

## ARTHROPOD MANAGEMENT AND APPLIED ECOLOGY

### Relative Efficacy of Cotton Events Expressing Cry1Ab and Vip3A Against Cotton Bollworm and Tobacco Budworm (Lepidoptera: Noctuidae)

Narendra Palekar\*, Ryan W. Kurtz, Frederick S. Walters, and David O'Reilly

#### ABSTRACT

**The most commonly accepted strategy for delaying insect resistance to transgenic crops is the high-dose plus refuge strategy. To augment this strategy and further reduce the likelihood of insect resistance developing, current and future transgenic insecticidal crops, such as VipCot™, routinely express two or more insecticidal proteins targeting a similar pest spectrum. In the present study, we have evaluated the relative efficacy of two transgenic cotton events, COT67B (which expresses a Cry1Ab protein) and COT102 (producing Vip3A protein), against the tobacco budworm (*Heliothis virescens*) and cotton bollworm (*Helicoverpa zea*). Our data indicate that both COT67B and COT102 have high levels of activity against both species. COT102 and COT67B have been combined in our new insect-resistant cotton product, VipCot™. The combined activity of the two component events in VipCot™ and Vip3A's unique mode of action will provide substantial benefit to cotton insect control and resistance management strategies.**

As the benefits of genetic engineering technology become better known, growers in more countries are adopting genetically engineered cotton varieties. An estimated 35 million hectares were cultivated worldwide with *Bacillus thuringiensis* Berliner (Bt) cotton varieties in 2007 (Gruere, 2007). Genetic engineering is an additional tool used by breeders to develop new varieties and has had successes in numerous applications complementing traditional breeding approaches for obtaining desirable traits. This technology involves insertion of a gene or genes of interest into crop varieties to provide specific traits such as insect resistance. Transgenic crops

offer innovative approaches for the development of integrated pest-management systems because they can eliminate or greatly reduce dependence on nonselective insecticide sprays for control of dominant pests and may include increased reliance on biological control of secondary pests (Roush, 1997). An important application of genetic engineering technology in cotton has been the adoption of Bt cotton for insect control (Perlak et al., 1990). Studies have shown an increased abundance of non-target species surviving in Bt cotton as compared to conventionally grown cotton (Fitt et al., 1994). In addition, as Bt-transgenic crops can be used to manage pest populations, the combination of under-utilized management tactics such as crop rotation and pheromone mating disruption became more attractive. In general, these tactics alone are not sufficiently effective at moderate-to-high pest densities (Roush, 1997).

A new generation of transgenic cotton has been developed with broad-spectrum lepidopteran insect resistance including control of major target pests threatening cotton production such as the cotton bollworm, *Helicoverpa zea* (Boddie), and the tobacco budworm, *Heliothis virescens* (Fabricius). Transgenic cotton, "VipCot™" (O'Reilly et al., 2007), comprises a pyramid of two insecticidal proteins that have been combined through conventional breeding, *cry1Ab* (from the event COT67B) and *vip3A* (from the event COT102). These events express a full-length version, sequence identification number 7 from Cayley et al. (2010) of the Cry1Ab delta-endotoxin protein and Vip3A vegetative-insecticidal protein (VIP), respectively. Both proteins are derived from Bt, the naturally-occurring soil bacterium found worldwide, but have two distinct modes of action (Lee et al., 2003, 2006), which is useful for proactively mitigating the occurrence of insect resistance.

Having a proactive strategy for insect resistance management in transgenic insecticidal crops has been a priority of Environmental Protection Agency (EPA) and industry since the initial commercial release of transgenic cultivars. The proactive approach of high-dose plus refuge strategy to insect resistance management has, in part, contributed to the continued

---

N. Palekar\*, R.W. Kurtz, F.S. Walters, and D. O'Reilly,  
Syngenta Biotechnology, Inc., 3054 Cornwallis Road,  
Research Triangle Park, NC 27709

\*Corresponding author: shank.palekar@syngenta.com

efficacy of Bt crops; however, all current transgenic insecticidal crops fail to meet some or all of the assumptions underlying high-dose plus refuge strategy for one or more pest species. To augment the high-dose plus refuge strategy and further reduce the likelihood of insect resistance developing, current and future transgenic insecticidal crops, such as VipCot™ cotton, are routinely expressing two or more insecticidal proteins targeting a similar pest spectrum. Roush (1997, 1998) concluded that the most effective way to reduce the overall levels of resistance development is through pyramided varieties using two or more toxins in the presence of an appropriate refuge. Pyramiding Bt toxins exploit “redundant killing,” where totally susceptible insects are killed by both toxins and insects resistant to one toxin are killed by the other (Comins, 1986; Gould, 1986). The computer simulation modeling performed by Roush (1998) demonstrated that pyramided Bt crops with component proteins causing greater than 95% mortality of susceptible homozygotes and 70% mortality of resistant heterozygotes will be effective with relatively small amount of non-Bt refuge. The pyramiding strategy was empirically validated by Zhao et al. (2003).

Previously, Kurtz et al. (2007) described the efficacy of COT102 and COT67B cotton combined as VipCot™ cotton against *H. virescens* and *H. zea* and showed that VipCot™ cotton expressed Cry1Ab and Vip3A at a sufficient combined level to kill all susceptible insects of both species in field trials and laboratory assays. To better understand the contribution the pyramided VipCot™ cotton brings to resistance management for Heliiothines, field trials and/or laboratory assays were conducted to assess the relative insecticidal activity of each component event, COT102 and COT67B cotton.

