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DETERMINATION OF THE HIGH DOSE STATUS OF COTTON EVENTS EXPRESSING CRY1AB

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Abstract

Syngenta is currently developing cotton varieties that express both Vip3A and Cry1Ab toxins. The mode-of-action of Vip3A is different to that of Cry1Ab, and there is no cross-resistance between Vip3A and Cry toxins, so that a stack of Vip3A and Cry1Ab will represent an excellent product from the Insect Resistance Management (IRM) perspective. We are currently evaluating the high dose status of the components of this stack to help formulate the IRM strategy that we will propose for the product. In this paper, laboratory and field data assessing the high dose status of candidate Cry1Ab components of the stack are presented.

Introduction

The first commercially available transgenic insect-resistant cotton varieties were based on the Monsanto product Bollgard®, and expressed the insecticidal toxin Cry1Ac derived from the bacterium *Bacillus thuringiensis* (Bt). Bollgard® is extremely effective at controlling tobacco budworm (TBW) *Heliothis virescens* and pink bollworm (PBW) *Pectinophora gossypiella*, but it is less effective against the cotton bollworm (CBW) *Helicoverpa zea*. As a consequence, growers have had to continue to use insecticides as part of their lepidopteran insect control program to avoid yield losses (Bacheler and Mott 1997; Burd *et al*. 1999; Layton *et al*. 1997, 1998, 2000; Leonard *et al*. 1997, 1998). More recently, two-gene transgenic cottons, BollgardII® and Widestrike® have been introduced that have a broader spectrum of activity, including significantly higher levels of activity against CBW (Jackson *et al*. 2004). However, both of these two-gene transgenic cottons continue to rely exclusively on Cry toxins and in particular on Cry1Ac, as the insecticidal active ingredients. BollgardII® expresses both Cry1Ac and Cry2Ab, whereas Widestrike® expresses Cry1Ac and Cry1F. Thus, there is a real need for greater toxin diversity in the marketplace. To meet this need, Syngenta has been working to develop cotton varieties that express the novel insecticidal toxin Vip3A. Syngenta's VipCot™ cotton will comprise Vip3A stacked with the Cry toxin, Cry1Ab. Vip3A is unrelated to the Cry toxins and targets a distinct binding site in the insects midgut (Lee *et al.*, 2003; Chen and Lee, 2005). Increasing evidence is becoming available that there will be no cross-resistance between Vip3A and any of the Cry toxins (Marcus *et al*., 2005; Jackson *et al.*, 2006; Lee *et al.*, 2006; McCaffery *et al*., 2006). The combination of Vip3A and Cry1Ab is expected to provide both exceptional spectrum as well as excellent insect resistance management (IRM) attributes.

It will be imperative to implement a robust insect resistance management strategy to preserve the effectiveness of this valuable insect control tool. Syngenta's proposed insect resistance management program for VipCot™ is likely to be based on a combination of the plants expressing a high dose of insecticidal activity for key target pests, and the grower planting an appropriate refuge of non-lepidopteran control cotton (McCaffery *et al*., 2005, 2006). In this paper, we describe research evaluating the high dose status of potential Cry1Ab components of VipCot™. The EPA Science Advisory Panel SubPanel on *Bacillus thuringiensis* (Bt) Plant Pesticides and Resistance Management defined a high dose as 25 times the toxin concentration needed to kill susceptible larvae (Science Advisory Panel, 1998). They described 5 methods that could be used to determine whether a transgenic event expresses a high dose against a target pest, and required demonstrations of high dose using at least 2 of these 5 methods. Here, we present

laboratory and field data assessing the high dose status of 2 Cry1Ab events, Cot67B and Cot69D, against TBW, CBW and *Pectinophora gossypiella* (PBW).

