance in *H. virescens* have been reported across the cotton belt (Allen et at 1987, Plapp et al. 1987, Roush and Luttrell 1989). In Louisiana,

pyrethroid resistance in *H. virescens* was documented in 1986. In subsequent years, pyrethroid alternatives such as organophosphates and carbamates failed to give satisfactory control of *H. virescens* (Leonard et al. 1993, Layton et al. 1996). Pyrethroids are still effective against *H. zea.* However, *H. zea* resistance to pyrethroids has been reported in some areas of the cotton belt (Walker et al. 1998). Bagwell et al. (2000) indicated increased *H. zea* survival to pyrethroid insecticides in 1998. The mean survival of *H. zea* increased from 27% in 1998 to 34% in 2002 (Bagwell et al. 1999, Cook et al. 2003).

Introduction of Genetically Engineered (Bt) Cotton

The severity of *H. virescens* and *H. zea* infestations and control failures due to insecticide resistance supported the development of alternative control strategies. Developing genetically transformed cotton plants to express insecticidal proteins has been viewed as an alternative to conventional insecticide use strategies for these pests. Recent advances in genetic engineering technology have enabled the introduction of novel genes in plants to confer insect resistance. One technology was approved by the environmental protection agency for commercialization in the United States during 1996. Transgenic cotton expresses an insecticidal protein (delta-endotoxin) from the naturally occurring soil bacterium, *Bacillus thuringiensis* (Bt) var. *kurstaki* (De Spain et al. 1993). Bt produces a crystal-like protein that disrupts the digestive system of selected lepidopteran larvae. The toxin binds to specific receptors in the insect midgut, forming pores, and leading to cell lysis, leakage of the midgut contents, paralysis, and death of the insect (Gill et al. 1992). In 1996, the first commercial Bt cotton approved by environmental protection agency (EPA) marketed in United States was Bollgard® cotton, which carries the Cry1Ac protein. Over 1.7 million acres were planted to Bollgard® cotton in the United States during 1996 (Gould 1998)

and now Bt cotton acreage in US during 2006 is 8.46 million acres (Williams 2007). More than 93% of cotton acreage in Louisiana is planted with Bt cotton varieties.

Bollgard® cotton exhibits some weakness against *H. zea*, and supplemental applications of insecticides are often necessary to manage *H.zea* (Leonard et al. 2001). Therefore Monsanto developed Bollgard II® cotton by inserting the Cry2Ab gene into Bollgard cotton varieties. As a result, Bollgard II® cotton expresses two proteins, Cry1Ac and Cry2Ab. Bollgard II® has proven to be more active against *H. zea* and other lepidopteran pests (Greenplate et al. 2000, Stewart et al. 2001).

Dow AgroSciences introduced a new transgenic cotton variety known as WideStrike® during 2004. WideStrike® cotton lines express two separate insecticidal Bt proteins (Cry1Ac and Cry1F) which may aid in resistance management and also broaden the spectrum of activity beyond that of a single insecticidal protein (Lorenz et al. 2005, Leonard et al. 2005).

Currently, Syngenta is developing a novel transgenic cotton technology that expresses the Vip3A (vegetative insecticidal protein) from Bt (Lee et al. 2003). In VipCot™ cotton lines, the insecticidal protein is secreted during the vegetative phase of bacterial development and referred to as an exotoxin. In Bollgard®, Bollgard II®, and WideStrike® cotton, the insecticidal protein endotoxin is produced during the bacterium reproductive phase and is enclosed in crystal (Micinski and Waltman 2005). The Vip3A protein expressed in VipCot™ cotton lines is distinguished from the endotoxins expressed by Bollgard®, Bollgard II®, and WideStrike®. Therefore cross-resistance between Vip3A and Cry toxins should be unlikely (McCaffery et al. 2005). Their initial cotton lines only expressed Vip3A as a single protein, but the new VipCot™ plants express both Vip3A and Cry1Ab proteins (McCaffery et al. 2005).

Efficacy of Bollgard® Cotton on *Helicoverpa zea*/*Heliothis virescens*

The Cry1Ac protein produced by Bollgard® cotton has demonstrated satisfactory control of *H. virescens* and pink bollworm, *Pectinophora gossypiella* (Maclntosh et al. 1990, Stewart et al. 2001). However, *H. zea* is more tolerant to the Cry1A(c) protein than *H. virescens* (Burd et al. 1999, Mahaffey et al. 1995). Control of *H. zea* by the insecticidal Cry1A(c) protein has been less successful and economically damaging infestations of this pest can occur on Bt cotton (Stewart and Knighten 2000). No significant differences were observed in the number of *H. zea* larvae on Bollgard® and non-Bollgard flower buds at 20-49 hours after infestation (De Spain et al. 1993). The Bollgard® cotton has demonstrated excellent control of the *H. virescens* (Stewart et al. 2001). Jech and Henneberry (2005) observed 100% mortality of *H. virescens* larvae on Bollgard® cotton compared to 0% mortality on conventional cotton. Less than 2% *H. virescens* larvae survived when Bollgard® plants were infested with neonates for 10 days. Larval weight and injury to small bolls were reduced on Bollgard® compared to the conventional cotton (Benedict et al. 1993). Late instar heliothine larvae typically are less susceptible to Cry proteins and more difficult to control than early instars. Bollgard® plants are toxic to only first through fourth instars of *H. zea*, but not to fifth instar. Movement of fifth instar larvae from conventional cotton to Bollgard® cotton could result in feeding injury to Bollgard® cotton (Halcomb et al. 1996). Mortality of the third instar *H. zea* larvae was similar between Bollgard® and non-Bt cotton squares (Leonard et al. 1997). Later instar stages (> third) of heliothines usually exhibit higher survivability on Bt cotton tissues compared to neonate and early stages of larvae (Jenkins et al. 1993). Parker et al. (2000) reported that 7 d–old *H. virescens* larvae demonstrated lower susceptibility to the Cry1Ac protein in a Bt cotton line when compared with 1 d and 4 d-old larvae. A similar study examining development of several larval instars (L1-L5 stages) of *H. zea*

on Bt cotton showed that only fifth instars were capable of successfully pupating (De Spain et al. 1993).

Neonate *H. virescens* survivorship on Bollgard® cotton ranged from 0 to 8% at six days after infestation (Jenkins et al. 1993). However 7-d-old larvae exhibited no significant reduction in survivorship when exposed to Bollgard® cotton for 48h (Parker et al. 2000). Gore et al. (2000) observed a delay in crop maturity and reduction in seedcotton yields of both Bollgard® and conventional cotton cultivars from *H. zea* injury. *H. zea* larvae are often observed feeding in flowers of Bollgard® plants and can result in relatively high levels (more than 50%) of boll abscission (Smith 1998, Gore et al. 2000). A reduction in larvae and damaged fruiting forms was achieved with pyrethroid over-sprays in both conventional and Bollgard® cotton cultivars. Since the introduction of Bollgard® cotton in 1996, foliar insecticide applications have been required to suppress *H. zea* infestations in Texas, as well as the Mid-South and Southeastern cotton production states (Leonard et al. 1997, 1998, 2001). In Louisiana, cotton growers treat Bollgard® cotton with ca. one to three insecticide over-sprays to control *H. zea*. Non-Bollgard cotton receives ca. four to eight insecticide applications per year (Leonard et al. 2001). Overall, the insecticidal activity of Bollgard® cotton expressing Cry1Ac protein provides sufficient control of *H. virescens* (Benedict et al. 1992, 1996, Jenkins et al. 1993, Stewart et al. 2001).

An additional issue influencing the efficacy of cry proteins in cotton plants against *H. zea* has been associated with the plant age and location of plant structure. In general, as the plant matures during the season, cry protein levels decrease (Greenplate 1999, Adamczyk et al. 2001, Olsen et al. 2005, Wan et al. 2005). In a study examining several Bollgard® cultivars, seasonal expression levels varied five-fold (Adamczyk and Sumerford 2001). In addition, vegetative tissue

of Bollgard® plants expresses higher Cry protein levels compared to floral structures such as pollen and flower petals (Greenplate 1999, Adamczyk et al. 2001, Gore et al. 2001).

Several studies have confirmed changes in heliothine movement and feeding on plants either treated with a foliar spray of Bt or on transgenic plants expressing cry proteins of Bt. On intact non-Bt cotton plants, *H. zea* larvae migrated from terminal leaves treated with a foliar Bt spray to adjacent expanded leaves or completely away from the plant terminal region (Jyoti et al. 1996). Moreover, *H. zea* avoid plant structures such as terminals and squares that exhibit high cry protein expression and feed on structures such as flowers and bolls that have been associated with lower cry protein expression levels (Greenplate 1999, Adamczyk et al. 2001, Gore et al. 2002). *H. virescens* larvae placed on Bt cotton plants frequently abandon those plants more often than larvae on non-Bt plants (Benedict et al. 1992, 1993, Parker and Luttrell 1999). *H. zea* larvae are more mobile on Bollgard® plants than on conventional non-Bt cotton (Gore et al. 2002). They reported that *H. zea* larvae moved approximately 3.3 nodes farther down on Bollgard® cotton compared to larvae on a non-Bollgard® line in 24 h. *H. zea* larvae are more frequently observed feeding in white flowers than on other Bt cotton structures (Smith 1998, Pietrantonio and Heinz 1999). Survival and development of *H. virescens* and *H. zea* larvae on Bt cotton plants appears to be influenced by this intra-plant variation in the expression of Cry proteins.

Efficacy of Bollgard II® Cotton on *Helicoverpa zea*/*Heliothis virescens*

Studies comparing the efficacy of the Cry1Ac protein in Bollgard® to the two proteins (Cry1Ac + Cry2Ab) in Bollgard II® against one or more target pests also have shown higher levels of mortality from the combination of two proteins compared to that produced by the single protein. Bollgard II® cotton cultivars (two proteins) are more toxic to *H. zea* and other noctuid pests such as fall armyworm and beet armyworm than Bollgard® cultivars (Adamczyk et al.

2001, Stewart et al. 2001). *H. zea* infestations do not appear to delay maturity or reduce yield of Bollgard II® cotton (Gore and Adamczyk 2004). Gore et al. (2001) reported higher levels of mortality for *H. zea* on Bollgard II® (49.0%) squares compared to levels on Bollgard® (8.0%) squares. Survival of *H. zea* second instars was 16.0% on Bt cotton expressing a single protein (Cry1Ac) compared to 2.0% on cotton tissue expressing two (Cry1Ac + Cry2Ab) proteins (Stewart et al. 2001). Jackson et al. (2003) reported that Bollgard® plants expressing a single cry protein (Cry 1Ac) had more squares (4.6%) and bolls (9.3%) damaged than squares (1.8%) and bolls (1.3%) of Bollgard II® plants expressing two cry proteins (Cry1Ac + Cry 2Ab). In another study, significantly fewer damaged squares were recorded on Bollgard II® (0.7) plants compared to those on Bollgard® (6.2%) and non-Bt (7.7) plants (Adamczyk et al. 2001). *H. zea* survivorship on floral structures of Bollgard II® plants was lower than on similar structures of Bollgard® plants (Gore et al. 2001). Wan et al. (2005) also documented lower *Helicoverpa armigera* (Hubner) larval densities on Bt cotton lines GK19 (Cry1Ac+ Cry1Ab) and BG1560 (Cry1Ac) throughout the season compared to that on non-Bt cotton. Defining the amount and type of cotton fruiting form injury produced by an individual larva can be necessary information for ultimately establishing economic injury levels. In a field study, an individual *H. zea* larva injured an average of 3.5 fruiting forms on Bollgard®, and 0.8 fruiting forms on Bollgard II® plants (Gore et al. 2003).

Efficacy of WideStrike® Cotton on *Helicoverpa zea*/*Heliothis virescens*

The Cry1Ac protein in WideStrike® cotton is similar to that found in Bollgard®, and provides satisfactory control of *H. virescens* (Leonard et al. 2005). WideStrike® cotton varieties express a second protein (Cry1F) and will improve control of *H.zea* and secondary Lepidopteran pests compared to Bollgard® (Willrich et al. 2005). Fruiting form damage by *H.zea* was low in

WideStrike® plots compared to conventional plots. WideStrike® plots yielded 1321 lb/acre as opposed to 472 lb/acre in conventional plots (Lorenz et al. 2005). Leonard et al. (2005) documented that WideStrike® cotton lines resulted in lower heliothine infested and damaged fruiting forms compared to that in conventional cotton. The number of *H.zea* and *H. virescens* larvae and damaged bolls were significantly lower in WideStrike® plots when compared to that in conventional cotton lines (Parker and Livingston 2005). Cotton tissue with a single protein Bt (Cry1F) was less efficacious against *H. zea* compared to tissue expressing WideStrike® (Cry1Ac and Cry1F) technology (Pellow et al. 2002).

Efficacy of VipCot™ Cotton on *Helicoverpa zea*/*Heliothis virescens*

The Vip3A protein expressed in VipCot™ exhibits a broad spectrum of activity against lepidopteran insect pests and is similar to Bollgard II® and WideStrike (Cloud et al. 2004, Mascarenhas 2004). Micinski and Waltman (2005) reported that *H.zea* and *H. virescens* caused significantly less damage and larval infestation in squares, flowers, and bolls of VipCot™ lines compared to that in conventional cotton (Coker 312). Another study suggests that VipCot™ plots have significantly fewer larval infested and damaged squares compared to that in conventional cotton plots (Leonard et al. 2005). Three VipCot™ cotton lines Cot 102, 202, and 203 appeared to have significantly fewer squares and bolls damaged from heliothine feeding than did Coker 312. However Cot 200 lines performed significantly better than Cot 100 lines on heliothines (Burd et al. 2005).

Considerable research has documented heliothine biology and ecology on Bt cottons that express single or multiple cry proteins. Currently, little information is available concerning the level of fruiting form injury from heliothine larval feeding on VipCot™ reproductive structures. Also, no data is available on sensitivity of selected larval age-classes of *H. zea* and *H. virescens*

and on the bioactivity of structures from plants transformed to express the Vip3A or VipCot™ traits on target pests in cotton. Before the VipCot™ technology can be fully integrated into a cotton pest management system; the consistency of performance against the primary heliothine targets should be documented.

The following objectives were proposed:

Objectives

I. To evaluate *H. zea*/*H. virescens* intra-plant movement and behavior in conventional and VipCot cotton lines.

II. To quantify the age specific mortality of heliothine larvae on VipCot cotton lines and conventional non-transgenic cotton.

III. To quantify *H.zea*/*H. virescens* survivorship on selected structures of VipCot cotton lines and conventional non-transgenic cotton.

IV. To determine injury caused by heliothine feeding on conventional non-transgenic and VipCot cotton lines.

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CHAPTER 2

HELIOTHINE LARVAL BEHAVIOR ON TRANSGENIC COTTON EXPRESSING A *BACILLUS THURINGIENSIS* INSECTICIDAL EXOTOXIN, VIP3A *

Introduction

Transgenic cotton cultivars that express ∂ - endotoxin from the bacterium *Bacillus thuringiensis* (Bt) have been widely adopted as alternative IPM strategies to conventional foliar sprays for management of specific lepidopteran pests. Two of the primary targets of the Bt cotton technologies in the U.S. are commonly referred to as heliothines and include the *Helicoverpa zea* (Boddie), and *Heliothis virescens* (F.). These species have historically been significant economic pests of cotton across the U.S. cotton belt, either from the cost of control strategies or associated yield losses (Williams 2006).

All commercial Bt cotton cultivars provide excellent control of *H. virescens*, but supplemental applications of insecticides are often necessary to manage *H.zea* (Leonard et al. 2001). In cage studies, more than 2% of *H. virescens* larvae survived on Bt cottons expressing a single crystal (Cry) insecticidal protein endotoxin (Benedict et al. 1993). The results of field studies showed that survival of *H. virescens* ranged from 0 to 8% on Bt plants, whereas on non-Bt plants, larval survival ranged from 49% to 88% (Jenkins et al. 1993). On commercial Bollgard® plants expressing only the Cry1Ac protein, high *H.zea* populations produced 14% boll injury (Mahaffey et al. 1995). *H.zea* larvae are often observed feeding in flowers of Bollgard® plants and can result in more than 50% of boll abscission (Smith 1998, Gore et al. 2000).

Expression levels and distribution of Cry1Ac protein are influenced by plant age as well as location of vegetative and fruiting structures on plants. Relative expression levels of this

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protein decrease as the cotton plant ages during the season (Greenplate 1999). In addition, not all plant structures (i.e. leaves, squares, flowers and bolls) express similar concentrations of Cry1Ac protein (Adamczyk et al. 2001, Gore et al. 2001, Olsen et al. 2005). Therefore, the behavior and distribution of *H. virescens* and *H.zea* larvae on a Bt cotton plant may be influenced by this intra-plant variation in protein expression. *H. virescens* larvae placed on Bt plants frequently abandon those plants more often than larvae on non-Bt cotton plants (Benedict et al. 1992, 1993, Parker and Luttrell 1999). A reduction in feeding activity by *H.zea* larvae compared to larvae offered the diet without Bt proteins was observed in two studies using meridic diets containing purified Bt proteins (Greenplate et al.1998, Akin et al. 2001). On intact cotton plants, *H.zea* larvae migrated from terminal leaves treated with a foliar Bt spray to adjacent expanded leaves or completely away from the plant terminal region (Jyoti et al. 1996). In another study, *H.zea* larvae placed on Bollgard® plants migrated from the site of infestation more often and more rapidly compared to larvae on non-Bollgard® plants (Gore et al. 2002).

Transgenic Bt technologies have advanced during recent years and cotton cultivars that express two insecticidal proteins (Bollgard II® and WideStrike®) have improved control of *H.zea* and other lepidopteran pest targets above that provided by the single protein expressed in Bollgard® (Stewart et al. 2001, Willrich et al. 2005). Scientists at Syngenta Crop Protection (Greensboro, NC) also have used genetic engineering protocols to develop novel transgenic cotton technology that expresses the Vip (vegetative insecticidal protein) 3A from Bt (Lee et al. 2003). Their initial cotton lines only expressed Vip3A as a single protein, but the new VipCot™ plants express both Vip3A and Cry1Ab proteins (McCaffery et al. 2006). The Vip3A protein has demonstrated significant levels of toxicity to lepidopteran targets, but also has exhibited

considerable selectivity to non-target invertebrates (Mascarenhas 2004, Micinski and Waltman 2005, Whitehouse et al. 2007).

The Vip3A protein is different from the Cry proteins expressed in Bollgard®, Bollgard II®, and WideStrike® cotton cultivars. It is secreted during the vegetative phase of bacterium development, whereas the insecticidal Cry proteins are produced during the bacterium reproductive phase, enclosed in crystals, and classified as endotoxins (Micinski and Waltman 2005, Yu et al. 1997). These differences between cotton plants expressing the Vip3A protein and those cotton plants expressing Cry proteins could provide a basis for reducing the potential of insect cross-resistance (McCaffery et al. 2005).

No research has examined *H. virescens* or *H.zea* larval behavior on transgenic cotton plants expressing Vip3A or VipCot™ proteins. The objective of this study was to observe and record the behavior of both pests on cotton plants expressing these proteins which is necessary to validate or refine the current IPM strategies for transgenic Bt cotton.

Materials and Methods

Test Site and Plant Material. This study was performed at the Louisiana State University Agricultural Center Macon Ridge Research Station near Winnsboro, LA (Franklin Parish) during 2005 and 2006. The conventional non-Bt cotton cultivar, 'Coker 312', and Bt cotton lines expressing either a single protein (Vip3A) or combination of proteins (Vip3A + Cry 1Ab [VipCot™]) were planted in a Gigger-Gilbert silt loam soil every two weeks from 9 June to 10 July during both years. This temporal seeding pattern of planting provided a wide range of plant maturities at the appropriate stages for infestations. Normal cultural practices and integrated pest management strategies recommended by the Louisiana Cooperative Extension Service were used to optimize plant development across the test site (Bagwell et al. 2005).

Insects. *H.zea* and *H. virescens* larvae were collected from sweet corn, *Zea mays* L., and garbanzo beans, *Cicer arietinum* L., during early June of each year. Colonies from those collections were established in the laboratory and reared for a minimum of one generation to eliminate parasitoids and pathogens and to obtain sufficient numbers at the proper stages of larval development. *H.zea* larvae were fed an artificial soy protein, wheat germ based diet (*Heliothis* premix, Ward Natural Science, Rochester, NY). *H. virescens* larvae were fed a pinto bean based diet (Leonard et al. 1987) in individual 29.5 ml plastic cups (Solo Co., Urbana, IL). Heliothine larvae were maintained at 27° ± 2°C and 85 ± 2% relative humidity with a 14:10 light:dark photoperiod until pupation. Adults of both species were held in 2.79 L cylindrical cardboard/plastic containers and fed 10% sucrose solution. A single layer of cheesecloth was placed on top of the containers to provide an adequate surface for oviposition. The oviposition sheets were harvested daily and placed into plastic bags until larval eclosion. Upon eclosion, larvae were offered the meridic diet for ≈ 48 h.

