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Susceptibilities of Geographic Populations of *Helicoverpa zea* (Lepidoptera: Noctuidae) in Mexico to Bt ∂-Endotoxins Cry1Ac and Cry2Ab: An 18-Yr Study

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Abstract

An insect resistance monitoring program was developed for Mexico to accommodate the commercial introduction and stewardship of Bt cotton. Between 1998 and 2015, field-collected geographic populations of *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) were evaluated against Cry1Ac and Cry2Ab proteins of *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae) to establish baseline susceptibility data before the commercial use of Bollgard (Cry1Ac) and Bollgard II (Cry1Ac and Cry2Ab) cotton. An annual monitoring program was subsequently established in which a single diagnostic concentration of each Bt protein was used in a diet overlay bioassay. The diagnostic concentration represented the concentration where larvae, evaluated in baseline studies, were reduced in weight by ≥97% relative to untreated controls or failed to molt to third instar after 5 d. In the monitoring study, populations were tested against Cry1Ac from 1998 through 2015, and against Cry2Ab from 2002 through 2004 and again from 2007 through 2015. None of the Cry1Ac-exposed larvae tested during the 18-yr period reached the third larval instar after an exposure of 5 d, and weight reduction relative to untreated control larvae was uniform at about 98–99%. For the 12 yr of Cry2Ab monitoring, no larvae reached third instar, and weight reduction was uniform at >97% relative to controls. These results indicate that *H. zea* susceptibility to Cry1Ac and Cry2Ab has not changed during the period Bollgard and Bollgard II have been cultivated in Mexico.

Resumen

Un programa de monitoreo de resistencia en insectos se desarrolló para México para acompañar la introducción comercial del algodonero Bt. Entre 1998 y 2015, se recolectaron, en campo, poblaciones de *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) y se evaluaron con las proteínas Cry1Ac y Cry2Ab de *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae) para determinar la línea de base de susceptibilidad antes del uso comercial del algodonero Bollgard® (Cry1Ac) y de Bollgard® II (Cry1Ac y Cry2Ab). Posteriormente, implementó un programa anual de seguimiento y se utilizó una concentración de diagnóstico de cada proteína Bt en bioensayos de capa. La concentración de diagnóstico representa aquella la cual las larvas reducen su peso en ≥97% respecto a los testigos no tratados o no mudan al tercer instar después de 5 días de exposición. El monitoreo con Cry1Ac se hizo desde 1998 hasta 2015, y con Cry2Ab de 2002 a 2004 y nuevamente desde 2007 hasta 2015. Ninguna de las larvas expuestas a la proteína Cry1Ac durante 18 años alcanzó el tercer estadio larval después de 5 d de exposición, y la reducción de peso en relación con las larvas del testigo no tratado fue de 98–99%. A los doce años de monitoreo de la Cry2Ab, ninguna de las larvas tratadas alcanzó el tercer instar, y la reducción de peso fue uniforme en >97% respecto a los testigos sin tratar. Estos resultados indican que la susceptibilidad de H. *zea* a Cry1Ac y Cry2Ab no ha cambiado durante el período que Bollgard® y Bollgard® II se han cultivado en México.

Key words: *Helicoverpa zea*, Bt protein, cotton, diagnostic concentration, insect resistance monitoring

In Mexico, prior to the 1960s, cotton was known as 'white gold' because it generated much income and many jobs. As many as 630,000 ha of cotton were cultivated in Tamaulipas alone (Vargas et al. 1979). To protect this valuable commodity in the face of growing insect pest pressure, mostly from caterpillars (Lepidoptera), applications of insecticides increased in volume and frequency until the 1970s, when 80% of all insecticides used in Mexican agriculture were applied to cotton (Jaimes 1972). Resistance issues developed during this time due to the overuse of insecticides, eventually precipitating a national crisis (Bujanos 1983, Lagunes 1992). The loss of insecticide effectiveness led to the disappearance of cotton from Apatzingán (state of Michoacán), Tapachula (state of Chiapas), and Matamoros (state of Tamaulipas) (Vargas et al. 1979, Bujanos 1983, Pérez 1983, Lagunes 1992). The slow recovery of the Tamaulipas region was halted by a subsequent crisis caused by resistance to pyrethroids by the tobacco budworm, *Heliothis virescens* (F.) (Lepidoptera: Noctuidae), in the mid-1990s (Terán-Vargas 1996). Other factors such as low yields, low lint prices, drought, increasing production costs, lack of adequate subsidies, and competition from cheaper imported fiber had kept cotton cultivation in Mexico below 140,000 ha per year (ASERCA 2005, Martínez-Carrillo and Díaz-López 2005, SAGARPA 2005).

Bollgard cotton, which expresses the Bt δ-endotoxin Cry1Ac (Perlak et al. 1990, 1991), was introduced into this environment in an attempt to improve lepidopteran pest control (Terán-Vargas et al. 2005). In 1996, 896 ha of Bollgard cotton were planted in the south of Tamaulipas (Martínez-Carrillo and Díaz-López 2005); in 2005, 78,550 ha of Bt cotton were grown, representing 60.3% of Mexico's cultivated cotton (SAGARPA 2005); and in 2011, 161,500 ha of Bt cotton were planted, representing an adoption rate of 87% (James 2011). In the mid-2000s, the Bt cotton landscape began to change as Bollgard II (containing both Cry1Ac and Cry2Ab) was introduced in the Mexican marketplace.

Bollgard cotton is effective against the key lepidopteran pests of Mexican cotton. It provides excellent activity against tobacco budworm, *H. virescens*, and pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae); it also shows good to moderate activity against bollworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) (Meeusen and Warren 1989), which is widely distributed in Chihuahua, Coahuila, Durango, Sonora, and Tamaulipas. Because the Cry1Ac in Bollgard does not provide an extremely high dose against *H. zea*, and survivors are not uncommon, the history of repeated sublethal exposure may represent a scenario for selection of resistance (Martínez-Carrillo and Díaz-López 2005).