Materials and Methods

Method 1: Lyophilized tissue bioassay

Terminal leaf tissue was used for TBW and CBW assays, whereas carpal wall tissue from young bolls was used for PBW assays. Terminal leaves were harvested approximately 4 weeks after planting, whereas young bolls (approx. 2.5 cm diameter) were harvested from 9 to 11 weeks after planting. Leaf samples were frozen at -80 ^{o}C immediately following harvest. Boll samples were cut into 4 segments, the lint removed, and the carpal wall segments frozen at -80 ^{o}C within 30 mins of harvest. Tissue samples were collected from Cot67B and Cot69D plants and from non-transgenic Coker 312 plants grown in parallel. The frozen tissue was stored at -80 ^{o}C until required and then lyophilized until completely dry. The lyophilized material was ground to a fine powder using a mortar and pestle, and stored at –20 ^{o}C until use. For the leaf tissue samples, 8.25 mls of artificial diet was dispensed into the appropriate number of 25 ml plastic containers and allowed to set. 4% (wt/vol), 0.8% (wt/vol) and 0.16% (wt/vol) suspensions of lyophilized tissue were prepared in 0.2% agar, and 1.7 mls of the appropriate suspension dispensed into each pot. For the boll tissue samples, the appropriate weight of powdered tissue was homogenized into 50 mls of solidified artificial diet to give final concentrations of 4% (wt/vol), 0.8% (wt/vol) or 0.16% (wt/vol) of lyophilized tissue in the diet. 1.4 mls of the diet with tissue incorporated was then dispensed into each well of a 24 well plate. For both assay formats, 20 replicates were prepared for each treatment and each was infested with a single neonate larva. The tests were held at 25 ^{o}C, 60% RH in the dark until all insects exposed to the test treatment had either died or pupated. The % mortality was then recorded for each treatment. The mortality data obtained for each Cot67B or Cot69D sample were corrected to allow for the mortality observed with the corresponding Coker 312 sample using Abbott's correction (Abbott, 1925). Each test was repeated a minimum of 3 times, with independent tissue preparations.

Method 4: Artificial infestation field trials

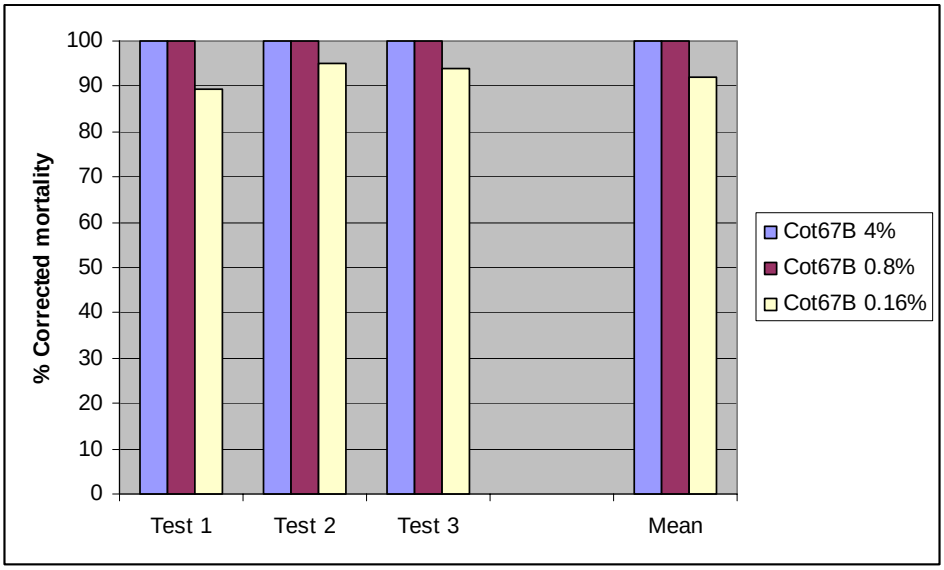
Cot67B or Cot69D cotton plants were artificially infested with tobacco budworm eggs, which were obtained from the Southern Insect Management Laboratory in Stoneville, MS 24 to 36 hours prior to artificial infestation. Eggs were mixed into a xanthan gum solution and sprayed onto the terminal area of the cotton plants utilizing a conventional CO_2 backpack sprayer. Eggs were sprayed through a flat fan 8006 nozzle at approximately 10 psi. The trial was carried out at 2 locations, Syngenta's Southern Regional Technical Centre at Leland, MS and Vero Beach Research Centre at Vero Beach, FL. At both locations, unreplicated, solid blocks of approximately 2240 plants of Cot67B and Cot69D, as well as smaller blocks of approximately 224 plants of non-transgenic Coker 312 were utilized for the infestation. If populations of natural enemies were deemed to be sufficiently high to interfere with infestation, the study area was over sprayed with acephate (Orthene®) at 0.5 lb ai/A 24 to 48 hours before scheduled infestation. The non-transgenic Coker 312 cotton block was used to estimate the infestation technique effectiveness and to determine field fitness of the tobacco budworm strain utilized in these studies. At each location, four artificial infestations were made to both Cry1Ab events and Coker 312 cotton with one quarter of the available plants being infested each time. The infestations were carried out between mid-squaring and early bloom. Egg hatch rate was estimated by collecting several leaves containing eggs from Coker 312 plants and placing them into Petri dishes. Eggs on the collected leaves were counted and two to three days later, successful larval eclosion was assessed. Assessments were carried out 7 days after infestation. One half of all infested plants were assessed at the Leland, MS location, whereas three quarters of all infested plants were assessed at the Vero Beach, FL location. In each case, the assessment involved a thorough whole plant search for surviving larvae. Square damage ratings were also taken from the Leland trial. Where surviving larvae were found on Cot67B or Cot69D, the fruiting structures containing the larva were tagged. 4 to 7 days later, these fruiting structures, plus all adjacent structures were thoroughly assessed again to evaluate whether the larvae were still surviving. Similar later assessments were not carried out on the Coker 312 plots because by this stage many of the larvae that had been on these plants had begun to search for pupation sites in the soil.