## MATERIALS AND METHODS

**Bioassays Using Lyophilized Tissue.** Lyophilized tissue of single events were diluted in an agar suspension and overlaid on artificial insect diet to reduce the exposure of a target insect species relative to the living plant (considered 100% concentration). The field trials described in Kurtz et al. (2007) showed the pyramided VipCot™ cotton plants resulted in 100% insect mortality. Therefore, diluting the single-event tissue in an agar suspension provided insight into the relative insecticidal activity of the single events compared to the combined activity in living plant tissue. Greenplate (1999) demonstrated the use of

lyophilized cotton tissue to develop a sensitive, quantitative bioassay for measuring Cry1Ac in Bollgard™ cotton plant tissue. He described the use of lyophilized cotton tissue (2% slurry), rather than plant extract to reduce the potential for possible extraction efficiency artifacts associated with plant tissue type or age. The ease of handling and storage of lyophilized cotton tissue compared to fresh cotton tissue enabled the use of lyophilized tissue for this study. The background germplasm used for this study was non-transgenic isoline Coker312 as it is indicative of isogenic backgrounds for COT102 and COT67B. Non-transgenic isoline (Coker312), COT102 (*vip3A* gene producing Vip3A protein), and COT67B (*cry1Ab* gene producing Cry1Ab protein) cotton plants were seeded in 4.5-in plastic pots containing Metro 380 potting soil (Sungro, McCormick, SC) and covered with 2 tablespoons of Sierra Fertilizer Mix 17-6-12 plus minors. Plants were hand watered daily as necessary and were not treated with any pesticide or plant-growth regulator. Plants were grown in the greenhouse with supplemental light to provide a 16:8 h light:dark cycle at 29:23 °C.

Terminal leaves (third leaf from top) were harvested from non-transgenic isoline (Coker312), COT102, and COT67B transgenic cotton plants 4 wk after planting. Leaves from 15 plants were placed in 50-ml tubes (Fisher Scientific, Pittsburgh, PA) and stored at -80 °C. The frozen tissue was then placed in a freeze-drier (VirTis Wizard 2.0, SP Industries, Gardiner, NY) and lyophilized gradually over a step-wise cycle. The lyophilized material was then routinely ground to a fine powder using a mortar and pestle and stored at -80 °C until use.

We depended on the dry-weight basis of leaf material rather than wet weight (Greenplate, 1999), and considered the lyophilate of fresh leaf to be 100% and calculated 4% dilution (25-fold) as described below. One and one-half ml of artificial multiple-species Lepidopteran larval diet (Southland Products, Inc., Lake Village, AR) was dispensed into individual wells of 24-well plates (Costar brand; Fisher Scientific) and allowed to set at room temperature. Suspensions of lyophilized leaf tissue at 4% (wt/vol) and 0.8% (wt/vol), were prepared in 0.2% bactoagar (Fisher Scientific). A 4% suspension is a 25-fold dilution of test material (2 g leaf powder/50 ml of 0.2% agar) and a 0.8% suspension is a 125-fold dilution of leaf powder (0.4 g leaf powder/50 ml of 0.2% agar). The suspensions were dispensed (0.45 ml per well) into each well of separate treatment plates and allowed to dry at room temperature. Plates were closely monitored

to avoid over-drying and subsequent cracking of the diet surface prior to insect infestation.

Each well of the 24-well treatment plate was infested with a single neonate *H. virescens* or *H. zea* [(eggs of both species purchased from known susceptible colonies from the NC State University insectary) (van Kretschmar, 2010)], and all plates were held in an incubator at 25 °C in the dark. Treatments (24 neonates per dilution in four independent experiments) were evaluated at 3- or 6-d intervals until mortality reached 100% in transgenic plants (COT102 and COT67B). Visibly inactive larvae that did not move when touched with blunt tip forceps were considered dead. Four experiments per pest were conducted with independent preparations of leaf tissue from 15 separate plants.

**Artificial Infestation of Field Trials.** To support the findings described in Kurtz et al. (2007), plants in field trials were artificially infested with laboratory strains of pest insects to determine if full-length Cry1Ab is expressed by COT67B at a dose that kills at least 95% of susceptible *H. virescens*. The trial was carried out at two locations, Syngenta's Southern Regional Technical Center at Leland, MS and Vero Beach Research Center in Vero Beach, FL. Unreplicated, solid blocks of COT67B cotton plants as well as smaller blocks of non-transgenic Coker312 plants were grown at each location using standard regional agricultural practices. To eliminate the natural-enemy interference with the infestation, the study area was over-sprayed with acephate (Orthene<sup>®</sup>, Valent U.S.A. Corporation, Walnut Creek, CA) at 0.09 kg ai/ha, 24 to 48 h before scheduled infestation (Jackson et al., 2003). The non-transgenic Coker312 cotton block was

used to estimate the infestation technique effectiveness and to determine field fitness of the *H. virescens* strain utilized in these studies. Because insect survival was expected to be low on COT67B cotton, more COT67B cotton plants were grown and infested than non-transgenic Coker312 plants, to increase the chance of being able to quantify surviving insects.