Several species of Lepidoptera have developed resistance to Bt crops in the field. These include pink bollworm in India (Cry1Ac cotton); fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), in Puerto Rico (Cry1F corn); and the African stalk borer, *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae), in South Africa (Cry1Ab corn) (van Rensburg 2007, Matten et al. 2008, Storer et al. 2010, Dennehy et al. 2011, Dhurua and Gujar 2011). Claims of resistance to Bt cotton (Cry1Ac) in *H. zea* (Tabashnik et al. 2008) have been made in the United States; however, there has been significant disagreement within the academic community regarding these claims (Moar et al. 2008, Luttrell and Ali 2009). At least some of this disagreement has centered on the use of multiple sources of data collected under different methodologies to make the argument for resistance in the face of little field-associated evidence of a change in Bt cotton efficacy or grower practices (Moar et al. 2008, Tabashnik et al. 2008). More recent publications indicate that Bt corn products in the southern United States do not manage *H. zea* as well as when they were launched (Reisig and Reay-Jones 2015, Dively et al. 2016). As a consequence, greater damage to Bt cotton products containing Cry1 proteins is being reported in the southern United States, after 20 yr of commercial availability. In the laboratory, numerous species of Lepidoptera have been selected for resistance to Bt toxins (Ferré and Van Rie 2002). It is reasonable, therefore, that the potential for development of resistance to Bt cotton by *H. zea* in Mexico be considered and assessed.

A strategy for managing insect resistance to Bollgard cotton, and to Bt crops in general, has been to employ a high dose of the Bt protein in the plant along with deployment of conventional cotton nearby to act as a refuge (Alstad and Andow 1995). The refuge allows the development and survival of a Bt-susceptible insect population which, at the adult stage, will mate with any rare resistant individuals that may have emerged in the Bt field. This will serve to 'dilute' Bt resistance alleles that are selected for survival in Bt fields and maintain low frequencies of these alleles over time in the general population, thus maintaining Bt crop effectiveness. In Mexico, regulatory authorities from the Agriculture Ministry allow Bt cotton growers two options for refuge deployment. One option consists of 80% Bt cotton and 20% conventional, in which the conventional may be sprayed with insecticides for lepidopteran control; a second option consists of 96% Bt cotton and 4% conventional, in which the refuge may not be sprayed for lepidopteran control (Martínez-Carrillo and Díaz-López 2005). One way to measure the success of a resistance management strategy is to periodically assess the susceptibility of geographic populations of the pest in question to the Bt protein present in the plant. Laboratory baseline susceptibility studies have been used to quantify the 'normal' mortality responses of target pest populations prior to the introduction of Bt crops (Stone and Sims 1993, Luttrell et al. 1999). The baseline study can be used to develop an appropriate testing concentration for future testing; this is called the diagnostic concentration or diagnostic dose. The diagnostic concentration is typically one that produces a total response or something close to it, such as 98–100% mortality or 98–99% growth inhibition (Sims et al. 1996). After a selection pressure is imposed, populations of the target pest can be periodically evaluated against the diagnostic concentration, increasing the chances of detecting the emergence of modest levels of resistance and allowing implementation of remediation tactics before field failure issues arise (ffrench-Constant and Roush 1990). However, detecting changes in allele frequencies at low levels remains a challenge (Caprio and Sumerford 2007).

The objective of this study was to evaluate populations of *H. zea* collected from areas of Bollgard and Bollgard II adoption in Mexico during the period from 1998 to 2015. Baseline susceptibilities for Cry1Ac and Cry2Ab were established among geographic populations of *H. zea*. From those data, a single diagnostic monitoring concentration, designed to elicit a 97–100% growth inhibition response, was developed and subsequently presented to neonate larvae of field-collected populations in feeding assays over an 18-yr period (Sims et al. 1996, Hardee et al. 2001). Growth inhibition, rather than a mortality response, was utilized for two main reasons. The first is that previous work with Bt proteins and U.S. *H. virescens* and *H. zea* populations found that sublethal responses (weight reduction and larval development retardation) were less variable among geographically distinct populations than were mortality responses, as is generally true for a less susceptible pest (Sims et al. 1996, Siegfried et al. 2000). The second is that the amount of characterized standard Cry1Ac protein needed to generate a full or nearly full sublethal response in *H. zea* was close to 100 times less than that required to generate a similar mortality response (Sims et al. 1996), and for Cry2Ab, the amount needed to generate a nearly full sublethal response yielded about 34% mortality (Aguilar-Medel et al. 2007).

The aim of this research was to determine the baseline susceptibility of Mexican field populations of *H. zea* from Chihuahua, Tamaulipas, Coahuila, and Durango to both Cry1Ac and Cry2Ab Bt proteins, and to Cry1Ac in Sonora, and then to monitor the response in field populations to these proteins from 1998 to 2015 (Cry1Ac) and from 2002 to 2004 and 2007 to 2015 (Cry2Ab).

Materials and Methods

Cry1Ac Baseline

In 1998, a total of eight geographic Mexican populations of *H. zea* were collected from the states of Chihuahua, Tamaulipas, Coahuila, Durango, and Sonora (Fig. 1). A ninth population was provided by the International Center for Wheat and Maize Improvement (CIMMYT), located in the state of Mexico, and used as the susceptible reference population (this population was used 15 out of 18 yr for Cry1Ac, and 11 out of 12 yr for Cry2Ab). This population had been in culture, under laboratory conditions, for over 20 yr, and was maintained free of Bt-selection pressure. For Cry1Ac, the diagnostic dose was 5 µg ml^{-1}, the concentration established by Sims et al. (1996).

