DIET OVERLAY BIOASSAYS WITH CRY2AB REDUCE EXPERIMENT-WISE VARIATION AND ENHANCE RESISTANCE DISCRIMINATION IN *HELICOVERPA ZEA*, RELATIVE TO DIET INCORPORATION BIOASSAYS: CONCLUSIONS FROM MONITORING OF RESISTANCE IN THE US IN 2008

Konasale J. Anilkumar
Nancy Adams
Thomas L. Clark
Timothy J. Dennehy
Graham P. Head
Paula A. Price
Monsanto Company
Chesterfield, MO

Abstract

Bioassays were conducted on 26 field strains of *Helicoverpa zea*, collected in 2008 to uphold EPA registration requirements. Contrasts of diet overlay bioassays with diet-incorporation bioassays highlighted shortcomings of this latter method for *H. zea.* The goal of diagnostic-concentration bioassays used for resistance monitoring is to detect pests with susceptibility that is significantly below that of the distribution of baseline responses existing prior to field use of an insecticide. Ideally, the bioassay method, duration of exposure, and mortality criterion used will consistently cause susceptible pests to be effected (i.e., death and/or developmental impairment) by the insecticide of interest, while having little or no visible effect on resistant individuals. It is difficult to meet this goal with bioassays of pests like *Helicoverpa zea* that are not highly susceptible to the Cry toxins currently produced by *Bt* cotton products. Bioassays in which *Bt* toxins are thoroughly incorporated into insect diet have frequently yielded highly variable numbers of survivors of *H. zea* of high concentrations of Cry1Ac and Cry2Ab, even with susceptible laboratory colonies. To overcome these limitations of diet incorporation bioassays, researchers in Australia, China, Mexico and India have resorted to a diet-overlay assay method that dispenses toxin over the top of insect diet. Interpretation of diet-incorporation bioassays in the US has been further complicated by use of dissimilar mortality criteria. This paper demonstrates the advantages for monitoring *H. zea* resistance to Cry2Ab using diet-overlay bioassays, combined with scoring larvae as dead if they do not develop to the third instar. Results demonstrate the absence of major-gene resistance to Cy2Ab in the strains evaluated.

Introduction

Bollworm, *Helicoverpa zea* (Boddie) is one of the target pests of *Bt* cotton in the US. This pest is naturally more tolerant (10 to 40-fold) and highly variable (>100-fold) in its response to both Cry1Ac and Cry2Ab proteins expressed in *Bt* cotton than other target pests (Ali et al. 2006, Ali and Luttrell, 2007, Sivasupramaniam et al. 2008). The presence of colonies of differing mean susceptibility does not necessarily demonstrate resistance evolution or development of field-evolved resistance (Moar et al. 2008, Tabashnik et al. 2008). It remains to be demonstrated that the colony of interest is significantly outside of the natural range (i.e., distribution) of baseline susceptibility of the pest. Moreover, any claim of field-relevant resistance must include evidence of increased larval survival on plants in the field (Moar et al. 2008). Lastly, gene frequencies in the field can change unpredictably and must be measured over the course of multiple seasons. For example, relatively high resistance allele frequency was reported in *Pectinophora gossypiella* (Saunders) in Arizona in 1997 (Patin et al. 1999) but it declined subsequently (Tabashnik et al. 2005) and has not ever been reported to impact field performance of *Bt* cotton against this species.

Resistance to *Bt* is a major concern for durability of *Bt* crops. Therefore, regulatory agencies such as the US EPA require mandatory monitoring of *Bt* susceptibilities in targeted pests, such as described in this paper (EPA 2001, Matten and Reynolds 2003). These studies are conducted annually by collecting insect populations from different geographical regions, and testing them with diagnostic concentrations. Diagnostic concentrations are intended to detect unusual survivorship, i.e. reductions in survivorship exceeding those found in baseline responses. Therefore, diagnostic concentrations should be selected carefully. Additionally, assay method, duration of exposure, and mortality criterion are equally important, and depend on both the insect and *Bt* protein of interest.