Results

Method 1: Lyophilized tissue bioassay

The results of the bioassays of lyophilized Cot67B and Cot69D leaf tissue against TBW are shown in Figures 1A and B. Data from each repeat of the test, and the mean data across all 3 tests are presented. For both events, 4% and 0.8% leaf tissue caused 100% mortality of the target insects in all tests. Exposure of the insects to 0.16% Cot67B and Cot69D leaf tissue resulted in 92% and 96.5% mean corrected mortality respectively.

A



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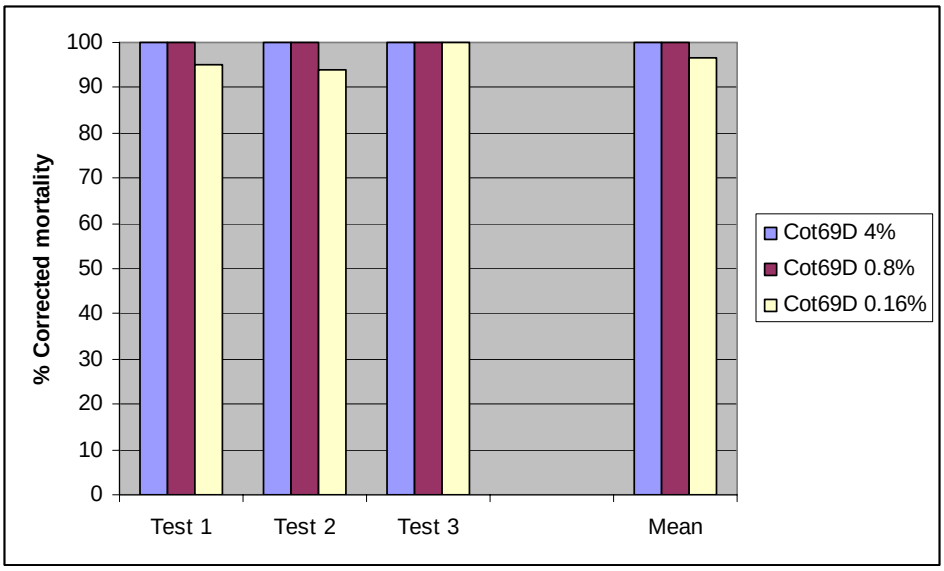
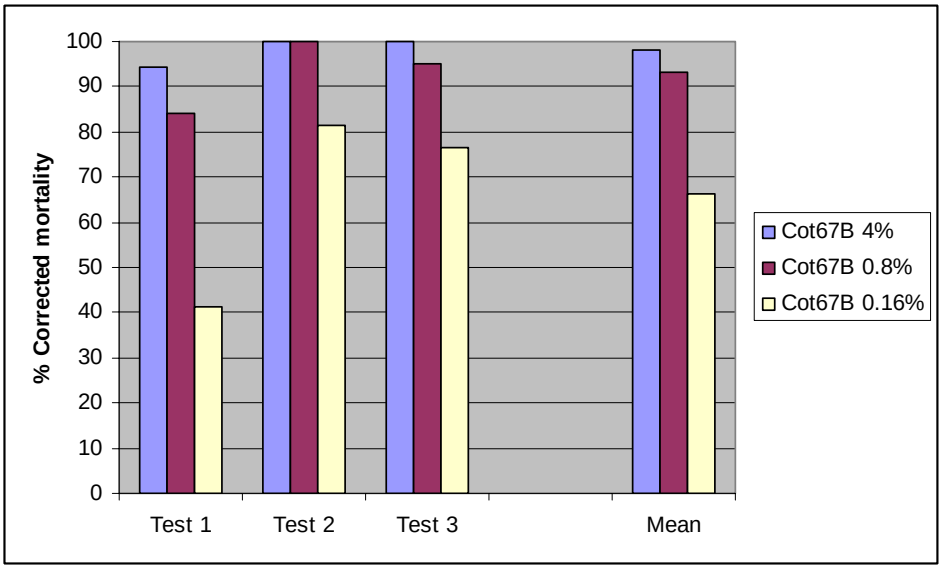


