MONITORING HELICOVERPA ZEA SUSCEPTIBILITY TO BT TOXINS: RESULTS OF 2007 STUDIES

Ibrahim Ali
University of Arkansas – Fayetteville
Fayetteville, AR
Craig Abel
USDA-ARS
Stoneville, MS
J. R. Bradley
North Carolina State University
Raleigh, NC
Graham Head, Monsanto
St. Louis, MO
Ryan Jackson
USDA-ARS
SIMRU
Stoneville, MS
Ryan Kutz
Syngenta Biotechnology, Inc.
Raleigh, NC
B. R. Leonard
LSU AgCenter
Winnsboro, LA
Juan Lopez
Southern Plains Agricultural Research Center
College Station, TX
R. G. Luttrell
University of Arkansas
Fayetteville, AR
William J. Moar
Auburn University
Auburn, AL
Walt Mullins
Memphis, TN
John Ruberson
University of Georgia
Tifton, GA
Sakuntala Sivasupramaniam
Monsanto
Saint Louis, MO
N.P. Storer
Dow AgroSciences
Indianapolis, IN

Abstract

Bollworm, *Helicoverpa zea* (Boddie) field populations collected on an array of host plants from seven U.S. Cotton Belt states were exposed to Cry1Ac and Cry2Ab2 insecticidal toxins in diet incorporated assays and to Cry1F on diet overlay assays at the University of Arkansas in 2007. After 7 d of exposure, observations were made on number of dead larvae and the number of larvae alive but remaining as first instars. Paired assays were conducted with the University of Arkansas susceptible laboratory colony (LABZA) of *H. zea*. Response data at diagnostic concentrations recommended by industries were studied by descriptive statistics with results compared to historical benchmark information and paired results from the laboratory susceptible colony. Using 95% upper confidence limits, eight of the 34 *H. zea* field populations tested at the highest testing diagnostic concentration of Cry1Ac (250 µg/ml of diet) had mortality ratios (mortality of a field colony divided by the mortality of the LABZA) significantly lower than that of LABZA, however when mortality plus stunting responses were considered, all results were similar that to the LABZA. At the highest diagnostic concentration of Cry2Ab2 (150 µg/ml of diet), four of 31 field

populations had lower mortality and lower mortality plus stunting responses than LABZA, and all results were similar to that of LABZA when mortality plus stunting responses were considered. Among 22 field populations tested at the highest diagnostic concentration of Cry1F (4 µg/cm^2), 12 of 22 field populations had lower mortality ratios than the LABZA and 11 populations had mortality plus stunting response than that of LABZA. Across all three Bt toxins tested, measurable variability in mortality response was observed in field populations exposed to the suggested diagnostic concentrations. Variability was reduced when mortality plus stunting was used as the response variable. Observed response variability in field populations of *H. zea* to Cry1Ac, Cry2Ab2 or Cry1F did not appear to be related to host plants, geographic locations or seasonal time of collection.

Introduction

Transgenic cottons expressing diverse insecticidal proteins from *Bacillus thuringiensis* have been adopted worldwide (Brookes and Barfoot 2006) and generally provide excellent crop protection against targeted pests including bollworm, *Helicoverpa zea* (Boddie). However, Bollworm has been one of the more difficult pests to control with Bt crops and Bt cotton is often sprayed with insecticides as supplemental control measure. (Jackson et al. 2003). Increased acreages of Bt cottons expressing these insecticidal proteins (Cry1Ac in Bollgard®, Cry1Ac and Cry2Ab2 in Bollgard®II, Monsanto Company and Cry1Ac and Cry1F in WideStrike®, Dow AgroSciences) may pose increased risk of resistance development in *H. zea,* although the increased diversity of different proteins may lessen selection for resistance to Cry1Ac, the only Bt toxin in commercial cottons prior to 2005 and the one present in all commercial Bt cottons. Another Bt cotton cultivar expressing Vip3A (Syngenta Biotech), is expected to be commercialized in the near future. Resistance to Bt toxins is a major concern for researchers and regulatory agencies worldwide, and the US EPA requires mandatory monitoring of Bt susceptibilities in targeted cotton and corn pests in U.S. (EPA 2001, Matten and Reynolds 2003).

We previously established baseline susceptibilities for monitoring Cry1Ac and Cry2Ab2 resistance in heliothines (Luttrell et al. 1999, Ali et al. 2005, 2006, 2007, Ali and Luttrell 2007), and beginning in 2006 we monitored field populations of *H. zea* collected across the US Cotton Belt for resistance to Cry1Ac and Cry2Ab2. This work has been done largely under a cooperative agreement with the USDA, ARS Southern Field Crops Insect Management Unit at Stoneville, MS. In 2007, we began assaying field populations for response to three Bt insecticidal proteins, Cry1Ac, Cry2Ab2 and Cry1F. Preliminary results of 2007 Bt resistance monitoring studies are summarized in this report.

Methods and Materials

Insects

In 2007, susceptibilities of 42 field populations of *H. zea* to diagnostic doses of three Bt insecticidal proteins were determined. These populations were collected from 15 counties of seven states. Of these, 25 field populations were collected from different regions of Arkansas and 17 field populations were received from research collaborators located in Alabama (N = 3), Georgia (N = 2), Louisiana (N = 2), Mississippi (N = 1), North Carolina (N = 3), and Texas (N = 6). Colonies were established from field insects on a wide array of host plants including cotton, corn, sweet corn, soybean, grain sorghum, peanut, chickpea, tobacco, Bt-corn, cotton and various wild host plants (Table 1). A few colonies were established from female moths captured in light traps. All colonies 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 subsequent generations (1-2) of each colony were used for bioassays. A longtime laboratory-susceptible colony of *H. zea* (LABZA) at the University of Arkansas was used as reference experimental control in all assays.

Table 1. Number of *Helicoverpa zea* colonies collected from Cotton Belt states for Bt resistance monitoring during 2007.