Diet incorporation and diet overlay bioassays have been used by monitoring laboratories in the US and elsewhere (e.g., Ali and Luttrell 2009). Both of these methods produce accurate estimates of mean lethal concentrations resulting in 50% mortality (LC_{50}). However, with pests of low to moderate susceptibility, the diet incorporation

method consistently has problems with reliable discrimination of resistance. This problem is particularly noticeable with monitoring of *H. zea* resistance to Cry2Ab. Because of technical problems with Cry2Ab produced in microbial expression system (Sivasupramaniam et al. 2007), Cry2Ab is produced in experimental lines of *Bt* corn plants. However, Cry2Ab is typically expressed at levels below 10 mg/g of corn leaf tissue. With this concentration of toxin, it is difficult to achieve diet-incorporation bioassay concentrations of ≥ 150 µg/ml of diet (Anilkumar et al. 2008).

The criterion used to record *H. zea* responses to *Bt* toxins vary widely in the literature. Most of lepidopteran-active *Bt* toxins require at least 4-5 days to kill even highly susceptible insects. With insects of low-to-moderate susceptibility, such as *H. zea*, an assay period of 7 days frequently will not kill even highly susceptible insects. Yet, it is frequently not practical or technically feasible to increase mortality by extending assay duration beyond 7 days. Instead, researchers have used a variety of response criteria other than mortality, *sensu stricta*. These have included: 1) larvae weighing less than 10 mg (Siegfried et al. 2000), 2) larvae failing to molt to second-instar (Ali et al. 2006, Ali and Luttrell 2007, Anilkumar et al. 2008, Sivasupramaniam et al. 2008) and 3) larvae failing to reach third-instar (Bird and Akhurst 2007, Blanco et al. 2008). From these have come a confusing and sometimes contradictory collection of terms for median lethal concentration (LC) values, including: lethal concentrations (LC), molt inhibitory concentrations (MIC), and inhibitory concentrations (IC).

In this paper results of two bioassay methods to detect major-gene resistance to Cry2Ab in 26 US collections of *H. zea* supported by Monsanto in 2008 were contrasted. Diet incorporation assays were conducted at the University of Arkansas (Ali and Luttrell 2009). Diet overlay assays were conducted at the Monsanto research facilities in Missouri. Contrasts with diet-incorporation bioassays highlighted shortcomings of this latter method for *H. zea.* Different mortality criteria were evaluated with each method. Results showed that 7 day diet overlay assays, in which survivorship is scored as the ability to develop to at least the third instar, offer clear advantages over diet incorporation tests of pests such as *H. zea,* with low to moderate susceptibility to the toxin of interest. Diet-overlay assays yielded no evidence of major-gene resistance to Cry2Ab in the 26 field strains of *H. zea* evaluated.

Materials and Methods

Insects

In 2008, 26 *H. zea* field populations, collected from different hosts, were sent to Monsanto Company's insect rearing facility at Union City, TN. These populations were collected from over 20 counties of seven states during April to September, 2008. They comprised collections from the following states: Alabama (N = 3), Arkansas (N = 5), Georgia (N = 3), Louisiana (N = 3), Mississippi (N = 5), North Carolina (N = 4), and Texas (N = 3). Most collections were made from field corn and sweet corn (N = 21). Three populations were collected from clover, one from soybean and one was from grain sorghum (Table 1). Neonates of each population, typically from F1 to F5 generations, were used for bioassays. Eggs collected from oviposition cages in the laboratory on different days were tested in different replications. Monsanto Company's laboratory strain of *H. zea* (MonZa) was used as an internal reference in all assays (Moar et al. 2008). This population was established in 2004 and reared with ≥ 1,000 individuals per generation. To limit problems with inbreeding depression, Cry2Ab-susceptible individuals from field collections were added to this strain during fall 2007.

Table 1. Details of *Helicoverpa zea* colonies collected from Cotton Belt states for *Bt* resistance monitoring during 2008.