Cry2Ab Baseline

In 2003, four geographic populations were used for baseline studies with Cry2Ab, which have since been published (Aguilar-Medel et al. 2007). These populations came from the states of Coahuila, Durango, Tamaulipas, San Luis Potosí, Veracruz, and Chihuahua (Fig. 1). Again, the CIMMYT population was used as a susceptible reference.

Monitoring Populations

For diagnostic concentration testing against Cry1Ac and Cry2Ab, populations of *H. zea* were collected from different locations through multiple years across the states of Chihuahua, Tamaulipas, Coahuila, Durango, Sinaloa, and Baja California (Table 1).

Rearing

For each population, at least 300 larvae at various stages of development were collected at a site and allowed to reproduce under laboratory conditions to obtain enough F1 individuals to carry out the bioassays (Aguilar-Medel et al. 2007). Larvae from field collections were quarantined individually in glass vials containing 10 ml of artificial diet prepared according to the manufacturer's instructions (Cotton bollworm diet, Southland Products, Inc., Lake Village, AR). Larvae were kept at 27 ± 1°C, 70 ± 5% relative humidity, and 14 hr of light per day. Larvae were observed daily; sick and/or parasitized individuals were discarded. Pupae were bulk mated in wire cages (25 × 25 × 35 cm), and emergent adults were fed with a 10% honey solution in tap water. At least 250 healthy larvae were obtained to generate each F1 generation. Oviposition cloths were laid over the cages and eggs were collected daily for 8 d. These egg sheets were placed in emergence boxes and held under larval rearing conditions until hatching. Active neonate larvae were used for testing.

Proteins

The Cry1Ac protein standard was a freeze-dried powder preparation of MVPII (Mycogen Corp., San Diego, CA), a devitalized formulation of a transgenic *Pseudomonas* that had been engineered

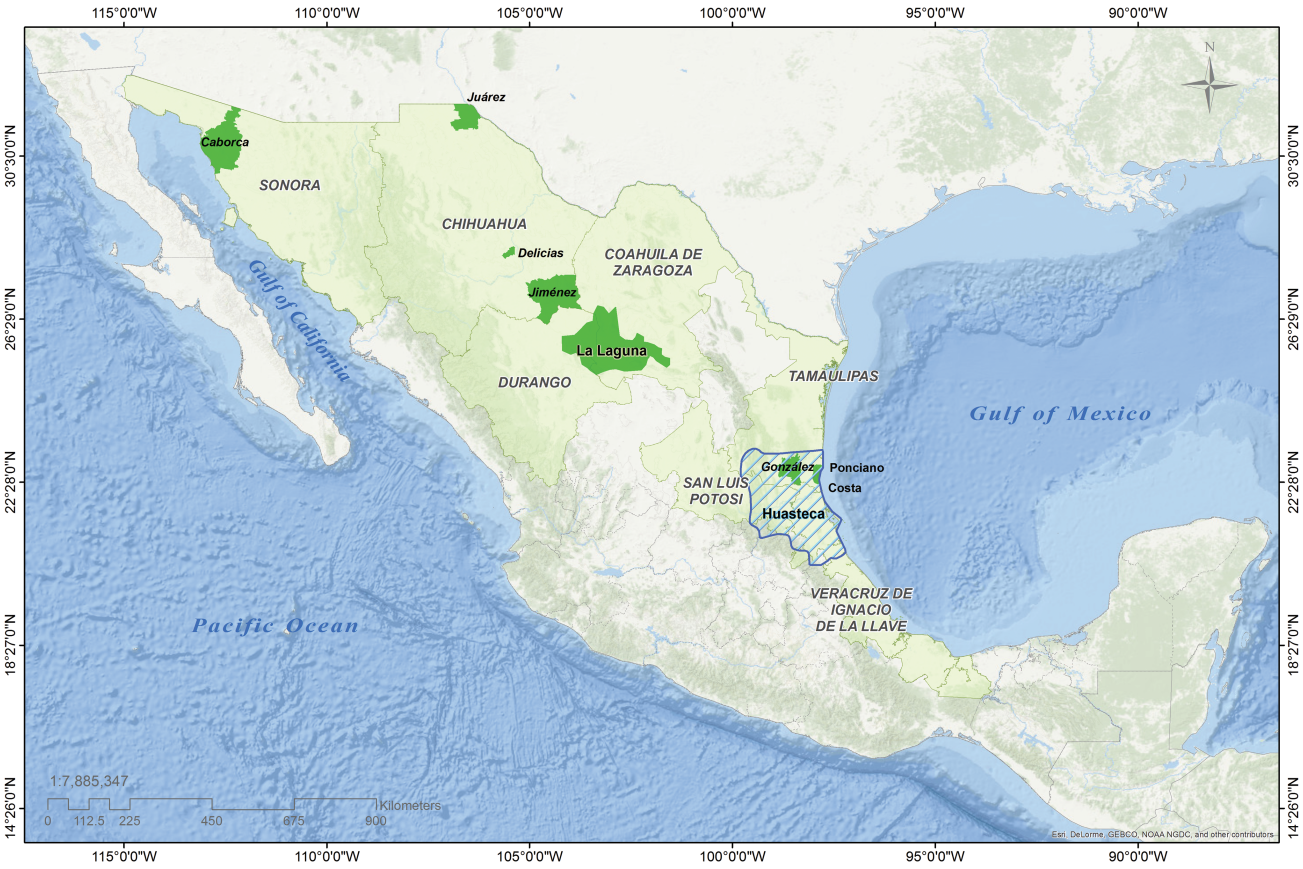

Fig. 1. Locations where *H. zea* geographic populations were collected to establish Cry1Ac and Cry2Ab diagnostic concentrations. Dark green areas correspond to the populations listed in Tables 2 and 3.

to express Cry1Ac (Gilroy and Wilcox 1992); the concentration of Cry1Ac in the freeze-dried powder was 19.1% by weight. The Cry2Ab standard was a freeze-dried powder of a maize inbred line transformed to express Cry2Ab (Monsanto, St. Louis, MO); the concentration of Cry2Ab was 0.6% by weight of dry powder. Both standard protein powders were stored in sealed containers at –14°C.