Colony	Coll Date	Location	County	State	Crop
F0507	05/04/07	Monticelo	Drew	AR	Clover
F0607	05/04/07	Magnolia	Columbia	AR	Clover
F0807	05/04/07	Garland	Miller	AR	Clover
F0907	05/14/07	Foreman	Little River	AR	Light trap
F1007	06/01/07	College Station	Brazos	TX	Sweet corn
F1107	05/17/07	Leachville	Mississippi	AR	Geranium
F1207	05/31/07	Foreman	Little River	AR	Light trap
F1307	06/05/07	College Station	Brazos	TX	Sweet corn
F1407	06/14/07	College Station	Brazos	TX	Bt corn
F2407	06/26/07	College Station	Brazos	TX	Light trap
F2507	06/20/07	Foreman	Little River	AR	Corn
F2607	06/21/07	Pickens	Desha	AR	Corn
F2707	05/06/07	Foreman	Little River	AR	Clover
F2807	05/17/07	Leachville	Mississippi	AR	Geranium
F3307	07/05/07	Foreman	Little River	AR	Bt corn
F3407	06/20/07	Foreman	Little River	AR	Bt corn
F3607	07/13/07	Foreman	Little River	AR	Light trap
F3907	07/20/07	J Webber	Escambia	AL	Sweet corn
F4007	07/12/07	Winnsboro	Franklin	LA	Sweet corn
F4907	07/12/07	Winnsboro	Franklin	LA	Sweet corn
F5107	06/18/07	J Webber	Escambia	AL	Field corn
F5207	06/18/07	Pratville	Autauga	AL	Field corn
F5307	06/18/07	J Webber	Escambia	AL	Field corn
F5507	07/16/07	Foreman	Little River	AR	Sorghum
F5707	07/05/07	Jamesville	Martin	NC	Corn
F5807	07/05/07	Clayton	Johnston	NC	Corn
F6307	08/05/07	Roger	Martin	NC	Light trap
F7707	08/14/07	Foreman	Little River	AR	Peanut
F7907	08/04/07	Fayetteville	Washington	AR	Chick pea
F8107	08/04/07	Fayetteville	Washington	AR	Sorghum
F8207	08/21/07	Tillar	Drew	AR	Cotton
F8307	08/15/07	College Station	Brazos	TX	Light trap
F8507	08/26/07	Fayetteville	Washington	AR	Chick pea
F8707	08/04/07	Tillar	Drew	AR	Sorghum
F8907	08/20/07	Tillar	Drew	AR	Soybean
F9207	08/04/07	Fayetteville	Washington	AR	Chick pea
F10007	09/09/07	Fayetteville	Washington	AR	Bt corn
F10207	09/11/07	Col Station	Brazos	TX	Corn
F10807	09/13/07	Stoneville	Washington	MS	Corn
F11507	09/25/07	Foreman	Little River	AR	Light trap
F11707	August	Blairsville	Union	GA	Corn
F11807	06/10/07	Tifton	Tift	GA	Sweet corn
F12107	10/05/07	Foreman	Little River	AR	Light trap

Bt insecticidal proteins

The sources of Cry1Ac and Cry2Ab2 were lyophilized (freeze-dried) formulations of MVP II (Mycogen Corporation, San Diego, CA) containing ~20% Cry1Ac toxin of *Bacillus thuringiensis* variety *kurstaki*) and Bt-corn

leaf powder expressing 6 mg of Cry2Ab2 protein/g of powder, respectively. Both proteins were supplied by Monsanto Company, St. Louis, MO. Truncated Cry1F protein containing ~14% of Cry1F was received from Dow AgroSciences, Indianapolis, IN. All lyophilized materials were stored at -80°C until used. They were allowed to warm to room temperature before weighing and use in assays.

Bioassays

Progenies of field colonies (1st to 2nd generation in laboratory colonization) were exposed to two diagnostic concentrations of Cry1Ac (100 and 250 µg/ml) and Cry2Ab2 (100 and 150 µg/ml) in diet incorporation bioassays and susceptibility was measured as in the baseline studies of Ali et al. (2005, 2006a, 2006b) and Ali and Luttrell (2007). Neonate *H. zea* were individually exposed to Bt toxins incorporated into pinto bean diet and dispensed into 128 wells bioassay trays (C-D International). The susceptibility of field colonies of *H. zea* to two diagnostic concentrations of Cry1F (1 and 4 µg/cm^2) was measured in diet overlay assays (Siegfried et al. 2000). One ml of freshly prepared diet was poured into bioassay trays. Once the diet has cooled and dried, 50 µl of protein solution dissolved in 0.1% Triton-X100 was pipetted onto the diet surface of a single well in the tray and allowed to dry. For each protein, a set of bioassay trays were prepared per week (batch). Along with assays of field populations, paired assays were run with the designated laboratory susceptible (LABZA) colony as an experiment control of variable assay conditions. For each dose there were 16 larvae, and assays were typically replicated four times. For all assays, untreated controls, distilled water for Cry1Ac, lyophilized non Bt corn leaf powder for Cry2Ab2 and 0.1% Triton X-100 for Cry1F were included. For each control (0 concentration), 16 larvae were used per replicate. Controls were replicated two to four times depending upon the availability of neonates.

Data and statistics

Larval mortality and mortality plus those that failed to molt to second instars were recorded after 7 d of exposure to the treated diet. Percent mortalities for *H. zea* at concentrations of 0, 100 and 250 µg/ml of diet for Cry1Ac; and 0, 100 and 150 µg/ml of diet for Cry2Ab2; and 0, 1 and 4 µg/cm^2 of diet for Cry1F were measured. Corrected responses for diagnostic concentrations were computed using Abbott's (1925) formula and the paired (batch) response of LABZA. Response data at the diagnostic concentrations for each assay batch were studied by descriptive statistics with results compared to paired results from the laboratory susceptible colony. Colonies with response ratios less than LABZA (i.e., 95% upper confidence limit did not include 1.0 or corresponding response of LABZA) were noted as potentially less susceptible colonies.

Results

Susceptibility of *H. zea* to Cry1Ac

H. zea neonates from 34 field populations were exposed to diagnostic concentrations of Cry1Ac. Mortality of laboratory susceptible *H. zea* (LABZA) ranged from 48.4 to 100% with a mean (± SE) of 76.9 (± 6.2)% at the 100 µg concentration of Cry1Ac and 53.2 to 100% with a mean of 76.9 (± 6.2)% at the 250 µg concentration of Cry1Ac among 14 test batches. Mean mortality plus stunting of LABZA was 100% in all tests (Fig. 1).