Colony	Date of Collected	Place of Collection	County	State	Host Plant
S-1	4/29/2008	Foreman	Little River	AR	Clover
S-2	5/16/2008	College Station	Brazos	TX	Sweet corn
S-3	5/02/2008	Pine Bluff	Jefferson	AR	Clover
S-4	5/15/2008	Possum Grope	Jackson	AR	Clover
S-5	5/30/2008	College Station	Brazos	TX	Field corn
S-6	6/18/2008	College Station	Brazos	TX	Corn
S-7	6/13/2008	Moultrie	Colquitt	GA	Corn
S-8	6/11/2008	Tifton	Tift	GA	Corn
S-11	7/2/2008	Plains	Sumter	GA	Corn
S-12	6/20/2008	Prattville	Autauga	AL	Sweet corn
S-13	6/22/2008	Webber Farm	Atmore	AL	Field corn
S-14	7/2/2008	Webber Farm	Atmore	AL	Field corn
S-15	7/11/2008	Jamesville	Martin	NC	Sweet corn
S-16	7/18/2008	Verona	Lee	MS	Field corn
S-17	7/21/2008	Monroe	Richland	LA	Soybean
S-18	7/22/2008	Ecru	Pontotoc	MS	Field corn
S-19	7/15/2008	Roper	Washington	NC	Corn
S-20	7/18/2008	Knightdale	Wake	NC	Corn
S-21	7/22/2008	Edenton	Chowan	NC	Corn
S-22	7/25/2008	Hamilton	Monroe	MS	Field corn
S-23	7/28/2008	Winnsboro	Franklin	LA	Sweet corn
S-25	7/3/2008	Stoneville	Washington	MS	Field corn
S-26	7/30/2008	Dumas	Desha	AR	Grain sorghum
S-27	8/21/2008	Fayetteville	Washington	AR	Corn
S-28	7/18/2008	Clarksdale	Coahoma	MS	Field Corn
S-30	9/18/2008	Winnsboro	Franklin	LA	Field Corn

_Bt_ protein

The Cry2Ab toxin evaluated in bioassays was produced and tested by Monsanto. It was in the form of lyophilized (freeze-dried) *Bt*-corn leaf powder expressing ~6 mg of Cry2Ab2 protein/g (herein, identified as Cry2Ab). Corn leaf powder was stored at -80°C and allowed to warm to room temperature before weighing and use in assays.

Bioassays for comparing diet overlay and diet incorporation methods

Responses to the two assay methods of the susceptible laboratory culture, MonZa and the S19 field strain (collected in July, 2008) were contrasted at Monsanto Company's laboratories in Chesterfield Village, MO. Artificial diet (Southland Multispecies, Lake Village, AR) was prepared as per instructions. The diet incorporation bioassay was conducted according to the method previously described by Sivasupramaniam et al. (2008). The diet overlay bioassay was conducted according the method previously described by Greenplate (1999). Seven concentrations of Cry2Ab were tested: 50.0, 10.0, 5.00, 2.50, 1.25, 0.63, 0.31 µg/cm^2 and 150, 75.0, 37.5, 18.7, 9.38, 4.69, 2.34 µg/ml for diet overlay and diet incorporation, respectively. However, the third replication of diet incorporation assay was conducted with different concentrations of Cry2Ab (100, 50, 25, 12.5, 6.25, 3.13, 1.56 µg/ml). Untreated controls consisted of lyophilized non-*Bt* corn leaf powder equivalent to that of the highest Cry2Ab concentration tested. In the diet overlay assay, one ml of freshly prepared diet was poured into 128 wells bioassay trays (C-D International). Once the diet cooled and solidified, 300 µl of protein solution suspended in 0.1% Agar was overlaid onto the diet surface of each well and allowed to air dry (Siegfried et al. 2000). In the diet incorporation assay, corn leaf powder was suspended in 20% (v) water and mixed thoroughly with 80% (v) artificial diet. Approximately, one ml of diet

was poured into each well of bioassay trays. Sixteen larvae were tested for each concentration, and assays were replicated three times. Assays were incubated at 27±1^{0}C, 70% relative humidity and 24 hr dark conditions. Larval mortality and surviving larval instars were recorded after 7 days of exposure to the treated diet.