Transgenic COT67B and non-transgenic Coker312 cotton plants were artificially infested with *H. virescens* eggs obtained from the Southern Insect Management Research Unit, USDA, ARS, Stoneville, MS. This strain of *H. virescens* is believed to have LC<sub>50</sub> values similar to field strain because the colony was infested with feral insects in 2002 (Blanco et al., 2009). Twenty-four to 36 h before hatching, eggs were mixed into a xanthan gum solution (11g xanthan powder/3000 ml DI water) and sprayed onto the terminal area of the cotton plants using a conventional CO<sub>2</sub> backpack sprayer. Eggs were sprayed through a Teejet flat fan 8006 nozzle (Spraying Systems Co., Wheaton, IL), at approximately 10 psi. To reduce the sampling time necessary for evaluating the total number of infested plants on a given date, four artificial infestations were made at each location on different dates with one quarter of the available plants being infested each time. The infestations were carried out after initiation of flowering. Egg hatch rate was estimated by collecting several leaves containing eggs from Coker312 plants and placing them into Petri dishes. Eggs recovered from leaves were counted and allowed to hatch in Petri dishes. The number of neonates was counted a day after hatching to determine eclosion rates. The total numbers of insects infested and plants assessed at each location are given in Table 1.

**Table 1. Artificial infestation of *H. virescens* in field trials carried out in 2005 on COT67B cotton plants at two locations in MS and FL indicating number of plants assessed, number of insects infested, and number of insects observed at day 7 and day 14.**

Location	Treatment	Total larvae infested <sup>z</sup>	Number of plants assessed <sup>y</sup>	Number of insects observed 7 DAI	Number of insects observed 14 DAI
MS	Coker312	3075	112	314 (3140) <sup>x</sup>	
	COT67B	30,755	1120	1	0
FL	Coker312	5208	168	141 (1410) <sup>x</sup>	
	COT67B	52,080	1680	2	0
Totals	Coker312	8283	280	455	
	COT67B	82,835	2800	3	0

<sup>z</sup> Estimated based on the number of eggs applied and the observed hatch rate

<sup>y</sup> Actual number of plants examined for live larvae, number of plants within treatments between locations differ due to plot size

<sup>x</sup> Estimated number of larvae that would have been observed if an equal number of plants were sampled in COT67B and Coker312

Field assessments of larval survival were carried out 7 d after infestation (DAI). In each case, the assessment involved a whole plant search for surviving larvae. Where surviving larvae were found on COT67B plants, the fruiting structures containing the larvae were tagged. Four to 7 d later, these fruiting structures, plus all adjacent structures were thoroughly reassessed to observe any surviving larvae.

**Bioassays of Neonate and Fourth Instar Larvae.** These assays involved the identification of the instar that is at least 25-fold more tolerant to the toxin than neonates, and determining mortality of older instar insects as compared to neonates. The more tolerant older instars serve as surrogate heterozygous neonates because neonates heterozygous for resistance are rarely greater than 25-fold less susceptible than homozygous susceptible neonates (US EPA, 1998). Bioassays with Cry1Ab and Vip3A were performed using neonates and fourth instar larvae to verify if older *H. zea* was at least 25-fold more tolerant to the toxins than neonates. We chose to use purified protein for these bioassays, which would be indicative of the intrinsic activity of the protein. In addition, the use of purified toxins from a bacterial source in the artificial-diet bioassay method was chosen as it was logistically much more practical than obtaining purified protein from plant tissue. Purified Vip3A protein (from a recombinant *E. coli* over-expression system) was solubilised in 1x phosphate-buffered saline (PBS) at pH 7.7 and centrifuged for 5 min at 25,000 x g at 4 °C. Purified full-length Cry1Ab (from a recombinant *E. coli* over-expression system) was solubilized in 50 mM Tris-HCl, 2 mM EDTA, at pH 9.5 and centrifuged as above. The *cry1Ab* gene used encodes the same full-length Cry1Ab protein as produced by *Bacillus thuringiensis* subsp. *kurstaki* HD-1, except that it contains an additional 26 amino acids (described as the Geiser motif) in the C-terminal portion of the protein (Geiser et al., 1986). The concentration of Vip3A and full-length Cry1Ab proteins in each supernatant was then determined using the BCA™ Protein Assay Kit (Pierce, Rockford, IL).

Sterile 12-well plates (Fisher Scientific) were prepared with 1.5 ml per well of artificial multiple-species Lepidopteran larval diet (Southland Products). One-hundred-twenty µl of an appropriate dilution of Vip3A or full-length Cry1Ab was applied to the surface of each diet well and allowed to dry at room temperature. Each well was then infested with a single neonate or fourth instar *H. zea* larva with 24 insects used per dilution. Eggs and fourth instar

*H. zea* larvae were obtained from the NC State University insectary (Raleigh, NC). Fourth instar *H. zea* were identified based on head-capsule size (Archer and Bynum, 1994). The bioassay plates were held at 25 °C in the dark and mortality read at days five through seven. Each test was conducted independently on different days using fresh stock solutions and test substance dilutions and different batches of larvae. Final LC<sub>50</sub> determinations were expressed in terms of ng protein/cm<sup>2</sup> diet surface. Each bioassay was evaluated in terms of its dose response, and LC<sub>50</sub> values were determined by probit analysis (Finney, 1971) using the EPA Probit Analysis Program (version 1.5) for those assays affording a dose response.

