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Susceptibility of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) field populations to the Cry1F *Bacillus thuringiensis* insecticidal protein

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Abstract

The fall armyworm, *Spodoptera frugiperda* (Smith & Abbot) (Lepidoptera: Noctuidae), is a polyphagous insect pest affecting multiple crops. Fall armyworm in corn is managed with insecticides and corn hybrids expressing insecticidal proteins derived from *Bacillus thuringiensis* Berliner (Bt). The early detection of insect resistance is important for making appropriate management decisions and for implementing integrated pest management and insect resistance management recommendations. The objective of this study was to estimate susceptibility of fall armyworm populations to the Cry1F Bt insecticidal protein, emphasizing collections from locations where fall armyworm overwinters in the U.S. Fall armyworm neonates were exposed to artificial diet treated with increasing Cry1F concentrations, and mortality and growth inhibition were evaluated after 7 d. Differences in Cry1F susceptibility between the most susceptible and the most tolerant field populations were 2- and 6-fold for 2012 and 2013, respectively. These results are consistent with other susceptibility studies of Bt toxicity in other species although reduced susceptibility in one population collected from Florida may suggest resistance development.

Key Words: fall armyworm; resistance management; transgenic corn; Bt toxin

Resumo

A lagarta do cartucho, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), é uma praga polífaga que afeta várias culturas. A lagarta do cartucho no milho é controlada com o uso de inseticidas em milho híbrido que expressa proteínas com poder inseticida derivadas da bactéria *Bacillus thuringiensis* Berliner (Bt). A detecção precoce de casos de resistência em insetos é importante para decisões de manejo apropriadas e na implementação do manejo integrado de pragas e manejo de resistência de insetos. O objetivo desse estudo foi de estimar a susceptibilidade da lagarta do cartucho para a toxina Cry1F, enfatizando coletas de populações em diferentes locais dos Estados Unidos onde essa lagarta sobrevive durante o inverno. Neonatas da lagarta do cartucho foram expostas em dieta artificial tratada com aumento contínuo de concentrações da proteína Cry1F, e a mortalidade e a inibição de crescimento foram avaliados após 7 d da infestação. Diferenças entre a população de campo mais suscetível e a mais tolerante da lagarta do cartucho do milho foram 2 e 6 vezes em 2012 e 2013, respectivamente. Estes resultados são consistentes com estudos de susceptibilidade de Bt em outras espécies, embora a redução de susceptibilidade observada em uma população coletada na Florida possa sugerir o desenvolvimento de resistência.

Palavras Chave: lagarta do cartucho; manejo de resistência; milho transgênico; toxina Bt

The fall armyworm, *Spodoptera frugiperda* (Smith & Abbot) (Lepidoptera: Noctuidae), is one of the most important lepidopteran pests in the United States. It is native to the tropical regions of the western hemisphere from the United States to Argentina and is an important pest of corn (*Zea mays* L.; Poaceae) and many other crops throughout its distribution (Sparks 1979). The fall armyworm is a migratory pest and does not diapause (Luginbill 1928). Because it does not survive prolonged freezing, annual infestations affecting most of North America result from migrants that fly north from southern Texas and Florida, where winter temperatures are mild and host plants are continuously available (Nagoshi et al. 2012). This species displays a very wide host range but prefers grasses, including corn, sorghum (*Sorghum vulgare* Pers.; Poaceae), and several turf grass varieties (Sparks 1979; Capinera 1999).

Transgenic corn and cotton (*Gossypium hirsutum* L.; Malvaceae) that express genes from *Bacillus thuringiensis* Berliner (Bt) encoding insecti-

cidal proteins to control specific target pests have been deployed widely in the United States and globally since 1996 (Storer et al. 2010). The introduction of the transgenic corn event TC1507 (Herculex® I insect protection technology developed jointly by Dow AgroSciences and Dupont Pioneer), which expresses Cry1F protein, has provided a new opportunity to manage *S. frugiperda* populations. This product was launched in the United States and Canada in 2003, Argentina in 2005, Colombia in 2006, and Honduras and Brazil in 2009 (Storer et al. 2012). In 2006, potential resistance to TC1507 corn in Puerto Rico was first reported, and populations collected from several sites were largely unaffected by the Cry1F protein in bioassays with resistance ratios in excess of 1,000 (Storer et al. 2010). Resistance to Cry1F was shown to be autosomally inherited and highly recessive (Storer et al. 2010; Vélez et al. 2013).