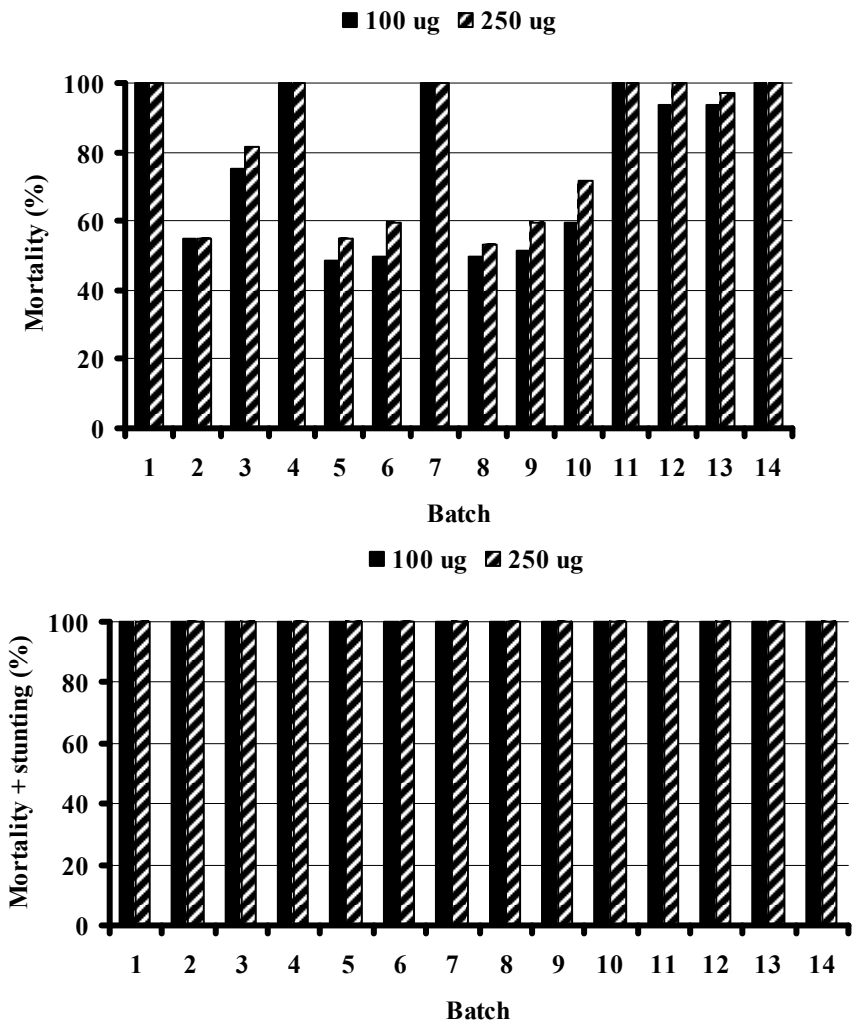

Figure 1. Percent corrected mortality (top) and mortality plus stunting (bottom) of laboratory susceptible *H. zea* (LABZA) larvae exposed to diagnostic concentrations (µg/ml) of Cry1Ac.

Mortalities of field populations ranged from 19.4 to 98.4% (mean ± SEM of 47.9 ± 4.0%) and 29.0 to 98.4% (mean ± SEM of 63.7 ± 3.1%) in 100 and 250 µg/ml concentrations of Cry1Ac, respectively. Mortalities plus stunting of the field populations ranged from 48.4 to 100% (mean ± SEM of 96.4 ± 1.4%) and 97.7 to 100% (mean ± SEM of 99.9 ± 0.1%), respectively at 100 and 250 µg/ml concentrations.

Ratios of mortality (mortality of a field population divided by mortality of LABZA) and mortality plus stunting (mortality plus stunting of a field population divided by mortality plus stunting of LABZA) for field populations at the 100 µg/ml concentration ranged from 0.2 to 1.4 and 0.5 to 1.0, respectively (Fig. 2). Those for the 250 µg/ml concentration ranged from 0.4 to 1.6 and 0.9 to 1.0, respectively (Fig. 3). At the highest tested diagnostic concentration, eight field populations had lower ratios of mortality than LABZA. One had a lower ratio of mortality plus stunting than LABZA.

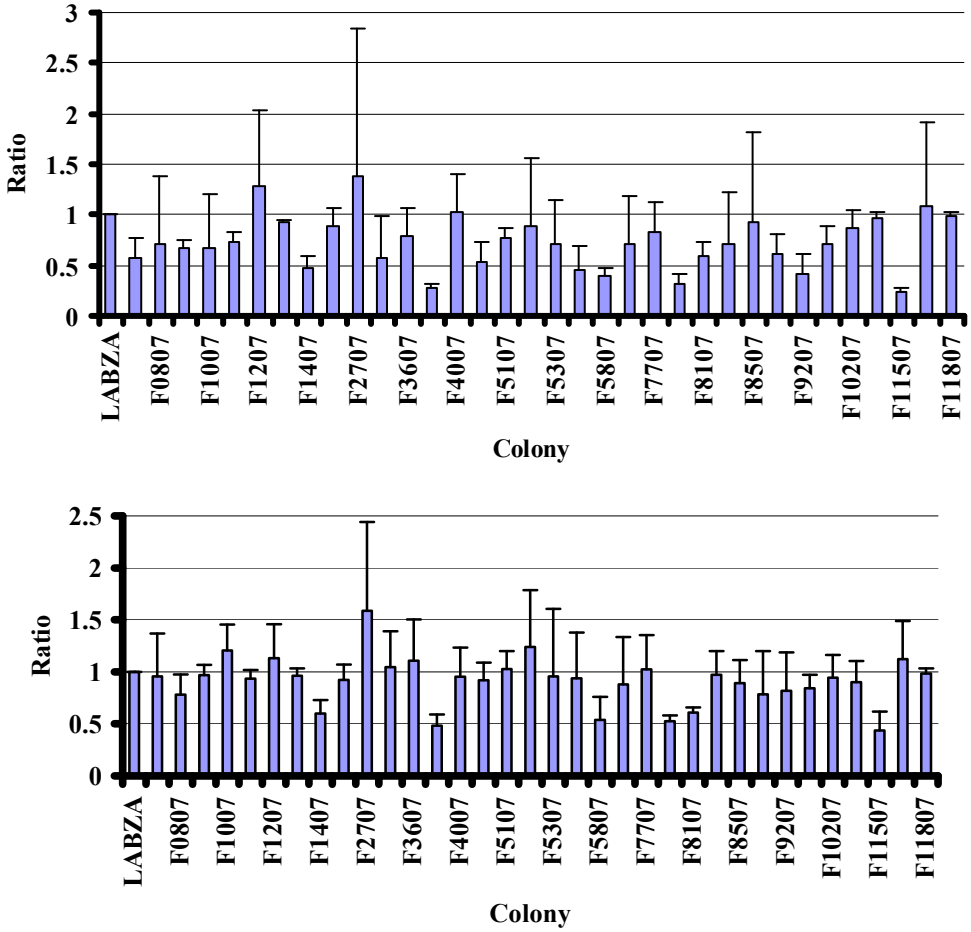

Figure 2. Ratios (mortality or mortality plus stunting response of a field colony divided by same response of laboratory colony- LABZA) (95% upper confidence limits) of mortality (top) and mortality plus stunting (bottom) of *H. zea* field colonies exposed to 100 µg/ml of Cry1Ac in diet incorporated assays in 2007.

