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Susceptibility of *Helicoverpa zea* and *Heliothis virescens* (Lepidoptera: Noctuidae) to Vip3A insecticidal protein expressed in VipCot™ cotton

M.I. Ali *, R.G. Luttrell [1]

Department of Entomology, Division of Agriculture, University of Arkansas, Fayetteville, AR, United States

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ABSTRACT

Susceptibility of laboratory and field colonies of *Helicoverpa zea* (Boddie) and *Heliothis virescens* F. to Vip3A insecticidal protein was studied in diet incorporation and diet overlay assays from 2004 to 2008. Responses of field populations were compared to paired responses of University of Arkansas laboratory susceptible *H. zea* (LabZA) and *H. virescens* (LabVR) colonies. After 7 d of exposure, observations were made on number of dead larvae (*M*) and the number of larvae alive but remaining as first instars (L1). Regression estimates using *M* (LC_{50}) and *M* plus L1 (MIC_{50}) data were developed for laboratory and field populations. Susceptibility of laboratory and field populations exposed to Vip3A varied among different batches of protein used over the study period. Within the same batch of Vip3A protein, susceptibilities of laboratory colonies of both species (LabZA and LabVR) were similar. Field colonies were significantly more susceptible to Vip3A than the respective reference colonies of both species. Within field populations, susceptibility to Vip3A varied up to 75-fold in *H. zea* and 132-fold in *H. virescens* in LC_{50} estimates. Variabilities in MIC_{50}s were up to 59- and 11-fold for *H. zea* and *H. virescens*, respectively.

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1. Introduction

A number of different proteins derived from *Bacillus thuringiensis* (Bt) have insecticidal activity (Hofte and Whiteley, 1989). The vast majority of these are Cry proteins which are very effective against Lepidoptera and Coleoptera (Bravo et al., 2006). Several have been incorporated into transgenic cotton (Bt cotton) for control of tobacco budworm, *Heliothis virescens* F. and bollworm, *Helicoverpa zea* (Boddie). Bollgard® (Monsanto Company, St. Louis, MO) was the first commercial transgenic insect protected cotton. It expresses a single Bt toxin, Cry1Ac, and was introduced into US cotton in 1996. Second generation Bt cottons that express two insecticidal proteins (Cry1Ac and Cry2Ab2 in Bollgard®II, Monsanto Company; Cry1Ac and Cry1F in WideStrike® insect protected cotton, Dow AgroSciences and Cry1Ab and Vip3A in VipCot™ cotton, Syngenta Biotechnology) have been more recently commercialized. It is believed that Bt resistance development is lessened with these pyramided protein cottons due to their dual action and higher effective dose (Gould, 2003). A higher effective dose should theoretically reduce survival of heterozygous individuals carrying recessive genes for resistance.

VipCot cotton differs from Bt cottons in that one of the insecticidal proteins, Vip3A, is not a Cry protein. VipCot cotton also expresses Cry1Ab, a Cry toxin. Field trials have shown that VipCot cotton provides crop protection against heliothine species comparable to that observed with other cotton cultivars that express pyramided Cry toxins (Bollgard II and WideStrike) (Bradley et al., 2004; Burd et al., 2005; Adamczyk and Mahaffey, 2008; Bommireddy and Leonard, 2008). Vip3A is derived from *B. thuringiensis* in the vegetative and sporulation phase and has insecticidal activity against several Lepidoptera (Estruch et al., 1996; Liao et al., 2002; Lee et al., 2003). Cry1Ac, Cry1Ab and Cry2Ab2 proteins are derived during the sporulation phase only (Schnepf et al., 1998). Due to its unique mode of action, different receptor sites than Cry1Ac (Lee et al., 2003, 2006), Vip3A may be more effective against Cry tolerant or resistant pest populations.