Infestation of Larvae on Pre-Flowering Cotton Plants. Seedlings in the Coker 312, Vip3A, and VipCot™ plots were thinned to one plant per row-foot before infestation to prevent interplant movement of larvae. Those plants designated for infestation were examined for the presence of eggs and larvae. Only those plants without a natural heliothine infestation were used in these studies. A trap (40.6 × 40.6-cm cardstock sheet) coated with Tanglefoot (Tanglefoot Company, Grand Rapides, MI) was placed on the soil beneath each plant prior to infestation. This trap placement was designed to capture any larva that exhibited "spin-down' behavior from the site of infestation and to demonstrate larval avoidance of the Bt toxin(s). A single first-instar (48 ± 6 h old) heliothine larva was placed in the terminal region of each plant using a small camel's hair brush. The infested plants were rated at 1, 3, 6, and 24 h after infestation by whole-

plant inspection. The number of nodes that a larva migrated from the original infestation site and avoidance (larval collection on the sticky trap) were recorded for each infested plant. This study consisted of 10 replications during the 2 yr (2005 and 2006) period. The cotton lines were arranged in a completely randomized design across the test area. Replications were represented by the day of infestation. Twenty plants of the Coker 312, Vip3A, and VipCot™ cotton lines were infested on each day. A total of 200 plants were infested during both years. All data were converted to percentages based on the number of plants infested on a given day and analyzed using repeated measures analysis of variance (PROC MIXED, Littell et al. 1996). Data were analyzed separately for larval species.

Infestation of Larvae on Flowering Cotton Plants. Cotton plants representing the Coker 312, Vip3A, and VipCot™cotton lines during flowering stages (8-9 main stem nodes above a first position sympodial white flower to the terminal [NAWF]) were infested with first instar (48 ± 6 h old) *H.zea* or *H. virescens* larvae. The procedures and experimental design for larval infestations during the flowering stages of development were similar to those described for pre-flowering cotton except that the sticky trap used to measure larval avoidance was not used. Plants were rated at 1, 3, 6, and 24 h after infestation by visually examining infested plants. The number of larvae that migrated from the original infestation site (plant terminal) and plant structure (terminal, square, flower, or boll) infested with larvae were recorded. Data were analyzed using repeated measures analysis of variance (PROC MIXED, Littell et al. 1996).

Results

Heliothine Larval Movement on Pre-Flowering Cotton Plants. *H.zea* larval behavior was significantly different among the three cotton lines. Cotton line (F = 35.19; df = 1, 12; P = 0.001), time of evaluation (F = 42.20; df = 3, 36; P = 0.001) and the cotton line by time of

evaluation interaction (F = 3.38; df = 6, 36; P = 0.01) effects were significant for percentages of larval-infested plant terminals (Fig. 2.1). The percentages of plant terminals that contained *H.zea* larvae in the Coker 312 (77.3 to 97.7%) plants were significantly higher compared to that for the Vip3A (32.8 to 75.0%) and VipCot™ (30.9 to 85.0%) cotton plants at all rating intervals. *H.zea* larvae migrated from the plant terminals of all three cotton lines, but most of the larval movement in the Coker 312 line had occurred by 3 h after infestation. On Vip3A and VipCot™ plants, larval movement from the site of infestation declined rapidly until 6 h after infestation.

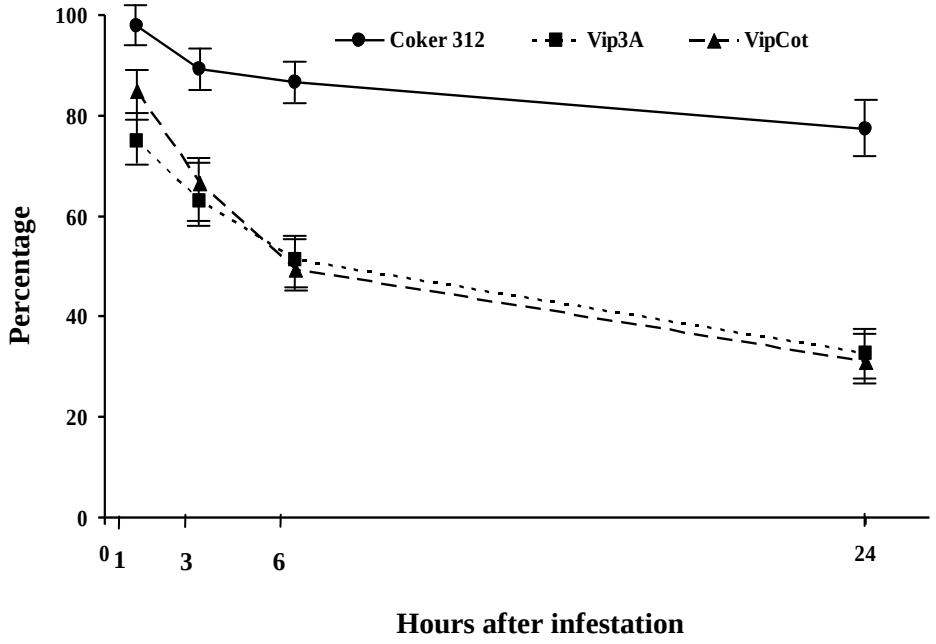

Figure 2.1. Percentage (± SE) of *H.zea* larval-infested non-Bt and Bt cotton plant terminals.

Cotton line (F = 25.19; df = 1, 12; P = 0.001), time of evaluation (F = 14.65; df = 3, 12; P = 0.0003) and the cotton line by time of evaluation interaction (F = 6.90; df = 6, 36; P = 0.01) was significant for percentages of *H.zea* larvae recovered from sticky traps beneath pre-flowering cotton plants (Fig. 2.2). Higher percentages of *H.zea* larvae were recovered on traps beneath

Vip3A (24.3 to 55.3%) and VipCot™ (15.0 to 58.3%) plants compared those on traps below the Coker 312 (2.8 to 17.7%) plants at all rating intervals ($P = 0.001$). There was no significant difference in percentage of larvae collected on traps below the Vip3A and VipCot™ plants during any evaluation period.

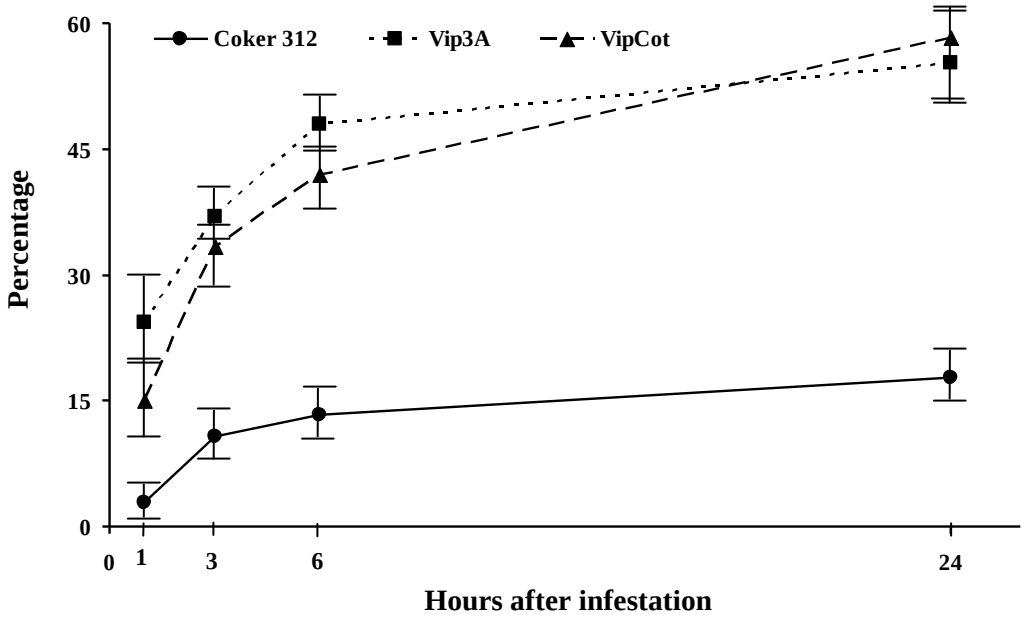

Figure 2.2. Percentage (± SE) of *H.zea* larvae recovered from sticky traps beneath non-Bt and Bt cotton plants.

Intra-plant vertical migration of *H.zea* larvae was influenced by cotton line ($F = 4.54$; df = 2, 7; $P = 0.01$) and time of evaluation ($F = 13.92$; df = 3, 21; $P < 0.0001$). Larvae migrated significantly farther from the infestation site on the Vip3A and VipCot™ plants compared with that on the Coker 312 plants at 3, 6, and 24 h after infestation (Fig. 2.3). Within 24 h, larvae were recorded on sympodia at 0.8, 1.5, and 2.8 main stem nodes below the terminal on the Coker 312, Vip3A, and VipCot™ plants, respectively. In addition, larval migration increased from the time of infestation to the endpoint (24 h after infestation) of the experiment. There was a significant interaction ($F = 4.49$; df = 6, 21; $P < 0.004$) between cotton line and time of evaluation for *H.zea*

larval movement. Larvae dispersed farther on VipCot™ plants compared with those on the Vip3A plants at 6 and 24 h after infestation.

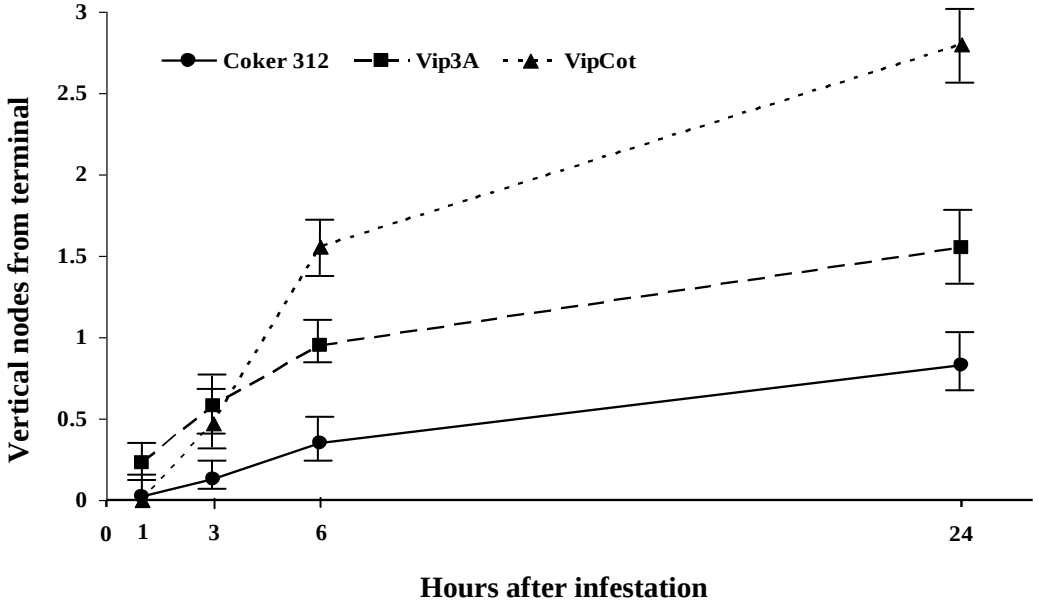

Figure 2.3 Distribution (± SE) of *H.zea* larvae recorded on main stem node sympodia below plant terminals on non-Bt and Bt cotton plants.

H. virescens larval behavior also was significantly influenced by the Vip3A and VipCot™ cotton lines. Cotton line (F = 29.49; df = 2, 17; P < 0.0001), time of evaluation (F = 42.76; df = 3, 51; P < 0.0001) and the cotton line by time of evaluation interaction (F = 12.31; df = 6, 51; P < 0.0001) affected percentages of larval-infested plant terminals (Fig. 2.4). *H. virescens* larvae were more common in the plant terminals of the Coker 312 plants than in the plant terminals of Vip3A and VipCot™ cotton plants at 3, 6, and 24 h after infestation. The percentages of larval-infested plant terminals for all three cotton lines declined across the complete evaluation period. By 24 h after infestation, percentages of Coker 312, Vip3A, and VipCot™ plant terminals that contained larvae were 72.4%, 41.0%, and 38.6%, respectively. Similar frequencies of larvae were observed in Vip3A and VipCot™ terminals within each evaluation interval.

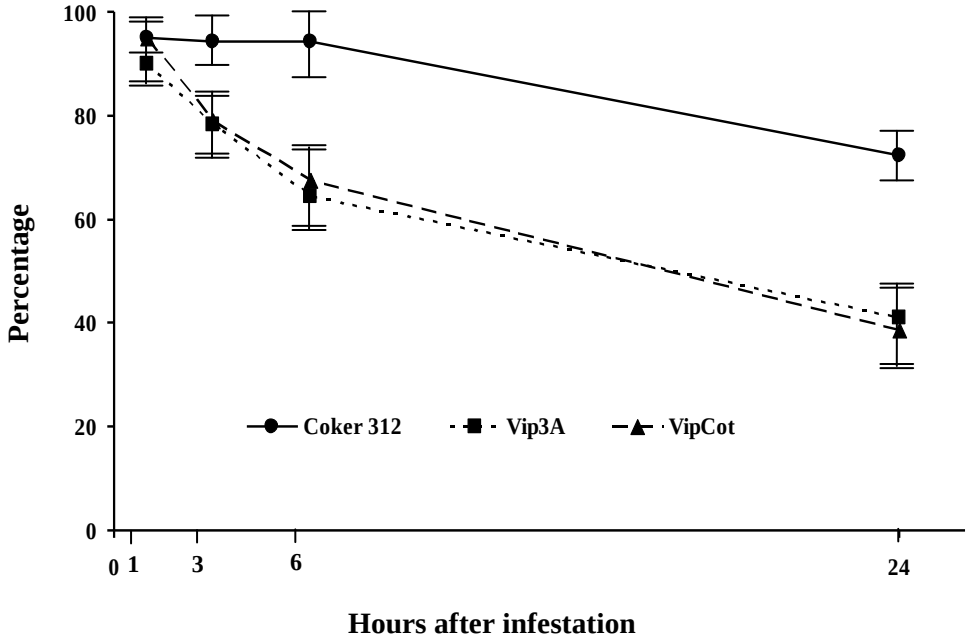

Figure 2.4. Percentage (± SE) of *H. virescens* larval-infested non-Bt and Bt cotton plant terminals.

The percentages of *H. virescens* larvae collected on sticky traps placed beneath the plants were significantly affected by cotton line ($F = 22.19$; df = 2, 17; $P < 0.0001$), time of evaluation ($F = 46.30$; df = 3, 51; $P < 0.0001$) and the cotton line by time of evaluation interaction ($F = 8.13$; df = 6, 51; $P < 0.0001$). The percentage of larvae found on traps beneath Vip3A and VipCot™ plants was significantly higher compared with that on traps beneath Coker 312 plants at 3, 6, and 24 h after infestation (Fig. 2.5). The percentage of *H. virescens* larvae observed on traps beneath Coker 312, Vip3A, VipCot™ cotton plants ranged from 5.0 to11.4%, 10.0 to 41.0%, and 5.0 to 50.0%, respectively, across all rating intervals. Vip3A and VipCot™ cotton lines did not differ in percentages of larvae recovered from traps at any evaluation interval.

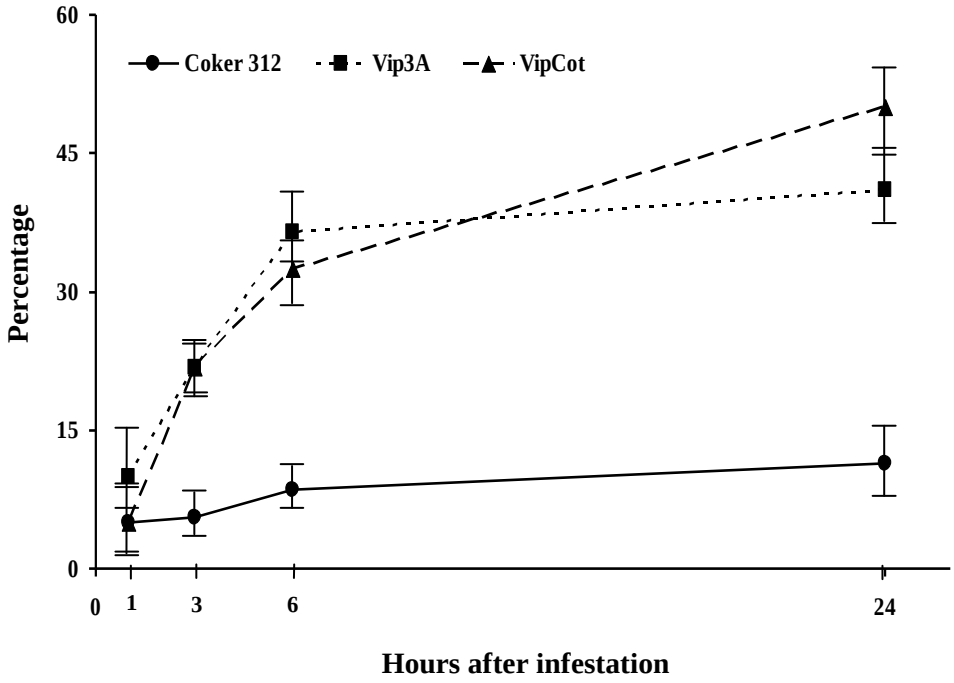

Figure 2.5. Percentage (± SE) of *H. virescens* larvae recovered from sticky traps beneath non-Bt and Bt cotton plants.

H. virescens larval migration down the main stem was influenced by cotton line (F = 24.66; df = 2, 9; P = 0.0002), time of evaluation (F = 59.71; df = 3, 27; P < 0.0001), and by the cotton line and time of evaluation interaction (F = 11.86; df = 6, 27; P < 0.0001). Larvae migrated significantly farther from the infestation site on the Vip3A and VipCot™ plants compared with larvae on the Coker 312 plants at 3, 6, and 24 h after infestation (Fig. 2.6). Larvae were found on sympodia at an average of 0.9, 2.0, and 2.8 main stem nodes below the terminal on the Coker 312, Vip3A, and VipCot™ plants, respectively, at 24 h after infestation. *H. virescens* larvae migrated significantly farther on VipCot™ plants compared with that on Vip3A plants by 24 h after infestation.

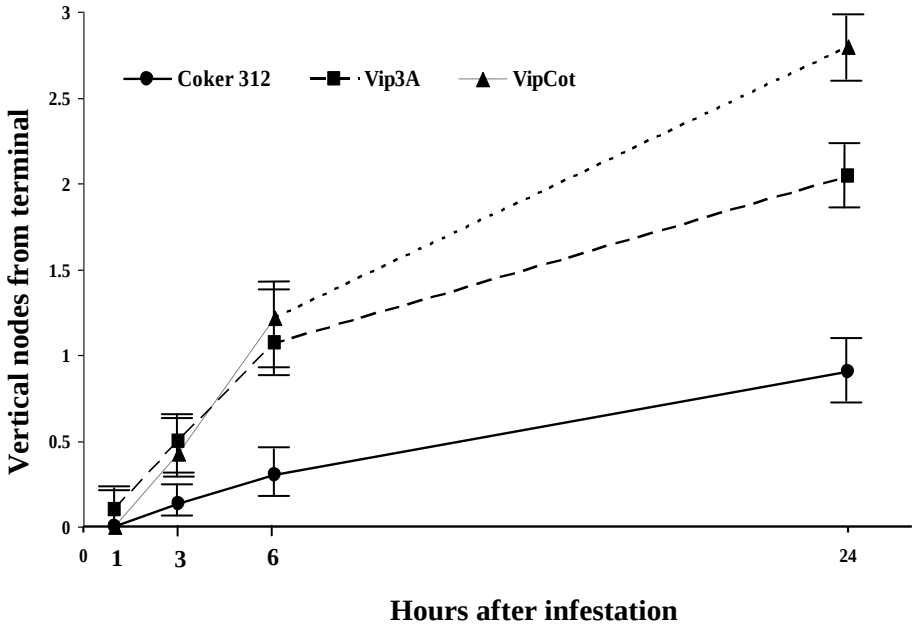

Figure 2.6. Distribution (± SE) of *H. virescens* larvae recorded on main stem sympodia below plant terminals on non-Bt and Bt cotton plants.

Heliothine Larval Movement on Flowering Cotton Plants. Intra-plant movement and preferred feeding sites for *H.zea* larvae were significantly different among the non-Bt, Coker 312, Vip3A and VipCot™ plants (Table 2.1). Cotton line (F = 48.64; df = 2, 9; $P < 0.001$), time of evaluation (F = 14.40; df = 3, 27; $P < 0.001$), and the cotton line by time of evaluation interaction (F = 24.58; df = 6, 27; $P < 0.001$) effects were significant for numbers of plant terminals infested with *H.zea* larvae. Higher numbers of larvae were recorded on Coker 312 plant terminals compared to numbers on the Vip3A and VipCot™ cotton lines at 6 and 24 h after infestation. Numbers of larvae in Coker 312 plant terminals decreased by two-fold at 24 h after infestation. A similar decrease was observed by 6 h after infestation on the Vip3A and VipCot™ plants.

Cotton line (F = 11.46; df = 2, 9; $P < 0.001$), time of evaluation (F = 19.99; df = 3, 27; $P < 0.001$), and the cotton line by time of evaluation interaction (F = 13.10; df = 6, 27; $P < 0.001$)

also had significant effects on *H.zea* in cotton squares (Table 2.1). Numbers of larvae on Coker 312 squares were significantly higher compared to number of larvae on Vip3A and VipCot™ cotton squares at all rating intervals. At 1 h after infestation numbers of *H.zea* larvae were approximately two-fold greater on Coker 312 squares compared to numbers on Vip3A and VipCot™ squares. There were no differences in numbers of *H.zea* on Vip3A and VipCot™ cotton squares. *H.zea* larvae found on Coker 312 squares increased about two-fold within 24 h after infestation. However, there was no significant change in the numbers of *H.zea* on Vip3A and VipCot™ squares across the entire sampling period.