Figure 1. Bioassay of neonate TBW on lyophilized Cot67B (A) and Cot69D (B) leaf tissue.

Figures 2A and B present similar data from the bioassay of lyophilized Cot67B and Cot69D leaf tissue against CBW. Exposure of the insects to a 4% suspension of Cot67B or Cot69D leaf tissue resulted in greater than 95% mean corrected mortality in both cases.

A



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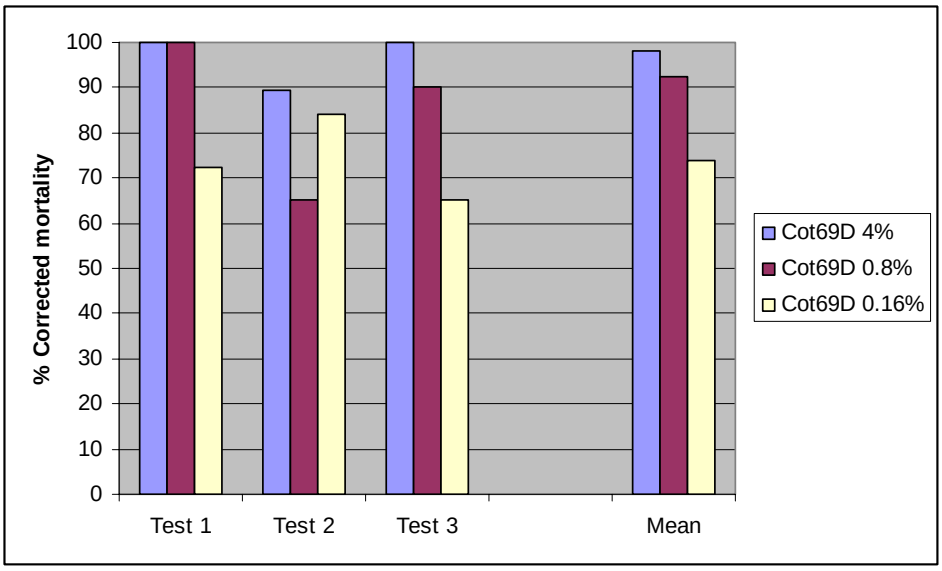
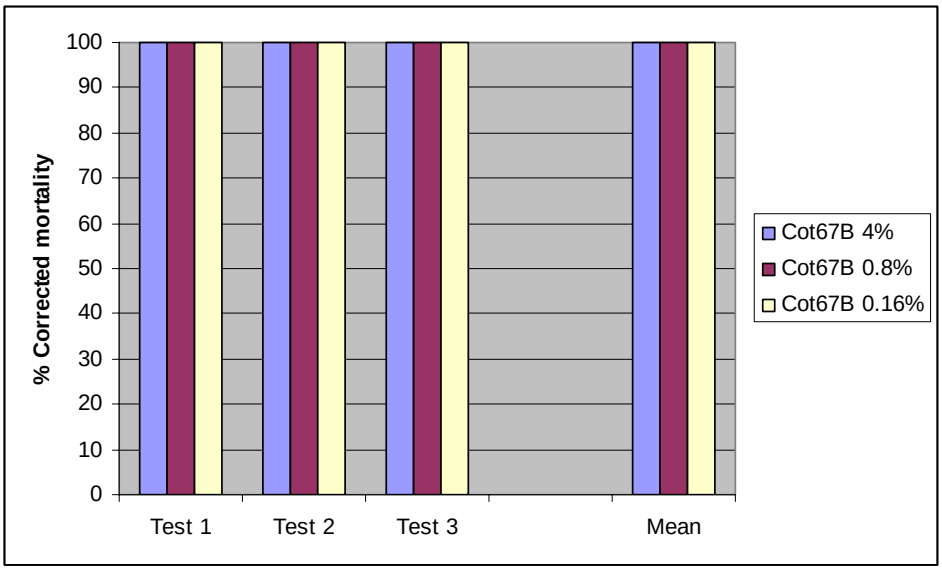


Figure 2. Bioassay of neonate CBW on lyophilized Cot67B (A) and Cot69D (B) leaf tissue.