Risks of resistance development in targeted pest populations have been a concern for the wide-scale use of Bt insecticidal toxins expressed in transgenic crops expressing Bt insecticidal toxin for more than a decade (Mellon and Rissler, 1998; US EPA, 1999), and routine monitoring of pest populations has been an EPA requirement for registration of commercial transgenic plants. Traditionally, monitoring observations were compared to baseline information generated from treated diet assays to detect shift in susceptibility of the pests to a Bt toxin. It is theoretically assumed that insect response to diet assays reflects activity/performance of the toxin in the field. Baseline studies are usually completed prior to wide-spread commercial deployment of Bt toxins to serve as a

* Corresponding author. Address: FDA-ORA, Arkansas Regional Laboratory, 3900 NCTR Road, Building 26, Jefferson, AR 72079, United States. Fax: +1 870 543 4041.
E-mail address: Ibrahim.Ali@fda.hhs.gov (M.I. Ali).
[1] Present address: USDA, ARS, Southern Insect Management Research Unit, 141 Experiment Station Road, Stoneville, MS 38776, United States.

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reference point for suspected resistance evolution (Liu and Tabashnik, 1997; Luttrell et al., 1999; Siegfried et al., 1995, 2005; Marcon et al., 1999; Trisyono and Chippendale, 2002). We previously developed baseline information on field populations of *H. zea* and *H. virescens* exposed to Cry1Ac (Luttrell et al., 1999; Ali et al., 2006) and Cry2Ab2 (Ali and Luttrell, 2007). This report summarizes baseline studies conducted with field and laboratory populations of *H. zea* and *H. virescens* exposed to Vip3A from 2004 to 2008.

2. Materials and methods

2.1. Colony information

Fifty-six *H. zea* and 19 *H. virescens* field populations were exposed to Vip3A insecticidal protein from 2004 to 2008. Colonies were established from collections made on different host plants from 33 counties of seven cotton-growing states in the US. Among the *H. zea* colonies, 24 were collected from different locations of Arkansas, six from Alabama, four from Georgia, four from Louisiana, four from Mississippi, eight from North Carolina and six from Texas. Of the *H. virescens* colonies, 17 were from Arkansas and two were from Texas. Number of moths per colony ranged from 7 to 234 with a mean of 95.07 ± 6.23 (Table 1). All populations were maintained on a semi-synthetic diet (Burton, 1969) in the Margaret McClendon Insect Rearing Facility, Department of Entomology, University of Arkansas and held until pupation in a walk-in temperature-controlled room at 26 °C, 70% RH and 14:10 (L:D) photoperiod. Progenies resulting from mass mating of 1st and 2nd generations of each colony in the laboratory were used for bioassays. Laboratory-susceptible colonies of *H. zea* (LabZA) and *H. virescens* (LabVR) at the University of Arkansas were used as reference experimental controls. These colonies have been maintained in laboratory culture for more than a decade (100+ generations) with no infusion of field insects and are considered to be extremely susceptible to Bt toxins and traditional insecticides.

2.2. Protein sources

The sources of Vip3A were four different batches of lyophilized proteins (Vip3Aa19) supplied by Syngenta Biotechnology Inc, Research Triangle Park, Raleigh, NC. All batches were produced through an *Escherichia coli* expression system and the Vip3A was purified by anion exchange chromatography prior to lyophilization. Purity was assessed/quantified by Sypro Orange-stained SDS–PAGE (Ryan Kurtz, Syngenta Biotechnology, Inc., Raleigh, NC, Personal communication). Vip3A materials were stored at −80 °C, but were allowed to warm to room temperature before weighing and use in assays.

2.3. Bioassays

Over the five years of study, multiple experiments were conducted with different batches of proteins, solvents and assay methods. Bioassay procedures evolved and were modified based on preliminary results. All concentrations used in developing regression estimates, mortality (*M*) or mortality plus stunting (ML1) were based on active ingredient.