Table 2.1. Number (mean ± SE) of *H.zea* observed on flowering stage non-Bt and Bt plant structures at selected time intervals after infestation of 2 d-old larvae in the plant terminal.

	Plant terminal (h after infestation)				Square (h after infestation)			
Line	1	3	6	24	1	3	6	24h
Coker 312	9.75 ± 0.41a	8.12 ± 0.25a	7.75 ± 0.25a	3.75 ± 0.32a	4.87 ± 0.25a	8.00 ± 0.18a	8.12 ± 0.23a	9.25 ± 0.47a
Vip3A	9.12 ± 0.29a	8.25 ± 0.24a	3.25 ± 0.09b	2.62 ± 0.25b	2.50 ± 0.27b	3.00 ± 0.41b	2.75 ± 0.28b	2.62 ± 0.16b
VipCot™	9.18 ± 0.25a	8.44 ± 0.30a	3.18 ± 0.20b	2.69 ± 0.25b	2.25 ± 0.25b	2.75 ± 0.19b	2.75 ± 0.24b	2.50 ± 0.29b

Means within a column followed by same letter are not significantly different ($\alpha = 0.05$, Tukey's Studentized Range Test).

The results for *H. virescens* larval movement recorded on Coker 312, Vip3A, and VipCot™ plants were similar to those for *H.zea* larvae (Table 2.2). Cotton line ($F = 19.31$; df = 2, 9; $P = 0.006$), time of evaluation ($F = 31.08$; df = 3, 27; $P < 0.001$), and the cotton line by time of evaluation interaction ($F = 20.60$; df = 6, 27; $P < 0.001$) effects were significant for numbers of plant terminals infested with *H. virescens*. Fewer *H. virescens* larvae remained in terminals of Vip3A and VipCot™ plants compared with that on Coker 312 plants at 6 and 24 h after infestation. Similar numbers of larvae were recorded on Vip3A and VipCot™ cotton terminals at

all sampling intervals. Numbers of *H. virescens* larvae in Coker 312, Vip3A, and VipCot™ cotton terminals decreased with each successive rating interval.

Table 2.2. Number (mean ± SE) of *H. virescens* observed on flowering stage non-Bt and Bt plant structures at selected time intervals after infestation of 2 d-old larvae in the plant terminal.

	Terminal (h after infestation)				Square (h after infestation)			
Line	1	3	6	24	1	3	6	24
Coker 312	9.50 ± 0.31a	7.75 ± 0.20a	7.25 ± 0.25a	3.85 ± 0.18a	5.00 ± 0.41a	7.50 ± 0.29a	8.25 ± 0.25a	9.25 ± 0.25a
Vip3A	9.25 ± 0.23a	8.75 ± 0.28a	3.00 ± 0.17b	2.62 ± 0.25b	2.00 ± 0.09b	2.50 ± 0.25b	3.00 ± 0.11b	2.50 ± 0.39b
VipCot™	9.25 ± 0.25a	8.50 ± 0.16a	3.50 ± 0.28b	3.00 ± 0.45b	2.25 ± 0.24b	2.75 ± 0.23b	2.50 ± 0.43b	2.50 ± 0.08b

Means within a column followed by same letter are not significantly different ($\alpha = 0.05$, Tukey's Studentized Range Test).

Cotton line ($F = 20.33$; df = 2, 9; $P < 0.0001$), time of evaluation ($F = 30.56$; df = 3, 27; $P < 0.0001$), and the cotton line by time of evaluation interaction ($F = 13.22$; df = 6, 27; $P < 0.0001$) also had significant effects *H. virescens* in squares (Table 2.2). Numbers of larvae were significantly higher on Coker 312 squares compared with those on Vip3A and VipCot™ squares at all rating intervals. At 1 h after infestation, numbers of *H. virescens* larvae were more than two-fold higher on Coker 312 squares compared to that on Vip3A and VipCot™ squares. There were no differences in numbers of *H. virescens* on Vip3A and VipCot™ squares. Similar to the observation for *H.zea*s, *H. virescens* larvae found on Coker 312 squares increased >1.8-fold by 24 h after infestation, but no differences in larvae on Vip3A and VipCot™ squares were recorded across the entire sampling period.

Discussion

H.zea and *H. virescens* larval movement from cotton plant terminals was significantly influenced by the Bt cotton lines evaluated in the present study. Both heliothine species dispersed

more rapidly on the two Bt lines compared to that on non-Bt (Coker 312) plants. In addition, by 24 h after infestation, *H.zea* and *H. virescens* larvae were detected farther down the plant on Vip3A (0.5 to 1.0 nodes) and VipCot™ (1.4 to 1.75 nodes) plants compared to Coker 312 plants. Larval dispersal was generally similar between Vip3A and VipCot™ lines with the exception of larval distribution on sympodia of flowering plants at 6 and 24 h after infestation. Gore et al. (2002) reported that *H.zea* larvae moved approximately 3.3 nodes farther on Bollgard® cotton compared to larvae on a non Bollgard® line at 24 h after infestation. In the present study, significantly more larvae were observed on traps beneath Vip3A and VipCot™ plants compared to those on traps beneath Coker 312 plants. Gore at al. (2002) recovered approximately three-fold more *H.zea* larvae on traps beneath Bollgard® cotton plants compared to traps beneath non-Bollgard® cotton plants. *H. virescens* larvae exhibited similar behavior on Bt plants and spent five-fold more time in spin-down behavior on Bt plants than on non-Bt cotton plants (Benedict et al. 1992). In a similar study, more *H. virescens* s (13%) infested on Bollgard® plants migrated to adjacent plants than larvae infested on non Bollgard® plants (Parker and Luttrell 1999). Finally, Benedict et al. (1992, 1993) observed that higher numbers of *H. virescens* migrated from terminals of Bt cotton compared to that on non-Bt cotton.

On flowering stage cotton plants, *H.zea* and *H. virescens* were observed on approximately 35 to 40% of the terminals on Coker 312, Vip3A, and VipCot™ cotton plants at 3 h after infestation. On Vip3A and VipCot™ plants, few larvae were observed in terminals compared to that on Coker 312 plants at 6 and 24 h after infestation. Heliothine larvae either began to disperse rapidly after infestation or attempted to avoid feeding on the Vip3A or VipCot™ plants by exhibiting spin-down behavior. In addition, heliothine larval numbers were significantly higher on Coker 312 squares than on Vip3A and VipCot™ squares at all rating intervals. Heliothine

larvae were not observed in flowers and bolls on infested plants for any of the cotton lines in this study. On the Vip3A and VipCot™ plants many of those insects likely either left the plant or were killed by the Bt protein(s). On Coker 312 plants, the insects appeared to remain on squares or in the terminal. Gore et al. (2002) observed more larvae on non Bollgard® squares at 24 h after infestation, which is consistent with the results of the present study. Pietrantonio and Heinz (1999) also observed more heliothine larvae in the top 20 nodes of non-Bt cotton plants compared to that on Bt cotton plants.

These differences in the intra-plant migration patterns and distribution of heliothine larvae are likely related to the avoidance behavior caused by Bt protein(s) present in the transgenic cotton plants. After 24h, 88% of *H.zea* larvae were found in cups containing non-Bt leaf tissue, whereas only 68% and 53% of larvae were observed in cups containing single and dual toxin plant tissue, respectively (Akin et al. 2001). Prior to the development of cotton transformed to express Bt protein, Jyoti et al. (1996) showed *H.zea* larvae dispersed from plant terminals to nearby expanded leaves within 6 h of a Bt spray application to those cotton terminals. Heliothine detection of Bt insecticidal proteins appears to occur for both foliar spray residues on leaf surfaces or expression throughout leaf tissue. The avoidance behavior is probably a survival mechanism that forces the insects to migrate more rapidly and farther to locate suitable non-toxic plant structures.

The results of the present study with Vip3A and VipCot™ are similar to the previous reports of Bt Cry proteins in cotton plants and their significant effects on heliothine behavior. Currently there has been no published report documenting heliothine larval behavior on transgenic cotton expressing the vegetative insecticidal protein, Vip3A. As a result of the behavioral effects of Bt on heliothines, *H.zea* larvae have been commonly found feeding in white

flowers in Bollgard® cotton fields. The current scouting protocols appropriate for sampling commercial Bt cultivars will likely be sufficient for cotton plants expressing the VipCot™ technologies. Several of the cotton production states' cooperative extension services recommend sampling fruiting structures such as flowers and bolls to detect *H.zea* infestations in commercial Bt cotton fields. Scouting for heliothines in commercial cotton fields expressing the Vip3A and VipCot™ traits should include an examination of vegetative and reproductive structures below the plant terminal. The heliothine complex of *H.zea* and *H. virescens* are primary targets of Bt traits in cotton and satisfactory control is usually obtained without foliar oversprays. However, in those instances where additional control is warranted, accurately detecting heliothine larval distribution in Bt cotton plants is necessary to provide the information for appropriate management decisions.

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CHAPTER 3

AGE-SPECIFIC MORTALITY OF *HELICOVERPA ZEA* AND *HELIOTHIS VIRESCENS* LARVAE ON FLOWER BUDS OF TRANSGENIC COTTON EXPRESSING VIP3A AND VIPCOT™ INSECTICIDAL PROTEINS

Introduction

The bollworm, *Helicoverpa zea* (Boddie), and tobacco budworm, *Heliothis virescens* (F.), are collectively known as the heliothine pest complex and are common across Southern United States cotton production regions (Williams 2003, 2006). Until the mid-to-late 1990's, foliar applications of insecticides were the primary control strategy used to manage heliothines and other important lepidopteran pests of cotton (Leonard et al. 1988, Graves et al. 1999). However, numerous instances of resistance to conventional insecticides and the occurrence of severe pest infestations resulted in significant economic losses for the cotton industry (Herzog et al. 1996). These persistent annual problems forced the development of alternative management strategies for lepidopteran pests which were necessary for cotton to remain a profitable crop.

Novel developments in cotton pest management strategies were the result of advances in genetic engineering. Cotton plants were transformed using transgenic technologies to express insecticidal proteins from the soil bacterium, *Bacillus thuringiensis* Berliner var. *kurstaki* (Bt). The initial commercial Bt product, Bollgard®, expresses a single crystal (Cry Ac) protein and was approved for use in cotton during 1996. The Bollgard technology has been highly effective against *H. virescens*, but *H. zea* is significantly less susceptible to this insecticidal protein than the former species (Luttrell et al. 1999, Stewart et al. 2001). Successful control of *H. zea* populations has been inconsistent with Bollgard® cotton lines. Supplemental foliar applications of conventional insecticides are occasionally required for *H. zea* management, especially during

the flowering stages of plant development (Mahaffey et al. 1995, Stewart et al. 2001, Gore et al. 2005).

H. zea larvae are often observed feeding on flowers of Bollgard® plants. This injury can induce greater than 50% abscission of cotton fruit (bolls) if those larvae are allowed to develop on floral and fruit structures (Smith 1998, Gore et al. 2000). The indeterminate growth habit of cotton plants provides insect pests with a flowering and boll development period that persists for several weeks during the production season (Oosterhuis and Jernstedt 1999). This relatively long period of cotton plant susceptibility, coupled with the fact that three to four generations of heliothines can occur during each season, increases the possibility of economic injury and yield losses.

Late instar heliothine larvae typically are less susceptible to insecticides and more difficult to control than early instars. Considerable work has demonstrated that conventional chemical insecticides are most active against early instars of several lepidpotera (Mink and Luttrell 1989, Shafique and Luttrell 1992, Bouvier et al. 2002). A similar response has been observed for susceptibility of heliothine larvae to Cry proteins in Bt cotton lines. Later instar stages (> third) of heliothines usually exhibit higher survivability on Bt cotton tissues compared to neonate and early stages of larvae (Jenkins et al. 1993). Parker et al. (2000) reported that 7 d–old *H. virescens* larvae demonstrated lower susceptibility to the Cry1Ac protein in a Bt cotton line when compared with 1 d and 4 d-old larvae. A similar study examining development of several larval instars (L1-L5 stages) of *H. zea* on Bt cotton showed that only fifth instars were capable of successfully pupating (De Spain et al. 1993).

The successful commercialization of Bollgard® cotton plants and the need to manage a wider spectrum of lepidopteran cotton pests prompted the agrochemical industry to develop

cotton cultivars that express two insecticidal Cry proteins. Two such products are Bollgard II® and WideStrike®, which have demonstrated significantly higher efficacy in controlling several more lepidopteran pest targets compared to that of Bollgard® (Stewart et al. 2001, Willrich et al. 2005). The most recent Bt cotton technology under development expresses the Vip (vegetative insecticidal protein) 3A protein (Lee et al. 2003). This insect pest management technology has been named VipCot™ and includes cotton lines that have been transformed to express both Vip3A and Cry1Ab proteins (McCaffery et al. 2006). The VipCot™ technology has also demonstrated efficacy against a range of lepidopteran pests (Mascarenhas 2004, Micinski and Waltman 2005, Whitehouse et al. 2007).

The Vip3A protein is produced by Bt in cotton tissues in a slightly different manner than the Cry proteins found in other Bt cottons (Yu et al. 1997, Micinski and Waltman 2005). Differences between cotton plants expressing the Vip3A protein and those plants expressing Cry proteins may also lead to differences in susceptibility of target pests such as heliothines. No research has examined the sensitivity of selected larval age-classes of *H. zea* and *H. virescens* to transgenic cotton plants expressing Vip3A or VipCot™ proteins. Therefore, this study was conducted to quantify heliothine larval mortality on VipCot™ cotton lines. This information should be useful in supporting insect resistance management plans for VipCot™ cotton and also to promote the integration of this technology into the current Bt cotton use strategies.

Materials and Methods

This study was performed at the Louisiana State University Agricultural Center's Macon Ridge Research Station near Winnsboro, LA (Franklin Parish) during 2005 and 2006. The conventional non-Bt cotton cultivar, 'Coker 312', and Bt cotton lines expressing either a single protein (Vip3A) or combination of proteins (Vip3A + Cry1Ab [VipCot™]) were planted in a

Gigger-Gilbert silt loam soil every two weeks from 9 June to 10 July during both years. This temporal seeding pattern of planting cotton seed provided a wide range of plant maturities at the appropriate stages for heliothine infestations. Normal cultural practices and integrated pest management strategies recommended by the Louisiana Cooperative Extension Service were used to optimize plant development across the test site (Bagwell et al. 2005, Stewart et al. 2007).

Insects. Late-instars of *H. zea* and *H. virescens* were collected from sweet corn; *Zea mays* L., and garbanzo beans; *Cicer arietinum,* respectively, during early June of each year. Colonies were established in the laboratory and reared for a minimum of one generation to eliminate parasitoids and pathogens and to obtain sufficient numbers at the selected age-classes of larval development. All tests were completed within three to four generations of colony establishment. Larvae of *H. zea* and *H. virescens* were fed an artificial soy protein and wheat germ meridic diet (*Heliothis* premix, Ward Natural Science, Rochester, NY) and a pinto bean-based meridic diet (Leonard et al. 1987), respectively, and reared in individual 29.5 ml plastic cups (Solo Co., Urbana, IL). These larvae were maintained at 27 ± 2°C and 85 ± 2% relative humidity with a 14:10 light:dark photoperiod until pupation. Adults were held in 2.79 L cylindrical cardboard/plastic containers and fed a 10% sucrose solution. A single layer of cotton gauze (cheesecloth, Grade 50) was placed on top of the containers to provide an adequate surface for oviposition. The sheets of gauze that contained eggs were harvested daily, placed into plastic bags, and sealed until larval eclosion. Upon eclosion, larvae were offered the meridic diet until they reached the proper age-class for infestations.

Plant Tissue Collection and Heliothine Infestations. Cotton squares were harvested from plots of the conventional non-Bt (Coker 312) and the two Bt (Vip3A and VipCot™) cotton lines, transported to the laboratory, and immediately offered to heliothine larvae. Squares were

harvested from 3 August to 23 August during both years. Pre-candle stage squares were collected from first position sites on sympodial branches in the upper one-third of the plant canopy. These squares were placed into 29.5 ml plastic cups.

Larval age-classes were determined by monitoring eclosion from eggs and transfer to the meridic diets within 12 h of hatching. One (2 d-old, 4 d-old, 6 d-old, and 8 d-old) larva was transferred into each cup containing a square and the cups were sealed with a plastic lid. Two squares were placed in each cup for older (8 d-old) larvae. The larval age-classes of 2, 4, 6, and 8 d generally corresponds to L1, L2, L3, and L3-L4 instars, respectively (Fye and McAda 1972). The larva in each cup was inspected every 2 d until complete mortality (100.0%) or pupation was observed on both Bt cotton lines. Mortality data for each species group (cotton line and larval age-class combination) were recorded every 2 d. A larva was considered dead if it was unable to assume an up right position when placed on its dorsal surface. All surviving larvae were offered additional squares every two days until they died or successfully pupated.

Experimental Design and Data Analysis. The plots of cotton lines were arranged in a completely random design across the field site. Treatment combinations (cotton lines and larval age-classes) for each species (*H. zea* and *H. virescens*) were arranged in a randomized block design with four blocks. Each date of infestation for a larval age-class within a species represented a block. Each year, thirty cups were infested for each cotton line and larval combination. A total of 240 larvae of each age-class for each species were infested for each cotton line over two years. Larval mortality data were converted to percentages for each species. Data were analyzed separately for the larval species and percentage mortality was compared among the cotton lines by larval age-class using the repeated measures procedures with ANOVA (PROC MIXED, Littell et al. 1996).

The time intervals from initial infestation to the maximum observed mortality (cumulative mortality) on both Vip3A and VipCot™ cotton lines were analyzed using a two-way factorial analysis by comparing larval age-classes and cotton lines within each species (PROC MIXED, SAS Institute 2003).

Results

Helicoverpa zea. Mortality levels of 2 d-old larvae were significantly influenced by cotton line (F = 140.4; df = 2,6; P < 0.0001), time of evaluation (*F* = 86.4; df = 4,36; *P* < 0.001), and the cotton line by time of evaluation interaction (*F* = 35.3; df = 8,36; *P* < 0.001) (Fig. 3.1A). Larval mortality on Vip3A and VipCot™ squares were higher than that on Coker 312 squares at 2 to 8 d after infestation. Larval mortality rapidly increased from 2 to 4 d after infestation on Vip3A (11.1 to 90.0%) and VipCot™ (24.4 to 90.5%) squares with little change in mortality after 4 d. Complete mortality (100.0%) was observed on Vip3A and VipCot™ squares at 8 and 6 d after infestation, respectively. However, larval mortality on Coker 312 was only 13.3% at 8 d after infestation. Larval mortality was similar on Vip3A and VipCot™ cotton squares at 4 and 6 d after infestation.

Cotton line (F = 81.9; df = 2,6; P < 0.0001), time of evaluation (*F* = 67.1; df = 4,36; *P* < 0.0001), and the cotton line by time of evaluation interaction (*F* = 25.9; df = 8,36; *P* < 0.0001) significantly affected mortality of 4 d-old *H. zea* larvae (Fig. 3.1B). Both Vip3A and VipCot™ squares produced significantly higher larval mortality compared to that for Coker 312 squares at all rating intervals. A rapid increase in larval mortality was observed from 2 to 4 d after infestation on Vip3A (31.8 to 70.9%) and VipCot™ (55.8 to 98.0%) cotton squares. Complete larval mortality (100.0%) was observed on Vip3A and VipCot™ cotton squares at 10 and 6 d

after infestation, respectively. *H. zea* larval mortality on Coker 312 squares at 10 d after infestation was 9.0%.

Mortality levels for 6 d-old larvae were significantly affected by cotton line ($F = 98.7$; df = 2,6; $P < 0.0001$), time of evaluation ($F = 19.8$; df = 4,36; $P < 0.0001$), and the cotton line by time of evaluation interaction ($F = 25.6$; df = 8,36; $P < 0.0001$). Larval mortality on Vip3A and VipCot™ squares was significantly higher than that for larvae offered Coker 312 squares at all rating intervals (Fig. 3.1C). At 2, 4, and 6 d after infestation, larval mortality (45.0, 95.8, and 100.0%, respectively) was significantly higher on VipCot™ squares compared to that (13.7, 56.5, and 77.5%, respectively) on Vip3A squares. Although initial larval mortality at 2 d after infestation was lower on Vip3A squares compared to that on VipCot™ squares, the rate of increase in larval mortality was similar between the two Bt cotton lines at 4 d after infestation. Complete *H. zea* mortality (100.0%) was observed on Vip3A and VipCot™ cotton squares at 10 and 6 d after infestation, respectively. Larval mortality observed on Coker 312 squares was only 9.4% at 10 d after infestation.

Larval mortality trends for *H. zea* within the 8 d age-class were similar to that for the earlier age-classes (Fig. 3.1D). Cotton line ($F = 52.8$; df = 2,6; $P < 0.0001$), time of evaluation ($F = 38.2$; df = 4,36; $P < 0.0001$), and the cotton line by time of evaluation interaction ($F = 30.54$; df = 8,36; $P < 0.0001$) significantly affected the mortality of 8 d-old larvae. Vip3A and VipCot™ squares resulted in significantly higher mortality compared to that on Coker 312 squares at all rating intervals. At 2, 4, 6, and 8 d after infestation, larval mortality (45.0 to 100.0 %) on VipCot™ squares was significantly higher than that (23.9 to 88.7%) on Vip3A squares. Complete larval mortality (100.0%) was observed on Vip3A and VipCot™ squares at 10 and 8 d after infestation, respectively.