Bioassay

A surface contamination diet bioassay was used (after Hardee et al. [2001]). One milliliter of molten artificial diet was pipetted into each well of an insect diet bioassay tray (Bio-Ba-128, C-D International, Pitman, NJ). After surface drying, 200 µl of standard protein suspended in a 0.2% agar solution at room temperature (<25°C; Bacto Nutrient Agar; Difco Laboratories, Detroit, MI) was pipetted onto the diet surface of each well; untreated control wells received 200 µl of 0.2% agar only. After the diet surface had dried, one active neonate was placed in each well using a small camel-hair brush. Infested bioassay trays were sealed using clear, perforated, self-adhesive Mylar panels (Pull N' Peel Tab Bio-CV-16; C-D International). Bioassays were incubated for 5 d under the larval rearing conditions described above. For baseline susceptibility studies, 12 Cry protein test concentrations were replicated 5 times (on different days); each replicate consisted of 32 larvae exposed to each Bt test concentration and 32 control (untreated) larvae. At 5 d, mortality responses were recorded, as were the number of test larvae failing to reach third instar (this included dead larvae and surviving first- and second-instar larvae). Weights of surviving larvae were recorded. Inhibition of weight gain (IW) was calculated as the difference in average weight between treatment and control for each concentration, expressed as a percentage ((1 – (average weight of treated larvae/average weight of control larvae)) × 100). For a replicate to be included in the analysis, the maximum mortality allowed in the untreated control was 10%; control mortality was adjusted by Abbott's formula (Abbott 1925). For the subsequent annual diagnostic concentration monitoring assays, all procedures were the same as for the baseline work except that a single concentration was used and that each replicate contained 96 larvae exposed to Bt and 32 control larvae.

Table 1. Locations and years in which populations of *H. zea* were collected to be tested with the diagnostic concentrations of Cry1Ac and Cry2Ab

Location, State	Years tested with Cry1Ac	Years tested with Cry2Ab
Jiménez, Chihuahua	1998–2001	
Delicias, Chihuahua	2000–2006	2002, 2004
Juárez, Chihuahua	2000–2014	2003–2004, 2007–2014
Ojinaga, Chihuahua	2007–2012	2007–2012
Aldama, Chihuahua	2015	2015
González, Tamaulipas	1998, 2000–2001	
Costa, Tamaulipas	1998	
Ponciano, Tamaulipas	1998	
Huasteca, Tamaulipas.		2002
Tamaulipas Nte., Tamaulipas	2014–2015	2014–2015
Coahuila and Durango[a]	1998–2015	2002, 2004, 2007–2015
Caborca, Sonora	1999	
Valle del Yaqui, Sonora	2009–2013, 2015	2009–2013, 2015
Sonoyta, Sonora	2015	2015
Valle del Fuerte, Sinaloa	2015	2015
Mexicali, Baja California	2014	2014

[a]The states of Coahuila and Durango constitute La Laguna region.

Statistical Analysis

For the baseline study, data were analyzed using the probit model (Finney 1971). The 50% and 95% response values for each population were calculated with their corresponding 95% confidence limits. Inhibition of development (ID) was used to describe the response measured by the failure to reach third instar in the 5-d assay. IW was used to describe the response measured as the reduction in surviving larval weight relative to untreated control larvae in the 5-d assay. For the monitoring study, mortality, larval development, and weight reduction data were transformed using the arcsine of the square root of the proportion of response relative to the untreated control response. An ANOVA (PROC ANOVA; SAS Institute 2000) and means comparisons (Tukey's $\alpha = 0.05$) were utilized to evaluate differences between years within population collection sites.

Results

Baseline Susceptibility

Baseline larval development inhibition and weight reduction responses for Cry1Ac appear in Tables 2 and 3, respectively. Values of ID_{50} (concentration at which 50% of the test larvae failed to reach third instar) among the wild populations ranged from 0.0104 to 0.06 µg ml^{-1}, while values of ID_{95} (concentration at which 95% of the test larvae failed to reach third instar) ranged from 2.32 to 4.72 µg ml^{-1} (Table 2). Values of IW_{50} (concentration at which mean

Table 2. ID by the ∂-endotoxin Cry1Ac of *Bacillus thuringiensis* var. *kurstaki* in Mexican populations of *H. zea* larvae

Population	*n*	Slope ± SEM	ID_{50} (95% CL)[a]	ID_{95} (95% CL)[a]	χ^2 value	df	$P > \chi^2$
Costa	1,120	0.66 ± 0.04	0.0104 (0.0072–0.0208)	4.72 (2.48–10.32)	5.11	4	0.2760
González	968	0.93 ± 0.06	0.04 (0.0312–0.0568)	2.48 (1.60–4.64)	5.30	3	0.1469
Jiménez	968	0.93 ± 0.06	0.04 (0.0328–0.0592)	2.48 (1.60–4.64)	8.3	4	0.0812
Ponciano	1,120	0.90 ± 0.06	0.04 (0.0232–0.0520)	2.64 (1.52–4.88)	12.0	4	0.0173
La Laguna	968	1.03 ± 0.07	0.06 (0.0424–0.076)	2.32 (1.44–4.08)	8.9	3	0.0301
Caborca	960	0.70 ± 0.07	0.0104 (0.0048–0.0280)	2.72 (0.56–13.84)	13.9	4	0.0076
Delicias	960	0.81 ± 0.11	0.0304 (0.0128–0.0760)	3.28 (0.64–18.24)	19.8	4	0.0005
Juárez	960	0.85 ± 0.10	0.04 (0.0168–0.0864)	3.44 (0.80–16.16)	18.1	4	0.0012
Susceptible	1,120	0.71 ± 0.04	0.004 (0.0032–0.0064)	0.88 (0.48–1.76)	4.4	4	0.3492

ID_{50} and ID_{95} represent the concentrations of Cry1Ac (expressed in µg ml^{-1}) that inhibited 50% or 95% of the larvae, respectively, from reaching third instar after 5 d of exposure.