***Bt* diet overlay bioassays for testing field colonies**

The susceptibility of field colonies (Table 1) of *H. zea* to two diagnostic concentrations of Cry2Ab (10 and 50 µg/cm^2) was measured in diet overlay assays at Monsanto Company's laboratories in Chesterfield Village, MO. Two untreated controls comprising non-*Bt* corn leaf powder equivalent to that found in 10 and 50 µg/cm^2 Cry2Ab treatments were included. Sixteen larvae were tested for each concentration, and assays were replicated at least four times. On all testing dates except on Oct 2nd and Oct 13th 2008, assays were run with MonZa as an experiment control. Assay incubation conditions were the same as above.

Data Analysis

In order to avoid the aforementioned confusion and ambiguities in defining mortality and estimating median lethal concentrations, two new terms are proposed and used herein. MIC-2 is defined as the median lethal concentration computed from responses of larvae failing to reach the second-instar after 7days (hereafter referred to as Mortality-2). This response criterion scores live individuals as those that develop to ≥ the 2nd instar. MIC-3 is defined as the median lethal concentration computed from responses of larvae failing to reach the 3rd instar after 7 days (hereafter referred to as Mortality-3). This response criterion scores live individuals as those that develop to ≥ the 3rd instar. Response data were analyzed by probit analysis using Polo Plus software (LeOra Software, CA). Responses of field strains failing to reach the second instar in diet incorporation and diet overlay methods of the highest concentrations were analyzed for differences in sample means and distributions using Z-tests in (JMP8.0 SAS institute). T-tests were conducted to evaluate significant differences in mean mortalities of field colonies and MonZa in diet overlay assays, using both response criteria detailed above.

Results

Comparison of diet overlay and diet incorporation assays: Mean Inhibitory Responses

Results indicated no significant differences in mean molt-inhibitory concentrations (MIC-2 or MIC-3) between colonies, within each method (Table 2). Due to dissimilar units of measurement used in diet overlay and diet incorporation methods, numerical differences in susceptibility estimates from these methods are expected. MIC-2s for both colonies were 7 to 10-fold lower in diet overlay compared to diet incorporation assays. Both MIC-2_{75} (321 and 303µg/ml for MonZa and S19, respectively) and MIC-2_{90} (876 and 1,105µg/ml for MonZa and S19, respectively) estimates were higher than the highest (150µg/ml) concentration tested in diet incorporation, whereas only MIC-2_{90} (108 and 103µg/cm^2 for MonZa and S19, respectively) values were beyond the range of concentrations tested in diet overlay method.

MIC-3 estimates were lower (10 to 15-fold) in diet overlay compared to diet incorporation assays. Importantly, all MIC-3 values (MIC-3_{50}, MIC-3_{75} and MIC-3_{90}) from diet overlay assays were within the range of concentrations tested. This was not the case with diet incorporation assays, for which MIC-3_{75} and MIC-3_{90} estimates exceeded the highest concentration tested. One additional practical implication of the aforementioned differences between diet overlay and diet incorporation assays is that, the former method required less than 50% as much toxin as the latter method.

Table 2. Lethal concentrations of *Cry2Ab* to *H. zea* estimated with diet overlay (DO) and diet incorporation (DI) methods.