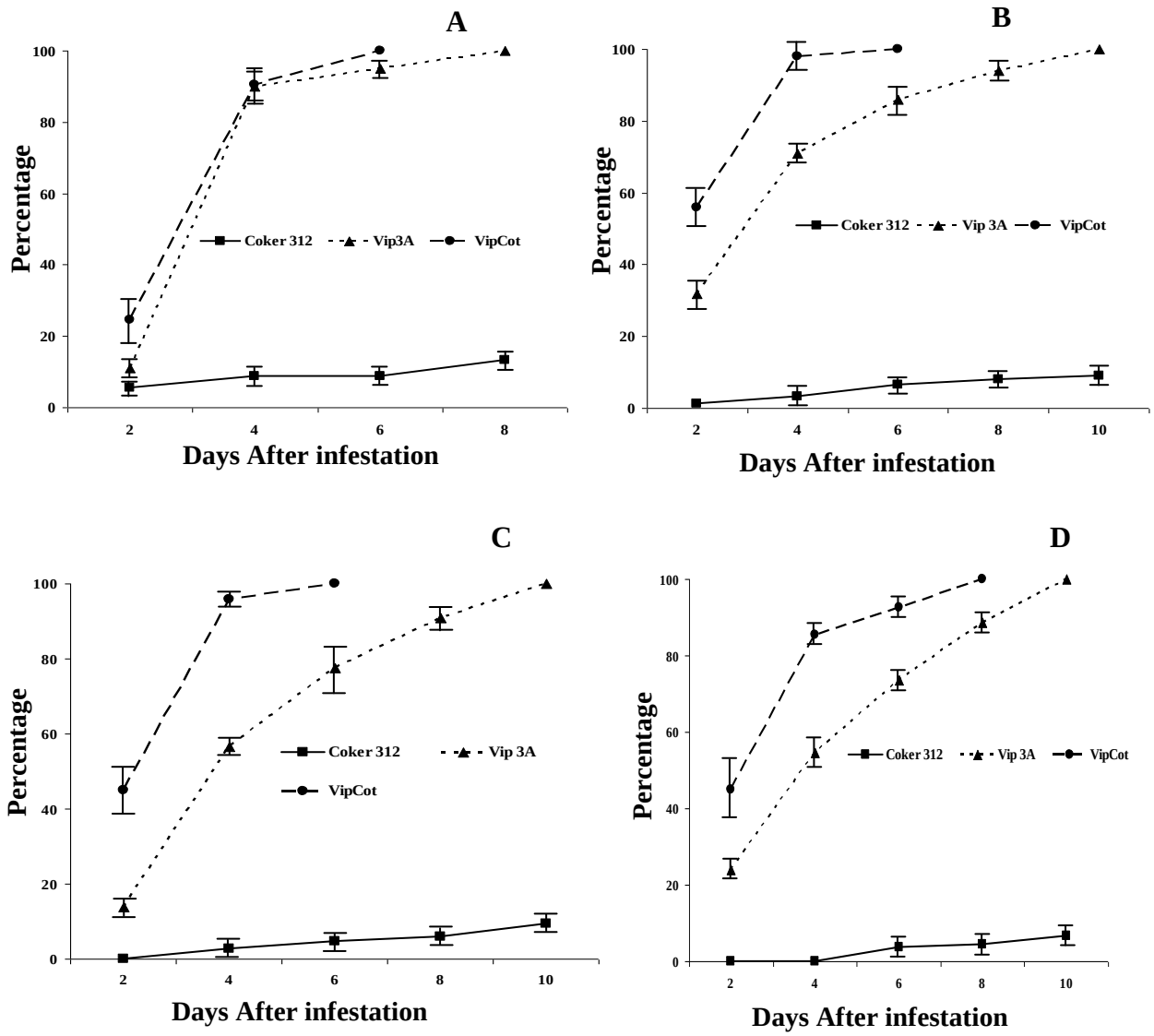

Figure 3.1. Cumulative percentage mortality (± SE) of four age-classes (A. 2 d, B. 4 d, C. 6 d, D. 8 d) of *Helicoverpa zea* (Boddie), larvae infested on flower buds of conventional non-Bt (Coker 312) and Bt (Vip3A and VipCot™) cotton lines.

Significant differences among age-classes of *H. zea* larvae in the post-infestation interval for complete larval mortality were observed on VipCot™ squares (F = 6.88; df = 3,21; P = 0.002), but not for Vip3A (F = 2.43; df = 3,21; P = 0.12) squares (Fig. 3.2). The time interval for complete mortality (100.0%) across all age-classes ranged from 8.5 to 9.9 d on Vip3A squares.

The temporal larval mortality pattern among age-classes on VipCot™ squares was generally similar to that on Vip3A squares, and ranged from 6.5 to 8.0 d. However, larvae in the 8 d age-class required significantly more time for complete mortality to occur compared to that for the other age-classes on VipCot™ squares.

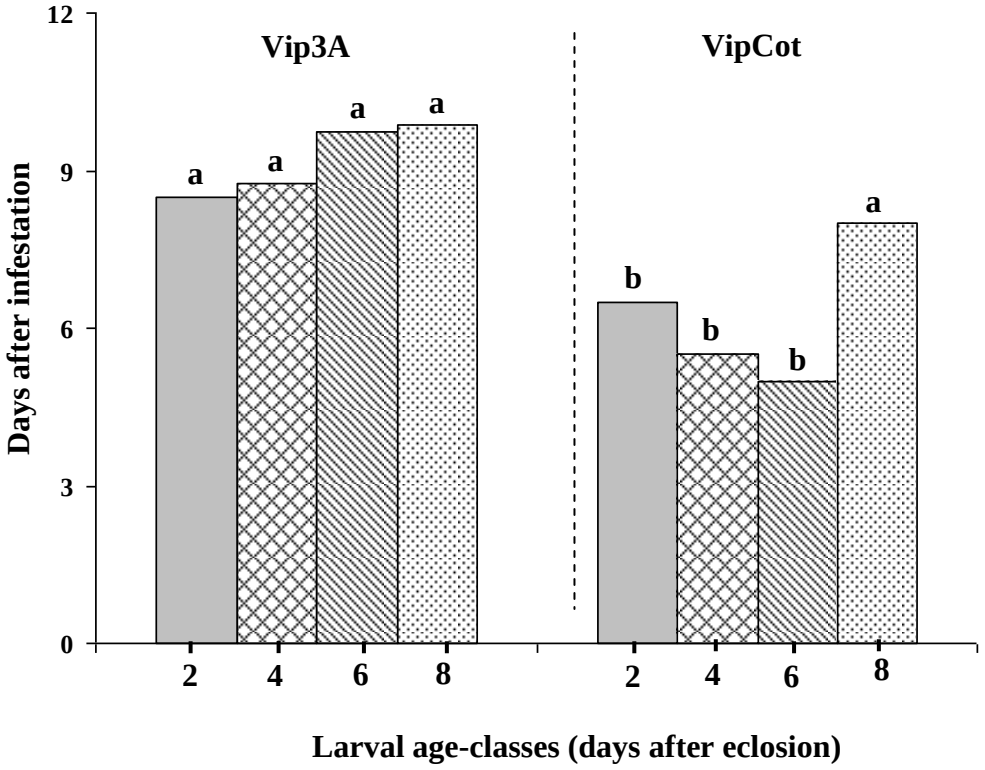

Figure. 3.2. Mean post-infestation interval (d) until maximum observed percentage mortality of *Helicoverpa zea* (Boddie), larvae on Vip3A and VipCot™ flower buds. Bars with the same letter indicate no significant differences among ages within cultivar ($P > 0.05$).

***Heliothis virescens*.** Mortality levels of 2 d-old larvae were significantly influenced by cotton line ($F = 93.6$; df = 2,6; $P < 0.0001$), time of evaluation ($F = 37.7$; df = 3,27; $P < 0.0001$), and the cotton line by time of evaluation interaction ($F = 61.1$; df = 6,27; $P < 0.0001$) (Fig. 3.3A). Larval mortalities on both Vip3A and VipCot™ squares were higher than for larvae on Coker 312 squares at all rating intervals. VipCot™ cotton squares produced significantly higher

larval mortality compared to that on Vip3A squares at all rating intervals. Larval mortality rapidly increased from 2 (46.1%) to 6 d (100.0%) after infestation on VipCot™ cotton squares. Complete mortality (100.0%) was observed on Vip3A and VipCot™ squares at 14 and 6 d after infestation, respectively. However, *H. virescens* larval mortality on Coker 312 squares was only 15.5% at 14 d after infestation.

Cotton line ($F = 82.8$; df = 2,6; $P < 0.0001$), time of evaluation ($F = 61.8$; df = 5,45; $P < 0.0001$), and the cotton line by time of evaluation interaction ($F = 24.1$; df = 10,45; $P < 0.0001$) significantly affected mortality of 4 d-old *H. virescens* larvae (Fig. 3.3B). Larval mortality was significantly higher on squares of Vip3A and VipCot™ compared to that on Coker 312 squares at all rating intervals. A rapid increase in larval mortality was observed from 2 (35.8%) to 6 d (100.0%) after infestation on VipCot™ cotton squares. On Vip3A squares, the maximum observed mortality at 22 d after infestation was 94.7%. All surviving larvae (5.3%) pupated. Complete larval mortality (100.0%) was observed on VipCot™ cotton squares at 6 d after infestation. *H. virescens* larval mortality on Coker 312 squares was only 14.9% at 22 d after infestation and all surviving larvae had successfully pupated.

Mortality values for 6 d-old *H. virescens* larvae were significantly affected by cotton line ($F = 75.1$; df = 2,6; $P < 0.0001$), time of evaluation ($F = 97.6$; df = 6,54; $P < 0.0001$), and the cotton line by time of evaluation interaction ($F = 24.4$; df = 12,54; $P < 0.0001$). Vip3A and VipCot™ cotton squares produced significantly higher larval mortality compared to that on Coker 312 squares at all rating intervals (Fig. 3.3C). At 2 d after infestation, larval mortality (61.2%) was significantly higher on VipCot™ squares compared to that (16.7%) on Vip3A squares. The maximum observed mortality on Vip3A squares was 95.1% at 26 d after infestation with all surviving larvae successfully pupating. Complete *H. virescens* mortality (100.0%) was observed

on VipCot™ cotton squares at 6 d after infestation. The final larval mortality observed on Coker 312 squares was 11.9% at 26 d after infestation by which time all surviving larvae had successfully pupated.

Larval mortality trends for *H. virescens* larvae within the 8 d age-class were similar to those for the other age-classes (Fig. 3.3D). Cotton line ($F = 68.7$; df = 2,6; $P < 0.0001$), time of evaluation ($F = 73.6$; df = 5,45; $P < 0.0001$), and the cotton line by time of evaluation interaction ($F = 47.6$; df = 10,45; $P < 0.0001$) significantly affected percentage mortality of 8 d-old larvae. Mortality levels were significantly higher on Vip3A and VipCot™ squares compared to that on Coker 312 squares at 2 d after infestation. Larval mortality was more rapid on VipCot™ squares compared to that on Vip3A squares. On Vip3A squares, the maximum observed mortality was 94.1% at 22 d after infestation, and as with the previous age-classes, all surviving larvae pupated. Complete larval mortality (100.0%) was observed on VipCot™ squares at 10 d after infestation. The final larval mortality observed on Coker 312 squares was 12.3% at 22 d after infestation by which time all surviving larvae had successfully pupated.

Significant differences among age-classes of *H. virescens* larvae in the post-infestation interval to achieve complete larval mortality or pupation were observed on Vip3A ($F = 98.87$; df = 3,21; $P < 0.0001$) and VipCot™ ($F = 29.25$; df = 3,21; $P < 0.0001$) squares (Fig. 3.4). The time interval for maximum observed mortality across all age-classes ranged from 13.8 to 24.6 d on Vip3A squares. Complete larval mortality of larvae in the 2 d age-class on Vip3A squares was more rapid compared to that for all other age-classes. In addition, the 4 d and 6 d age-classes required more time to reach maximum observed mortality or pupate compared to that for 8 d-old larvae on Vip3A squares. The time interval for complete (100.0%) larval mortality ranged from 5.7 to 9.2 d across all age-classes on VipCot™ squares, and was generally less than that for larval

mortality on Vip3A squares. In addition, the oldest age class (8 d) of *H. virescens* larvae required significantly more time to reach maximum observed mortality compared to that for the other age-classes on VipCot™ squares.

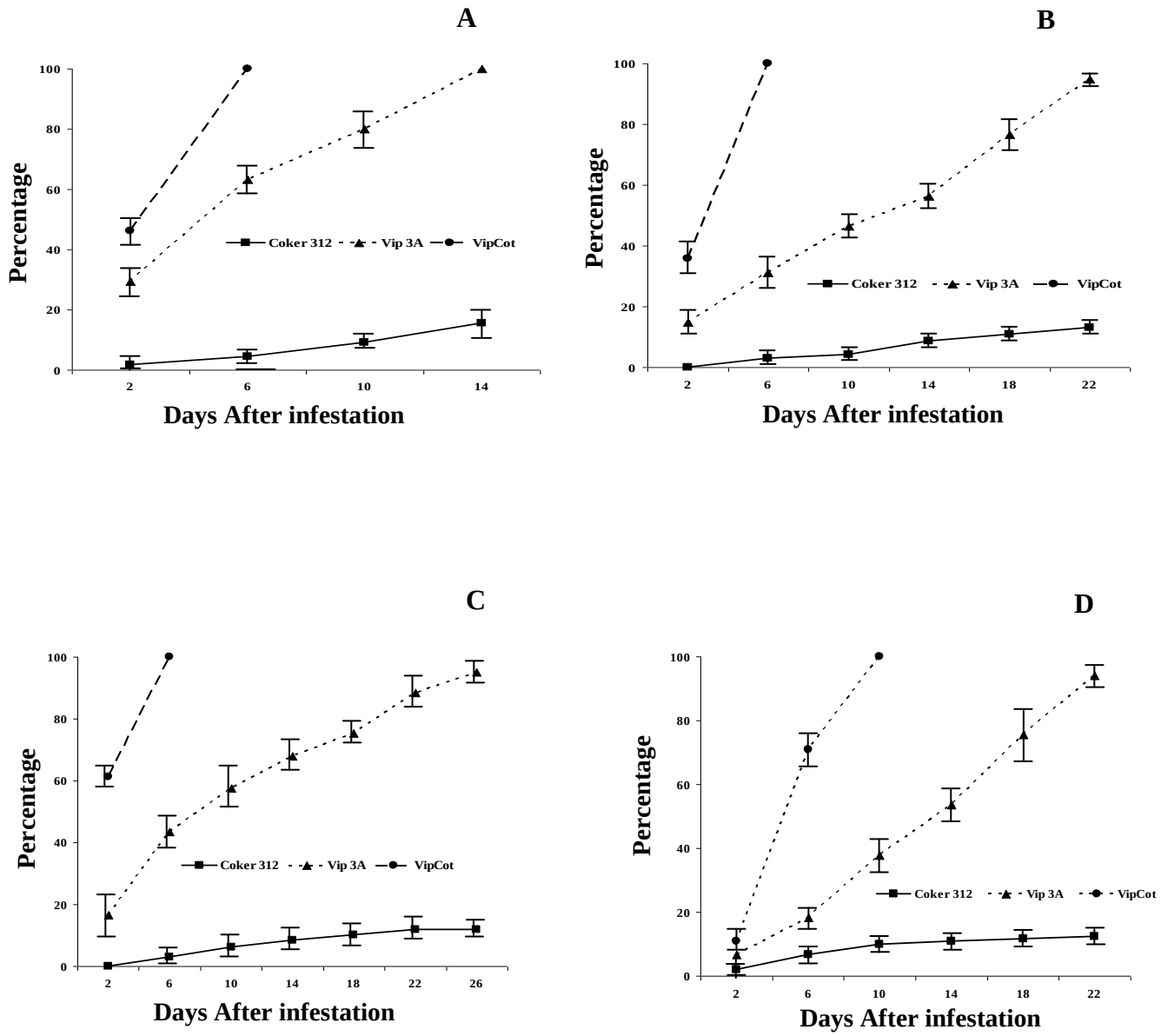

Figure 3.3. Cumulative percentage mortality (± SE) of four age-classes (A. 2 d, B. 4 d, C. 6 d, D. 8 d) of *Heliothis virescens* (F.), larvae infested on flower buds of conventional non-Bt (Coker 312) and Bt (Vip3A and VipCot™) cotton lines.

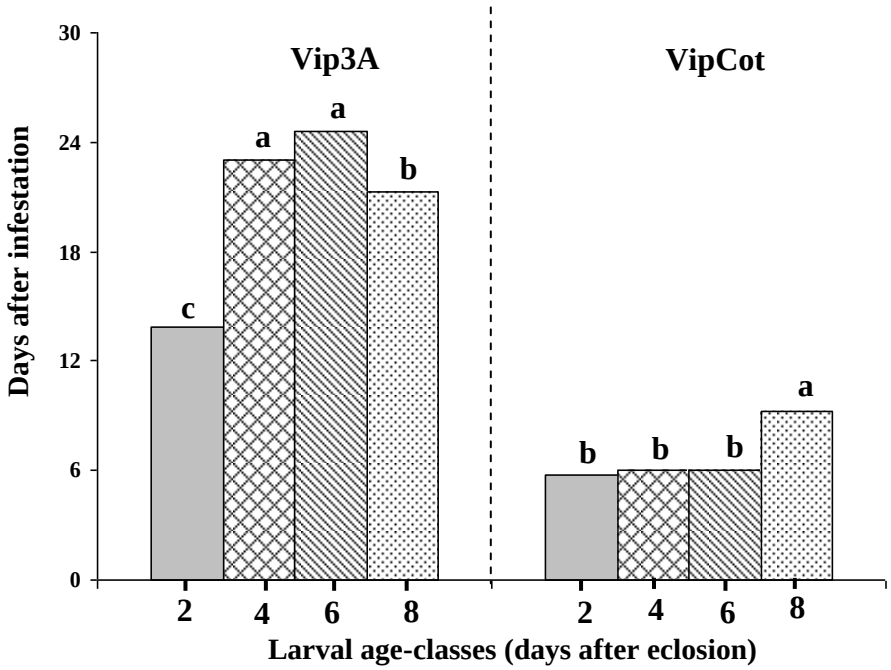

Figure. 3.4. Mean post-infestation interval (d) until maximum observed percentage mortality of *Heliothis virescens* (F.), larvae on Vip3A and VipCot™ flower buds. Bars with the same letter indicate no significant differences among ages within cultivar ($P > 0.05$).

Discussion

The cotton lines expressing both Vip3A and VipCot™ insecticidal proteins produced relatively high mortality levels of *H. zea* (100.0%) and *H. virescens* (94.1%- 100.0%). On Vip3A squares, complete mortality (100.0%) of all age-classes of *H. zea* was observed, but for *H. virescens* larvae in the 4 d, 6 d, and 8 d age-classes, mortality levels did not exceed 95.0%. Larval mortality levels for all age-classes, regardless of heliothine species, eventually reached 100.0% on VipCot™ cotton squares, but 8 d-old larvae required a longer interval before complete mortality was observed. At nearly every evaluation interval, heliothine larval mortality levels were lower on Vip3A squares compared to those on VipCot™ cotton squares. The combined

effects of the two proteins in VipCot™ (Vip3A + Cry1Ab) appear to be necessary to produce complete mortality on the heliothine complex. The geographic range of these pests overlap across much of the United States cotton production regions and transgenic Bt traits that control both species will be a more effective IPM tool accepted by the cotton industry.

Studies comparing the efficacy of the Cry1Ac protein in Bollgard® to the two proteins (Cry1Ac + Cry2Ab) in Bollgard II® against one or more target pests also have shown higher levels of mortality from the combination of two proteins compared to that produced by the single protein. Gore et al. (2001) reported higher levels of mortality for *H. zea* on Bollgard II® (49.0%) squares compared to levels on Bollgard® (8.0%) squares. Survival of *H. zea* second instars was 16.0% on Bt cotton expressing a single protein (Cry1Ac) compared to 2.0% on cotton tissue expressing two (Cry1Ac + Cry2Ab) proteins (Stewart et al. 2001). Similar results have been observed when heliothine mortality on one of the cry protein components in WideStrike® was compared to that for a combination of the two cry proteins found in commercial WideStrike® cotton cultivars. Cotton tissue with a single protein Bt (Cry1F) was less efficacious against *H. zea* compared to tissue expressing WideStrike® (Cry1Ac and Cry1F) technology (Pellow et al. 2002).

Only small differences in the final observed larval mortality values among age-classes within each species and Bt cotton line were observed in the present study. Complete mortality eventually occurred for all age-classes of both species, except for 4 d, 6 d, and 8 d age-classes of *H. virescens* larvae. Considerable work has shown that larger, late instar larvae are usually more tolerant of toxicants either as conventional insecticides or Bt proteins. In a laboratory bioassay, when *H. virescens* larvae were exposed to plant tissues treated with chemical insecticides, lethal concentration $(LC)_{50}$ values dramatically increased as larval age is increased. The LC_{50} values

were higher for *H. virescens* third instars compared to those for first instars (Shafique and Luttrell 1992). De Spain et al. (1993) found complete mortality (100.0%) of first to fourth instars of *H. zea* on Bt (Cry protein) cotton squares, but only 49.0% mortality of fifth instars. In a study by Parker at al. (2000), neonate, 4 d and 7 d-old *H. virescens* fed Bt (Cry1Ab) cotton tissue, 97.0% mortality of neonate *H. virescens* larvae was observed at 14 d after exposure, but only 38.0% and 52.0% mortality levels were observed for 4 d and 7 d-old larvae, respectively. Mortality of *H. zea* second and third instars after exposure to fruiting structures of Bollgard II® was 93.0% and 36.0%, respectively (Stewart et al. 2001). The relatively narrow range of high mortality values across heliothine larval age-classes in the present study is probably related to the fact that these cumulative mortality values were not recorded until all larvae in that age-class either died or pupated, rather than examination at a single time point after infestation.