[a]95% confidence limits.

survivor weight was reduced by 50% relative to untreated control larvae) among the wild populations ranged from 0.002 to 0.005 µg ml^{-1}, while values of IW_{95} (concentration at which mean survivor weight was reduced by 95% relative to untreated control larvae) ranged from 0.24 to 1.16 µg ml^{-1}(Table 3). Among the eight wild populations, the mean ID_{95} for Cry1Ac was approximately 3.01 µg ml^{-1}, while the mean IW_{95} was approximately 0.758 µg ml^{-1}. When considering a single diagnostic concentration for future monitoring, attention was given to earlier work performed in the United States. Sims et al. (1996) suggested the utilization of a diagnostic monitoring concentration that would elicit a sublethal response in the 98–99% range. It was estimated that the concentration of 5 µg ml^{-1} reported by Sims et al. (1996) would be suitable for Mexican monitoring as well, since it represented a value that could be expected to elicit 97–99% responses for both larval arrest before third instar and weight reduction (Tables 2 and 3). An examination of Mexican *H. zea* Cry2Ab response curves (Aguilar-Medel et al. 2007) suggested that the same concentration (5 µg ml^{-1}) could be expected to elicit about a 99% response for both larval arrest before third instar and weight reduction. For this reason, a single diagnostic concentration of 5 µg ml^{-1} was used for both Cry1Ac and Cry2Ab proteins in subsequent monitoring assays.

Monitoring of Cry1Ac Resistance

All populations across all years showed about a 98–99% reduction in weight relative to untreated control larvae (Table 4). Although

Table 3. IW by the ∂-endotoxin Cry1Ac of *B. thuringiensis* var. *kurstaki* in Mexican populations of *H. zea* larvae

Population	*n*	Slope ± SEM	IW_{50} (95% CL)[a]	IW_{95} (95% CL)[a]	χ^2 value	df	$P > \chi^2$
Costa	1,120	0.62 ± 0.04	0.002 (0.00104–0.004)	0.928 (0.288–3.008)	4.1	4	0.3907
González	968	0.80 ± 0.05	0.002 (0.00104–0.004)	0.24 (0.104–0.592)	3.8	4	0.4342
Jiménez	968	0.63 ± 0.02	0.002 (0.00104–0.004)	0.656 (0.472–1)	0.4	4	0.9821
Ponciano	1,120	0.66 ± 0.02	0.00304 (0.002–0.004)	0.816 (0.592–1.184)	1.5	4	0.8180
La Laguna	968	0.75 ± 0.07	0.004 (0.002–0.008)	0.56 (0.144–2.224)	8.1	4	0.0869
Caborca	960	0.66 ± 0.05	0.004 (0.002–0.00504)	1.16 (0.568–2.84)	1.8	4	0.7770
Delicias	960	0.74 ± 0.05	0.00504 (0.002–0.00704)	0.824 (0.44–1.8)	1.2	4	0.8704
Juárez	960	0.75 ± 0.05	0.00504 (0.002–0.00704)	0.88 (0.2–1.912)	3.09	4	0.5429
Susceptible	1,120	0.58 ± 0.02	0.002 (0.00104–0.00304)	1.216 (0.832–1.864)	1.4	4	0.8352

IW_{50} and IW_{95} represent the concentrations of Cry1Ac (expressed in µg ml^{-1}) that reduced mean survivor weight by 50% or 95%, respectively, relative to untreated control larvae after 5 d of exposure.

[a]95% confidence limits.

Table 4. Percent reduction in weight relative to the untreated control in neonate larvae of *H. zea* exposed for 5 d to the diagnostic concentration of the Cry1Ac protein (5 µg ml^{-1}) of *B. thuringiensis*

