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LABORATORY STUDIES OF VIPCOTTM SUPPORT HIGH DOSE

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Abstract

Syngenta proposes to implement a high dose / structured refuge progam to prevent or delay the development of resistance to the Vip3A toxin expressed in its VipCot™ transgenic cotton varieties. This paper describes work to test whether the 2 VipCot™ events, Cot202 and Cot203, express a high dose of toxin against the tobacco budworm *Heliothis virescens* and the cotton bollworm *Helicoverpa zea*. Data is presented from 2 laboratory-based methods to show that these VipCot™ events display high dose toxin expression against both these key target pests

Introduction

Current commercially available transgenic insect-resistant cotton varieties rely exclusively on Cry toxins, and in particular on Cry1Ac, as the insecticidal active ingredients. The Monsanto products Bollgard® and BollgardII® express Cry1Ac alone, or Cry1Ac and Cry2Ab respectively, whereas Dow's Widestrike® expresses Cry1Ac and Cry1F. Thus, there is a pressing need for greater toxin diversity in the marketplace. Syngenta's VipCot™ cotton expresses a novel insecticidal toxin, Vip3A, that is unrelated to the Cry toxins used in other manufacturers' products. This protein has a particularly broad spectrum of activity (Estruch *et al*. 1996, Mascarenhas *et al*. 2003, Cook *et al*. 2004, Cloud *et al*. 2004, Mascarenhas 2004) and targets a distinct binding site to the Cry toxins (Lee *et al.*, 2003; Chen and Lee, 2005). The deployment of VipCot™ will reduce selection pressure from Cry toxins at a macro level and will bring associated resistance management benefits for all technologies. 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 based on a combination of the plants expressing a high dose of the toxin for key target pests, and the grower planting an appropriate refuge of non-lepidoptera control cotton (McCaffery *et al*., 2005). A key component of delivering this program is demonstrating that the selected transgenic varieties express Vip3A at an appropriate high dose. 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. In this paper, we present data obtained for 2 VipCot™ events, Cot202 and Cot203, using EPA methods 1 and 5 to demonstrate high dose against the tobacco budworm (TBW) *Heliothis virescens* and the cotton bollworm (CBW) *Helicoverpa zea*. Method 1 involves the bioassay of lyophilized tissues from transgenic plants to confirm that such tissues retain activity even when diluted 25-fold, whereas method 5 involves identifying a later larval instar that is at least 25-fold less susceptible to the toxin than neonate insects, and then demonstrating that the event shows at least 95% control of such later instar insects.

Materials and Methods

Method 1: Lyophilized tissue bioassay

Terminal leaves were harvested from Cot202, Cot203 & Coker312 plants 4 weeks after planting and were snap-frozen by placing immediately on dry ice. The frozen leaf tissue was then placed immediately in a freeze-drier and lyophilized overnight. The lyophilized material was ground to a fine powder using a mortar and pestle, and stored at –20 ^{o}C until use. 8.25 mls of artificial diet was dispensed into the appropriate number of 25 ml plastic pots and allowed to set. 4% (wt/vol), 0.8% (wt/vol) and in some experiments 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. 20 pots were prepared for each treatment. Each pot was infested with a single neonate TBW or CBW larva. The tests were held at 25 ^{o}C, 60% RH in the dark until all insects had either died or pupated. The % mortality was then recorded for each treatment. The mortality data obtained for each Cot202 or Cot203 sample was corrected to allow for the mortality observed with the corresponding Coker312 sample using Abbott's correction (Abbott, 1925). Each test was repeated 3 to 6 times, with independent preparations of leaf tissue.