For both species and across all age-classes, the rates of larval mortality were somewhat more rapid on VipCot™ cotton squares compared to that on Vip3A squares. Although complete (100.0%) or nearly complete (>94.0%) mortality of heliothine larvae in all age-classes was observed on both Bt cotton squares, the final post-infestation evaluation on Vip3A squares was delayed by an average of 2.9 d for *H. zea* and 13.4 d for *H. virescens* compared to that for each species on VipCot™ squares. These differences in time to maximum observed mortality between the two species were not directly compared, but these results also appear to illustrate considerable differences in susceptibility of *H. zea* and *H. virescens* to Vip3A squares. In a similar study, Gore et al. (2001) reported rapid mortality of *H. zea* larvae on Bollgard II® bracts and squares compared to similar structures on Bollgard®. Larval mortality rapidly decreased from 24 to 72 h after infestation on Bollgard II® squares (3.0 to 92.0%, respectively) compared to that on Bollgard® (4.0 to 51.0%, respectively) squares.

These results suggest that both species of heliothine larvae were extremely susceptible to squares of the VipCot™ line. None of the larvae, regardless of age, were capable of successfully pupating on VipCot™ squares. The observed cumulative rates of larval mortality were similar for 2 d, 4 d, and 6 d-old *H. zea* and *H. virescens* on this cotton line, as well. These data further suggest that older larvae (8 d-old) survived longer on Vip3A and VipCot™ squares compared to younger larvae (≤6 d-old). The ability of the VipCot™ technology to generate complete and rapid mortality across a range of heliothine larval age-classes should allow this technology to provide comparable performance to that of the standard commercial products and become another useful tool for the cotton industry.

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CHAPTER 4

SURVIVORSHIP OF *HELICOVERPA ZEA* AND *HELIOTHIS VIRESCENS* ON COTTON PLANT STRUCTURES EXPRESSING A *BACILLUS THURINGIENSIS* VEGETATIVE INSECTICIDAL PROTEIN

Introduction

Genetically engineered plants have rapidly become alternate management strategies to conventional chemical control in integrated pest management programs in many crops. *Bacillus thuringeinsis* crops including corn, *Zea mays*, cotton, *Gossypium hirsutum* L., and potato, *Solanum tuberosum*, were approved for registration in 1995 by the United States Environmental Protection Agency. This adoption of transgenic insect pest protection traits has resulted in an overall reduction in use of foliar insecticides against the target pests in these crops (Shelton et al. 2002). This effect has been especially noticeable in cotton where the adoption of cultivars expressing Bt proteins has reduced foliar sprays from 2.4 in 1995 to 0.39 in 2006 for an overall reduction of 92% (Williams 1996, 2007).

Since the first transgenic Bt cotton, Bollgard®, was commercialized in the United States in 1996, acreage planted to transgenic cotton cultivars has increased dramatically. In Louisiana, the percent Bt cotton acreage planted has increased from 38% in 1997 to 97% in 2006 (Williams 1998, 2007). Bollgard® cotton has provided excellent control of *Heliothis virescens* (F.) and pink bollworm, *Pectinophora gossypiella* (Saunders) (Stewart et al. 2001). However, *Helicoverpa zea* (Boddie), management has been inconsistent with the single crystal protein (Cry1Ac) in Bollgard® cotton cultivars. Supplemental applications of foliar insecticides have been occasionally required for satisfactory control (Leonard et al. 1998, Stewart et al. 2001, Gore et al. 2005) particularly during flowering stages of plant development and when these insect populations persist at moderate to high levels.

The insecticidal performance of Bt proteins expressed by plants on the cotton heliothine complex has been reported in numerous studies. Survivorship of *H. zea* during a native infestation on Bollgard® cotton cultivars was sufficient to cause >30% capsule (boll) damage (Mahaffey et al. 1995). Gore et al. (2000) reported >50% abscission of bolls when *H. zea* larvae were allowed to feed on floral structures of Bt cotton expressing the Cry1Ac protein. In some instances, late instars of *H. zea* and *H. virescens* infested on selected plant tissues of Bt cotton successfully pupated (De Spain et al. 1993, Parker et al. 2000). However, *H. virescens* is extremely susceptible to the Cry1Ac protein and native infestations of this pest have not established in Bollgard® cotton fields or cause economic injury (Stewart et al. 2001). *H. zea* is inherently more tolerant to the Cry1Ac protein than *H. virescens*. Susceptibility of *H. virescens* larvae is 20 to 30-fold greater to Cry1Ac than *H. zea* (Luttrell et al. 1999).

An additional issue influencing the efficacy of cry proteins in cotton plants against *H. zea* has been associated with the plant age and location of plant structure. In general, as the plant matures during the season, cry protein levels decrease (Greenplate 1999, Adamczyk et al. 2001, Olsen et al. 2005, Wan et al. 2005). In a study examining several Bollgard® cultivars, seasonal expression levels varied five-fold (Adamczyk and Sumerford 2001). In addition, vegetative tissue of Bollgard® plants expresses higher cry protein levels compared to floral structures such as pollen and flower petals (Greenplate 1999, Adamczyk et al. 2001, Gore et al. 2001).

Several studies have confirmed changes in heliothine movement and feeding on plants either treated with a foliar spray of Bt or on transgenic plants expressing cry proteins of Bt. On intact non-Bt cotton plants, *H. zea* larvae migrated from terminal leaves treated with a foliar Bt spray to adjacent expanded leaves or completely away from the plant terminal region (Jyoti et al. 1996). Moreover, *H. zea* avoid plant structures such as terminals and squares that exhibit high

cry protein expression and feed on structures such as flowers and bolls that have been associated with lower cry protein expression levels (Greenplate 1999, Adamczyk et al. 2001, Akin et al. 2002, Gore et al. 2002). *H. virescens* larvae placed on Bt cotton plants abandon those plants more often than do larvae on non-Bt plants (Benedict et al. 1992, 1993, Parker and Luttrell 1999). *H. zea* larvae are more mobile on Bollgard® plants than on conventional non-Bt cotton (Gore et al. 2002). *H. zea* larvae are more frequently observed feeding in white flowers than on other Bt cotton structures (Smith 1998, Pietrantonio and Heinz 1999). Survival and development of *H. virescens* and *H. zea* larvae on Bt cotton plants appears to be influenced by this intra-plant variation in the expression of cry proteins.

Transgenic cotton cultivars that express two insecticidal proteins (Bollgard II® and WideStrike®) have improved control of *H. zea* and other lepidopteran pest targets above that provided by the single cry protein expressed in Bollgard® (Stewart et al. 2001, Willrich et al. 2005). Scientists with Syngenta Crop Protection have used similar transgenic engineering protocols to develop cotton lines that express a novel vegetative insecticidal protein,Vip3A (Lee et al. 2003). The Vip3A is different from the cry proteins found in the current commercial products (Bollgard®, Bollgard II®, and WideStrike®). Vip is secreted during the vegetative phase of bacterium development, where as the insecticidal cry proteins are produced during the bacterium reproductive phase, enclosed in crystals, and classified as endotoxins (Micinski and Waltman 2005, Yu et al. 1997). The initial transformation events included plants that expressed Vip3A as a single protein, but recent advances led to the development of VipCot™ plants that express Vip3A and also the Cry1Ab protein (McCaffery et al. 2006). Considerable research has documented heliothine biology and ecology on Bt cottons that express single or multiple cry proteins. Currently, no information is available on the bioactivity of structures from plants

transformed to express the Vip3A or VipCot™ traits on target pests in cotton. Therefore, the objective of this study was to quantify heliothine survivorship on selected plant structures of Vip3A and VipCot™ cotton lines.

Materials and Methods

This study was performed at the Louisiana State University Agricultural Center's Macon Ridge Research Station near Winnsboro, LA (Franklin Parish) during 2005 and 2006. The procedures for these studies followed the general outline previously described by Bommireddy et al. (2007). The conventional non-Bt cotton cultivar, 'Coker 312', and Bt cotton lines expressing either a single protein (Vip3A) or combination of proteins (Vip3A + Cry 1Ab [VipCot™]) were planted in a Gigger-Gilbert silt loam soil every two weeks from June 9 to July 10 during both years. This temporal seeding pattern provided a wide range of plant maturities at the appropriate growth stages for harvesting plant structures. Normal cultural practices and integrated pest management strategies recommended by the Louisiana Cooperative Extension Service were used to optimize plant development across the test site (Bagwell et al. 2005, Stewart et al. 2007).

Insects. *H. zea* and *H. virescens* larvae were collected from sweet corn, *Zea mays* L., and garbanzo beans, *Cicer arietinum* L., during early June of each year. Colonies from those collections were established in the laboratory and reared for a minimum of one generation to eliminate parasitoids and pathogens and to obtain sufficient numbers at the proper stages of larval development. *H. zea* larvae were fed an artificial soy protein, wheat germ-based diet (*Heliothis* premix, Ward Natural Science, Rochester, NY). *H. virescens* larvae were fed a pinto bean-based diet (Leonard et al. 1987) in individual 29.5 ml plastic cups (Solo Co., Urbana, IL). Heliothine larvae were maintained at 27° ± 2°C and 85 ± 2% relative humidity with a 14:10 light:dark photoperiod until pupation. Adults of both species were held in 2.79 liter cylindrical

plastic containers and fed a 10% sucrose solution. A single layer of cotton gauze (cheesecloth, Grade 50) was placed on top of the containers to provide an adequate surface for oviposition. The sheets of gauze containing eggs were harvested daily, placed into plastic bags, and sealed until larval eclosion. Upon eclosion, larvae were transferred to cups containing a meridic diet for ≈ 48 h. Larvae in the F2, F3 or F4 generations removed from the original field collections were used in these studies.

Cotton Plant Structures and Heliothine Infestations. Selected plant tissues were harvested from plots of conventional Coker 312, Vip3A, and VipCot™, and were immediately transported to the laboratory. All tissues were harvested from cotton plants that had seven to nine main stem nodes above the upper-most first position white flower on a sympodial branch. This period of harvest corresponded to plants at 60 to 75 days after planting. The plant structures harvested from cotton plants included: 1) vegetative tissue (first fully expanded terminal leaf), 2) bracts removed from squares, 3) whole (intact) flower buds (squares) with bracts removed (pre-candle size), 4) white flower petals, 5) flower anthers (style and stigma), and 6) small intact bolls (<4-d-old). The structures were placed individually into 29.5 ml plastic cups. Flower petals were replaced with fresh petals 2 d after infestation to avoid desiccation. First position intact white flowers were harvested from plants and transported to the laboratory where petals and anthers were removed before larval infestation. Squares were harvested in a similar manner and placed as intact structures in cups. Additional squares were harvested such that bracts could be removed and also placed in cups. Squares, white flowers, and bolls were collected from first position sites on sympodial branches in the upper one-third of the plant canopy.

Three *H. zea* or *H. virescens* (<3-d-old; L2 stage) larvae were transferred to each cup containing a plant structure and then cups were sealed with a plastic lid. The larvae were allowed

to feed for 96 h. The larvae in each cup were inspected daily and mortality was recorded at 24, 48, 72, and 96 h after initial exposure. A larva was considered dead if it was unable to assume an up right position when placed on its dorsal surface.

Treatments (species and cotton line) were arranged in a randomized complete block design with four blocks. Each date of infestation represented a block. A minimum of fifteen cups were infested for each cotton line and species combination on a given date. A total of 360 larvae of each species were infested on each structure for each cotton line over two years. Survivorship data for the vegetative plant tissue (terminal leaf) were analyzed using repeated measures analysis of variance (PROC MIXED, Littell et al. 1996). Means were separated according to Fisher's protected least significant difference (SAS Institute 2003). Percent survivorship within each rating interval was analyzed using a two factor (3 cotton lines x 5 reproductive structures) analysis of variance (PROC MIXED, Littell et al. 1996). Data were analyzed separately for each larval species.

Results

***Helicoverpa zea* Survivorship on Terminal Leaves.** Cotton line ($F = 88.76$; df = 2,6; $P < 0.0001$), time of evaluation ($F = 52.37$; df = 3,27; $P < 0.001$), and the cotton line by time of evaluation interaction ($F = 19.37$; df = 6,27; $P < 0.001$) influenced larval survivorship on terminal leaf tissue (Fig. 4.1). *H. zea* survivorship was significantly higher on Coker 312 leaf tissue than that on Vip3A and VipCot™ tissues at all rating intervals. Larval survivorship declined very rapidly during the 0 to 48 h period after infestation on Vip3A (100 to 28%) and VipCot™ (100 to 20%) leaves. At 96 h after infestation on Vip3A and VipCot™ leaves, survivorship was only 4% and1%, respectively. However, larval survivorship on Coker 312 was

86% at 96 h after infestation. Survivorship was similar between Vip3A and VipCot™ leaves at all rating intervals.

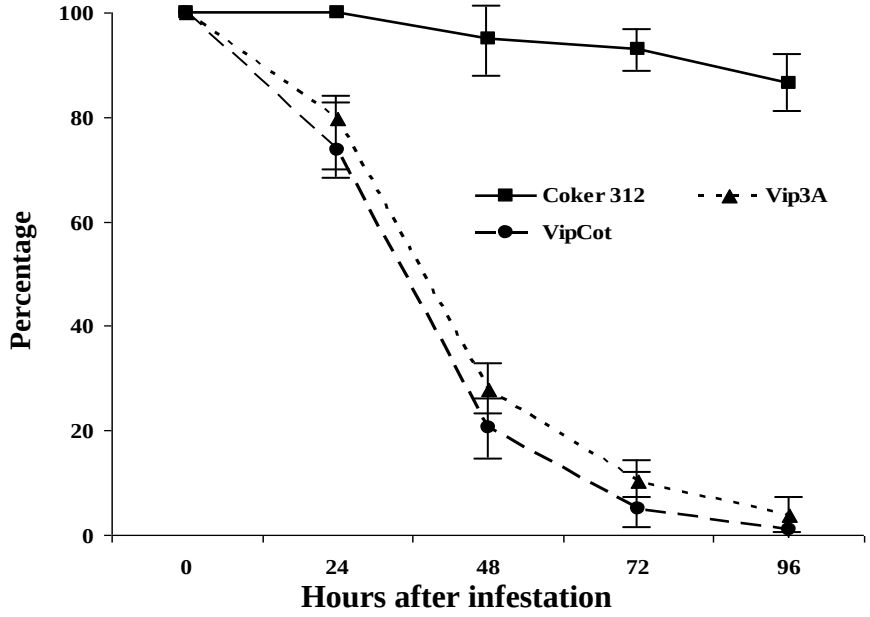

Figure 4.1. Survivorship (mean ± SE) of *H. zea* larvae on vegetative tissue (terminal leaves) of conventional non-transgenic (Coker 312) and transgenic Bt cotton (Vip3A and VipCot™) lines.

***Helicoverpa zea* Survivorship on Reproductive Structures of Cotton.** The cotton line by plant structure interaction ($P = 0.04$, $P = 0.02$, $P = 0.04$, and $P = 0.003$ at 24, 48, 72, 96 h, respectively) significantly influenced larval survivorship on reproductive structures (Table 4.1). *H. zea* survivorship was not different among reproductive structures of non-Bt (Coker 312) cotton within any rating interval. Larval survivorship ranged from 98-100%, 93-96%, 84-95%, and 81-87% at 24, 48, 72, and 96 h after infestation, respectively. Significant differences in larval survivorship were observed among Vip3A reproductive structures at 48, 72, and 96 h after infestation. Survivorship ranged from 82-92%, 47-68%, 17-48%, and 7-28% at 24, 48, 72, and 96 h after infestation, respectively (Table 4.1). Survivorship was higher on flower anthers and bolls compared to that on other structures at 48 h after infestation.

Table 4.1. Survivorship (mean ± SE) of *H. zea* larvae on reproductive structures of conventional non-Bt (Coker 312) and transgenic Bt cotton (Vip3A and VipCot™) lines.

Hours after infestation	Plant structure	Percent					
		Coker 312	Vip3A	VipCot	*F*	df	*P* > *F*
24							
	Square bracts	98 ± 1aA	87 ± 4aA	87 ± 4aA	2.67	2,42	0.08
	Whole squares	98 ± 1aA	82 ± 8bA	84 ± 5bA	4.64	2,42	0.01
	Flower petals	99 ± 1aA	87 ± 3bA	87 ± 3bA	3.10	2,42	0.05
	Flower anthers	100 ± 0aA	92 ± 1aA	86 ± 2aA	3.05	2,42	0.07
	Intact boll	99 ± 1aA	87 ± 7bA	75 ± 5cA	9.07	2,42	0.005
	F	0.05	0.94	1.69			
	df	4,42	4,42	4,42			
	P > *F*	0.99	0.45	0.17			
48							
	Square bracts	94 ± 2aA	47 ± 3bB	35 ± 7cB	23.73	2,42	< 0.0001
	Whole squares	93 ± 5aA	54 ± 7bB	48 ± 7bA	11.41	2,42	0.0001
	Flower petals	96 ± 1aA	53 ± 9bB	37 ± 8cB	23.82	2,42	< 0.0001
	Flower anthers	95 ± 4aA	68 ± 5bA	52 ± 8cA	12.40	2,42	< 0.0001
	Intact boll	96 ± 1aA	63 ± 4bA	35 ± 5cB	8.90	2,42	0.0006
	F	0.35	3.69	2.94			
	df	4,42	4,42	4,42			
	P > *F*	0.84	0.011	0.03			
72							
	Square bracts	89 ± 2aA	17 ± 4bB	13 ± 1bA	45.88	2,42	< 0.0001
	Whole squares	95 ± 3aA	23 ± 8bAB	7 ± 3cB	36.47	2,42	< 0.0001
	Flower petals	89 ± 3aA	29 ± 6bA	12 ± 2cAB	40.81	2,42	< 0.0001
	Flower anthers	90 ± 1aA	48 ± 4bA	28 ± 6cA	25.30	2,42	< 0.0001
	Intact boll	84 ± 3aA	38 ± 8bA	19 ± 8cA	28.08	2,42	< 0.0001
	F	0.55	5.68	2.42			
	df	4,42	4,42	4,42			
	P > *F*	0.70	0.001	0.05			
96							
	Square bracts	83 ± 1aA	11 ± 4bBC	6 ± 3cB	49.42	2,42	< 0.001
	Whole squares	87 ± 1aA	14 ± 4bB	1 ± 1cC	45.40	2,42	< 0.001
	Flower petals	86 ± 1aA	7 ± 4bC	3 ± 1cC	51.77	2,42	< 0.001
	Flower anthers	81 ± 2aA	28 ± 4bA	18 ± 4cA	28.19	2,42	< 0.001
	Intact boll	81 ± 2aA	20 ± 6bB	7 ± 2cB	38.43	2,42	< 0.001
	F	0.16	2.32	3.85			
	df	4,42	4,42	4,42			
	P > *F*	0.92	0.04	0.003			

Means within a row followed by same lower case letter and within a column and time interval followed by same upper case letter are not significantly different ($\alpha = 0.05$) according to Fisher's protected least significant difference.

At 72 h after infestation, survivorship was lower on square bracts compared to that on all other structures. At 96 h after infestation, larval survivorship was higher on flower anthers compared to that on all other structures.

VipCot™ reproductive structures also influenced larval survivorship during the 48 to 96 h interval after infestation. Survivorship among structures ranged from 75-87%, 35-52%, 7-28%, and 1-18% at 24, 48, 72, and 96 h after infestation, respectively (Table 4.1). Survivorship was highest on squares and flower anthers compared to that on other structures at 48 h after infestation. Larval survivorship was lowest on squares at 72 h after infestation which was different from survivorship on all structures except that on flower petals. At 96 h after infestation, flower anthers produced significantly higher larval survivorship compared to that for the other structures.

H. zea survivorship was influenced by cotton type and reproductive structure at each rating intervals (Table 4.1). At 24 h after infestation, survivorship was significantly higher on squares, flower petals, and bolls of Coker 312 plants compared to corresponding structures of Vip3A and VipCot™ plants. At 24 h after infestation, survivorship was higher on Vip3A bolls compared to that on VipCot™ bolls. Higher survivorship was recorded on Vip3A square bracts, flower petals, flower anthers, and bolls compared to corresponding structures of VipCot™ plants at 48 h after infestation. Survivorship on Vip3A squares, flower petals, flower anthers, and bolls was significantly higher compared to that for similar structures of VipCot™ plants at 72 h after infestation. Survivorship was significantly higher on all Vip3A structures compared to that on similar structures of VipCot™ plants at 96 h after infestation.

***Heliothis virescens* Survivorship on Terminal Leaves.** Cotton line ($F = 46.54$; df = 2,4; $P = 0.002$), time of evaluation ($F = 28.28$; df = 3,18; $P < 0.001$), and the cotton line by time of

evaluation interaction (F = 30.28; df = 6,18; P < 0.001) influenced larval survivorship on terminal leaf tissue (Fig. 4.2). *H. virescens* survivorship was higher on Coker 312 leaves compared to that on Vip3A and VipCot™ leaves at all rating intervals. Larval survivorship declined very rapidly from 0 to 48 h after infestation on Vip3A (100 to 51%) and VipCot™ (100 to 44%) leaves. At 72 and 96 h after infestation, larval survivorship was higher (34% and 20%, respectively) on Vip3A leaves compared to that (11% and 4%, respectively) on VipCot™ leaves. However, larval survivorship on Coker 312 was 83% at 96 h after infestation.