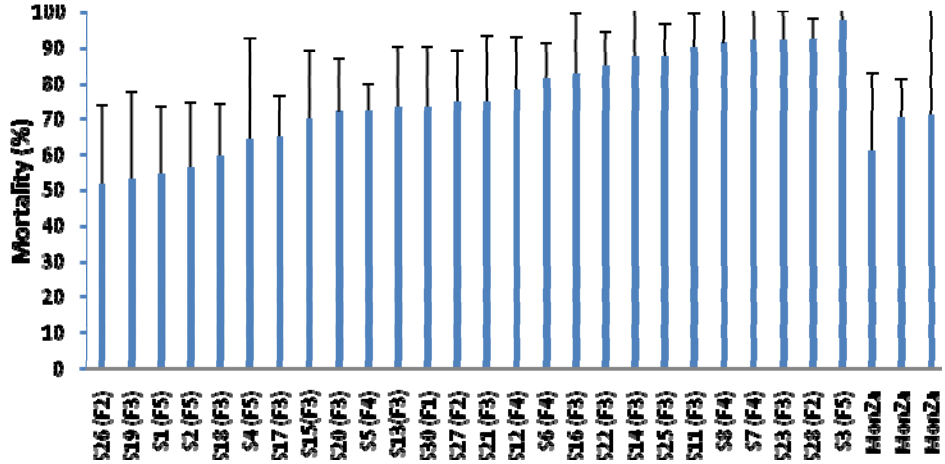
Colony	Method	Slope ± SE	MIC_{50}* (95% CI)	MIC_{75}* (95% CI)	MIC_{90}* (95% CI)	χ^2
Mortality-2: Scores as dead larvae that fail to develop to the 2nd instar						
MonZa	DI	1.39 ± 0.18	105 (71.7- 188)	321 (181- 842)	876 (405- 3,340)	21.5
	DO	1.45 ± 0.17	14.1 (10.3 - 20.8)	41.35 (26.80 -77.04)	108 (60- 261)	18.0
S-19	DI	1.08 ± 0.16	72.1 (46.9 – 138)	303 (153 – 1,049)	1,105 (421- 6,882)	21.5
	DO	1.13 ± 0.14	7.52 (4.79 -13.4)	29.88 (16.17 – 85.0)	103.39 (43.8 - 495)	29.4
Mortality-3 : Scores as dead larvae that fail to develop to the 3rd instar.						
MonZa	DI	1.54 ± 0.17	63.7 (46.8 – 95.8)	174 (113 – 342)	431 (238 –1,119)	22.2
	DO	1.49 ± 0.16	6.18 (4.29 – 9.51)	17.6 (11.1 – 35.9)	44.9 (24.2 – 128)	30.4
S-19	DI	1.53 ± 0.20	34.2 (25.79 – 45.89)	94.0 (66.8 – 156)	234 (144 – 511)	17.5
	DO	1.50 ± 0.18	2.31 (1.55 – 3.25)	6.52 (4.60 – 10.2)	16.6 (10.6 – 32.9)	20.4

* 1) Lethal concentrations are in µg/ml and µg/cm^2 for diet incorporation and diet overlay methods, respectively
2) Lethal concentrations are referred as molt inhibitory concentrations-2 (MIC-2) and molt inhibitory concentrations-3 (MIC-3), which were estimated from Mortality-2 and Mortality-3, respectively.

Susceptibility of *H. zea* to Cry2Ab in diet overlay assays

Mortality-2 of the laboratory susceptible culture (MonZa) ranged from 60.9 to 70.8%, with a mean ± SEM of 67.3 ± 3.21% in 10µg/cm^2 bioassays. At the concentration of 50µg/cm^2 Cry2Ab, mortality-2 of MonZa ranged from 81.2 to 85.4%, with a mean ± SEM of 82.6 ± 1.67%. For 26 field populations, Mortality-2 ranged from 51.2 to 92.5%, with a mean ± SEM of 75.8 ± 2.74% at the 10µg and 73.4 to 100%, with a mean ± SEM of 90.7 ± 1.32% at the 50µg/cm^2 Cry2Ab (Figure 1A and B). Grand mean Mortality-2 of field colonies at both concentrations were significantly ($P<0.05$) higher than MonZa. Nine and 11 of 26 field strains had mean mortality-2 significantly ($P<0.05$) greater than MonZa at 10µg/cm^2 and 50µg/cm^2 Cry2Ab, respectively. Mean mortality-2 of the remaining strains were not different from MonZa.