The results of the bioassays of lyophilized boll tissue from these two events against PBW are presented in Figures 3A and B. For both events, 100% mortality was observed in all tests following exposure of the insects to 4% or 0.8% lyophilized boll tissue.

A



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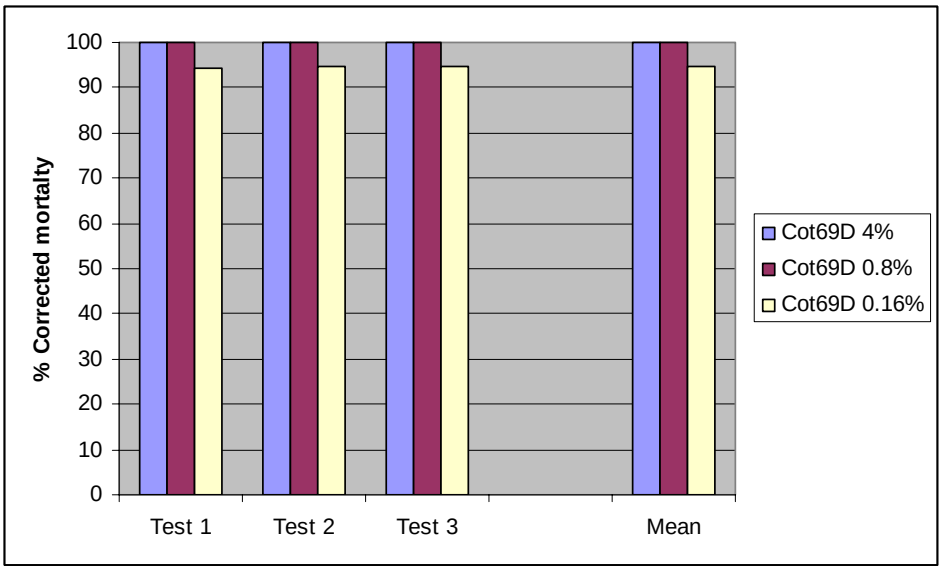

Figure 3. Bioassay of neonate PBW on lyophilized Cot67B (A) and Cot69D (B) leaf tissue.

Method 4: Artificial infestation field trials

Cot67B and Cot69D were evaluated in studies located at Leland Ms. and Vero Beach Fl. during 2005. Data from both studies are presented in Table 1. Robust infestations were established on the non-transgenic Coker 312 plots at both locations, with 314 and 141 larvae being recovered after 7 days at Leland and Vero beach respectively. 28% square damage was recorded on Coker 312 at Leland. These data confirm both that the infestation regime was effective and that the insects infested were vigorous and healthy. In contrast, only 1 and 3 live larvae were found on Cot67B and Cot69D respectively after 7 days at the Leland location, despite infestation of ten-fold the number of plants compared to Coker 312. Square damage on Cot67B and Cot69D was 1% or less in the Leland trial. Similarly, only 2 and 1 live larvae were found on Cot67B and Cot69D after 7 days at Vero Beach. At both locations, the larvae surviving on the Cry1Ab events after 7 days were very small, ranging from 1st to early 3rd instars. Fruiting structures that contained live larvae were tagged and assessed again 4 to 7 days later. Adjacent fruiting structures 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. This strongly suggests that the few small larvae that were still alive on the Cry1Ab events at 7 days had not survived.

Table 1: Artificial infestation of TBW on Cot67B and Cot69D

Location:	**Leland Ms**			**Vero Beach Fl**		
Genotype:	**Coker 312**	**Cot67B**	**Cot69D**	**Coker 312**	**Cot67B**	**Cot69D**
No. plants infested	112	1120	1120	168	1680	1680
No. larvae infested[a]	3080	30800	30800	5208	52080	52080
No. larvae recovered 7 days	314	1	3	141	2	1
No. larvae recovered 10-14 days	ND	0	0	ND	0	0
No. squares assessed	2760	26825	25589	ND	ND	ND
% squares damaged	28	1	0.9	ND	ND	ND

[a]Estimated based on the number of eggs applied and the observed hatch rate. ND: Not determined.