Region	Percent weight reduction relative to untreated control ± SEM								
	1998	1999	2000	2001	2002	2003	2004	2005	2006
Caborca		99.1 ± 0.1							
Costa	99.0 ± 0.2								
Delicias			98.8 ± 0.2$_{bc}$	99.2 ± 0.1$_{a}$	98.9 ± 0.1$_{bc}$	98.7 ± 0.3$_{c}$	99.1 ± 0.2$_{ab}$	99.0 ± 0.2$_{ab}$	98.9 ± 0.2$_{bc}$
González	99.1 ± 0.2$_{ab}$		99.0 ± 0.2$_{b}$	99.3 ± 0.2$_{a}$					
Jiménez	99.0 ± 0.1$_{b}$	99.1 ± 0.2$_{ab}$	99.0 ± 0.2$_{b}$	99.3 ± 0.2$_{a}$					
Juárez			98.8 ± 0.1$_{bc}$	99.3 ± 0.1$_{a}$	98.9 ± 0.2$_{bc}$	98.7 ± 0.2$_{cd}$	99.0 ± 0.2$_{bc}$	99.0 ± 0.2$_{bc}$	98.9 ± 0.2$_{bc}$
La Laguna	99.2 ± 0.0$_{ab}$	99.1 ± 0.2$_{abc}$	99.0 ± 0.1$_{abcd}$	99.2 ± 0.1$_{a}$	98.9 ± 0.2$_{cd}$	98.9 ± 0.2$_{cd}$	99.0 ± 0.2$_{abcd}$	99.0 ± 0.1$_{abcd}$	99.0 ± 0.2$_{abcd}$
Ponciano	99.2 ± 0.2								
Susceptible		99.0 ± 0.1$_{ab}$			99.0 ± 0.1$_{ab}$	98.7 ± 0.3$_{abc}$	99.1 ± 0.2$_{a}$	98.9 ± 0.3$_{abc}$	98.8 ± 0.2$_{abc}$
	2007	2008	2009	2010	2011	2012	2013	2014	2015
Aldama									98.6 ± 0.1
Juárez	99.0 ± 0.2$_{bc}$	98.8 ± 0.2$_{bc}$	99.0 ± 0.1$_{abc}$	99.0 ± 0.1$_{ab}$	98.9 ± 0.2$_{bc}$	98.8 ± 0.1$_{bc}$	98.5 ± 0.2$_{d}$	98.7 ± 0.1$_{cd}$	
La Laguna	98.9 ± 0.2$_{cd}$	98.8 ± 0.1$_{cd}$	98.9 ± 0.2$_{bcd}$	98.9 ± 0.2$_{cd}$	98.7 ± 0.1$_{de}$	98.8 ± 0.1$_{de}$	98.4 ± 0.1$_{e}$	98.6 ± 0.4$_{de}$	98.7 ± 0.1$_{de}$
Mexicali								98.8 ± 0.1	
Ojinaga	98.8 ± 0.2$_{a}$	98.9 ± 0.1$_{a}$	98.8 ± 0.1$_{a}$	98.8 ± 0.1$_{a}$	98.6 ± 0.1$_{a}$	98.9 ± 0.1$_{a}$			
Sonoyta									98.6 ± 0.1
Tamaulipas Nte.								98.4 ± 0.1$_{a}$	98.6 ± 0.1$_{a}$
Valle del Fuerte									98.8 ± 0.1
Valle del Yaqui			99.0 ± 0.1$_{a}$	98.9 ± 0.1$_{a}$	98.8 ± 0.1$_{ab}$	98.7 ± 0.1$_{b}$	98.4 ± 0.1$_{c}$		98.8 ± 0.1$_{ab}$
Susceptible	98.8 ± 0.2$_{abc}$	98.8 ± 0.1$_{abc}$	98.9 ± 0.1$_{abc}$	98.7 ± 0.1$_{abc}$	98.6 ± 0.2$_{abc}$	98.7 ± 0.1$_{abc}$	98.3 ± 0.1$_{bc}$	98.5 ± 0.2$_{abc}$	98.2 ± 0.1$_{c}$

For each population each year, 480 larvae were exposed to the diagnostic concentration (five replicate tests with 96 larvae each), and 160 larvae were untreated controls (five replicates of 32 larvae each). Values within rows (same regional population between years) followed by the same letter are not significantly different (Tukey, $\alpha = 0.05$). Significance testing was performed using unrounded values. Blank table cells indicate that the respective population was not evaluated in the respective year either because cotton was not cultivated or because the pest population was so small that it was not possible to field-collect enough individuals to carry out the bioassays.

a number of statistical differences were detected, the values were numerically similar and there was no clear trend over time. Similarly, the developmental response of arrest at second instar or smaller was complete (0% third instars) for all populations across all years (Table 5); untreated controls in the Susceptible population ranged from 78.1% to 93.1% third instars.

Monitoring of Cry2Ab Resistance

All populations across all years showed greater than 97% reduction in weight relative to control larvae with no statistical differences between years except for differences observed in the Susceptible and Ojinaga populations (Table 6). As with Cry1Ac, developmental arrest with Cry2Ab at the diagnostic concentration was complete; there were no third instars recorded for any field population, while more than 76% of the untreated larvae transitioned to third instar (data not shown).

Discussion

This report documents the use of growth inhibition responses to determine baseline levels of susceptibility among Mexican populations of *H. zea* to the Cry protein components of commercial Bt cotton grown in Mexico. Taken together, these data indicate that no marked changes in levels of susceptibility in the wake of broad commercial use of Bt cotton cultivars were detected during the seasons evaluated. It is one of only a few reports that present the results of a comprehensive Bt resistance monitoring program, using a standard

Table 5. Percentage of neonate larvae of *H. zea* that were able to reach the third instar after 5 d of exposure to the diagnostic concentration of the Cry1Ac protein (5 µg ml^{-1}) of *B. thuringiensis*