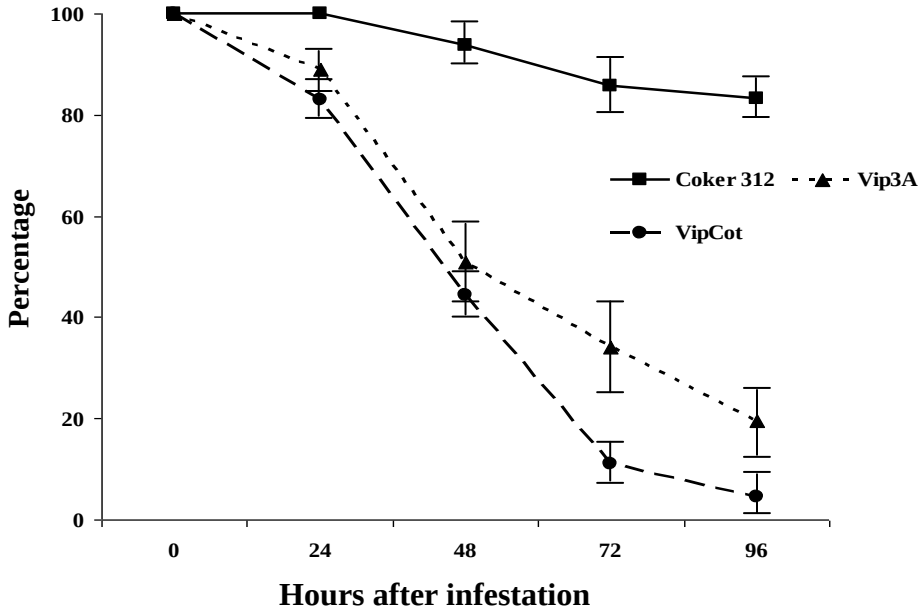

Figure 4.2. Survivorship (mean ± SE) of *H. virescens* larvae on vegetative tissue (terminal leaves) of conventional non-transgenic (Coker 312) and transgenic Bt cotton (Vip3A and VipCot™) lines.

***Heliothis virescens* Survivorship on Reproductive Structures of Cotton.** The cotton line by plant structure interaction (P = 0.002, P < 0.0001, P = 0.01, and P = 0.002 at 24, 48, 72, 96 h, respectively) influenced larval survivorship on reproductive structures (Table 4.2). *H. virescens* survivorship was not different among reproductive structures of conventional non-Bt (Coker 312) 91-98%, 88-91%, and 81-87% at 24, 48, 72, and 96 h after infestation, respectively.

Table 4.2. Survivorship (mean ± SE) of *H. virescens* larvae on reproductive structures of conventional non-Bt (Coker 312) and transgenic Bt cotton (Vip3A and VipCot™) lines.

Hours after infestation	Plant structure	Percentage					
		Coker 312	Vip3A	VipCot	*F*	df	*P* > *F*
24							
	Square bracts	100 ± 0aA	98 ± 2aA	97 ± 1aA	0.42	2,34	0.66
	Whole squares	100 ± 0aA	96 ± 4aA	74 ± 4bB	20.69	2,34	<0.0001
	Flower petals	99 ± 1aA	93 ± 4aA	84 ± 2bB	5.77	2,34	0.007
	Flower anthers	98 ± 2aA	84 ± 2bB	84 ± 4bB	5.52	2,34	0.008
	Intact boll	99 ± 1aA	91 ± 4aA	81 ± 2bB	7.11	2,34	0.003
	F	0.11	5.11	6.58			
	df	4,34	4,34	4,34			
	P > *F*	0.99	0.002	0.0005			
48							
	Square bracts	95 ± 2aA	80 ± 7bA	78 ± 5cA	2.45	2,34	0.011
	Whole squares	98 ± 1aA	83 ± 5bA	41 ± 6cC	22.70	2,34	< 0.0001
	Flower petals	94 ± 2aA	23 ± 6cC	47 ± 2bB	35.25	2,34	< 0.0001
	Flower anthers	96 ± 3aA	43 ± 5bB	49 ± 5bB	16.29	2,34	< 0.0001
	Intact boll	91 ± 2aA	64 ± 4bA	59 ± 3bB	5.64	2,34	0.007
	F	0.15	17.12	4.44			
	df	4,34	4,34	4,34			
	P > *F*	0.96	< 0.0001	0.005			
72							
	Square bracts	89 ± 3aA	53 ± 1bA	14 ± 4cB	30.53	2,34	< 0.0001
	Whole squares	90 ± 3aA	40 ± 7bB	13 ± 6cB	39.67	2,34	< 0.0001
	Flower petals	90 ± 1aA	17 ± 4bC	11 ± 2bB	48.90	2,34	< 0.0001
	Flower anthers	91 ± 2aA	36 ± 5bB	31 ± 4cA	21.73	2,34	< 0.0001
	Intact boll	88 ± 2aA	48 ± 5bA	20 ± 5cB	23.34	2,34	< 0.0001
	F	0.19	6.08	2.29			
	df	4,34	4,34	4,34			
	P > *F*	0.98	0.0008	0.05			
96							
	Square bracts	84 ± 2aA	43 ± 1bA	5 ± 1cB	90.99	2,34	< 0.0001
	Whole squares	81 ± 3aA	26 ± 3bB	6 ± 1cB	73.60	2,34	< 0.0001
	Flower petals	83 ± 1aA	10 ± 2bC	2 ± 1cC	68.06	2,34	< 0.0001
	Flower anthers	87 ± 2aA	28 ± 2bB	12 ± 2cA	60.54	2,34	< 0.0001
	Intact boll	84 ± 2aA	28 ± 3bB	7 ± 1cB	82.70	2,34	< 0.0001
	F	0.22	9.20	4.94			
	df	4,34	4,34	4,34			
	P > *F*	0.93	< 0.001	0.003			

Means within a row followed by same lower case letter and within a column and time interval followed by same upper case letter are not significantly different ($\alpha = 0.05$) according to Fisher's protected least significant difference.

Significant differences in larval survivorship were detected among structures of Vip3A plants at all rating intervals. Larval survivorship ranged from 84-96%, 23-83%, 17-53%, and 10-43% at 24, 48, 72, and 96 h after infestation, respectively (Table 4.2). At 24 h after infestation, survivorship was similar and higher on all Vip3A structures compared to that on flower anthers. Survivorship was higher on square bracts, squares, and bolls than that on other structures at 48 h after infestation. Higher survivorship was detected on Vip3A square bracts and bolls compared to that on other structures at 72 h after infestation. At 96 h after infestation, larval survivorship was higher on square bracts compared to that on all other structures.

VipCot™ reproductive structures also significantly influenced larval survivorship at all rating intervals. Survivorship ranged from 74-97%, 41-78%, 11-31%, and 2-12% at 24, 48, 72, and 96 h after infestation, respectively (Table 4.2). At 24 and 48 h after infestation, larval survivorship was significantly higher on square bracts compared to that on all other structures. At 72 h and at 96 h after infestation, survivorship was higher on flower anthers compared to all other structures.

H. virescens larval survivorship was influenced by cotton type and reproductive structure at each rating interval (Table 4.2). Larval survivorship on all structures of Coker 312 plants was higher than that of corresponding structures on Vip3A and VipCot™ plants at 48, 72 and 96 h after infestation. However at 24 h after infestation, survivorship was higher only on Coker 312 flower anthers compared to that on flower anthers of Vip3A plants. Also at 24 h after infestation, survivorship was higher on all Coker 312 structures compared to that on similar structures of VipCot™ plants, except for square bracts. In addition, higher survivorship was recorded on Vip3A squares, flower petals, and bolls compared to that on corresponding structures of VipCot™ plants at 24 h after infestation. Larval survivorship was higher on square bracts and

squares of Vip3A plants compared to that on corresponding structures of VipCot™ plants at 48 h after infestation. At 72 h after infestation, larval survivorship was higher on all Vip3A structures compared to that on similar structures of VipCot™ plants, except for flower petals. Larval survivorship was higher on all reproductive structures of Vip3A compared to similar structures of VipCot™ at 96 h after infestation.

Discussion

In the present study, larval survivorship of both species varied significantly among Vip3A, VipCot™, and Coker 312 plant structures. Larval survivorship on the non-Bt Coker 312 structures was higher than that on similar structures of both Bt cotton lines at all rating intervals, with a few exceptions at the 24 h after infestation rating period. Terminal leaf tissue from Vip3A and VipCot™ plants produced similar levels of *H. zea* survivorship, but *H. virescens* survivorship was different between terminal leaves of the two Bt cotton lines. Survivorship of *H. zea* and *H. virescens* on Vip3A reproductive plant structures ranged from 7-28% and 10-43% at the endpoint (96 h after infestation) of the experiment. Although many of these insects may have stopped feeding and would likely not have survived to pupation, any variation in protein expression within or among plants may create opportunities for these insects to become established and produce economic injury. Similar to previous experiences with Bollgard® cultivars, supplementary insecticide applications may be needed on Vip3A cotton lines to prevent economic losses. Fortunately, survivorship of both species was significantly lower on all VipCot™ reproductive structures compared to that on Vip3A plant structures at 96 h after infestation. With the exception of flower anthers, survivorship of *H. zea* and *H. virescens* on VipCot™ plant structures never exceeded 7%. The results of several field trials have

demonstrated that VipCot™ cotton lines have provided satisfactory control of heliothines under field conditions (Leonard et al. 2005, Micinski and Waltman 2005, Parker and Livingston 2005).

The sensitivity of *H. virescens* to Bt plants expressing cry proteins has been extremely high, regardless of plant structures. There currently have been no published reports of a collection of *H. virescens* survivors from a commercial field of pure Bt cotton plants expressing either one or two proteins in the United States. Based upon the relatively low *H. virescens* survivorship on VipCot™ plant structures in the present study at 96 h after infestation and results of field trials, it is unlikely that this species will become established in fields planted to commercial VipCot™ cotton lines.

The effects of plant structures expressing single and dual cry proteins on heliothine larval survivorship have been reported previously. Many of these studies have attempted to explain why heliothine larvae are commonly found on reproductive structures (primarily flowers) of commercial Bt cotton plants. Both *H. zea* and *H. virescens* larvae feed on vegetative and reproductive plant structures of conventional non-Bt cotton plants (Bohmfalk et al. 1982). The first three instars of both species feed on structures located in the upper portion of the cotton plant, whereas the last two instars feed on structures such as bolls lower in the plant canopy (Farrar and Bradley 1985). That same study showed *H. zea* larvae were found associated with flowers of conventional non-Bt cotton more than *H. virescens* larvae. The introduction of Bollgard® cotton cultivars prompted a series of similar studies that showed the feeding patterns of *H. zea* to be similar to that previously described on non-transgenic Bt cotton plants (Smith 1998, Pietrantonio and Heinz 1999). An important difference in *H. zea* behavior on conventional non-Bt and Bollgard® plants was the common field observation of early instars in flowers of Bollgard® plants. Gore et al. (2002) found that early instars of larvae migrated more rapidly to

flowers on Bollgard® plants compared to that on non-Bt plants. On Bollgard® plants, flowers may be preferred feeding structures for *H. zea* because cry protein expression is lower than in other vegetative or reproductive structures.

Variation in the Cry1Ac protein expression among Bollgard® plant parts could result in different levels of heliothine survivorship on specific plant structures (Adamczyk et al. 2001, Adamczyk and Gore 2004). Higher levels of a cry protein were detected in squares compared to white flowers of a Bt cotton lines and resulted in higher survivorship of *H. zea* larvae on white flowers (Adamczyk et al. 2001). Furthermore, Greenplate (1999) reported significantly higher levels of Cry1Ac in plant terminals (68 µg/g dry weight) compared to reproductive plant tissues (26 µg/g dry weight). The observed differences in larval survivorship among plant structures of the Vip3A and VipCot™ cotton lines in the present study could be attributed to variation in protein expression among those structures. However, no quantitative technique has been validated to accurately estimate Vip3A levels in cotton plant structures and relate concentrations to actual bioactivity of Vip3A.

Native infestations of *H. zea* have been able to become established in commercial Bt fields and were observed feeding on vegetative and reproductive plant structures. *H. zea* survivorship on Bt cottons plants expressing single (Cry1Ac) and dual (Cry1Ac + Cry2Ab) proteins was highest on pink flowers (48 and 18%) followed by leaves (34 and 7%), and 1 d-old bolls (10 and 0%), respectively, at 96 h after infestation (Stewart et al. 2001). In the present study, *H. zea* survivorship was consistently lower on plant structures of VipCot™ (Vip3A + Cry1Ab) cotton lines compared to survivorship on cotton lines only expressing the Vip3A protein. Interestingly, survivorship among reproductive structures was highest on flower anthers at 28% and 18% for Vip3A and VipCot™ plants, respectively, at 96 h after infestation. In a study

by Gore et al. (2001), *H. zea* survivorship on floral structures of Bollgard II® plants was lower than on similar structures of Bollgard® plants. Survivorship was highest on flower anthers and square anthers of Bollgard® and Bollgard II® plants compared to that on other plant structures. Higher *H. virescens* survival was observed on reproductive structures of Vip3A cotton lines and indicates that *H. virescens* could be less susceptible than *H. zea* to Vip3A. In contrast Bt cotton plants expressing cry proteins are extremely toxic to *H. virescens*, but less so to *H. zea.* The results reported herein suggest that the addition of Cry1Ab to Vip3A in VipCot™ cotton lines significantly increased efficacy against *H. virescens*. For both species, relatively low larval survivorship was observed on VipCot™ cotton structures compared to that on Vip3A. For optimum performance and sustainability of the Vip3A technology in commercial cotton cultivars, these results support the further development of the VipCot™ trait in cotton.

Furthermore, from a practical heliothine management issue, these results provide information for a sampling protocol to detect infestations of these species in cotton fields. Several of the cotton production states' cooperative extension services currently recommend sampling reproductive structures such as flowers and bolls to detect *H. zea* infestations in commercialtransgenic Bt cotton fields. A similar scouting strategy will likely be appropriate for VipCot™ cotton lines because the highest survivorship was recorded on flower anthers and bolls. To ensure season-long expression and efficacy against heliothine target pests, future work should focus on profiling the expression of Vip and cry proteins among plant structures of transgenic Bt plants.

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CHAPTER 5

FIELD PERFORMANCE AND SEASONAL DURABILITY OF A TRANSGENIC COTTON LINE VIPCOT AGAINST HELIOTHINE LARVAE UNDER NATIVE AND ARTIFICIAL INFESTATIONS

Introduction

Transgenic cottons, which express proteins from the soil bacterium, *Bacillus thuringiensis,* are the standard management strategy for primary lepidopteran pests. The first commercial transgenic Bt cotton, Bollgard® provides excellent control of *Heliothis virescens* (F.), but *Helicoverpa zea* (Boddie) control has not been consistent. *H. zea* are inherently less toxic to the Cry1Ac protein in Bollgard than *H. virescens* (Luttrell et al. 1999). Also, Cry1Ac levels decrease as the plant ages (Greenplate 1999, Adamczyk et al. 2001b, Olsen et al. 2005) and vegetative tissue of Bollgard® plants express higher cry protein levels compared to levels in floral structures such as pollen and flower petals (Greenplate 1999, Adamczyk et al. 2001b, Gore et al. 2001). *H. zea* larvae are often observed feeding in flowers of Bollgard® plants which can result in relatively high levels (more than 50%) of boll abscission (Smith 1998, Gore et al. 2000).

The inconsistent efficacy of Bollgard® against *H. zea* and other occasional lepidopteran pests prompted the agrochemical companies to develop more broad spectrum transgenic technologies. Therefore, in recent years, cotton cultivars that express two insecticidal proteins (Bollgard II® and WideStrike®) were commercialized. These products provide season long, broad spectrum control of major lepidopteran pests above that provided by the single protein expressed in Bollgard® Plants. Several studies with Bollgard II® and WideStrike® have demonstrated significantly better control of *H. zea* and other lepidopteran pests than Bollgard® cotton (Gore et al. 2001, Stewart et al. 2001, Leonard et al. 2005, Willrich et al. 2005).

Novel transgenic cotton plants which express a vegetative insecticidal protein, Vip3A, are being developed by Syngenta Crop Protection (Lee et al. 2003). This insect pest management technology has been trademarked VipCot™ and includes cotton lines that have been transformed to express both Vip3A and Cry1Ab proteins (McCaffery et al. 2006). The Vip3A is different from Cry proteins found in all current commercial products (Bollgard®, Bollgard II®, and WideStrike®). The insecticidal Cry proteins are produced during the bacterium reproductive phase, enclosed in crystals, and classified as endotoxins. Vip3A is secreted during the vegetative phase of bacterium development, and considered an exotoxin (Micinski and Waltman 2005, Yu et al. 1997).

Relatively few field studies have evaluated the efficacy of the VipCot™ technology against native and artificial infestations of heliothines and examined the seasonal durability against these lepidopteran targets. Before the VipCot™ technology can be fully integrated into a cotton pest management system; the consistency of performance against the primary heliothine targets should be documented. Therefore, the objective of this study was to evaluate the seasonal efficacy of VipCot™ cotton lines against *H. zea* and *H. virescens*. A second objective was to compare heliothine injury levels among fruiting structures of Coker 312, Vip3A, and VipCot™ cotton lines.

Materials and Methods

Native Infestations of Heliothines in Field Trials. These studies were performed at the Louisiana State University Agricultural Center's Macon Ridge Research Station near Winnsboro, LA (Franklin Parish) from 2005 to 2007. The conventional non-Bt cotton cultivar, 'Coker 312', and Bt cotton lines expressing either a single protein (Vip3A) or combination of proteins (Vip3A + Cry1Ab [VipCot™]) were planted in a Gigger-Gilbert silt loam soil on 8 Jun in 2005, on 20 Jun

in 2006, and on 16 Jun in 2007. Normal cultural practices and integrated pest management strategies recommended by the Louisiana Cooperative Extension Service were used to optimize plant development across the test site (Bagwell et al. 2005, Stewart et al. 2007). The plots in these studies were not treated with any insecticides specifically for heliothine control. However, control of non-target pests such as thrips, *Frankliniella* spp., cotton aphids, *Aphis gossypii* (Glover), and tarnished plant bugs, *Lygus lineolaris* (Palisot de Beauvois) during the study was accomplished with the insecticides aldicarb (Temik 15G, Bayer CropScience, Research Triangle Park, NC), dicrotophos (Bidrin 8EC, Amvac Chemical Corporation, Los Angeles, CA), imidacloprid (Trimax SC, Bayer CropScience, Research Triangle Park, NC), and thiamethoxam (Centric 40 WG, Syngenta Crop Protection, Greensboro, NC) which express minimum efficacy against heliothines.

Cotton lines were evaluated by examining 25 randomly selected fruiting forms, flower buds (squares) and bolls from the two center rows of each plot for incidence of heliothine damage and surviving larvae. Plots were sampled once to twice weekly from ≈40 days after planting to 100 days after planting (mid-Jul to late-Sept). Species composition across the test areas was estimated with pheromone trap captures of heliothine adults. In addition, collections of larvae were examined from adjacent plots (border rows) of non-Bt cotton to further support the seasonal trap capture data.

Treatments (cotton lines) were arranged in a randomized block design with four replications. The analysis was standardized to include only those dates on which average fruiting form injury was ≥ 5% in the Coker 312 plots. The number of sample dates included in the analysis during 2005, 2006, and 2007 were 6, 10, and 9, respectively. The numbers of damaged fruiting forms and surviving heliothine larvae were converted to percentages, averaged across all

samples and then subjected to ANOVA using PROC MIXED (SAS 2003). The seasonal durability data was subjected to a two-way factorial analysis with cotton line and days after planting as factors.

Artificial Infestations of Heliothines in Field Trials. These experiments also were performed at the Louisiana State University Agricultural Center Macon Ridge Research Station near Winnsboro, LA (Franklin Parish) in 2006. The non-Bt cotton cultivar, Coker 312, and Bt cotton lines Vip3A and VipCot™ were planted in a Gigger-Gilbert silt loam soil on 15 Jun, 2006. The cotton lines were arranged in a completely randomized design across the test area. The test area was maintained with agronomic and pest management practices in a manner similar to that previously described.

The procedures for the artificial infestation study followed the general outline described by Bommireddy et al. (2007). Late-instars (L4-L5 stages) of *H. zea* and *H. virescens* were collected from sweet corn; *Zea mays* L., and garbanzo beans; *Cicer arietinum* L., respectively, during early Jun. Colonies were established in the laboratory and reared for a minimum of one generation to eliminate parasitoids and pathogens and to obtain sufficient numbers at the selected age-classes of larval development. *H.zea* and *H. virescens* larvae were fed an artificial soy protein and wheat germ meridic diet (*Heliothis* premix, Ward Natural Science, Rochester, NY) and a pinto bean-based meridic diet (Leonard et al. 1987), respectively and reared in individual 29.5 ml plastic cups (Solo Co., Urbana, IL). These larvae were maintained at 27 ± 2°C and 85 ± 2% relative humidity with a 14:10 light:dark photoperiod until pupation. Adults were held in 2.79 liter cylindrical cardboard/plastic containers and fed a 10% sucrose solution. A single layer of cotton gauze (cheesecloth, Grade 50) was placed on top of the containers to provide an adequate surface for oviposition. The sheets of gauze that contained eggs were harvested daily,

placed into plastic bags, and sealed until larval eclosion. Upon eclosion, larvae were offered the meridic diet until they reached the proper stage for inoculation on plants. Field infestations were completed within three to four generations of colony establishment.