Discussion

Syngenta is committed to implementing a robust insect resistance management strategy to support VipCot™ following launch. Demonstrating that VipCot™ events express a high dose of insecticidal activity against key target pests will be an essential element of this IRM strategy. VipCot™ is a stack expressing two insecticidal toxins,

Vip3A and Cry1Ab. Because the insecticidal activity of a stack is in part dependent on the independent activity of the component toxins, we are assessing the high dose status of both Vip3A and Cry1Ab components of VipCot™. In this study, we present data evaluating the high dose status of two Cry1Ab events that are candidate components of the VipCot™ stack. The US EPA has set out 5 tests that can be used to determine whether a transgenic event is expressing at high dose, and requires that successful demonstrations of high dose be made with at least 2 of these 5 methods (Science Advisory Panel, 1998). In this paper, we present evidence that the Cry1Ab events Cot67B and Cot69D express a high dose of toxin for TBW, CBW and PBW larvae. EPA methods 1 and 4 were used: 1) the bioassay of dilutions of lyophilized plant tissue; and 4) the large-scale artificial infestation of field-grown plots of plants and assessment of larval survival.

For method 1, the aim is to demonstrate that lyophilized plant tissue that has been diluted at least 25-fold can still cause >95% mortality of the target insects. We used this method for TBW, CBW and PBW and in all cases demonstrated >95% mortality with a 4% (i.e. 25-fold dilution) suspension of leaf tissue. Indeed, in most cases, we observed >95% mortality with a 0.8% suspension of leaf tissue, corresponding to a 125-fold dilution.

Method 4 involves surveying large numbers of plants following artificial infestation in the field to confirm that the cultivar is at least an $LD_{99.9}$. To this end, blocks of 1120 or 1680 plants of each event were infested with TBW at two locations in 2005. The total number of larvae infested and the mean recovery rates across both locations are summarized in Table 2. A total of more than 80,000 larvae were infested onto each event across both locations. Despite carrying out comprehensive whole-plant searches of all infested plants, only 3 and 4 live larvae were recovered from Cot67B and Cot69D respectively. In all cases, these larvae were developmentally retarded and considered highly unlikely to survive to adulthood. To confirm this, fruiting structures containing the larvae were tagged, and these and surrounding structures thoroughly assessed again 4 – 7 days later. As expected, the live larvae could no longer be recovered, strongly suggesting they had not survived. In agreement with these data, the levels of square damage observed on either Cry1Ab event at the Leland location were extremely low (1% or less). In contrast, significant levels of square damage were observed on the Coker 312 non-transgenic controls, confirming that the TBW larvae used for these trials were vigorous and capable of establishing a robust infestation. Thus, these data provide strong evidence that both Cot67B and Cot69D express a high dose of insecticidal activity against TBW.

Table 2: Summary larval recovery rates following artificial infestation of Cot67B and Cot69 with TBW

Genotype	Total No. larvae infested	Total No. larvae recovered 7 days	Recovery rate 7 days	Total No. larvae recovered 10-14 days	Recovery rate 10-14 days
Coker 312	8288	445	0.054	ND	ND
Cot67B	82880	3	3.62×10^{-5}	0	0
Cot69D	103040	4	4.83×10^{-5}	0	0

In summary, we have presented evidence based on 2 of the EPA-recommended methods that both Cot67B and Cot69D express a high dose of insecticidal activity against TBW, and based on 1 method that these events express a high dose of toxin against CBW and PBW. Work is ongoing using a 2nd method to evaluate the high dose status of these events against CBW and PBW. Work is also in progress the evaluate the high dose status of potential Vip3A components of the VipCot™ stack, and of the stack itself. Together, these data will provide a robust foundation for the IRM strategy Syngenta will propose for VipCot™.

References

Abbott, W. S. 1925 A method for computing the effectiveness of an insecticide. Journal of Economic Entomology Vol. 18: 267-267.

Bacheler, J.S., and D.W. Mott. 1997. Efficacy of grower managed Bt cotton in North Carolina, pp. 858-861. *In* P. Dugger and D. A. Richter [eds.], Proceedings, 1997 Beltwide Cotton Conference. National Cotton Council, Memphis, TN.

Burd, T., J.R. Bradley, Jr. and J.W. Van Duyn. 1999. Performance of selected Bt cotton genotypes against bollworm in North Carolina, pp. 931-934. *In* P. Dugger and D.A. Richter [eds.], Proceedings 1999 Beltwide Cotton Conference. National Cotton Council, Memphis, TN.