Region	Treatment	Percent reaching third instar ± SEM								
		1998	1999	2000	2001	2002	2003	2004	2005	2006
Caborca	Diagnostic concentration		0.0							
	Control		88.8 ± 3.2							
Costa	Diagnostic concentration	0.0								
	Control	86.3 ± 3.2								
Delicias	Diagnostic concentration			0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Control			89.4 ± 4.2	90.3 ± 3.0	90.4 ± 3.3	87.1 ± 4.3	84.0 ± 1.3	90.2 ± 4.7	91.9 ± 4.2
González	Diagnostic concentration	0.0		0.0	0.0					
	Control	93.1 ± 2.3		89.7 ± 2.8	91.3 ± 3.6					
Jiménez	Diagnostic concentration	0.0	0.0	0.0	0.0					
	Control	90.0 ± 2.3	88.5 ± 4.2	89.0 ± 3.9	90.9 ± 3.6					
Juárez	Diagnostic concentration			0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Control			89.4 ± 3.2	91.3 ± 3.6	89.0 ± 4.6	87.5 ± 2.8	90.2 ± 4.8	91.7 ± 3.6	91.9 ± 4.2
La Laguna	Diagnostic concentration	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Control	93.8 ± 6.6	89.6 ± 4.4	89.6 ± 3.7	92.8 ± 4.9	89.4 ± 5.0	87.5 ± 4.3	90.2 ± 5.7	93.5 ± 3.9	93.1 ± 4.4
Ponciano	Diagnostic concentration	0.0								
	Control	90.0 ± 6.1								
Susceptible	Diagnostic concentration		0.0			0.0	0.0	0.0	0.0	0.0
	Control		90.6 ± 4.4			90.0 ± 4.2	90.6 ± 4.0	87.5 ± 4.4	93.1 ± 5.4	91.9 ± 3.2
		2007	2008	2009	2010	2011	2012	2013	2014	2015
Aldama	Diagnostic concentration									0.0
	Control									83.8 ± 2.6
Juárez	Diagnostic concentration	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
	Control	87.5 ± 6.6	86.2 ± 3.6	88.7 ± 4.2	87.5 ± 4.9	81.9 ± 7.1	78.8 ± 6.0	81.3 ± 6.6	81.9 ± 6.4	
La Laguna	Diagnostic concentration	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Control	88.1 ± 6.2	85.3 ± 6.3	90.6 ± 3.8	83.1 ± 4.7	80.6 ± 7.1	82.5 ± 3.6	80.6 ± 4.6	79.4 ± 5.7	80.6 ± 4.1
Mexicali	Diagnostic concentration								0.0	
	Control								80.6 ± 3.6	
Ojinaga	Diagnostic concentration	0.0	0.0	0.0	0.0	0.0	0.0			
	Control	87.5 ± 4.5	88.4 ± 3.0	86.9 ± 4.1	85.0 ± 4.6	83.8 ± 6.0	81.9 ± 6.0			
Sonoyta	Diagnostic concentration									0.0
	Control									83.1 ± 3.6
Tamaulipas Nte.	Diagnostic concentration								0.0	0.0
	Control								81.9 ± 5.7	76.9 ± 3.5
Valle del Fuerte	Diagnostic concentration									0.0
	Control									80.0 ± 3.6
Valle del Yaqui	Diagnostic concentration			0.0	0.0	0.0	0.0	0.0		0.0
	Control			88.8 ± 4.2	86.3 ± 4.7	80.6 ± 3.1	78.1 ± 6.8	78.8 ± 5.4		83.1 ± 3.2
Susceptible	Diagnostic concentration	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Control	83.1 ± 4.2	86.2 ± 2.5	85.0 ± 3.6	81.9 ± 3.6	81.3 ± 4.0	80.0 ± 3.2	78.1 ± 4.4	82.5 ± 4.2	80.0 ± 4.2

For each population each year, 480 larvae were exposed to the diagnostic concentration (five replicate tests with 96 larvae each), and 160 larvae were untreated controls (five replicates of 32 larvae each). Blank table cells indicate that the respective population was not evaluated in the respective year either because cotton was not cultivated or because the pest population was so small that it was not possible to field-collect enough individuals to carry out the bioassays.

Table 6. Percent reduction in weight relative to the untreated control in neonate larvae of *H. zea* exposed for 5 d to the diagnostic concentration of the Cry2Ab protein (5 μg ml^{-1}) of *B. thuringiensis*

Region	Percent weight reduction relative to untreated control ± SEM												
	2002	2003	2004	2007	2008	2009	2010	2011	2012	2013	2014	2015
Aldama												98.7 ± 0.1
Delicias	98.9 ± 0.2$_a$		98.9 ± 0.3$_a$									
Huasteca	98.9 ± 0.1											
Juárez		98.6 ± 0.4$_a$	98.3 ± 0.2$_a$	98.9 ± 0.2$_a$	98.8 ± 0.1$_a$	98.7 ± 0.2$_a$	98.7 ± 0.1$_a$	98.6 ± 0.2$_a$	98.6 ± 0.2$_a$	98.7 ± 0.1$_a$	98.7 ± 0.1$_a$	
La Laguna	98.8 ± 0.2$_a$		98.7 ± 0.2$_a$	98.2 ± 0.2$_a$	98.7 ± 0.2$_a$	98.7 ± 0.2$_a$	98.6 ± 0.2$_a$	98.5 ± 0.1$_a$	98.5 ± 0.2$_a$	98.6 ± 0.1$_a$	98.7 ± 0.3$_a$	98.5 ± 0.2$_a$
Mexicali											98.6 ± 2.8	
Ojinaga				98.6 ± 0.2$_a$	98.5 ± 0.3$_{ab}$	98.5 ± 0.1$_{ab}$	98.5 ± 0.2$_{ab}$	98.4 ± 0.2$_{ab}$	98.2 ± 0.1$_b$			
Sonoyta												98.7 ± 0.1
Tamaulipas Nte.											98.6 ± 0.1$_a$	98.6 ± 0.1$_a$
Valle del Fuerte												98.5 ± 0.1
Valle del Yaqui						98.7 ± 0.1$_a$	98.6 ± 0.1$_a$	98.1 ± 0.2$_a$	98.3 ± 0.2$_a$	97.7 ± 0.2$_a$		98.4 ± 0.1$_a$
Susceptible	98.9 ± 0.2$_a$		98.7 ± 0.4$_{abcd}$	98.8 ± 0.1$_{ab}$	98.7 ± 0.2$_{abc}$	98.8 ± 0.2$_{ab}$	98.6 ± 0.1$_{abcd}$	98.5 ± 0.1$_{bcd}$	98.3 ± 0.2$_d$	98.4 ± 0.1$_{cd}$	98.4 ± 0.1$_d$	98.3 ± 0.1$_d$

For each population each year, 480 larvae were exposed to the diagnostic concentration (five replicate tests with 96 larvae each), and 160 larvae were untreated controls (five replicates of 32 larvae each). Values within rows (same regional population between years) followed by the same letter are not significantly different (Tukey, $\alpha = 0.05$). Significance testing was performed using unrounded values. Blank table cells indicate that the respective population was not evaluated in the respective year either because cotton was not cultivated or because the pest population was so small that it was not possible to field-collect enough individuals to carry out the bioassays.

methodology over time, for a specific country or geography (Siegfried et al. 2007, Wu 2007). This work has shown that geographic wild populations of *H. zea* across Mexico have remained consistent in their susceptibility to the Bt Cry proteins Cry1Ac (18 yr of data) and Cry2Ab (12 yr of data), the components of commercial Bt cotton cultivars sold as Bollgard (Cry1Ac) and Bollgard II (Cry1Ac + Cry2Ab). Our use of a single testing concentration to elicit stable sublethal responses (weight reduction and developmental arrest) supports the idea proposed by Sims et al. (1996) of employing, as a key component in a Bt resistance monitoring program for heliothines, a diagnostic concentration that will induce close to a 98% reduction in larval weight (IW_{98}), which is highly correlative with the more easily measured response criterion of developmental arrest at second instar or smaller.