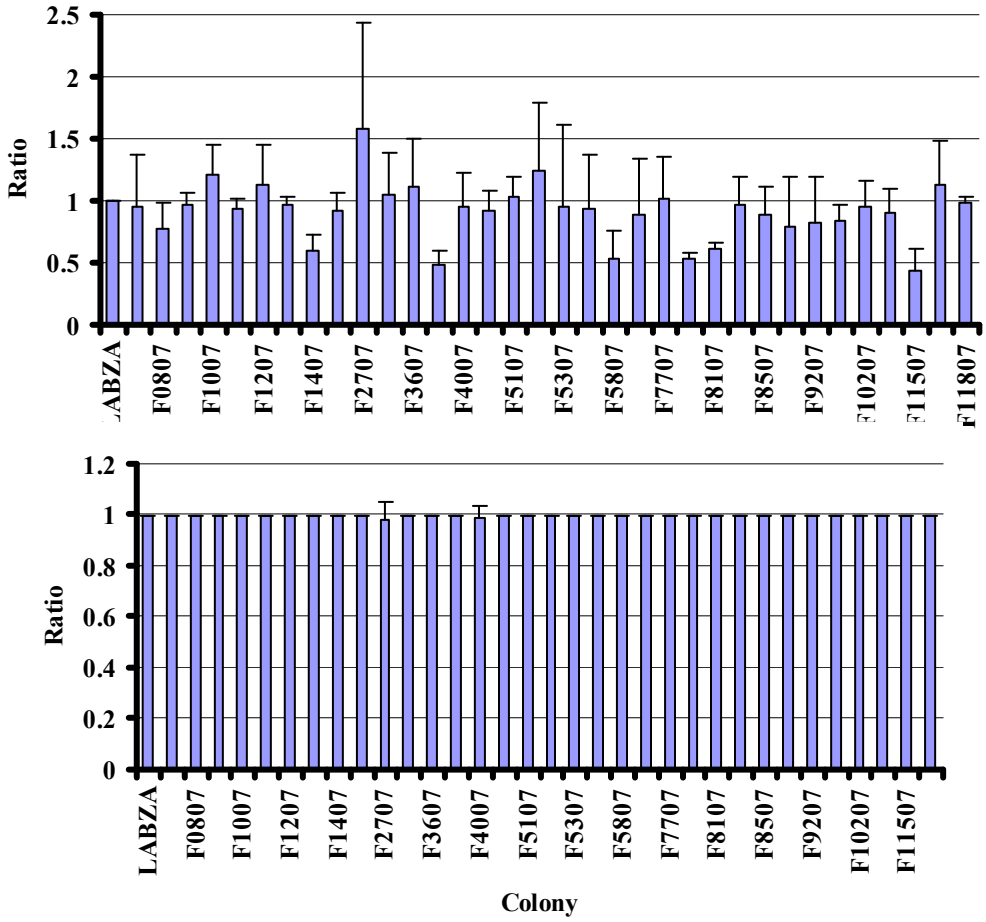

Figure 3. Ratios (mortality or mortality plus stunting response of a field colony divided by same response of laboratory colony- LABZA) (95% upper confidence limits) of mortality (top) and mortality plus stunting (bottom) of *H. zea* field colonies exposed to 250 µg/ml of Cry1Ac in diet incorporated assays in 2007.

Susceptibility of *H. zea* to Cry2Ab2

Neonates from 31 field populations were exposed to two diagnostic concentrations of Cry2Ab2 (100 and 150 µg/ml) and an untreated control. Mean mortalities of LABZA exposed to 100 µg of Cry2Ab2 ranged from 80.7 to 100% with a mean (± SEM) mortality of 95.1 (± 2.9)% among seven tests. At 150 µg Cry2Ab/ml of diet, mean (± SEM) mortality of LABZA was 97.1 (± 1.7)%. Mean (± SEM) mortality plus stunting of the laboratory colony was 97.6 (± 1.8)% and 99.9 (± 0.7)% at 100 and 150 µg of Cry2Ab2, respectively (Fig. 4).