Method 5: Bioassay of later instar CBW larvae

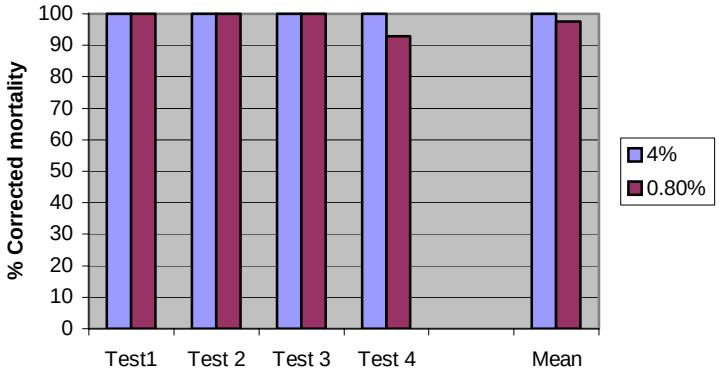
We have previously demonstrated that 3rd instar CBW larvae are at least 25-fold less susceptible to Vip3A than neonate insects (data not shown). Thus, they were chosen for use in this assay. 18 or 19 plants of Cot202 or Cot203 and the non-transgenic control Coker312 were sown, and 2 leaves were harvested from each plant 4 weeks later. The youngest leaves on the plant with a width of at least 4 cm were chosen for harvest. Leaves from 5 plants of each event were used for bioassay of 1st instar insects, and the remaining 13 or 14 plants were used for the 3rd instar bioassays. For the 1st instar bioassays, 4 leaf disks were punched from each leaf using a size 13 leaf cutter and placed on agar in 12 well plates (1 leaf disk per well). 45 µl of water was added to each well to maintain humidity. Thus, for each event, 8 leaf disks were sampled from each of 5 plants (40 leaf disks / event in total). 5 neonate CBW larvae were infested into each well. For the 3rd instar bioassays, a single 3rd instar larva was infested into each of 26 or 28 squat pots for each event. 1 leaf (axial surface down) was placed on top of each pot, and snapped in place with the lid of the pot. The test was held at 25 °C, 60% RH in the dark. % Mortality was recorded after 3 or 4 days. The 1st instar bioassays were stopped at this stage. Each leaf in the 3rd instar bioassay was replaced with a freshly harvested leaf from the same event, and the % mortality recorded again after 6 days. The mortality observed for Cot202 or Cot203 was corrected to allow for the mortality observed with the corresponding Coker312 sample as before (Abbott, 1925). Each test was repeated 3 to 4 times.

Results

Method 1: Lyophilized tissue bioassay

The results of the bioassays of lyophilized Cot202 tissue against TBW and CBW are shown in Figures 1A and B respectively. Data from each repeat of the test, and the mean data across all 4 tests are presented. In both cases, 4% Cot202 leaf tissue caused 100% mortality of the target insects in all tests. Exposure of the insects to 0.8% Cot202 leaf tissue resulted in 98% and 98.75% mean corrected mortality respectively.

A



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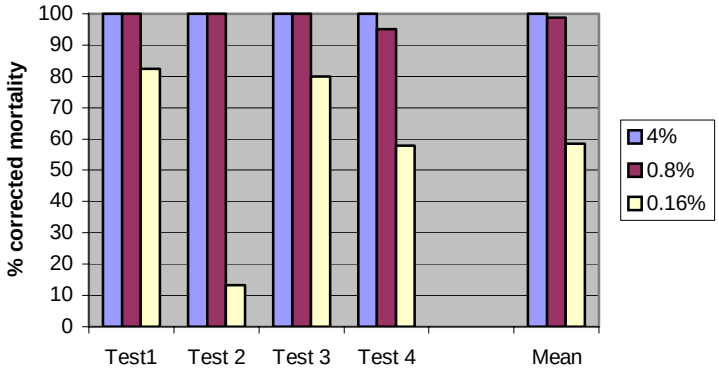
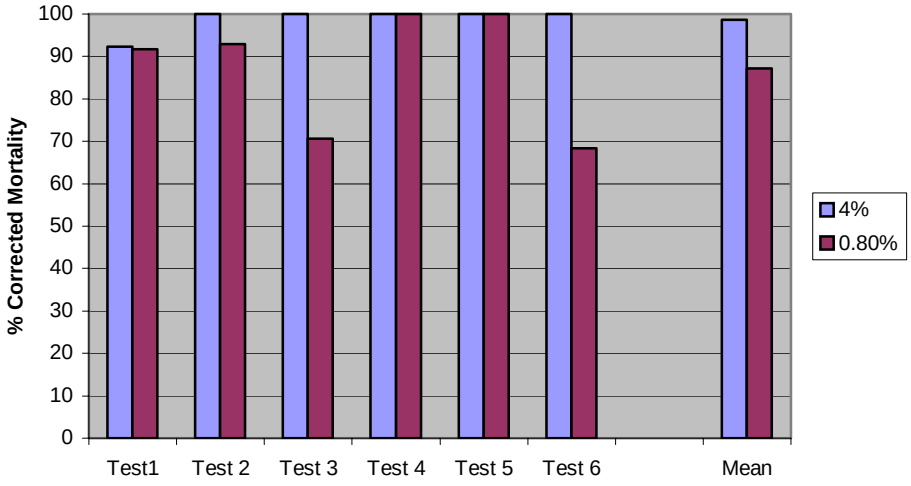


Figure 1. Bioassay of neonate TBW (A) and CBW (B) on lyophilized Cot202 leaf tissue.

The data from the bioassay of TBW and CBW larvae on lyophilized Cot203 leaf tissue are presented in Figures 2A and B respectively. Exposure of the insects to a 4% suspension of Cot203 leaf tissue resulted in greater than 95% mean corrected mortality in both cases.