Cotton plants were seeded and grown as described for the lyophilized tissue bioassays above. Four weeks after planting, the youngest leaves on the plant with a width of at least 4 cm were harvested for fresh leaf bioassay. For neonate *H. zea* larvae, two leaf disks were cut from respective leaves using a size 10 cork borer and placed on top of a Millipore™ glass fiber pre-filter (Millipore Corporation, Billerica, MA) that was pre-wetted with 0.08 ml sterile distilled water in each well of a sterile 12-well plate. One neonate was placed into each well. Twenty-four wells each were used for each leaf type from Coker312 (non-transgenic), COT102, and COT67B transgenic cotton and the plates were covered with a layer of Breathe-Easy™ (Diversified Biotech, Boston, MA) film, then plate lids clipped on with four large binder clips. Plates were held at 25 °C in the dark. Old leaf discs were removed from each well, and fresh water and new leaf discs added every 2 d. Percent mortality was recorded after 3 to 4 d and periodically thereafter until 100% mortality was observed in the transgenic samples. For fourth instar *H. zea* larvae, six to eight 15-mm squares of leaf tissue from Coker312, COT102, and COT67B cotton were cut and placed into each well of sterile 12-well plates prepared as described above. One fourth instar *H. zea* larva was placed into each well. Twenty-four wells were used for each leaf type to be tested and the plates were covered with plate lids clipped on with two large binder clips. The test was conducted at 25 °C in the dark. Surviving larvae were removed from each well and reset into newly prepared wells in a new sterile 12-well plate every 2 to 3 d. Percent mortality was recorded at each plate change until 100% mortality was noted in the transgenic event. Three independent tests were conducted with different batches of insects and leaf material.

**Statistical Analysis.** Percentage mortality was calculated and data were transformed prior to ANOVA and the Student-Newman-Keuls mean separation procedure for each respective set of pest species data for bioassays using lyophilized tissues and also for bioassays with neonate and fourth instar larvae (on leaf disks). Percentage mortality values were transformed using an arcsine  $\sqrt{\text{proportion}}$  procedure. An adjustment of  $+1/(4n)$ , where  $n$  = number of insects, was used for each respective sample falling between 0 to 14% mortality, whereas an adjustment of  $100 - (1/4n)$  was used for respective samples between 86 to 100% mortality. Each Least Significant Range (LSR) was determined per the procedure described in Sokal and Rohlf (1969):

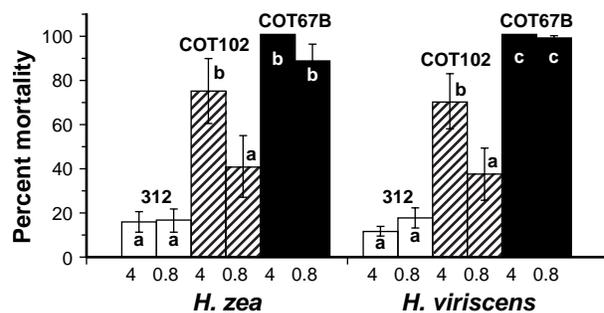
$$\text{LSR} = Q_{\alpha[k,v]} 0.05 \sqrt{\text{MS}_{\text{within}}/n}$$

Means assigned the same letter for each respective pest species (lyophilized tissue bioassay) or for each respective set of neonate or fourth instar *H. zea* larvae (leaf disk bioassay) are not significantly different.

## RESULTS

The three methods discussed (bioassays using lyophilized tissue, artificial infestation of field trials, and bioassays of neonate and fourth instar larvae) were used in combination to support the findings reported in Kurtz et al. (2007) for the commercial product VipCot™ cotton by evaluating biological activity of the single events, COT102 cotton and COT67B cotton, against *H. virescens* or *H. zea*. Both species were evaluated by using lyophilized tissue of each single event to provide insight into the relative insecticidal activity of single events compared to the combined activity in living plant tissue. Because *H. zea* has been more difficult to control historically (Greenplate et al., 1998; Stone and Sims, 1993), further assays were run with each single event to determine what impact each might have on survival of neonates heterozygous for resistance. Because *H. virescens* has been controlled easily with Cry1 expressing cotton events (Jackson et al., 2007), field assays were run to determine if COT67B provides similar control. Data from all methods performed for both pests on VipCot™ cotton can be found in Kurtz et al. (2007). Results show both the proteins, Vip3A and Cry1Ab in VipCot™ cotton, are highly effective against both the pests.

**Bioassays Using Lyophilized Tissue with *H. virescens* and *H. zea*.** For *H. zea*, the 25-fold dilution of lyophilized tissue of COT67B and COT102 caused 100 and 75% mean mortality, respectively, whereas non-transgenic Coker312 caused only 16% mean mortality (Fig. 1). Furthermore, even at a 125-fold dilution, COT67B tissue caused 89% mean mortality of *H. zea*, whereas Coker312 caused 17% mean mortality. Similar data were obtained for bioassays on *H. virescens*, with COT67B tissue causing 100% and 99% mean mortality at 25-fold and 125-fold dilutions, respectively, and with Coker312 causing only 12% and 18% mean mortality, respectively. COT102 resulted in 70% mean mortality to *H. virescens* when diluted 25-fold (Fig. 1). These data indicate that the COT67B and COT102 events exhibit insecticidal activity toward *H. zea* and *H. virescens* even when diluted into insect diet.