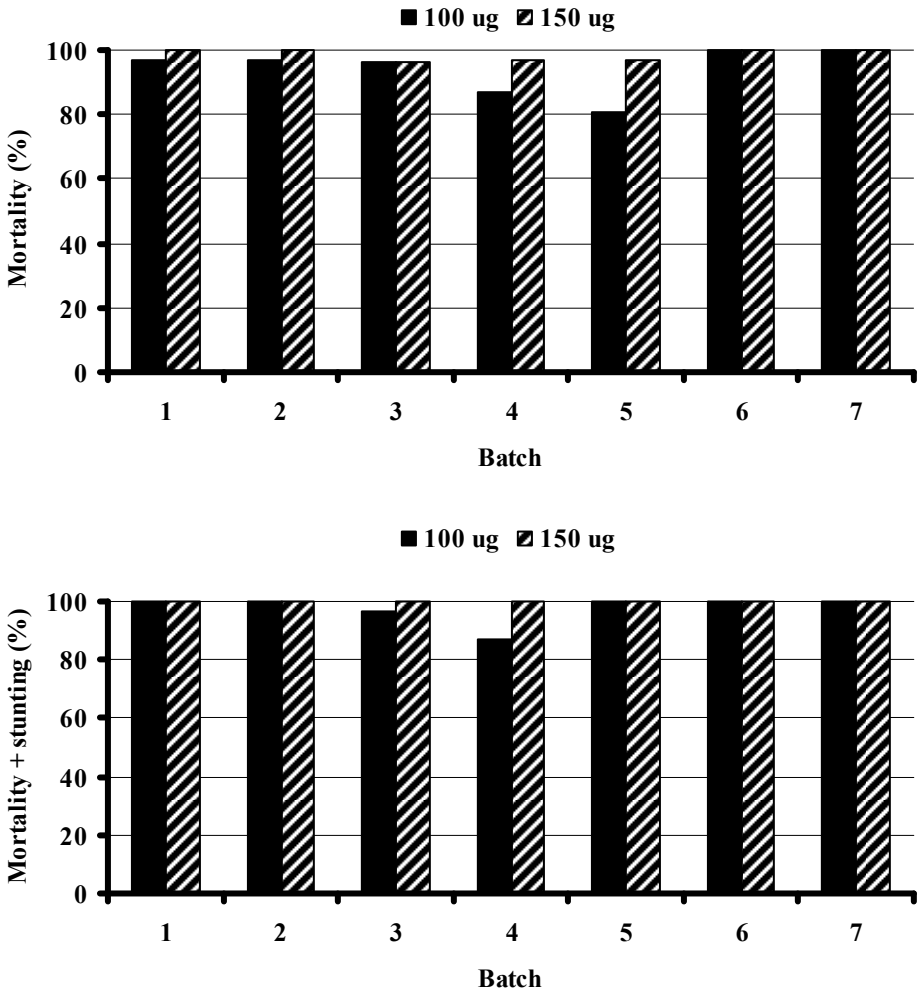

Figure 4. Percent corrected mortality (top) and mortality plus stunting (bottom) of laboratory susceptible *H. zea* (LABZA) larvae exposed to diagnostic concentrations (µg/ml) of Cry2Ab2.

Mean (± SEM) mortalities of field populations at 100 and 150 µg were 76.1 (± 3.7)% (range of 27.9 to 100%) and 82.8 (± 3.1)% (range of 41.2 to 100%), respectively. The ranges of mortality plus stunting for field populations were 51.5 to 100% with a mean (± SEM) of 88.1 (± 2.4)% and 73.3 to 100% with a mean (± SEM) of 94.4 (± 1.7)% for the 100 and 150 µg of Cry2Ab2/ml of diet concentration, respectively.

Ratios for mortality and mortality plus stunting for field populations at 100 µg ranged from 0.3 to 1.3 and 0.7 to 1.2, respectively (Fig. 6). At 150 µg, those ranges were 0.4 to 1.2 and 0.8 to 1.0, respectively (Fig. 5). At concentrations of 100 and 150 µg, respectively, 7 and 4 field populations had lower mortality ratios than the LABZA. Only two field populations had lower mortality plus stunting ratios than that for LABZA at 100 µg. At 150 µg, mortality and mortality plus stunting ratios of all field populations were similar to that for LABZA (Fig.7).

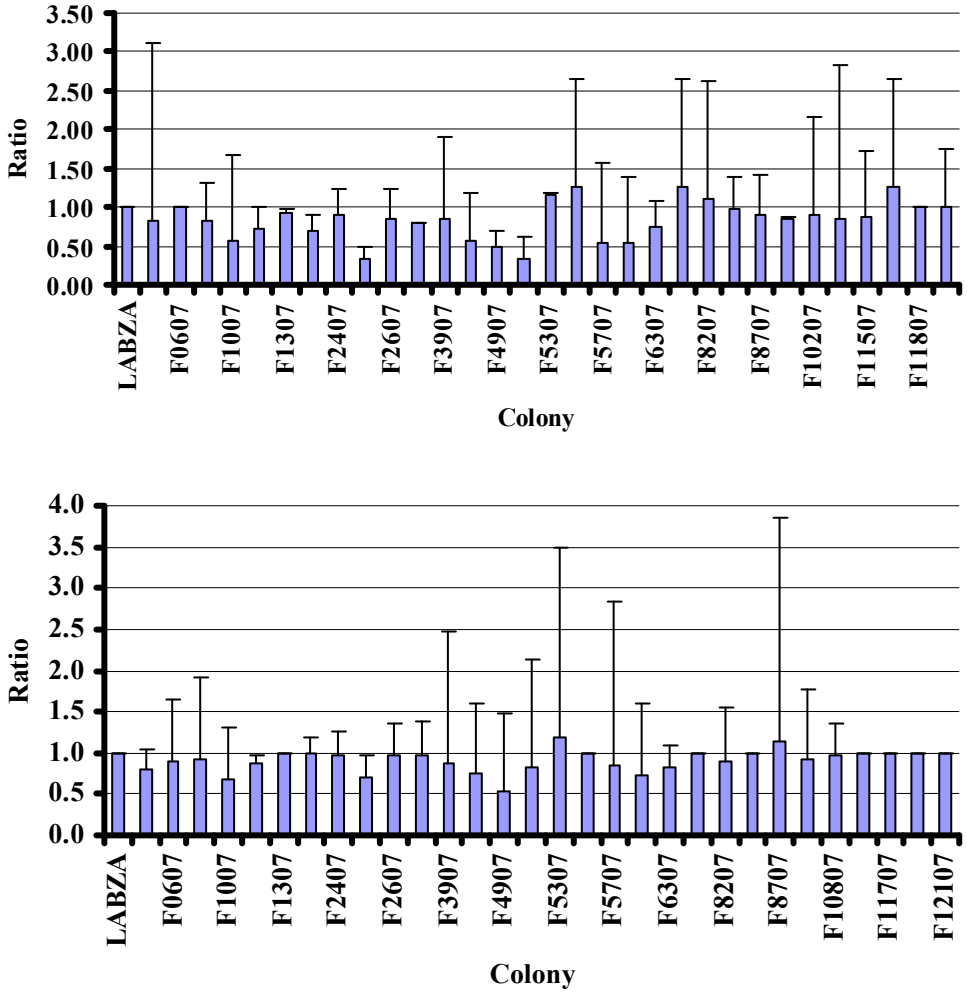

Figure 5. Ratios (mortality or mortality plus stunting response of a field colony divided by same response of laboratory colony- LABZA) (95% upper confidence limits) of mortality (top) and mortality plus stunting (bottom) of *H. zea* field colonies exposed to 100 µg/ml of Cry2Ab2 in diet incorporated assays in 2007.