In addition to Puerto Rico, resistance of *S. frugiperda* to Cry1F has been reported recently in Brazil (Farias et al. 2014). Similar to Puerto

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Rico, most Brazilian agriculture occurs in a tropical or subtropical climate, allowing corn production throughout the year. In 2011, *S. frugiperda* neonates were collected from damaged TC1507 corn fields in western Bahia after reports of reduced effectiveness of this trait. Results of bioassays showed that this population was able to survive on Cry1F corn plants under laboratory conditions and develop into normal adults (Farias et al. 2014).

High resistance ratios and the presence of Cry1F resistance alleles have also been reported in some populations from the southern United States (Vélez et al. 2013; Huang et al. 2014). Huang et al. (2014) reported significantly reduced efficacy of Cry1F corn in fields from Florida, Louisiana, and North Carolina where some of the field populations collected from non-Bt corn and from damaged Bt corn plants showed approximately 85-fold resistance. Further investigations based on F1 and F2 screens revealed the presence of Cry1F resistance alleles among populations from Florida, Louisiana, and Texas (Vélez et al. 2013; Huang et al. 2014). Recent findings suggest that fall armyworm populations from Puerto Rico have significant genetic exchange with populations in Florida and that there are migratory patterns involving substantial genetic exchange with the continental regions of the USA (Nagoshi et al. 2010, 2012). Such genetic exchange with Puerto Rico may result in the introduction of resistance alleles into Florida.

The possibility of resistance development in fall armyworm highlights the need for effective resistance monitoring programs that allow early detection of resistance and implementation of appropriate management decisions (Dennehy 1987). The first step in such programs involves monitoring the susceptibility among geographically distinct populations (Marçon et al. 1999). The objectives of this study were to estimate susceptibility of *S. frugiperda* populations of the USA to the Cry1F Bt insecticidal protein, and to determine the interpopulation variation in Cry1F susceptibility, emphasizing collections from areas where fall armyworm overwinters and populations that have been reported to have different origins and hosts (Nagoshi et al. 2012).

Materials and Methods

INSECT COLLECTIONS AND REARING

Eleven field-collected populations of fall armyworm were obtained from cooperators across the southern USA (Table 1). Populations were collected from either non-Bt corn or other grass species in 2012 and 2013 and from overwintering areas in Florida and Texas. Additionally, a migratory fall armyworm population was collected in Iowa. Field collections were delivered overnight to Custom Bio-Products, Maxwell, Iowa, or DM Crop Research, Polk City, Iowa, where the collections were maintained until egg production. The rearing process for field-collect-

ed populations consisted in the placement of pupae in 30 × 32 × 61 cm wire cages (Custom Bio-Products, Maxwell, Iowa) containing diet placed on a cotton pad inside of the bottom of a 57 g container (Dart Brand, Iowa-Des Moines Supply, Inc., Des Moines, Iowa) until emergence. Adult diet consisted of stale beer and was replaced every other day. Adults were allowed to mate and lay eggs on wax paper. Eggs were harvested daily and placed in 1 quart (0.946 L) food storage bags (Glad Products, Oakland, California) with moistened filter paper and held at 10 °C until shipping. Larvae were reared on multispecies lepidopteran diet (Southland Products, Lake Village, Arkansas).

For colony propagation, 2 neonate larvae were placed in 28 g translucent polystyrene soufflé portion cups (Iowa-Des Moines Supply, Inc., Des Moines, Iowa) with 7 mL of diet to minimize cannibalism, totalizing up to 300 cups. Pupation occurred within the cups. Pupae were transferred twice weekly to mating cages for adult emergence and egg production. Adults were held in an environmental chamber with a photoperiod of 15:9 h L:D at 30 ± 1 °C and 70 ± 10% RH during photophase and at 20 ± 1 °C and 60 ± 10% RH during scotophase. Larvae were held in 24 h scotophase at 26 ± 1 °C and 65 ± 10% RH. Eggs were harvested daily and neonates obtained from field-collected parents were considered the F1 generation and were used in most bioassays. In populations with low egg production, neonates from the F2 and F3 generations were used in some bioassays.