Bt crops express ∂-endotoxins throughout the plant, throughout the growing season, and, in many cases, across large acreages, creating a scenario for the selection of resistance to specific Bt proteins. From the earliest commercial release of Bt cotton (Cry1Ac-expressing Bollgard) in Mexico, as in other countries, a key response to the threat of resistance has included the implementation of non-Bt cotton refuges to maintain susceptible individuals of the target pests to dilute resistance alleles present in rare survivors from Bt fields. The size of the refuge needed to delay resistance is dependent on the sustained effective dose of the product. The refuge is considered most effective if the dose is high and the inheritance of resistance is functionally recessive, meaning that the Bt concentration in the plant is enough to kill all or nearly all individuals heterozygous for the resistance allele (Roush and McKenzie 1987, Roush and Daly 1990). The high-dose criterion for most commercial Bt cotton can be assumed for extremely sensitive species such as *H. virescens* and *P. gossypiella* (Bartlett et al. 1997, Gould 1998, Henneberry et al. 2000, Sivasupramaniam et al. 2008), both of which are key pests in certain areas of Mexico. Mexico's experience with these species in Bt cotton is consistent with this belief; efficacy studies have shown extreme sensitivity to Bt cotton (Nava-Camberos et al. 1999, Terán-Vargas et al. 2005) and published resistance monitoring studies have shown no changes in Bt protein susceptibility of geographic populations (Martínez-Carrillo and Berdegue 1999; Nava-Camberos et al. 1999, 2000; Martínez-Carrillo et al. 2000, 2004; Martínez-Carrillo and Díaz-López 2005, 2008). Another testament to the overall efficacy of Bt cotton in Mexico has been its economic success, which has translated to high levels of adoption among growers. Demonstrated economic gains due to increased yields and/or reduction of insecticide costs (Traxler and Godoy-Avila 2004, Qaim 2009) led to an 87% adoption rate by 2011 (James 2011).

H. zea is inherently less susceptible than either *H. virescens* or *P. gossypiella* to both Cry1Ac and Cry2Ab and cannot be included in the high-dose scenario (Stone and Sims 1993, Mahaffey et al. 1995, Greenplate et al. 1998, Sivasupramaniam et al. 2008). So why have Cry protein susceptibilities remained relatively stable in *H. zea* populations in Mexico (this study) in spite of the lack of a high dose and amid increasing adoption levels of Bt cotton? There are likely to be several reasons. First, even under circumstances where high-dose criteria are not met, a refuge will provide some benefit in reducing resistance allele frequencies (Roush 1994), and co-expressed (pyramided) toxins provide value in delaying resistance, especially if the toxins are independent in their method or site of action (no cross-resistance) and if efficacy is high, as is the case with Bollgard II (Tabashnik 1989, Caprio 1998, Roush 1998, Greenplate et al. 2003, Sivasupramaniam et al. 2008). Also, available refuge for the polyphagous species *H. zea* may include numerous non-cotton crops and wild hosts that supplement the structured refuge (Orth et al.

2007, Jackson et al. 2008, Head et al. 2010). Finally, it has been demonstrated that fitness costs are often associated with resistance to pesticides, including in laboratory-selected, Bt-resistant colonies of cotton pests. Among cotton pests, it appears that the ability to effectively deal with gossypol, a toxic terpene aldehyde, may be compromised in Bt-resistant *P. gossypiella* (Williams et al. 2011) and *H. zea* (Anilkumar et al. 2008, 2009), rendering Bt-resistant individuals relatively less likely to survive and pass on Bt resistance alleles. Although resistance has not yet developed in *H. zea*, it is important to continue the use of refuges and monitoring to ensure that Bt cotton continues to provide protection against this insect.

Recent reports of changes in the performance of Bt crops in the southern United States are consistent with resistance to Cry1 proteins in populations of *H. zea* (Reisig and Reay-Jones 2015, Dively et al. 2016). Cry1A proteins have been in the landscape >20 yr in both Bt cotton and Bt corn. Before the 2003 commercial availability of Bollgard II cotton, many generations of *H. zea* were exposed to first-generation Bt crops expressing single Bt proteins. Head et al. (2010) demonstrated that there was an abundance of non-Bt host plants that served as additional refuge for cotton. However, Head et al. (2010) also demonstrated that there are times of the year when *H. zea* feeds exclusively on corn. There has been a dramatic shift away from cotton in the southern United States towards corn acres (National Cotton Council 2017) due to changes in commodity prices. As a consequence, the acreages of Bt corn have increased in the southern United States. Because of poor refuge compliance there has been selection for resistance to Bt corn, and therefore Bt cotton, due to shared Bt proteins. Major changes in *H. zea* susceptibility to Bt proteins due to cross-crop interactions appear to be less likely in Mexico than in the United States due to characteristics of production cycles and small proportion of Bt cotton relative to conventional maize (only 27%; Martínez-Carrillo 2015, SIAP 2017). However, resistance monitoring efforts remain important to ensure that major changes in susceptibility are not occurring. Field observations on Bt cotton performance against target pests supplement the monitoring programs in place, the results of which indicate that *H. zea* susceptibility to Cry1Ac and Cry2Ab has not changed during the period Bollgard and Bollgard II have been cultivated in Mexico.

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