**Figure 1.** Lyophilized tissue bioassays with *H. zea* and *H. virescens*. Percent mortality for four bioassays are summarized as the mean mortality (error bars indicate the standard error of the mean). Means assigned the same letter for each respective pest species are not significantly different. Numbers below x axis indicate the percent lyophilized leaf material present in the assay. Open, diagonal, and solid boxes represent Coker 312, COT102, and COT67B, respectively.

**Artificial Field Infestations with *H. virescens*.** The insecticidal activity of COT67B cotton vs. *H. virescens* was evaluated in two studies located at Leland, MS and Vero Beach, FL during 2005. Artificial infestation resulted in the establishment of robust *H. virescens* populations on the non-transgenic Coker312 plots at both locations, with 314 and 141 larvae observed after 7 d at Leland and Vero Beach, respectively (Table 1). These data confirm that the infestation regime was effective and that the control mortality was comparable to the natural Heliothine mortality in cotton (Pustejovsky and Smith, 2006). In contrast, only one surviving larva was found on COT67B cotton 7 d after infestation at the Leland location, despite infestation of 10-fold the number

of plants compared to Coker312. Similarly, only two surviving larvae were found on COT67B 7 d after infestation at Vero Beach (Table 1). At both locations, the larvae surviving on COT67B cotton after 7 d were small, ranging from first to early third instars. Fruiting structures (with no damage to insignificant feeding) that contained surviving larvae were tagged and assessed again 4 to 7 d later. Adjacent plants were also thoroughly checked. In all cases, the larvae could no longer be recovered. Furthermore, all the tagged fruiting structures remained on the plants and were developing normally. These observations indicate that the few small larvae still alive on COT67B at 7 d likely did not survive to pupation, and that COT67B cotton plants showed 100% mortality of *H. virescens* 10 to 14 d after infestation.

**Neonate and Fourth Instar Bioassays for *H. zea*.** In two separate LC<sub>50</sub> bioassays, fourth instar *H. zea* larvae were 30- and 87-fold more insensitive than neonates to Cry1Ab (Table 2). In four

separate bioassays for Vip3A, while neonate *H. zea* larvae had a LC<sub>50</sub> value ranging from 487 to 1780 ng/cm<sup>2</sup> (Table 3), none could be calculated for fourth instar *H. zea* larvae in three bioassays. In these three bioassays, the mortality at the highest dose of approximately 58,000 ng/cm<sup>2</sup> was 75, 33, and 8%, respectively, with consistent low mortality and poor dose responses at the lower doses used. However, in a fourth bioassay, even though the mortality never rose above 42% even at the highest dose tested (100,000 ng/cm<sup>2</sup>), we were able to calculate an LC<sub>50</sub> that was 82-fold higher than neonates (Table 3). These data indicated that fourth instar *H. zea* were over 25-fold more insensitive to Vip3A than neonates. Therefore, fourth instar larvae were appropriate to use in older instar bioassays of either event. The variation in LC<sub>50</sub> values of neonate and fourth instar *H. zea* could be entirely a result of natural variation in cohorts of a population and subsequent generations (Robertson et al., 1995).

**Table 2.** LC<sub>50</sub> values of full-length Cry1Ab (in COT67B event) pure protein for first and fourth instar *H. zea* showing LC<sub>50</sub> ratio and 95% C. I. in two bioassays.

Bioassay	Instar	n <sup>z</sup>	Slope ± SE	LC <sub>50</sub> <sup>y</sup>	95% C. I.	df	χ <sup>2</sup>	LC <sub>50</sub> Ratio(L4:L1)
1	L1	72	1.076 ± 0.283	691	313-2001	3	1.158	30
	L4	60	0.809 ± 0.379	20,838	nc <sup>x</sup>	3	0.009	
2	L1	84	1.089 ± 0.243	219	82-439	4	0.178	87
	L4	84	0.785 ± 0.271	19,263	7764-240,451	4	0.151	

<sup>z</sup> Total number of insects

<sup>y</sup> Measured as ng/cm<sup>2</sup> concentration

<sup>x</sup> None calculated by EPA Probit Analysis Program (version 1.5), mortality was only at 50% at the highest dose of 60,000 ng/cm<sup>2</sup>

**Table 3.** LC<sub>50</sub> values of Vip3A (in COT102 event) pure protein for first and fourth instar *H. zea* showing LC<sub>50</sub> ratio and 95% C. I. in four bioassays.

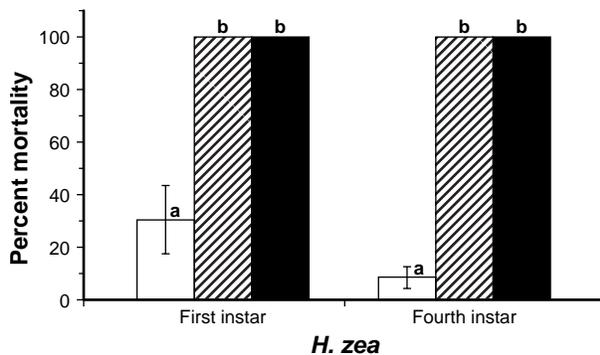
Bioassay	Instar	n <sup>z</sup>	Slope ± SE	LC <sub>50</sub> <sup>y</sup>	95% C. I.	df	χ <sup>2</sup>	LC <sub>50</sub> Ratio (L4:L1)
1	L1	108	0.818 ± 0.241	505	50-1186	6	5.613	>25
	L4	132	nc <sup>x</sup>	nc <sup>x</sup>	nc <sup>x</sup>	nc <sup>x</sup>	nc <sup>x</sup>	
2	L1	84	1.552 ± 0.427	1780	1040-3760	3	0.841	>25
	L4	108	nc <sup>x</sup>	nc <sup>x</sup>	nc <sup>x</sup>	nc <sup>x</sup>	nc <sup>x</sup>	
3	L1	192	2.04 ± 0.637	487	291-907	2	4.495	>25
	L4	96	nc <sup>x</sup>	nc <sup>x</sup>	nc <sup>x</sup>	nc <sup>x</sup>	nc <sup>x</sup>	
4	L1	192	0.767 ± 0.238	1599	479-4250	4	2.851	82
	L4	216	1.068 ± 0.238	130,341	69,484-515,061	6	5.668	