Chen, E. and M. Lee. 2005. *In vitro* cross-resistance studies with Vip3A to support the IRM strategy for VipCot™. *In* Proceedings, 2005 Beltwide Cotton Conference. National Cotton Council, Memphis, TN.

Jackson, R. E., J. R. Bradley, Jr. and J. W. Van Duyn. 2004. Bollworm population production and associated damage in bollgard and bollgard II cottons under insecticide-treated and non-treated conditions, pp. 1022-1025. *In* Proceedings, 2004 Beltwide Cotton Conference. National Cotton Council, Memphis, TN.

Jackson, R.E., M.A. Marcus, F. Gould, J.R. Bradley, A.R. McCaffery and J. Van Duyn. 2006. Cross-resistance levels between the Bacillus thuringiensis proteins Cry1Ac and Vip3A in *Heliothis virescens*. *In* Proceedings, 2006 Beltwide Cotton Conference. National Cotton Council, Memphis, TN.

Layton, M.B, M.R. Williams and S. Stewart. 1997. Bt cotton in Mississippi: the first year, pp. 861-863. *In* P. Dugger and D.A. Richter [eds.], Proceedings, 1997 Beltwide Cotton Conference. National Cotton Council, Memphis, TN.

Layton, M.B., S. D. Stewart and M.R. Williams. 1998. Performance of Bt cotton in Mississippi, 1997, pp. 970-973. In P. Dugger and D.A. Richter [eds.], Proceedings, 1998 Beltwide Cotton Conference. National Cotton Council, Memphis, TN.

Layton, M.B., M.R. Williams and J.L. Long. 2000. Performance of Bt cotton in Mississippi, 1999, pp. 1037-1039. In P. Dugger and D.A. Richter [eds.], Proceedings, 2000 Beltwide Cotton Conference. National Cotton Council, Memphis, TN.

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. Biochemical and Biophysical Research Communications. Vol 339: 1043-1047.

Lee, M.K., F. S. Walters, H. Hart, N. Palekar and J. S. Chen. 2003. The mode of action of Bacillus thuringiensis vegetative insecticidal protein Vip3A differs from that of Cry 1Ab delta-endotoxin. Applied and Environmental Microbiology. Vol. 69: 4648-4657.

Leonard, B.R., J.H. Fife, K. Torrey, J.B. Graves and J. Holloway. 1997. Helicoverpa/Heliothis management in Nu-COTN and conventional cotton cultivars in Louisiana, pp. 863-867. In P. Dugger and D.A. Richter [eds], Proceedings, 1997 Beltwide Cotton Council, Memphis, TN.

Leonard, B.R., J.H. Fife, K. Torrey, E. Burris and J.B. Graves. 1998. Evaluation of transgenic Bt cotton lines against Heliothines in Northeast Louisiana, pp. 967-970. *In* P. Dugger and D.A. Richter [eds.], Proceedings, 1998 Beltwide Cotton Conference. National Cotton Council, Memphis, TN.

Marcus, M., J.R. Bradley, F.L. Gould, and J.W. Van Duyn. 2005. Cross-resistance evaluations of Cry1Ac tolerant *Heliothis virescens* strains to the novel insecticidal protein Vip3A, *In* Proceedings, 2005 Beltwide Cotton Conference. National Cotton Council, Memphis, TN.

McCaffery, A., L. Artim, D. Negrotto, D. O'Reilly, T. Burd, V. Mascarenhas, D. Dickerson and J. Reed. 2005. Insect resistance management for VipCot™. *In* Proceedings, 2005 Beltwide Cotton Conference. National Cotton Council, Memphis, TN.

McCaffery, A., M. Caprio, R. Jackson, M. Marcus, T. Martin, D. Dickerson, D. Negrotto, D. O'Reilly, E. Chen and M. Lee. 2006. Effective IRM with the novel insecticidal protein Vip3A. *In* Proceedings, 2006 Beltwide Cotton Conference. National Cotton Council, Memphis, TN.

Science Advisory Panel (SAP), Subpanel on *Bacillus thuringiensis* (Bt) plant pesticides. 1998. Transmittal of the final report of the FIFRA scientific advisory panel subpanel on *Bacillus thuringiensis* (Bt) plant pesticides and resistance management (Docket number OPPTS-00231).