A



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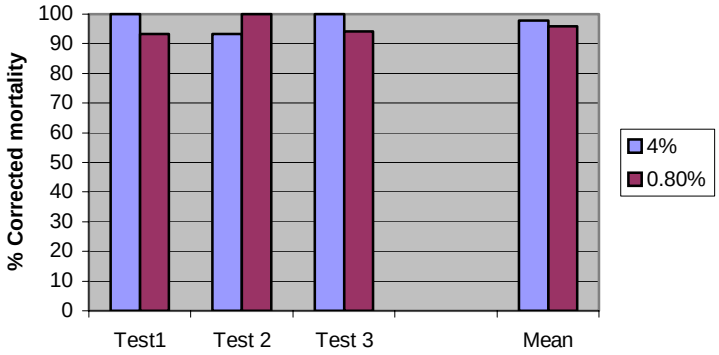
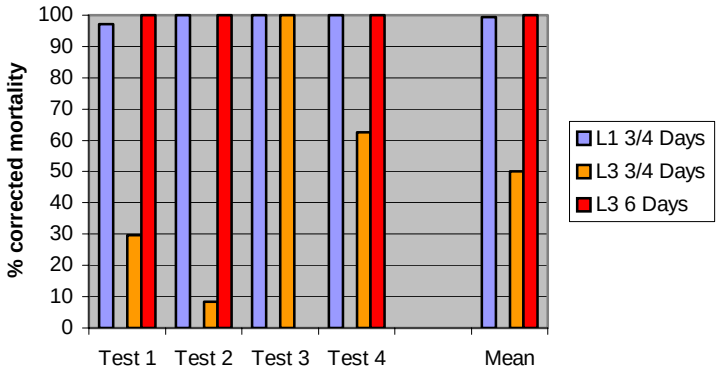


Figure 2. Bioassay of neonate TBW (A) and CBW (B) on lyophilized Cot203 leaf tissue.

Method 5: Bioassay of later instar larvae

Cot202 and Cot203 were tested for their ability to control 3rd instar CBW larvae. The data obtained are presented in Figures 3A and B respectively. Both events displayed excellent activity against these later instar CBW larvae, giving rise to 100% and 96% mean corrected mortality respectively after 6 days.

A



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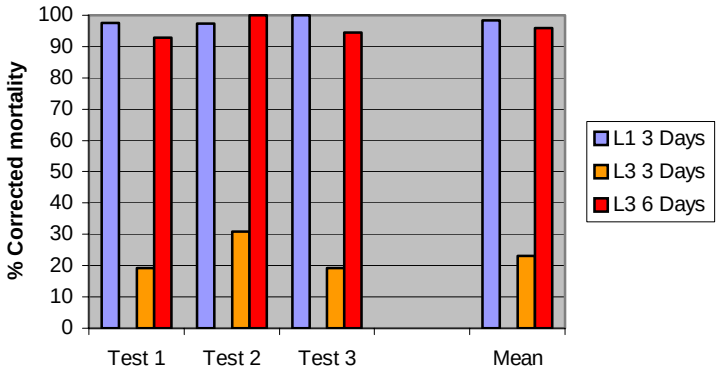

Figure 3. Bioassay of 3rd instar CBW larvae on Cot202 (A) and Cot203 (B) leaf tissue

Discussion

Demonstrating that VipCot™ events express a high dose of Vip3A against key target pests is essential to support the IRM strategy proposed by Syngenta for this product. 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 VipCot™ events Cot202 and Cot203 express a high dose of toxin for both TBW and CBW larvae. EPA methods 1 and 5 were used: 1) the bioassay of dilutions of lyophilized plant tissue; and 5) the bioassay of later-instar larvae that are less susceptible to the Vip3A toxin. 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 both TBW and CBW 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. The principle of method 5 is to identify a later larval instar that is at least 25-fold less susceptible to Vip3A than neonate insects and demonstrate that the transgenic event can effectively control such older insects. We have previously demonstrated that 3rd instar CBW larvae are at least 25-fold less susceptible to Vip3A than neonates (data not shown). Here we show that both Cot202 and Cot203 leaf disks are highly active against these 3rd instar larvae.

In summary, we have presented evidence based on 2 of the EPA-recommended methods that both Cot202 and Cot203 express a high dose of Vip3A toxin against CBW, and based on 1 method that these events express a high dose of toxin against TBW. In an accompanying paper, additional data are presented based on artificial infestation of field-grown plants that demonstrate that Cot202 and Cot203 express a high dose of toxin against TBW (Mascarenhas *et al*., 2005). Together, these data provide compelling evidence that VipCot™ is high-dose for both TBW and CBW, and strongly support the IRM strategy Syngenta is proposing for this product.

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