<sup>z</sup> Total number if insects

<sup>y</sup> Measured as ng/cm<sup>2</sup>

<sup>x</sup> None calculated

Three individual bioassays of neonate and fourth instar *H. zea* larval survival on COT102 and COT67B were performed on fresh cotton leaf disks. The data presented in Fig. 2 indicate that exposure to either COT102 or COT67B leaf material resulted in 100% mortality estimates of both neonate and fourth instar *H. zea*. This indicates that both events display high activity towards *H. zea*, based on this method.



**Figure 2.** Neonate and fourth instar bioassays with *H. zea*. Three leaf disk bioassays were conducted using fresh leaf disks of Coker312, COT102, and COT67B against neonate and fourth instar *H. zea*. Data for three bioassays are summarized as the mean mortality for all bioassays. Error bars indicate the standard error of the mean. Means assigned the same letter for each respective larval stage are not significantly different. Open, diagonal, and solid boxes represent Coker312, COT102, and COT67B, respectively.

## DISCUSSION

These results confirm the high insecticidal activity seen with pyramided VipCot™ cotton reported earlier (Kurtz et al., 2007). In our study, the data show that COT67B resulted in high mortalities of Heliiothine neonates when exposed to the three methods used, and COT102 caused high mortalities of first and fourth instars of *H. zea* when exposed to fresh leaf tissue in the laboratory study. Previously reported data showed that VipCot™ cotton killed all susceptible *H. virescens* and *H. zea* using similar methodology (Kurtz et al., 2007). The data presented herein reinforce the potential value that pyramiding COT67B and COT102 as VipCot™ cotton brings to resistance management. The computer simulation modelling performed by Roush (1998) demonstrated that pyramided Bt crops with component proteins causing greater than 95% mortality of susceptible homozygotes and 70% mortality of resistant heterozygotes will be effective with relatively small amount of non-

Bt refuge. Our data indicate that the component events comprising VipCot™ cotton meet the level of efficacy that simulation modelling predicts, will be highly effective for delaying resistance development in insect populations even with small amounts of non-Bt refuge.

To further evaluate the relative efficacy of the component events in VipCot™ cotton, field trials that included COT102 and COT67B alongside VipCot™ cotton were conducted in multiple locations in 2006 and 2007 (Minton et al., 2008) and the data presented here is not part of our study. It is reported here to emphasize the efficacy of individual component events as it has reflected in our laboratory study here. In all cases, the COT102 and COT67B events performed well providing nearly 97 to 98% protection of squares and bolls against Heliiothines. VipCot™ cotton provided excellent protection of both *H. virescens* and *H. zea*, resulting in little square damage (0.25% for *H. virescens* and 1% for *H. zea*) and boll damage (0.02% for *H. virescens* and 1.2% for *H. zea*) compared to 30% damage to non-transgenic Coker312 squares and bolls (Minton et al., 2008). These field trials indicate that COT67B and/or COT102 have satisfied the high-dose component for the insect resistance management. By pyramiding the COT67B and COT102 events in VipCot™ cotton, the maximum potential of these two proteins has been harnessed for cotton insect control and resistance management.

Lack of cross-resistance is another key component for good insect resistance management. Roush's simulation models (1998) predicted that no cross-resistance should exist between the two proteins to capture the most advantage of pyramiding. To that point, the Vip3A-expressing COT102 event brings an important option to manage resistance in lepidopteran species by offering a distinct mode of action. Cross-resistance could not be directly evaluated in the current study because no resistant insect colonies were available, but other studies have addressed this issue as cited below and reviewed in Kurtz (2010). The risk of cross-resistance is highest between proteins that share a similar structure (Gould, 1995; Tabashnik, 1994). The complete lack of sequence homology between Vip3A and the Cry proteins (Estruch et al., 1996) supports the two toxins as being structurally different. Furthermore, it has been demonstrated (Lee et al., 2003, 2006) that significant differences exist between Cry1Ab and Vip3A toxins with regard to binding to insect brush

border or known Cry1 receptors, pore formation in the midgut epithelium, and flux properties of the underlying channel structures in artificial membranes. The differences in receptor binding are particularly important, because changes in binding properties to the insect brush border are the most common mechanism of resistance to Bt toxins in lepidopterans (Baxter et al., 2005).