A



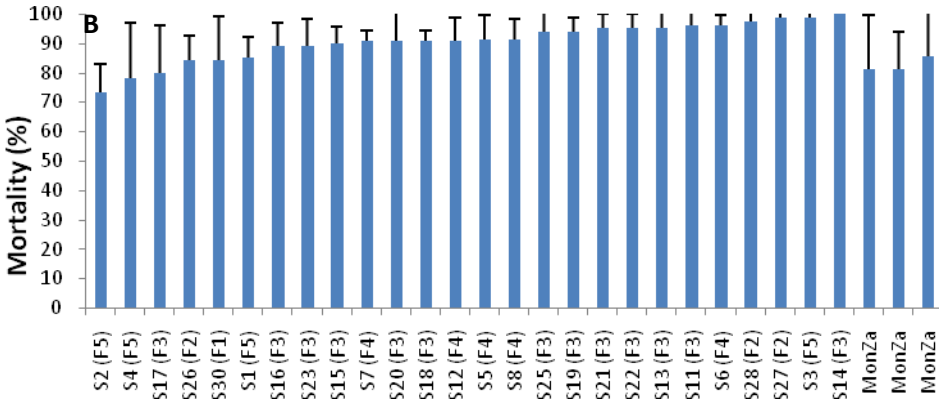
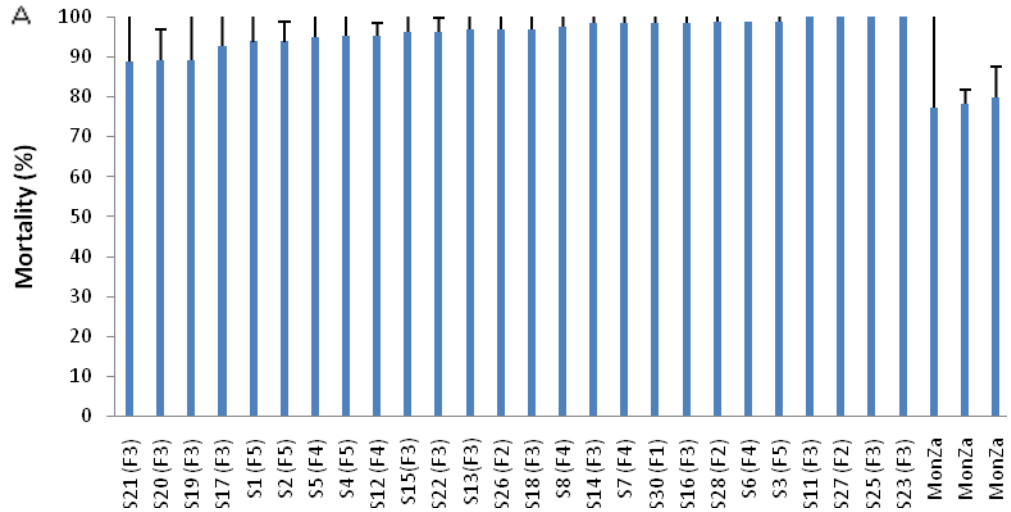


Figure 1. Mortality-2 of field colonies, and MonZa at diagnostic concentrations (A: 10 and B: 50 µg/cm2) of Cry2Ab in diet overlay assays. This criterion scores dead individuals as those failing to develop to the 2nd larval stage.

Mortality-3 of MonZa ranged from 77.0 to 79.6%, with a mean ± SEM of 78.3 ± 0.91% at the 10µg and 95.3 to 98.4% with a mean ± SEM of 96.5± 1.16% at the 50µg/cm2 Cry2Ab. For 26 field populations, Mortality-3 ranged from 88.7 to 100%, with a grand mean ± SEM of 96.2 ± 0.680% at the 10µg, and 98.4 to 100%, with a grand mean ± SEM of 99.5 ± 0.15% at the 50µg/cm2 Cry2Ab (Figures 2 A and B). Grand mean Mortality-3 of all field colonies at both concentrations were also significantly (P<0.05) higher than MonZa. All and 19 of 26 All strains had mean morality-3 greater than MonZa at 10µg/cm2 and 19 strains had mean Mortality-3 greater than MonZa at 50µg/cm2 Cry2Ab. None of the field strains were less susceptible than MonZa at either concentrations tested.