2.4. Preliminary bioassays with H. zea and H. virescens laboratory and field colonies in 2004 and 2005

During 2004 and 2005, preliminary baseline studies were conducted with laboratory and field populations of *H. zea* and *H. virescens* exposed to Vip3A (Vip3A batches 0104 and 0204 with 63% active ingredient) in diet overlay assays. Initial diet overlay bioassays included a range of eight serial concentration of Vip3A dissolved in 200 mM ammonium carbonate buffer (pH 9.5) to determine reasonable concentration–mortality ranges for LabZA and LabVR. Subsequently, concentration–mortality responses were determined in diet overlay bioassays. Concentrations for *H. zea* were 0, 2, 5, 15, 50, 150, and 500 ng/cm^2 (a.i.) of Vip3A and those for *H. virescens* were 0, 5, 15, 50, 150, 500 and 1500 ng/cm^2. A standard pinto-bean based meridic diet (Burton, 1969) was used and treatments were applied as overlays to the diet surface (Siegfried et al., 2000). One milliliter of freshly prepared diet was poured into individual wells of the 128 well bioassay trays (C. D. International). Once the diet has solidified, 50 µl of protein solution in 200 mM ammonium carbonate buffer was pipetted onto the diet surface and allowed to air dry. Neonate *H. zea* or *H. virescens* were exposed to the toxic diet individually as described in Ali and Luttrell (2007) with the exception that treatments were applied as diet overlay rather than diet incorporation. Each concentration was replicated two to four times and each replicate had 16 neonates. Additional studies were required as we observed that diet overlaid with ammonium carbonate buffer was darker in color and that neonates fed these treatments, including the 0 concentration, grew slower compared to those on diet not overlaid with the buffer.

2.5. Baseline studies with LabZA and LabVR in 2006

Additional diet overlay and diet incorporation bioassays were conducted in 2006 to more accurately determine the concentration ranges using a new batch of Vip3A protein (batch B2-J4364/168-002 with 90% active ingredient). Based on the initial observations in 2004–2005, comparison was made between protein sources dispensed in ammonium carbonate buffer relative to that in distilled water. In the overlay assays, concentrations 0, 1, 3, 10, 30, 100 and 300 ng/cm^2 were used against *H. zea* and *H. virescens* in the initial tests. Subsequent tests were conducted with 0, 0.1, 1, 3, 10, 30 and 100 ng/cm^2. For diet incorporation assays, one ml of freshly prepared diet was thoroughly mixed with the appropriate amount of protein dissolved in ammonium carbonate buffer or dispensed in distilled water and poured in bioassay trays. The concentrations for both species were 0, 0.1, 0.3, 1, 3, 10, 30 and 100 µg/ml. Neonate *H. zea* or *H. virescens* were exposed to the toxin incorporated diet individually as described in 2004 and 2005 studies and in Ali and Luttrell (2007). Each concentration was replicated at least four times on different dates, and each replicate included 16 neonates.

2.6. Baseline susceptibility of field populations of H. zea and H. virescens exposed to Vip3A in 2006 to 2007

Diet incorporation assays using distilled water as a diluent were used to determine susceptibility of 28 *H. zea* and 17 *H. virescens* populations to Vip3A in 2006 and 2007. Concentrations used for both species were 0, 0.1, 0.3, 1, 3, 10, 30 and 100 µg/ml. Diet incorporation was used because historical data with Cry proteins (Luttrell et al., 1999; Ali et al., 2006; Ali and Luttrell, 2007) utilized the diet incorporation method and our laboratory was structured for efficient use of this assay procedure. Our experiments comparing diet overlay and diet incorporation should provide a mechanism to relate the data to future observations with both assay methods.

2.7. Monitoring susceptibility of H. zea field populations to Vip3A in 2008

Based on baseline information generated in 2006 and 2007, susceptibilities of *H. zea* field colonies to three preliminary diagnostic zconcentrations (10, 30 and 100 µg) of Vip3A (batch B1-J4534/53-

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