Field plots of Coker 312, Vip3A, and VipCot™ cotton lines were thinned to 3 plants per meter (one plant per row-feet) before infestation to prevent interplant movement of larvae. Infestations were initiated when cotton plants across the test area had seven to nine main stem nodes above the upper-most first position white flower on a sympodial branch. All plants were in similar stages of plant development during this study. Those plants designated for infestation were examined for the presence of heliothine eggs and larvae. Only those plants without a native heliothine infestation were used in these studies. White flowers were selected for infestation and tagged with a yellow snap-on tag (A. M. Leonard, Inc., Piqua, OH). A single L2 stage heliothine larva (72 ± 6 h old) was placed in a first position white flower on a single plant of each cotton line using a small camel's hair brush. Twenty five plants of the Coker 312, Vip3A, and VipCot™ cotton lines were independently infested with each species on each of three days.

The infested plants were visually inspected 3 d after infestation for damage to the fruiting structure at the infested site and for the presence of surviving larvae. Thereafter, entire plants were inspected every 2 d for cumulative damage to fruiting structures (squares, white flowers, and bolls) until larvae were no longer detected. Non-infested plants adjacent to the infested plants were monitored for natural abscission of fruiting structures due to native heliothine populations. The effects of native heliothines during this period were suppressed by removing and destroying any eggs or small larvae not associated with the experiment. Numbers of damaged fruiting forms and surviving larvae were recorded from the same experimental units over independent rating intervals during the study; therefore, these data were subjected to a

repeated measures ANOVA (PROC MIXED, Littell et al. 1996). The total number of fruiting forms damaged by an individual larva for each species was subjected to ANOVA (PROC MIXED, SAS Institute 2003).

Results

Native Infestations of Heliothines in Field Trials. The number of fruiting forms damaged by heliothines was significantly higher in Coker 312 compared with Vip3A and VipCot™ cotton lines (Table 5.1, $F = 78.3$; df = 2,31; $P < 0.0001$). VipCot™ cotton had significantly fewer heliothine damaged fruiting forms compared with Vip3A cotton. Fruiting forms infested with surviving larvae were also significantly influenced by cotton line ($F = 58.4$; df = 2,31; $P < 0.0001$). Larval numbers were higher on Coker 312 than on Vip3A and VipCot™ cotton plants. The VipCot™ plants had significantly fewer heliothine larvae compared with that on Vip3A cotton plants.

Table 5.1. Seasonal (mean ± SE) percentage of fruiting forms damaged by heliothines (*Helicoverpa zea* [Boddie]; and *Heliothis virescens* [F.]) and infested with larvae for non-Bt, Coker 312, and Bt cotton, Vip3A and VipCot™ lines in Louisiana field trials, 2005-2007.

	Percent	
Cotton lines	Damaged forms[1]	Surviving larvae[1]
Coker 312	14.15 ± 0.6a	4.61 ± 0.4a
Vip3A	4.05 ± 0.5b	1.02 ± 0.2b
VipCot	0.93 ± 0.2c	0.12 ± 0.0c

Means within a column followed by same letter are not significantly different according to Fisher's protected LSD ($\alpha = 0.05$).
[1] Field trials sampled 6, 10, and 9 times during 2005, 2006, and 2007, respectively.

Pheromone trap captures and samples of larvae collected from non-Bt cotton plants adjacent to the test areas indicated that the *H. zea* was the most common species (>80% seasonal

composition) infesting plants during all three years. Populations of *H. virescens* were considerably lower than *H.zea* during each year, and this species was only common during the late season (81-100 DAP).

Heliothines damaged significantly more fruiting forms in Coker 312 plots compared with Vip3A and VipCot™ plots during the period of 40-90 days after planting (Fig. 5.1). The number of fruiting forms damaged by heliothines was significantly influenced by the cotton line (F = 117.0; df = 2,54; $P < 0.0001$), time of evaluation (F = 41.4; df = 5,54; $P < 0.0001$), and cotton line by time of evaluation interaction (F = 15.3; df = 10,54; $P < 0.0001$). A single defined peak was observed in heliothine damaged fruiting forms on Coker 312 and Vip3A plots at 71-80 DAP.

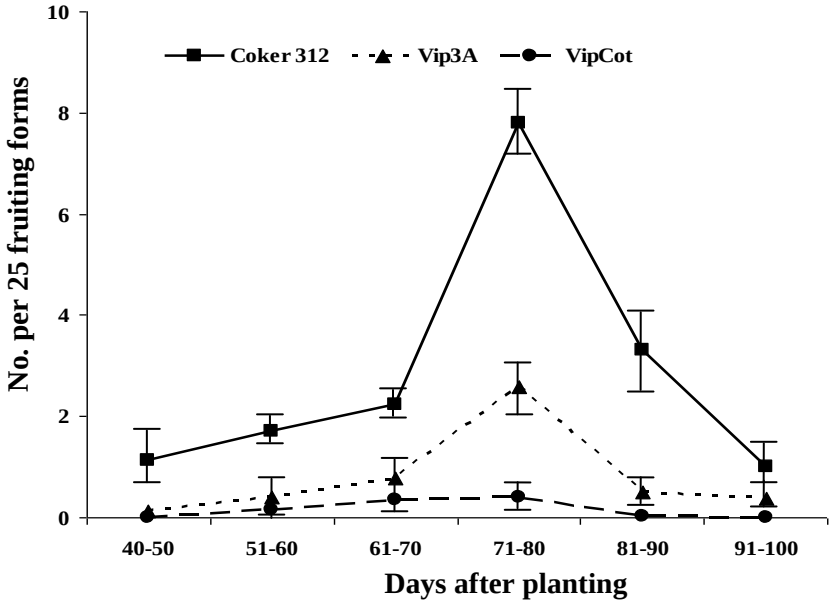

Figure 5.1. Seasonal distribution (mean ± SE) of*Helicoverpa zea* (Boddie), and *Heliothis virescens* (F.), damaged fruiting forms on non-Bt, Coker 312, and transgenic Bt cotton, Vip3A and VipCot™ lines in Louisiana field trials, 2005-2007.

During this period, the number of damaged fruiting forms was 7.8, 2.6, and 0.4 per 25 plants in Coker 312, Vip3A, and VipCot™ cotton, respectively. In addition, this was the only

period of time when a significant difference in damaged fruiting forms was detected between Vip3A and VipCot™ plants. The pheromone trap captures and samples of larvae from adjacent non-Bt plots indicated that the *H. zea* (>70%) was still the dominant species. However, low numbers of *H. virescens* were detected and began to increase during the 71-80 DAP period. *H. virescens* did not become the dominant species (≈65%) until overall heliothine populations declined at 81-100 DAP.

Artificial Infestations of Heliothines in Field Trials. Cotton line ($F = 93.4$; df = 2,4; $P < 0.0001$), time of evaluation ($F = 56.7$; df = 3,18; $P < 0.0001$), and cotton line by time of evaluation interaction ($F = 11.4$; df = 6,18; $P < 0.0001$) were significant for *H. zea* injured fruiting forms(Fig. 5.2). *H. zea* larvae injured more fruiting forms on Coker 312 compared with that on Vip3A and VipCot™ cotton plants at all rating intervals. Cumulative injury to fruiting forms also was significantly higher on Vip3A cotton compared with VipCot™ cotton at 3, 5, 7, and 9 d after infestation.

Cotton line ($F = 20.7$; df = 2,4; $P < 0.0001$), time of evaluation ($F = 28.2$; df = 3,18; $P < 0.0001$), and cotton line by time of evaluation interaction ($F = 16.5$; df = 6,18; $P < 0.0001$) was significant for surviving larvae remaining on plants (Fig. 5.3). Significantly more *H. zea* larvae were recorded on Coker 312 plants compared with that on both Bt cotton lines at all rating intervals. At 3, 5, and 7 d after infestation, fewer surviving *H. zea* larvae were detected on VipCot™ plants compared with that on Vip3A plants. By 7 d, no larvae were recorded on VipCot™ plants, but larvae were still found on Coker 312 and Vip3A plants. No *H. zea* larvae were found on Vip3A plants at 9 d after infestation, but 8.3 larvae per 25 plants were recorded on the Coker 312 cotton line.

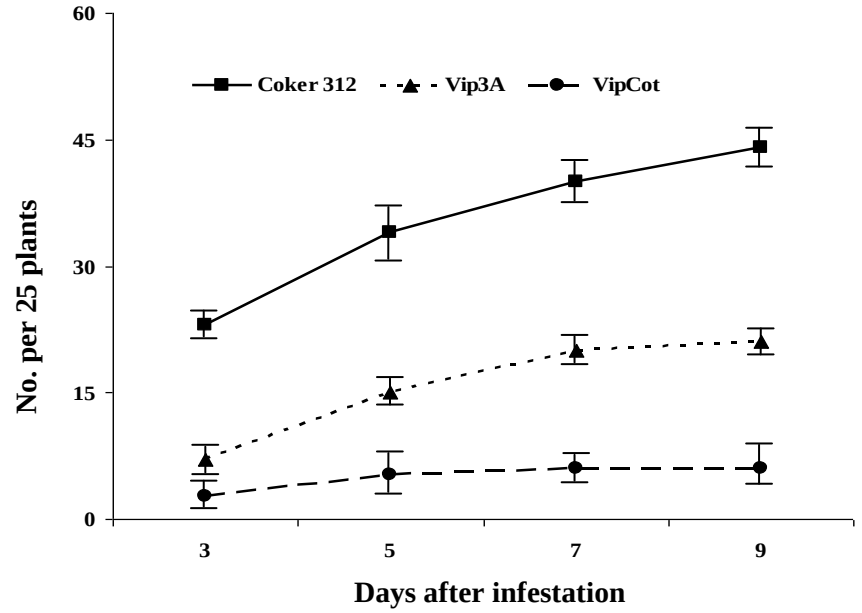

Figure 5.2. *Helicoverpa zea* (Boddie), damaged fruiting forms (mean ± SE) on non-Bt, Coker 312, and transgenic Bt cotton, Vip3A and VipCot™ lines.

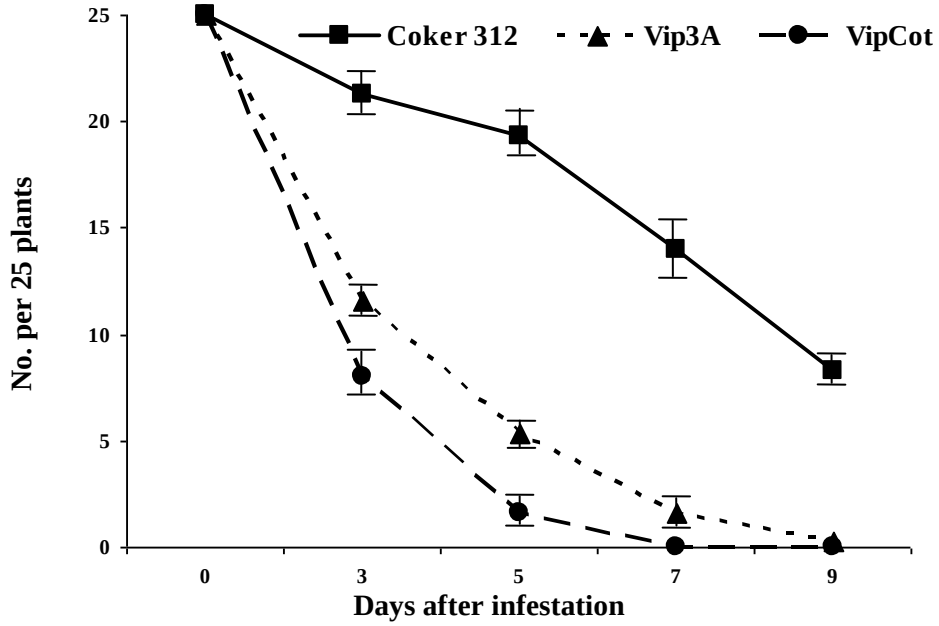

Figure 5.3. Surviving *Helicoverpa zea* (Boddie), larvae (mean ± SE) recovered on non-Bt, Coker 312, and transgenic Bt cotton, Vip3A and VipCot™ lines.

A *H. zea* larva injured more squares (F = 27.8; df = 2,4; P < 0.01), flowers (F = 75.5; df = 2,4; P < 0.01), and bolls (F = 40.7; df = 2,4; P < 0.01) on Coker 312 than on Vip3A and VipCot™ cotton lines (Fig. 5.4). An average of 8.6 fruiting forms (2.6 squares, 2.3 white flowers, and 3.5 bolls) on Coker 312 plants were injured per *H.zea* larva. On Vip3A plants, a *H. zea* larva injured 4.6 fruiting forms (2.1 squares, 0.5 white flowers, and 1.9 bolls). Although VipCot™ plants were damaged less by *H.zea* than Vip3A plants, a low level of fruiting form injury was recorded. A *H. zea* larva damaged an average of 1.0 fruiting forms (0.6 squares, 0.2 white flowers, and 0.2 bolls) during the 9 d evaluation period.

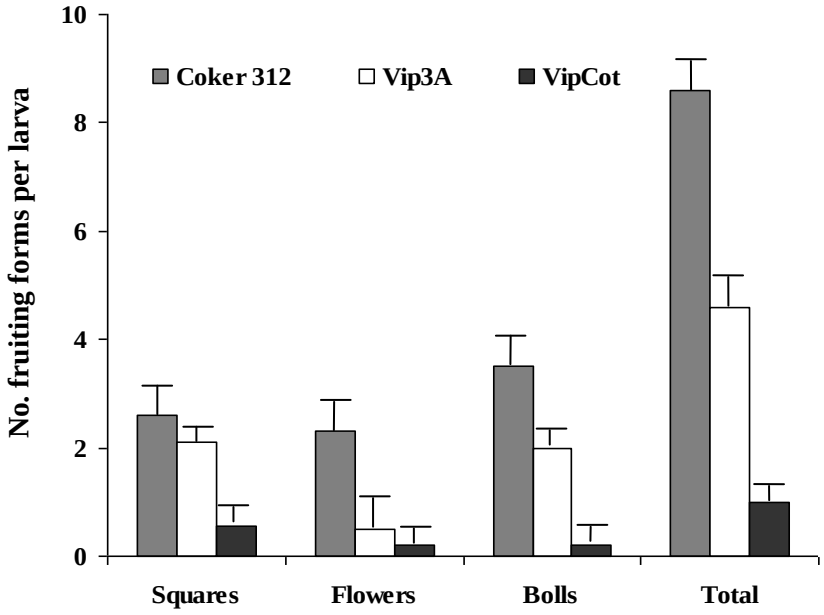

Figure 5.4. *Helicoverpa zea* (Boddie), injury to fruiting forms on non-Bt, Coker 312, and transgenic Bt cotton, Vip3A and VipCot™ lines at 9 d after inoculation in white flowers (bars represent mean ± SE of structures damaged by a single larva).

Cotton line (F = 77.8; df = 2,4; P < 0.0001), time of evaluation (F = 66.2; df = 3,18; P < 0.0001), and cotton line by time of evaluation interaction (F = 14.0; df = 6,18; P < 0.0001) were significant for *H. virescens* injured fruiting forms (Fig. 5.5). *H. virescens* larvae injured

significantly more fruiting forms on Coker 312 plants compared with that on Vip3A and VipCot™ plants at all rating intervals. Cumulative injury to fruiting forms also was significantly higher on Vip3A cotton compared to VipCot™ cotton at 3, 5, 7, and 9 d after infestation.

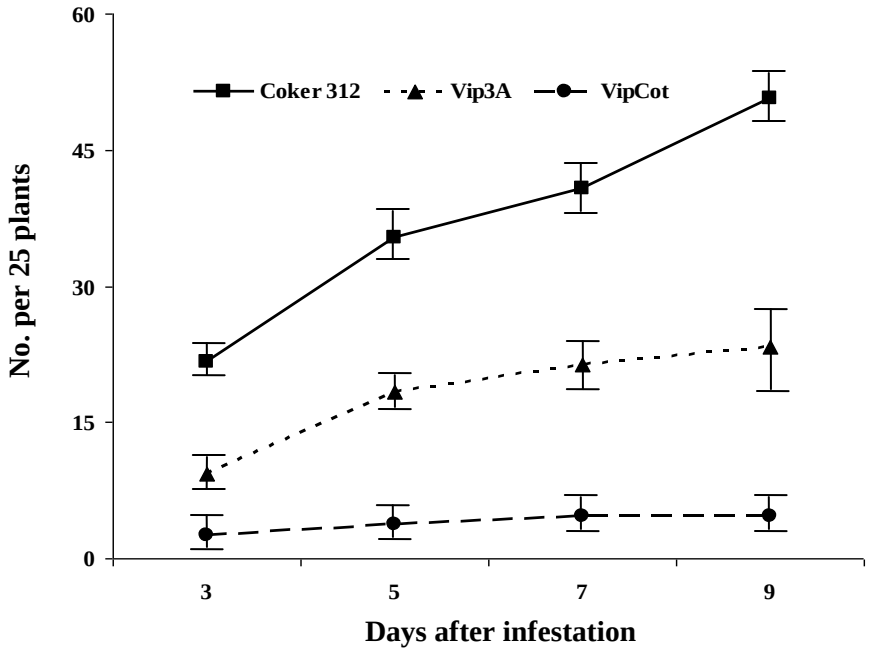

Figure 5.5. *Heliothis virescens* (F.), damaged fruiting forms (mean ± SE) on non-Bt, Coker 312, and transgenic Bt cotton, Vip3A and VipCot™ lines.

Cotton line (F = 27.5; df = 2,4; P < 0.0001), time of evaluation (F = 15.4; df = 3,18; P < 0.0001), and cotton line by time of evaluation interaction (F = 12.2; df = 6,18; P < 0.0001) was significant for surviving larvae remaining on plants (Fig. 5.6). Significantly more *H. virescens* larvae were recorded on Coker 312 compared with both Bt cotton lines at all rating intervals. In addition, fewer surviving *H. virescens* larvae were detected on VipCot™ plants compared with that on Vip3A plants at all rating intervals. At 7 d, no larvae were found on VipCot™ plants, but 13.3 and 4.7 larvae per 25 plants were recorded on Coker 312 and Vip3A plants. No larvae were found on Vip3A plants by 9 d after infestation.

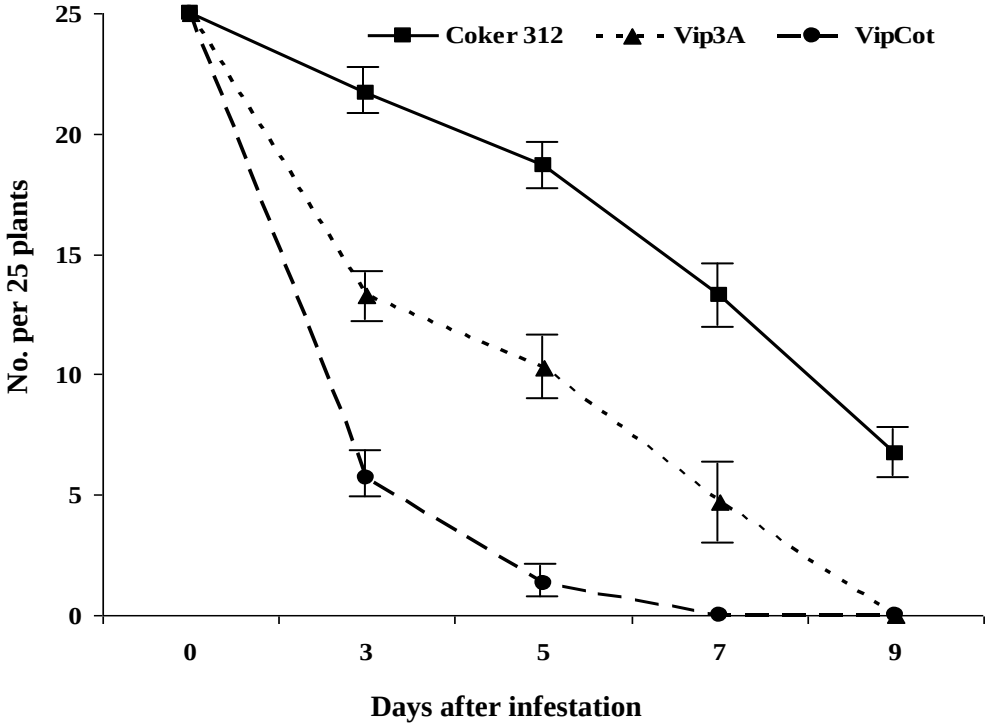

Figure 5.6. Surviving *Heliothis virescens* (F.), larvae (mean ± SE) recovered on non-Bt, Coker 312, and transgenic Bt cotton, Vip3A and VipCot™ lines.

A *H. virescens* larva injured more squares ($F = 70.8$; df = 2,4; $P < 0.01$), flowers ($F = 43.7$; df = 2,4; $P < 0.01$), and bolls ($F = 37.8$; df = 2,4; $P < 0.01$) on Coker 312 than on Vip3A and VipCot™ cotton lines (Fig. 5.7). On Coker 312 plants, a larva damaged 9.2 fruiting forms (2.6 squares, 3.3 white flowers, and 3.2 bolls). A larva damaged 5.9 fruiting forms (2.3 squares, 0.5 white flowers, and 3.0 bolls) on Vip3A plants, as observed with *H.zea*, total fruiting form injury by *H. virescens* was lower on VipCot™ plants compared with that on Vip3A plants. A *H. virescens* larva injured 0.9 fruiting forms (0.4 squares, 0.2 white flowers, and 0.3 bolls) on VipCot™ plants.