*In vivo* evidence for an absence of cross-resistance between Vip3A and Cry proteins has been obtained for both *H. virescens* and *H. zea* using assays with Cry1Ac-selected and susceptible strains. Jackson et al. (2007) demonstrated with concentration-mortality data that three Cry1Ac resistant *H. virescens* strains were not resistant to Vip3A. Their studies also showed that Cry1Ac-resistant strains of *H. virescens* are unable to survive on cotton varieties expressing Vip3A. Anilkumar et al. (2008) demonstrated that Cry1Ac-resistant *H. zea* was not resistant to Vip3A. These studies highlight the advantage of stacking two proteins with distinct modes of action. There are differences between the modes of action of Vip3A and Cry proteins, and a large margin of safety against the possibility of cross-resistance, which is important for insect resistance management and the stewardship of these proteins.

This study has shown independent cotton events (COT102 and COT67B) perform effectively by providing excellent protection of fruiting structures and cotton plants in general against Heliothines. The level of efficacy shown by these events combined with the lack of cross-resistance exceeds efficacy levels predicted to result in substantial resistance delays and requiring only a small amount of non-Bt refuge. This could be an important integrated pest management tool for future agriculture.

### ACKNOWLEDGMENTS

The authors wish to thank Ashley Boyer and Sharon Billings for assistance and support with lab insect bioassays and Victor Mascarenhas and Mike Green for assistance and support with the field studies. We appreciate the help of Kristin Keeler and the greenhouse staff for taking care of and maintaining the cotton plants. We also thank the protein production team at the Syngenta Jealott's Hill research station for the purified protein test substances. The support of Eric Chen is also acknowledged, and that of Andre Crespo for helpful comments and review. We extend our acknowledgements to Beverley Pagura of

North Carolina State University Insectary, Raleigh, NC and to the staff at Southern Insect Management Research Unit, Stoneville, MS for providing us with insect eggs.

### REFERENCES

- Anilkumar, K.J., A. Rodrigo-Simón, J. Ferré, M. Pusztai-Carey, S. Sivapuram, and W. Moar. 2008. Production and characterization of *Bacillus thuringiensis* Cry1Ac-resistant cotton bollworm *Helicoverpa zea* (Boddie). *Appl. Environ. Microbiol.* 74:462–469.
- Archer, T.L., and E.D. Bynum. 1994. Corn earworm (Lepidoptera: Noctuidae) biology on food corn on the high plains. *Environ. Entomol.* 23(2):343–348.
- Baxter, S.W., J.-Z. Zhao, L.J. Gahan, A.M. Shelton, B.E. Tabashnik, and D.G. Heckel. 2005. Novel genetic basis of field-evolved resistance to Bt toxins in *Plutella xylostella*. *Insect Mol. Biol.* 14:327–334.
- Blanco, C.A., F. Gould, P. Vega-Aquino, J.L. Jurat-Fuentes, O.P. Perera, and C.A. Abel. 2009. Response of *Heliothis virescens* (Lepidoptera: Noctuidae) strains to *Bacillus thuringiensis* Cry1Ac incorporated into different insect artificial diets. *J. Econ. Entomol.*, 102(4):1599–1606.
- Cayley, P.J., D.V. Negrotto, and J. Barnett. 2010. CE43-67B insecticidal cotton. US Patent 7834254-B27. Date issued: 16 Nov. 2010.
- Comins, H.N. 1986. Tactics for resistance management using multiple pesticides. *Agr. Ecosyst. Environ.* 16:129–148.
- Estruch, J.J., G.W. Warren, M.A. Mullins, G.J. Nye, J.A. Craig, and M.G. Koziel. 1996. Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. *Proc. Natl. Acad. Sci. USA.* 93:5389–5394.
- Finney, D.J. 1971. *Probit Analysis*. London: Cambridge University Press, Cambridge, UK.
- Fitt, G.P., C.L. Mares, and D.J. Llewellyn. 1994. Field evaluation and potential ecological impact of transgenic cottons (*Gossypium hirsutum*) in Australia. *Biocontrol. Sci. Tech.* 4:535–548.
- Geiser, M., S. Schweitzer, and C. Grimm. 1986. The hyper-variable region in the genes coding for entomopathogenic crystal proteins of *Bacillus thuringiensis*: nucleotide sequence of the *kurhdI* gene of subsp. *kurstaki* HDI. *Gene* 48:109–118.
- Gould, F. 1986. Simulation models for predicting durability of insect resistant germplasm; a deterministic diploid, two-locus model. *Environ. Entomol.* 15:1–10.