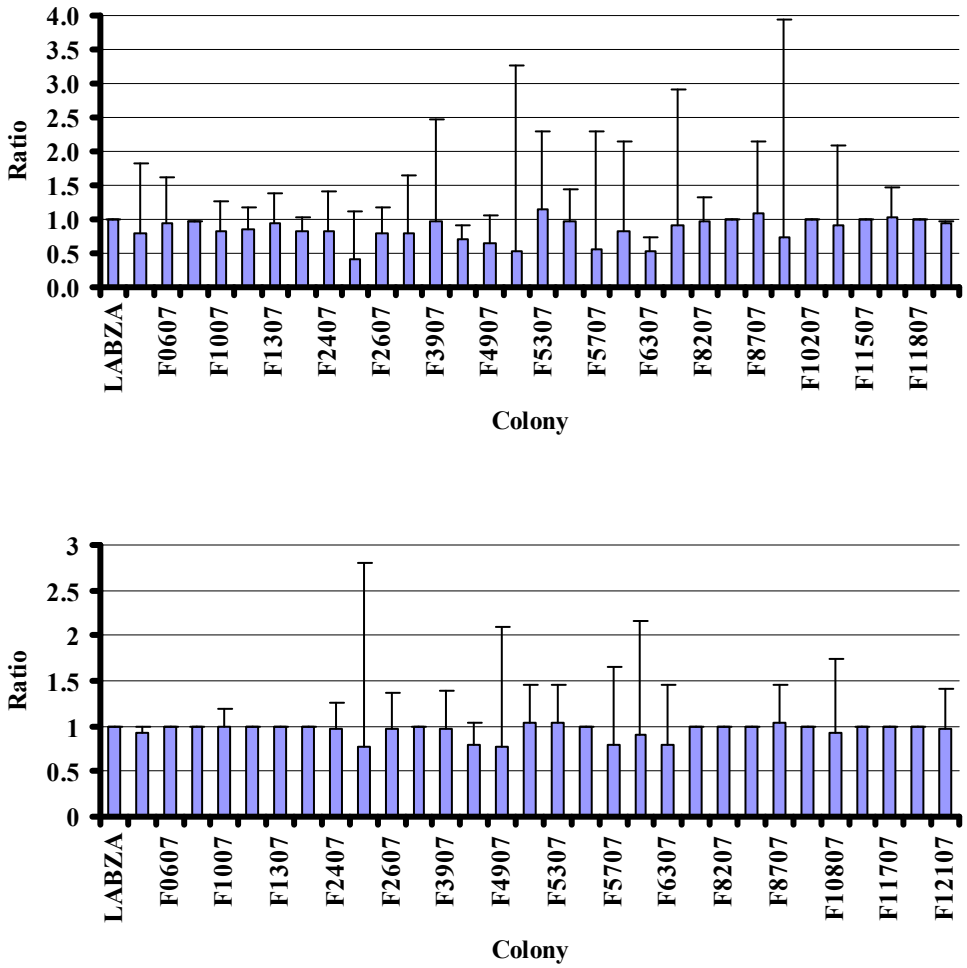

Figure 6. Ratios (mortality or mortality plus stunting response of a field colony divided by same response of laboratory colony- LABZA) (95% upper confidence limits) of mortality (top) and mortality plus stunting (bottom) of *H. zea* field colonies exposed to 150 µg/ml of Cry2Ab2 in diet incorporated assays in 2007.

Susceptibility of *H. zea* to Cry1F

Neonates from 22 field populations were exposed to two diagnostic concentrations (1 and 4 µg/cm^2) of Cry1F. Mortalities of LABZA exposed to 1 µg/cm^2 of Cry1F ranged from 36.7 to 82.7%. Mean (± SEM) mortality across six tests (batches) of LABZA was 54.4 (± 7.4)%. At the highest tested concentration of Cry1F (4 µg/cm^2), mortality of LABZA ranged from 65.6 to 91.4% with a mean of 80.7 (± 3.7)%. Mortality plus stunting of the laboratory colony ranged from 43.3 to 83.3% with a mean of 66.1 (± 6.6)% for the 1 µg/cm^2 concentrations, and 90.0 to 98.2% with a mean of 93.5 (± 1.2)% at the 4 µg/cm^2 concentration (Fig. 7).

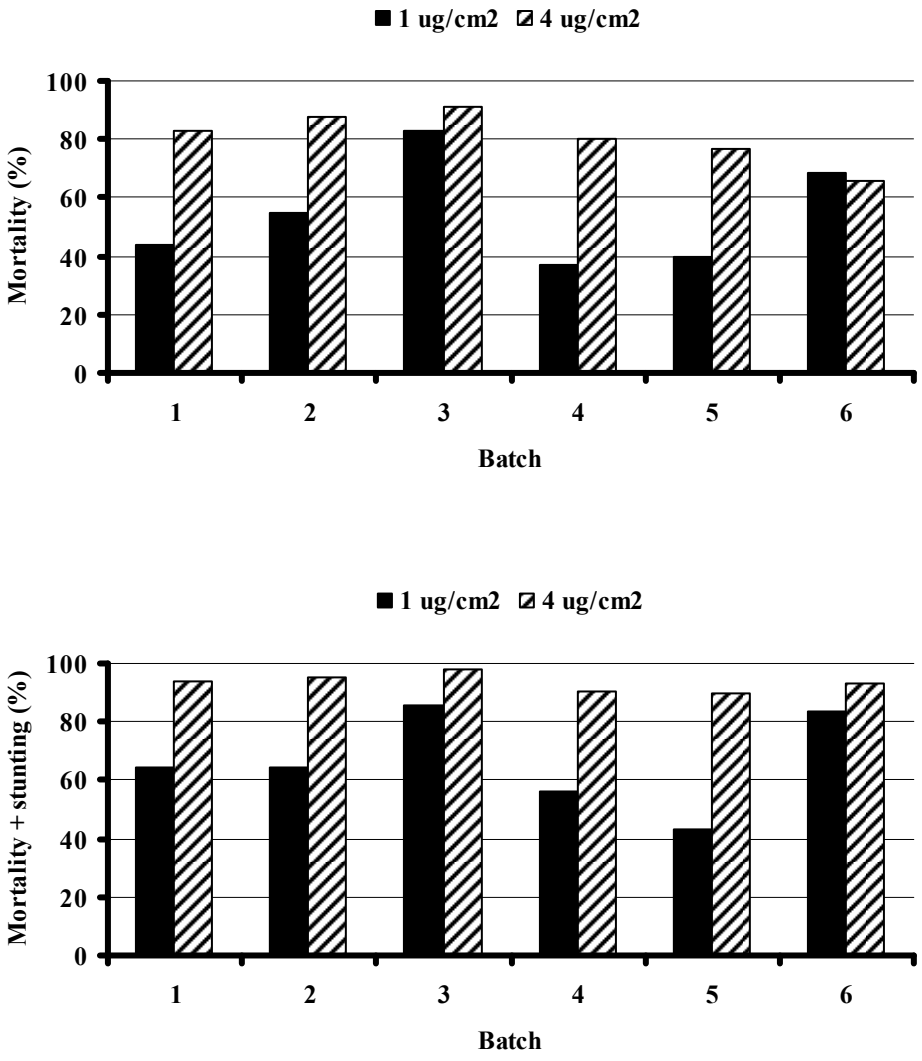

Figure 7. Percent corrected mortality (top) and mortality plus stunting (bottom) of laboratory susceptible *H. zea* (LABZA) larvae exposed to diagnostic concentrations (µg/ml) of Cry1F.