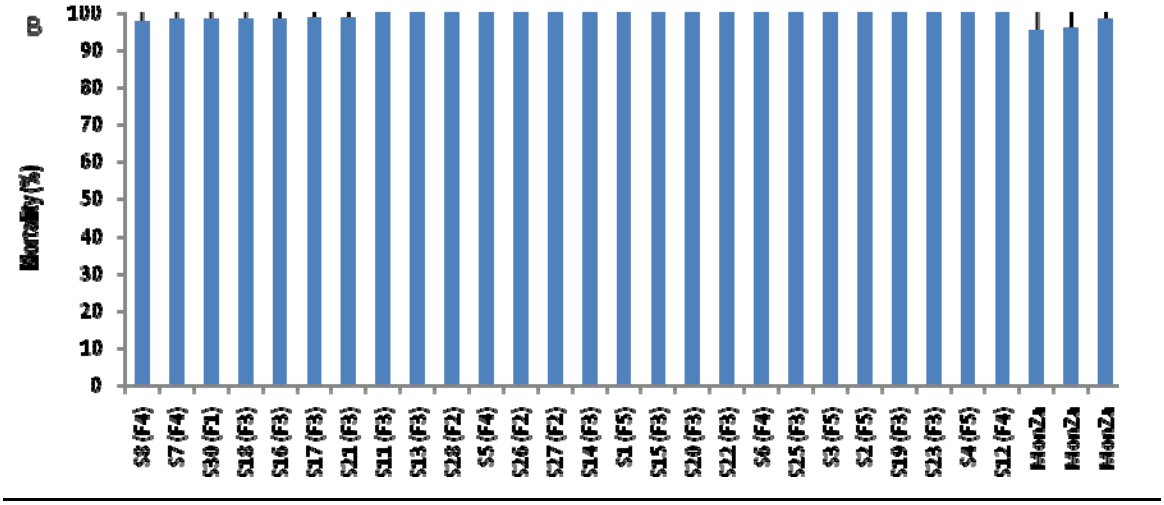


Figure 2. Mortality-3 of field colonies, and MonZa at diagnostic concentrations (A:10 and B:50 µg/cm^2) of Cry2Ab in diet overlay assays. This criterion scores dead individuals as those failing to develop to the 3rd larval stage.

Susceptibility of *H. zea* to Cry2Ab in diet incorporation assays

Data presented in this section were generated at University of Arkansas by Ali and Luttrell (2009), and results are discussed here with permission. For methods, refer to Ali and Luttrell (2009). Grand mean ± SEM mortality-2 of susceptible strains, LabZa and MonZa, were significantly ($P<0.0001$) different; at 150µg Cry2Ab/ml diet mean mortality was 99.7 ± 0.26% (range 96.9 to 100%) and 91.7 ± 1.50% (range from 90.2 to 93.2%), respectively (Figure 3). This greater susceptibility of LabZa reflects the much longer period that it has been isolated in laboratory culture (Ali and Luttrell, 2009). Only one replication (out of eight) of MonZa assays had mortality-2 equal to 100%. In contrast, only one replication (out of 48) of LabZa assays had mortality-2 less than 100% (87.5%). Mean mortality-2 of 24 field strains (including strain S4 tested in two generations) ranged from 56.9 to 100% with a grand mean ± SEM of 85.2 ± 2.61%. Grand mean mortality-2 of field strains was significantly ($P<0.05$) lower than both LabZa and MonZa. Mean mortality-2 of ten field strains (S1, S3, S4, S5, S7, S12, S17, S19, S20 and S23) were significantly ($P<0.05$) lower than grand mean mortality-2 of MonZa. Mean mortality-2 of remaining field strains were not different from MonZa.