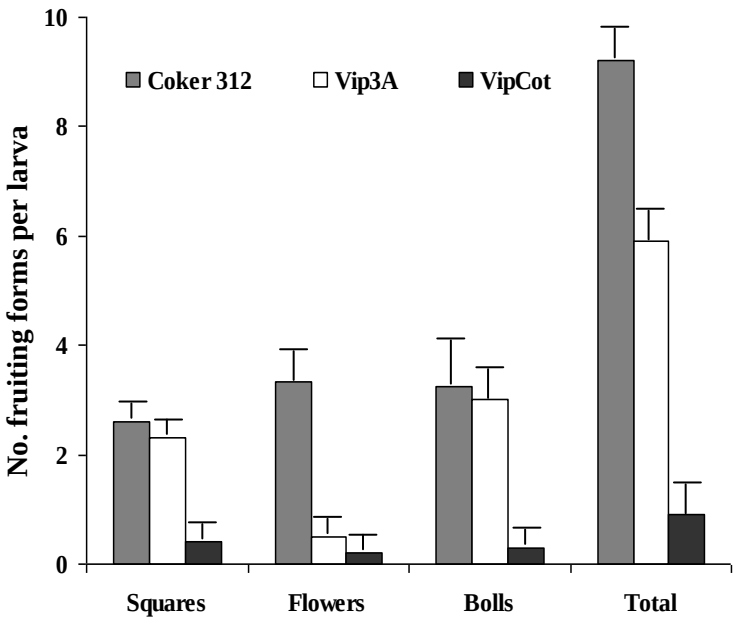

Figure 5.7. *Heliothis virescens* (F.), injury to fruiting forms on non-Bt, Coker 312, and transgenic Bt cotton, Vip3A and VipCot™ lines at 9 d after inoculation in white flowers (bars represent mean ± SE of structures damaged by a single larva).

Discussion

H. zea and *H. virescens* injured more fruiting forms on Coker 312 plants compared to that on the single protein, Vip3A, and stacked proteins, VipCot™ plants during the native and artificial infestation studies. Fruiting forms infested with surviving larvae also were lower on plants of both Bt cotton lines compared with that on Coker 312 plants. The VipCot™ line generally sustained significantly less injury to fruiting forms and maintained a lower larval infestation compared with that on the Vip3A cotton line. In field trials evaluating the performance of single and stacked Cry proteins expressed in cotton lines, Jackson et al. (2003) reported patterns of efficacy against *H. zea* similar to that presented in the present study. Bollgard® plants expressing a single cry protein (Cry 1Ac) had more squares (4.6%) and bolls

(9.3%) damaged than squares (1.8%) and bolls (1.3%) of Bollgard II® plants expressing two cry proteins (Cry1Ac + Cry 2Ab). Fruiting forms infested with larvae ranged from 0.9 to 2.9% on Bollgard® plants and 0.3 to 0.5% on Bollgard II® plants. Adamczyk et al. (2001a) found significantly fewer damaged squares (0.7) on Bollgard II® plants compared to those on Bollgard® (6.2) and non-Bt (7.7) plants. This reduction in fruiting form injury and larval survival on Bollgard II® plants compared with that on Bollgard® plants is directly related to the effects generated by the second protein (Cry2Ab). The combination of two proteins in Bollgard II® has increased activity against several lepidopteran target pests (Adamczyk et al. 2001a, Jackson et al. 2003). In the present study, the combining effects of the Cry1Ab protein to that of Vip3A enhanced the overall efficacy of the VipCot™ line against heliothines compared with that for the Vip3A line. The results of limited field trials also have demonstrated that VipCot™ cotton lines have provided satisfactory control of heliothines (Leonard et al. 2005, Micinski and Waltman 2005, Parker and Livingston 2005).

In addition, profiling the seasonal distribution of fruiting form damage indicates that the VipCot™ line sustained less injury during the heliothine peak infestation period (71-80 DAP) compared with injury to Vip3A and Coker 312 fruiting forms. During that period of peak infestation, *H. zea* was the dominant species, but low levels of *H. virescens* were present. Wan et al. (2005) also documented lower *Helicoverpa armigera* (Hubner) larval densities on Bt cotton lines GK19 (Cry1Ac+ Cry1Ab) and BG1560 (Cry1Ac) throughout the season compared with that on non-Bt cotton. For many of the commercial Bt cotton lines expressing Cry proteins, overall levels decrease as the plant ages during the season (Greenplate et al. 1999). Furthermore, a decline in efficacy of cry proteins in Bt cottons against *H. armigera* has been observed in Australia (Fitt et al. 1998). In the present study, the efficacy of the Vip3A line was not as

consistent as that in the VipCot™ during the season. This observation may be related to several of factors such as species selectivity, infestation pressure, plant genotype and environment interaction as well as a seasonal decline in protein expression.

The artificial infestation study isolated injury to individual fruiting forms and species specific survivorship of larvae. The conventional non-Bt, Coker 312 plants sustained significantly higher square damage at all rating intervals compared with that on Vip3A and VipCot™ plants, regardless of heliothine species. *H. zea*-damaged fruiting forms ranged from 23.0-44.1, 7.0-21.1, and 2.7-6.0 per 25 plants on Coker 312, Vip3A, and VipCot™ plants, respectively. However, *H. virescens* injured 21.7-50.7, 9.3-23.3, and 2.6-4.7 fruiting forms per 25 plants on Coker 312, Vip3A, and VipCot™ cotton, respectively. Though injury on Vip3A cotton was significantly lower compared with that on Coker 312 plants, significant numbers of damaged fruiting forms were observed on Vip3A plants for *H. zea* and *H. virescens*. In addition, heliothine injury on VipCot™ cotton was significantly low at all rating intervals. Studies evaluating the efficacy of Bollgard® and Bollgard II® against *H. zea* have shown little injury on Bollgard II® compared with that on Bollgard®. *H. zea* larvae injured a total of 25.0, 11.5, and 6.4 fruiting forms per 10 plants on non-Bt, Bollgard®, and Bollgard II® cotton plants, respectively, at 11 d after infestation (Gore et al. 2003).

In the present study, significantly more larvae were recorded on Coker 312 plants compared with that on Vip3A and VipCot™ plants. Similar to the results for injury to fruiting forms, significantly fewer larvae were recorded on VipCot™ plants compared with that on Vip3A plants for *H. zea* and *H. virescens*. No larvae of either species were found on VipCot™ and Vip3A plants at 7 and 9 d after infestation, respectively. A similar pattern of results has been observed with *H. zea* survivorship on Bollgard® and Bollgard II® plants. Significantly fewer *H.*

zea larvae were recovered on Bollgard II® (25.0, < 10.0, and 0.0%) compared with Bollgard® (73.6, 59.7, and 40.3%) at 5, 7, and 9 d after infestation, respectively (Gore et al. 2003). No *H. zea* larvae were found on Bollgard® and Bollgard II® plants beyond 9 d after infestation.

Defining the amount and type of cotton fruiting form injury produced by an individual larva can be necessary information for ultimately establishing economic injury levels. The results of the present study for *H. zea* and *H. virescens* injury to fruiting forms on the non-Bt Coker 312 plants are similar with that of a number of previous studies. A *H. zea* and *H. virescens* larva was found to injure an average of 8.6 and 9.2 fruiting forms, respectively, on Coker 312 plants at 9 d after infestation. Studies in Arkansas found that an individual *H. zea* larva injured 6.0 fruiting forms (Anonymous 1967). Finally, a single *H. virescens* was capable of damaging 12.1 fruiting forms during complete larval development (Heilman et al. 1981).

Fewer studies have examined the relationship of individual larval feeding and fruiting form injury on transgenic Bt plants. This results of the present study showed that for *H. zea* and *H. virescens*, an individual larva injured more fruiting forms on Coker 312 compared to that on Vip3A and VipCot™ cotton. A *H.zea* larva injured only 4.6 and 1.0 fruiting forms on Vip3A and VipCot™ plants, respectively at 9 d after infestation. Gore et al. (2003) found that an individual *H. zea* larva injured an average of 6.6 fruiting forms on non-Bt cotton, 3.5 fruiting forms on Bollgard®, and 0.8 fruiting forms on Bollgard II® plants at 11 d after infestation. The results for *H. virescens* are similar those previously mentioned for *H. zea*. A single larva was found to injure only 5.9 and 0.8 fruiting forms on Vip3A and VipCot™ plants, respectively.

These results suggest that both species of heliothine larvae were extremely susceptible to the VipCot™ technology. None of the larvae for either species produced significant injury to fruiting forms in the field trials. In addition, none of the larvae were capable of completing larval

development on the VipCot™ cotton line. However, a significant level of fruiting form injury was observed on Vip3A plants. In the artificial infestations, mortality of *H. virescens* was slower on Vip3A plants compared to that of *H. zea* and suggests differential susceptibility between species. The ability of the VipCot™ technology to sustain minimal injury against both species of heliothines should allow this technology to become another useful tool for the cotton industry. To ensure season-long expression and efficacy against heliothine target pests, future work should focus on profiling the seasonal expression of Vip3A and cry protein combination on the final lines released for commercialization and among plant structures. Additional studies also need to evaluate the efficacy of VipCot™ line against a multitude of lepidopteran target pests.

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CHAPTER 6

SUMMARY AND CONCLUSIONS

The bollworm, *Helicoverpa zea* (Boddie), and tobacco budworm, *Heliothis virescens* (F.), are collectively known as the heliothine pest complex and are common Lepidopteran pests across Southern United States cotton production regions. Traditionally, and until 1996, these pests were primarily controlled with foliar applications of insecticides. Genetically engineered plants have rapidly become alternate management strategies to the use of conventional chemical control strategies for heliothine control in cotton integrated pest management. All of the commercial transgenic cotton cultivars express the insecticidal proteins from the bacterium *Bacillus thuringiensis* (Bt) which are selectively toxic to certain Lepidopteran pests. The initial transgenic Bt cotton registered in the U.S. was labeled as Bollgard®. This technology expresses a single crystal (Cry1Ac) protein and has been highly effective against *H. virescens;* and pink bollworm, *Pectinophora gossypiella* (Saunders); and European corn borer, *Ostrinia nubilalis* (Hubner). However, the spectrum of target pests successfully controlled with Bollgard® plants was limited and supplemental foliar applications of conventional insecticides were still required for many non-target Lepidopteran pests. Commercialization of transgenic cotton cultivars that express two insecticidal Cry proteins (Bollgard II® and WideStrike®) have improved control of *H. zea* and other Lepidopteran pest targets above that provided by the single protein expressed in Bollgard®. Currently, Syngenta Crop Protection is developing another series of cotton cultivars (VipCot™) that express a novel vegetative insecticidal protein, Vip3A, and a second Cry1Ab protein. Limited work has been completed to characterize the effects of the (VipCot™) technology on heliothine pests. Therefore, the objective of these studies was to evaluate the larval behavior and survivorship of *H. zea* and *H. virescens* on Vip3A and VipCot™ cotton lines. A second objective

was to determine the relative injury potential of heliothine pests to cotton plants expressing Vip3A (single) and VipCot™ (two) proteins.

H. zea and *H. virescens* larval behavior was determined on a non-Bt cotton line, Coker 312, and compared with that on Bt cotton lines expressing Vip3A and VipCot™ proteins. *H. zea* or *H. virescens* larvae were placed on terminal leaf tissue of individual plants during pre-flowering and flowering stages of development. On pre-flowering cotton plants, significantly more *H. zea* and *H. virescens* larvae migrated from the site of infestation (plant terminal region) on Bt (Vip3A and VipCot™) cotton plants compared to those placed on non-Bt Coker 312 cotton plants. During the flowering stages of cotton plant development, similar numbers of both species were recovered from terminals of the non-Bt, Vip3A, and VipCot™ plants at 1 h and 3 h after infestation. However, significantly more *H. zea* and *H. virescens* larvae were observed on non-Bt cotton flower buds (squares) compared to those on Vip3A and VipCot™ squares at all evaluation intervals. Within 24 h, *H. zea* larvae moved 1.5, 2.8, and 0.8 main stem nodes below the terminal on Vip3A, VipCot™, and Coker 312, respectively. At the same time interval, *H. virescens* larvae moved 2.0, 2.8, and 0.9 main stem nodes below the terminal on Vip3A, VipCot™, and Coker 312, respectively. These differences in the intra-plant migration patterns and distribution of heliothine larvae between Bt and non-Bt plants are likely related to avoidance behavior caused by the Vip3A and Cry1Ab protein(s) present in the transgenic cotton plants. Larval avoidance is probably a survival mechanism that forces the insects to migrate more rapidly and farther to locate suitable non-toxic plant structures. In addition, there were no significant differences in *H. zea* and *H. virescens* behavior detected between the Vip3A and VipCot™ cotton lines. This similarity in species is important because heliothine populations in many U.S. cotton fields can be comprised entirely of a single species or a combination of both species. These results of this

study are the first documented reports of heliothine larval behavior on transgenic cotton expressing the vegetative insecticidal protein, Vip3A.

A series of tests quantified *H. zea* and *H. virescens* larval survival on plant structures of the non-Bt, Coker 312, and Bt, Vip3A and VipCot™, cotton lines. Vegetative and reproductive structures including terminal leaves; flower bud (square) bracts; intact, but debracted squares; flower petals; flower anthers; and intact capsules (bolls) were harvested from plants in field plots. Each structure was infested with larvae from one of the two heliothine species. Survivorship at 96 h after infestation was significantly lower on all structures of Vip3A and VipCot™ cotton lines compared to similar structures on Coker 312. VipCot™ plant structures generally resulted in lower larval survivorship compared with similar structures of the Vip3A cotton line. *H. zea* survivorship ranged from 4 to 28% and 1 to 18% on Vip3A and VipCot™ plant structures, respectively. *H. virescens* survivorship ranged from 10 to 43% and 2 to 12% on Vip3A and VipCot™ plant structures, respectively. *H. virescens* survivorship was higher on Vip3A plant structures compared that for *H. zea* on similar structures indicating that *H. virescens* may be less susceptible than *H. zea* to Vip3A. However, the differences between species were not observed on plants expressing the VipCot™ proteins. This difference is novel among transgenic Bt cotton plants, because commercial Bollgard® and Bollgard II® cultivars expressing Cry proteins are extremely toxic to *H. virescens*, but less so to *H. zea.* These results may serve to differentiate among Vip3A and Cry protein expressing plants, at least for the heliothine spp. included in the present study. Incorporating Cry1Ab with Vip3A in VipCot™ cotton lines significantly increased efficacy against *H. virescens*. The relatively low larval survivorship of both target species observed on VipCot™ cotton structures suggests that to

optimize and sustain Vip3A performance in commercial cotton cultivars, the VipCot™ trait (Vip3A + Cry1Ab) should be the candidate for future development.

Larval age-specific mortalities of *H. zea* and *H. virescens* were quantified on squares of Coker 312, Vip3A, and VipCot™ cotton plants. Squares were offered to larvae of each species in selected age-classes (2 d, 4 d, 6 d, and 8 d after eclosion). These larval age-classes (2, 4, 6, and 8 d-old) generally correspond to L1, L2, L3, and L3-L4 instars, respectively. Cumulative mortality levels for both species and larval age classes (2 d, 4 d, 6 d, and 8 d-old) were significantly higher on squares of the Vip3A and VipCot™ cotton lines than for Coker 312. Older larvae (8 d-old) survived longer on Vip3A and VipCot™ squares compared with younger larvae ($\leq$6 d-old). In general, *H. zea* and *H. virescens* larvae demonstrated significantly lower survivorship on VipCot™ squares compared with larvae that were offered Vip3A squares. The final cumulative mortality levels of *H. zea* larvae occurred more rapidly than that observed for *H. virescens* larvae on Vip3A squares. The effects of VipCot™ cotton squares on time to complete mortality (100.0%) were similar between species. No larvae of either species were capable of completing pupation on squares of the VipCot™ cotton line. The study also showed the combination of two insecticidal proteins expressed in the VipCot™ cotton line improved heliothine efficacy levels above that of the single protein in the Vip3A line.

Field studies evaluated the performance of non–Bt conventional (Coker 312) and Bt (Vip3A and VipCot™) cotton lines expressing against native and artificial infestations of *H. zea* and *H. virescens*. Both Bt cotton lines had significantly fewer damaged fruiting forms and surviving larvae compared with those found on Coker 312 plants. VipCot™ cotton plants had lower numbers of damaged fruiting forms and fruiting forms infested with larvae compared with that on Vip3A plants. The patterns of seasonal efficacy against native infestations generally showed

the VipCot™ plants to be more durable with less fruiting form injury than that recorded on Coker 312 and Vip3A, especially during periods of peak heliothine infestations. None of the larvae for either species produced significant injury to VipCot™ fruiting forms in the field trials. However, a significant level of fruiting form injury was observed on Vip3A plants. In addition, selected Coker 312, Vip3A, and VipCot™ plants in field plots were infested with either *H. zea* or *H. virescens* larvae. Lower levels of damaged fruiting forms and fewer larvae for both species were recorded on Vip3A, and VipCot™ plants compared to those on Coker 312 plants. A single *H. zea* larva injured an average of 8.6, 4.6, and 1.0 fruiting forms on Coker 312, Vip3A, and VipCot™ plants, respectively. A single *H. virescens* larva injured an average of 9.2, 5.9, and 0.9 fruiting forms on Coker 312, Vip3A, and VipCot™ plants, respectively. The VipCot™ technology in an advanced cotton line demonstrated relatively high levels of consistent efficacy against both species of heliothine larvae.

The larval behavior and survivorship of *H. zea* and *H. virescens* was significantly influenced by the Bt insecticidal proteins in Vip3A and VipCot™ cotton lines. Both species of heliothine larvae were extremely susceptible to the VipCot™ technology. However, cotton lines expressing the single Vip3A protein demonstrated lower overall efficacy levels against *H. virescens* compared with that observed for VipCot™ cotton lines. The combination of two insecticidal proteins expressed in the VipCot™ cotton line improved efficacy levels against the heliothine targets above that of the single protein in the Vip3A line. In addition, VipCot™ cotton lines generated complete and rapid mortality across a range of heliothine larval age-classes. These results with Vip3A and VipCot™ are similar to the previous reports of that for Bollgard® and Bollard II® cotton plants and their significant effects on heliothine behavior and survivorship. These studies have provided considerable information by characterizing Vip3A and VipCot™

effects on selected heliothines which can be used to support the further development of the VipCot™ technology and eventually become another useful tool in cotton integrated pest management.

To ensure season-long expression and efficacy against heliothine target pests, future work should focus on profiling the seasonal expression of Vip3A and cry protein combination on the final lines released for commercialization and among plant structures. Additional studies also need to evaluate the efficacy of VipCot™ line against a multitude of Lepidopteran target pests.

APPENDIX A

LETTER OF PERMISSION FOR CHAPTER 2

Letter of permission from the Journal of Cotton Science to reprint Chapter 2.

From: Colyer, Patrick D.
Sent: Tuesday, January 29, 2008 1:42 PM
To: Bommireddy, Padma L.
Subject: JCS07-039.approval.DOC

Ms. Padma Bommireddy
Louisiana State University
Department of Entomology
404 Life Sciences Building
Baton Rouge, LA 70803

Dear Ms. Bommireddy:

The *Journal of Cotton Science* grants you permission to include the article cited below as a chapter in your dissertation at Louisiana State University.

Bommireddy, P. L., B. R. Leonard, and K. Emfinger. 2007. Heliothine larval behavior on transgenic cotton expressing a *Bacillus thuringiensis* insecticidal exotoxin, Vip3A. Journal of Cotton Science11: 177-185.

If I can be of further assistance, please contact me.

Regards,
Patrick D. Colyer
Editor-in-Chief
Journal of Cotton Science

From: Bommireddy, Padma L.
Sent: Monday, January 14, 2008 2:42 PM
To: Colyer, Patrick D.
Subject: JCS07-039

Dr. Colyer

The following article "Heliothine larval behavior on transgenic cotton expressing a *Bacillus thuringiensis* insecticidal exotoxin, Vip3A" has been published in the Journal of Cotton Science (vol. 11: 177-185). The data from this manuscript will also be published as a chapter of my dissertation from Louisiana State University. This dissertation will be submitted to the graduate school this coming April. Could I request a letter or email stating that Journal of Cotton Science give me permission to reprint the data from this manuscript for my dissertation?

Thanks
Padma Bommireddy

VITA

Padma Latha Bommireddy is the daughter of Subbarathnamma and Kesava Reddy. She was born in Yerraguntla, Andhra Pradesh, India. Ms. Bommireddy is married to Raja Bellary. She attended and graduated from Kakateeya Junior College in 1989. Ms. Bommireddy earned her Bachelor of Science (agriculture major) and Master of Science (agricultural economics) from Andhra Pradesh Agricultural University, Tirupati, India, during 1993 and 1995, respectively. She completed a Master of Science (entomology) under the supervision of Dr. Megha Parajulee from Texas Tech University in 2004. Ms. Bommireddy entered graduate studies under the supervision of Dr. B. Rogers Leonard in the Department of Entomology at Louisiana State University and Agricultural and Mechanical College in 2004. She currently is a doctoral candidate in the Department of Entomology.