For field populations, mean mortalities were 31.2 (± 3.2)% with a range from 12.8 to 70.4% at the 1 µg/cm^2 concentration and 60.2 (± 3.0)% with range from 42.2 to 93.5% at the 4 µg/cm^2 concentration. Mortality plus stunting responses of these populations at lower (1 µg/cm^2) and higher (4 µg/cm^2) concentrations of Cry1F ranged from 12.8 to 85.1% with a mean of 45.1 (± 4.7)% for the lower concentration and 46.9 to 100% with a mean of 77.5 (± 3.8)% for the higher concentration, respectively.

Mean mortality and mortality plus stunting ratios at 1 µg/cm^2 ranged from 0.3 to 1.8 and 0.2 to 1.4, respectively (Fig. 8). At the 4 µg/cm^2 concentration, these ratios ranged from 0.5 to 1.2 for mortality and 0.5 to 1.1 for mortality plus stunting (Fig. 9).

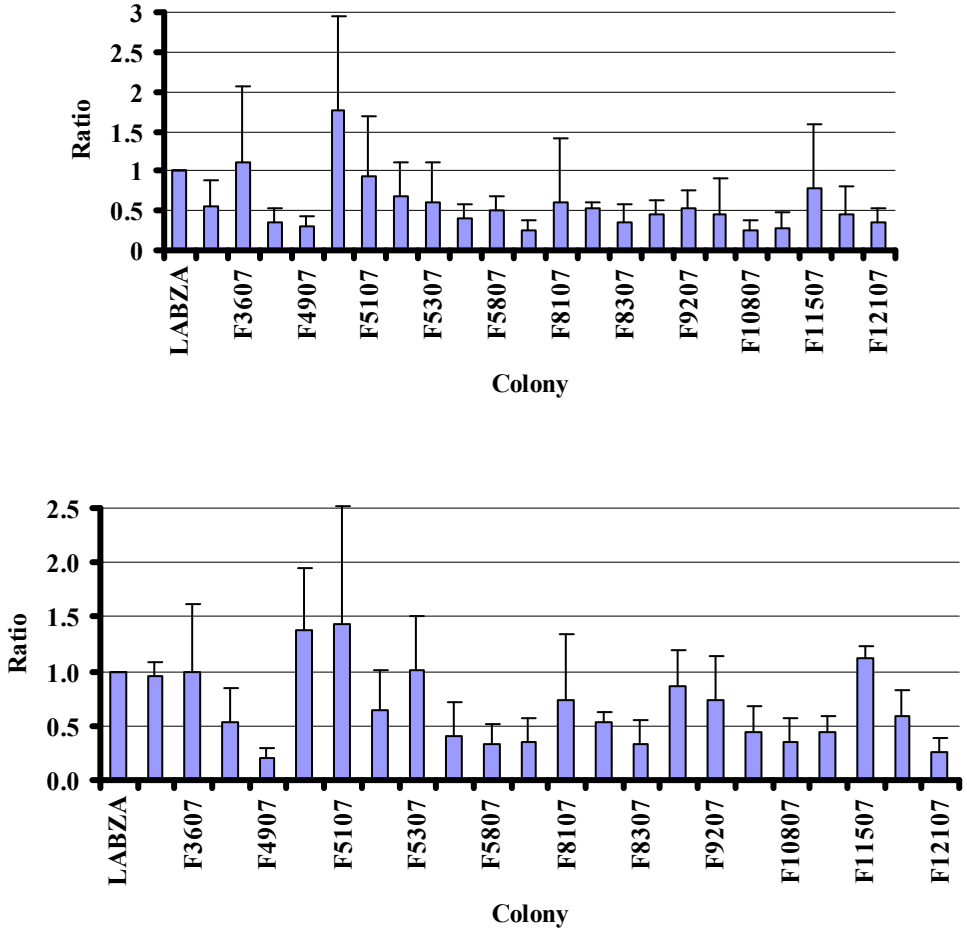

Figure 8. Ratios (mortality or mortality plus stunting response of a field colony divided by same response of laboratory colony- LABZA) (95% upper confidence limits) of mortality (top) and mortality plus stunting (bottom) of *H. zea* field colonies exposed to 1µg/cm^2 of Cry1F in diet overlay assays in 2007.