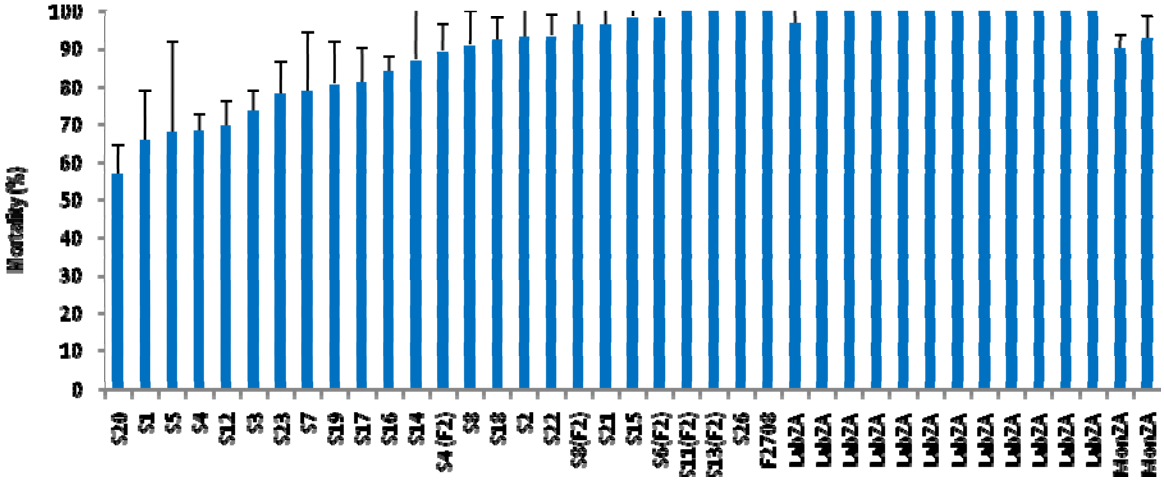


Figure 3. Mortality-2 of field colonies, LabZA and MonZa at 150µg/ml Cry2Ab in diet incorporation assays*.

*Data from Ali and Luttrell, 2009.

Comparison of diet overlay and diet incorporation assays

Substantially greater variation was observed in field colony response (Mortality-2) to Cry2Ab in diet incorporation assays than in diet overlay assays (Figure 1B and Figure 3). Mean Mortality-2 (±SEM) was significantly (P=0.0498) different between diet incorporation (85.2 ± 2.61%) and diet overlay (90.7 ± 1.29%) methods. Further, the coefficient of variation of mortality-2 in diet overlay was 7.23% relative to 15.0% in diet incorporation assays.

Discussion and Conclusions

Monitoring of resistance to Cry2Ab was done with 26 collections of *H. zea* made in the US in 2008. Irrespective of the concentration tested or mortality criterion used, no field strains had significantly lower susceptibility than the susceptible laboratory strain, MonZa. When tested with diet overlay assay, many field strains had significantly higher mortality than MonZa. This indicates the absence of major-gene resistance to Cry2Ab in the strains evaluated.

Contrasts made with diet-incorporation bioassays highlighted shortcomings of this method for testing susceptibility of *H. zea* to Cry2Ab. Diet incorporation bioassays conducted at the University of Arkansas on 23 of the same field strains (out of 26 strains tested in diet overlay assays) showed significantly greater variation in mortality. Also, the laboratory strain used in Arkansas, LabZa, was more susceptible to Cry2Ab than Monsanto's susceptible reference strains and most field collections. Effective diagnostic concentrations for monitoring should detect survivors that fall outside the distribution of baseline responses. To achieve this requires a bioassay that yields consistently high levels of responses of susceptible field strains and is able to reliably differentiate these from individuals with major-gene resistance. The highest concentration technically feasible in diet incorporation bioassays (150µg/ml Cry2Ab) still yielded substantial numbers of survivors of many field strains as well as the MonZa laboratory strain. This contrasted strikingly with the results of diet overlay bioassays employing the Mortality-3, but not the Mortality-2 criterion.

Diet overlay bioassays, in which larvae failing to develop to the L3 stage are recorded as responses (i.e., dead), offer promise for overcoming the problems associated with diet incorporation assays of low-to-medium susceptibility pests. Specifically, we found that diet overlay assays with diagnostic concentrations of 10 and 50 µg/cm^2 offer clear advantages over diet incorporation assays for monitoring *H. zea* resistance to Cry2Ab. Although not the subject of this paper, preliminary findings from 2008 trials indicate that the advantages of diet overlay assays extend also to Cry1Ac (Ali and Luttrell 2009). Diet overlay assays yielded a low number of *H. zea* surviving the upper discriminating concentration, 50 µg/cm^2. Studies are planned to investigate the genetic basis of resistance in these survivors to determine if they have enhanced ability to survive on *Bt* plants. To date, there has been no evidence that genetically-conferred resistance of *H. zea* has increased larval survival on Bollgard cotton in the field.

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