- Gould, F. 1995. Selection and genetic analysis of a *Heliothis virescens* (Lepidoptera: Noctuidae) strain with high levels of resistance to *Bacillus thuringiensis* toxins. *J. Econ. Entomol.* 88:1545–1559.
- Greenplate, J.T., G.P. Head, S.R. Penn, and V.T. Kabuye. 1998. Factors potentially influencing the survival of *Helicoverpa zea* on Bollgard® cotton. p. 1020–1033. *In Proc. Beltwide Cotton Conf., San Diego, CA. 5-9 Jan. 1998. Natl. Cotton Counc. Am., Memphis, TN.*
- Greenplate, J.T. 1999. Quantification of *Bacillus thuringiensis* insect control protein Cry1Ac over time in Bollgard cotton fruit and terminals. *J. Econ. Entomol.* 92(6):1377–1383.
- Gruere, A. 2007. 2007/08 World Cotton Outlook. Available at [www.icac.org/cotton\\_info/speeches/gruere/2007/gdynia\\_2007.pdf](http://www.icac.org/cotton_info/speeches/gruere/2007/gdynia_2007.pdf). (verified 1 July 2011).
- Jackson, R.E., J.R. Bradley, and J.W. Van Duyn. 2003. Field performance of transgenic cottons expressing one or two *Bacillus thuringiensis* endotoxins against bollworm, *Helicoverpa zea* (Boddie). *J. Cotton Sci.* 7:57–64.
- Jackson, R.E., M.A. Marcus, F. Gould, J.R. Bradley, and J.W. Van Duyn. 2007. Cross-resistance responses of Cry1Ac-selected *Heliothis virescens* (Lepidoptera: Noctuidae) to the *Bacillus thuringiensis* protein Vip3A. *J. Econ. Entomol.* 100:180–186.
- Kurtz, R.W. 2010. A review of Vip3A mode of action and effects on Cry protein-resistant colonies of Lepidoptera larvae. *Southwest. Entomol.* 35(3):391–394.
- Kurtz, R.W., A. McCaffery, and D. O'Reilly. 2007. Insect resistance management for Syngenta's VipCot™ transgenic cotton. *J. Invert. Path.* 95:227–230.
- Lee, M.-K., F.S. Walters, H. Hart, N. Palekar, and J-S. Chen. 2003. The mode of action of the *Bacillus thuringiensis* vegetative insecticidal protein Vip3A differs from that of Cry1Ab  $\delta$ -endotoxin. *Appl. Environ. Microbiol.* 69:4648–4657.
- Lee, M.-K., P. Miles, and J-S. Chen. 2006. Brush border membrane binding properties of *Bacillus thuringiensis* Vip3A toxin to *Heliothis virescens* and *Helicoverpa zea* midguts. *Biochem. Biophys. Res. Comm.* 339:1043–1047.
- Minton, B.W., H.S. McLean, V. Mascarenhas, D.V. Negrotto, and D.P. Dickerson. 2008. VipCot™ performance in the southeast and Texas upper Gulf coast: 2006–2007. p. 1081–1085. *In Proc. Beltwide Cotton Conf., Nashville TN. 8-11 Jan. 2008. Natl. Cotton Counc. Am., Memphis, TN.*
- O'Reilly, D., F. Walters, N. Palekar, A. Boyer, and R. Kurtz. 2007. Laboratory studies to determine the high dose status of VipCot™ cotton. p. 1087–1094. *In Proc. Beltwide Cotton Conf., New Orleans, LA. 9-12 Jan. 2007. Natl. Cotton Counc. Am., Memphis, TN.*
- Perlak, F.J., R.W. Deaton, T.A. Armstrong, R.L. Fuchs, S.R. Sims, J.T. Greenplate, and D.A. Fischhoff. 1990. Insect resistant cotton plants. *Bio/Tech.* 8:939–943.
- Pustejovski, D.E., and J.W. Smith, Jr. 2006. Partial ecological life table of immature *Helicoverpa zea* (Lepidoptera: Noctuidae) in an irrigated cotton cropping system in the Trans-Pecos region of Texas, USA. *Biocontr. Sci. Tech.* 16(7):727–742.
- Robertson, J.L., H.K. Preisler, S.S. NG, L.A. Hickle, and W.D. Gelernter. 1995. Natural variation: a complicating factor in bioassays with chemicals and microbial pesticides. *J. Econ. Entomol.* 88(1):1–10.
- Roush, R.T. 1997. Bt transgenic crops: just another pretty insecticide or a chance for a new start in resistance management? *Pestic. Sci.* 51:328–334.
- Roush, R.T. 1998. Two-toxin strategies for management of insecticidal transgenic crops: Can pyramiding succeed where pesticides mixtures have not? *Phil. Trans. R. Soc. Lond.* B353:1777–1786.
- Sokal, R.R., and F.J. Rohlf. 1969. p. 239–246. *In: J. Wilson, and S. Cotter (ed.), Biometry, 1<sup>st</sup> ed. W. H. Freeman and Co., New York, NY.*
- Stone, T.B., and S.R. Sims. 1993. Geographic susceptibility of *Heliothis virescens* and *Helicoverpa zea* (Lepidoptera: Noctuidae) to *Bacillus thuringiensis*. *J. Econ. Entomol.* 86:989–994.
- Tabashnik, B.E. 1994. Evolution of resistance to *Bacillus thuringiensis*. *Ann. Rev. Entomol.* 39:47–79.
- US EPA, 1998. Transmittal of the Final Report of the FIFRA Scientific Advisory Panel Subpanel on *Bacillus thuringiensis* (Bt) Plant-Pesticides and Resistance Management, Meeting held on February 9 and 10, 1998. United States Environmental Protection Agency, Washington, D.C. Available at <http://www.epa.gov/scipoly/sap/meetings/1998/february/finalfeb.pdf> (verified 1 July 2011).
- US EPA Probit Analysis, Version 1.5. Available at <http://www.epa.gov/nerleerd/stat2.htm> (verified 1 July 2011).
- van Kretschmar, J.B. 2010. Novel insecticide resistance-monitoring bioassays for Lepidopteran and Hemipteran cotton pests and 454 pyrosequencing to identify potential gene targets for RNAi silencing in Hemipteran cotton pests. Ph.D. diss., North Carolina State University.
- Zhao, J-Z., J. Cao, Y. Li, H.L. Collins, R.T. Roush, E.D. Earle, and A.M. Shelton. 2003. Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance evolution. *Nature Biotech.* 21(12):1493–1497.