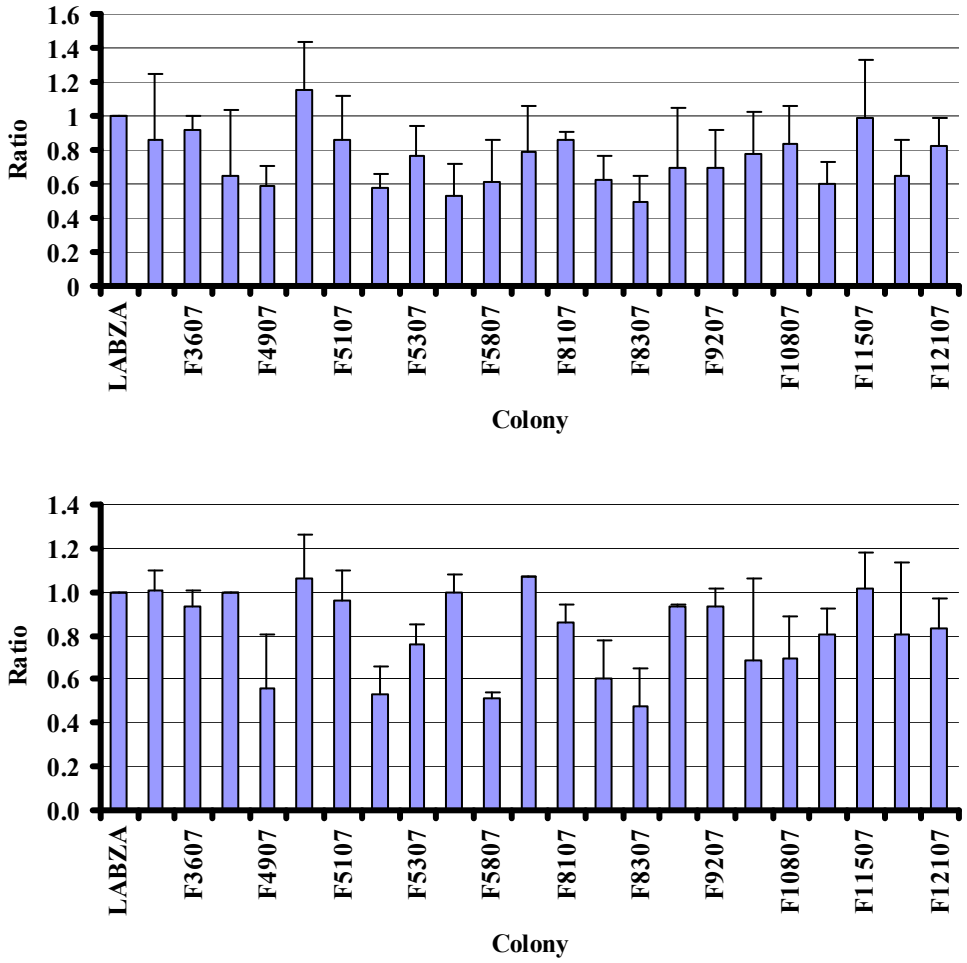

Figure 9. Ratios (mortality or mortality plus stunting response of a field colony divided by same response of laboratory colony- LABZA) (95% upper confidence limits) of mortality (top) and mortality plus stunting (bottom) of *H. zea* field colonies exposed to 4 µg/cm^2 of Cry1F in diet overlay assays in 2007.

At highest tested diagnostic concentration (4 µg/cm^2), 12 of 22 field populations had lower mortality ratios than the LABZA and 11 populations had mortality plus stunting response than that of LABZA.

Discussions

Variability in mortality of laboratory susceptible *H. zea* exposed to Bt toxins was observed. This variability was up to 2-fold at both diagnostic concentrations (100 and 250 µg/ml) of Cry1Ac and about 2-fold across two diagnostic concentrations of Cry1F. Similarly, mean mortality plus stunting response of the laboratory susceptible colony varied up to 2-fold at the lower concentration of Cry1F. Mean mortality plus stunting responses of the laboratory colony exposed to Cry1Ac and Cry2Ab2 were similar. In other experiments (Ali and Luttrell, unpublished), we have observed that using the stunting criteria as a response results in increased dose-response sensitivity and generally extends the range of effective assay doses more than 2-fold. Sims et al (1996) reported similar results.

Considerable variability in concentration-mortality response was observed in field populations exposed to both diagnostic concentrations of all three Bt insecticidal proteins. At highest tested concentrations, these variabilities

were about 4-fold in Cry1Ac, 3-fold in Cry2Ab2 and 2-fold in Cry1F. Less variability was observed in mortality plus stunting responses of field populations. Responses of field populations exposed to Cry1Ac and Cry2Ab2 were similar to LABZA; however those exposed to Cry1F varied 2-fold.

Results of 2007 studies again confirmed that the University of Arkansas laboratory colony of *H. zea* (LABZA) is a very susceptible laboratory strain. Previously, Ali et al. (2006) reported that LABZA is 1.2- and 9-fold more susceptible to Cry1Ac than other laboratory colonies of *H. zea* at the North Carolina State University and Monsanto Company, respectively. Ali and Luttrell (2007) also reported that LABZA is 6-fold more susceptible to Cry2Ab2 than a Monsanto Company laboratory colony of *H. zea.* Based on a comparison of 95% confidence intervals with those of LABZA, 53 of the 57 field colonies assayed in 2002-2004 would have been categorized as being less susceptible to Cry1Ac. In comparison to LABZA, observed reduced susceptibility of field populations to Bt toxins was more evident in mortality than in mortality plus stunting response. As per Ali et al. 2006, benchmark mortality of *H. zea* field populations collected on non-Bt crops during 2002 to 2004 at the 250 µg of Cry1Ac ranged from 78.6 to 79.4% with a mean of 80.2%. When results of current studies were compared to the results as in Ali et al. 2006a, 25 field populations had lower mortality than the benchmark for field populations. Similarly, as in Ali and Luttrell (2007), predicted benchmark mortality of *H. zea* field populations collected on non-Bt crops at 150 µg of Cry2Ab2 ranged from 76.4 to 78.3% with a mean of 77.4%. Seven of 31 field populations of current studies had lower mortality than the benchmark for field populations. No benchmark information for mortality of field populations of *H. zea* exposed to Cry1F was available. Mortality plus stunting responses of field populations of *H. zea* exposed to highest diagnostic concentrations of Cry1Ac and Cry2Ab2 were similar to LABZA. However, at the highest diagnostic concentration of Cry1F, 50% field populations had lower mortality ratios than the LABZA. When compared with the Dow AgoSciences provided benchmark information for *H. zea* (mean mortality plus stunting of 77.0% with a range of 62.0 to 96.0%) (Nick Storer, Dow AgroSciences, personal communication), 10 of 22 field populations (45.5%) had lower mortality and stunting responses than that the mean mortality plus stunting response of DowLabZA.

Based on comparisons of LC_{50}s, the response of field colonies to Cry1Ac as in Ali et al. 2006a and Cry2Ab2 as in Ali and Luttrell, 2007 was highly variable. Thus, the range of variabilities measured in these 2007 studies are well within the range of the variable responses measured by concentration-mortality regressions for Cry1Ac in 2002-2004 and Cry2Ab2 in 2002-2005.

Variability in concentration-mortality or concentration-mortality plus stunting responses observed among field populations in 2007 did not appear to be affected by geographic location of collection, collection time and host plants. In our earlier studies, we reported that *H. zea* colonies collected on Bollgard II cotton late in the season had elevated LC_{50}s (Ali et al. 2006b and Ali and Luttrell 2007, Ali et al. 2007). In 2007 studies, no *H. zea* colony was established from commercial Bt cottons.

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