The eggs were delivered overnight to the University of Nebraska Insect Toxicology Laboratory (Lincoln, Nebraska), where they were used as a source of 1st instars for bioassay. A susceptible population (UNL), reared continuously in the absence of selection since 1997 and regularly screened to monitor changes in insecticide susceptibility, was purchased from BioServ (Frenchtown, New Jersey) and established at the University of Nebraska Insect Toxicology Laboratory. Adults were placed in 31 × 23 cm hermit crab cages (Florida Marine Research, Sarasota, Florida) with adult diet placed on a cotton pad inside of the bottom of a 100 × 15 mm Petri dish (Fisherbrand, Waltham, Massachusetts) and replenished daily. Adult diet consisted of stale beer containing ascorbic acid (1.5 mg/mL), propionic acid (2.1 µL/mL), and aureomycin (0.5 mg/mL) (Vélez et al. 2013). Adults were held in an environmental chamber with a photoperiod of 14:10 h L:D at 27 ± 1 °C and 75 ± 10% RH during photophase and at 22.5 ± 1 °C and 60 ± 10% RH during scotophase. Adults were allowed to mate, and eggs were deposited on wax paper that surrounded the cage.

BT TOXIN

The Cry1F used in diet bioassays was expressed in BtG8 cells grown in CYS2 medium with tetracycline for 6 d at 30 °C. Cells were harvested by centrifugation, and the pellets were washed 5 times with 0.5 M NaCl and twice with water. Washed pellets were stored at -20 °C. Pellets

Table 1. Source description of *Spodoptera frugiperda* populations used to estimate susceptibility to Cry1F from *Bacillus thuringiensis*.

Population	Year of collection	Generation	Month of collection	Host plant	Initial number of larvae
Bradenton, FL	2012	F1	Jul	sweet corn	340
Lubbock, TX	2012	F1 & F2	Jun/Jul	corn	309
Muleshoe, TX	2012	F1	Aug	sorghum	260
Altoona, FL	2012	F1	Aug	corn	258
Bradenton II, FL	2012	F1	Oct	sweet corn	300
Colhoun, TX	2012	F3	Sep	Bermuda grass	375
Bradenton, FL	2013	F1 & F2	May	sweet corn	300
Palm Beach, FL	2013	F1	May	corn	300
Cameron, TX	2013	F1	May/Jun	sweet corn	300
Lubbock, TX	2013	F1	Sep	corn	300
Johnston, IA	2013	F1	Sep	corn	500

were lysed with 50 mM sodium carbonate pH 11.7, containing 10 mM dithiothreitol overnight at 4 °C. Aliquots of 1.6 mg and 40 mg were flash frozen in liquid nitrogen and then lyophilized.

Protoxin preparations were quantified by gel electrophoresis and densitometry (Crespo et al. 2008) and adjusted to 0.8 mg/mL based on the 60 to 65 kDa peptides observed after sodium dodecyl sulfate–polyacrylamide gel electrophoresis and compared to a standard curve prepared with a bovine serum albumin standard (>95% purity). Quantified preparations were stored at –80 °C.

BIOASSAYS

Bioassays were performed based on methods described by Marçon et al. (1999) in 128 well bioassay trays (CD International, Pitman, New Jersey). One mL of European corn borer wheat germ–based diet (Lewis & Lynch 1969) was dispensed into each well and allowed to solidify. Seven concentrations of the toxin were used for LC50 determinations. Dilutions were made in 0.1% Triton-X 100 non-ionic detergent to obtain uniform spreading on the diet surface, and 30 µL of dilution was applied in each well. The negative control wells were treated with 30 µL of 0.1% Triton-X 100 (Vélez et al. 2013). The highest concentration of the serial dilution was 270 ng/cm².

The treated wells were allowed to dry, and 1 randomly selected neonate (unfed and <12 h after hatching) was transferred to each well with a fine camel hair paintbrush. The wells were covered with vented lids (BIO-CV-16, C-D International), and trays were held at 27 °C, 24 h scotophase, and 80% RH. Mortality and group larval weights were recorded 7 d after infestation. Larvae that had not grown beyond 1st instar and weighed ≤0.1 mg were considered dead. Therefore, severe growth inhibition and death were considered as mortality. In each experiment, bioassays were replicated from 4 to 16 times for each population depending on availability of neonates. Each replicate used 16 larvae per concentration.

STATISTICAL ANALYSES

Mortality data were analyzed by probit analysis (Finney 1971) and POLO-PC (LeOra Software 1987) to estimate LC50 and LC90 values with their respective 95% confidence intervals, slopes, and standard errors. Sensitivity ratios were calculated with the concentration–response statistics based on mortality, by the ratio of the LC50 from the most tolerant and most susceptible field populations. These values were

considered significant if the 95% confidence limit (CL) of the ratio did not include 1.0 (Wheeler et al. 2005). The confidence intervals for each ratio were calculated based on the intercepts and slopes of 2 probit lines and estimates of their variance–covariance matrixes (Robertson et al. 2007). Larval weights were transformed to percentage of growth inhibition relative to the controls, and these data were analyzed by nonlinear regression (PROC NLIN, SAS 9.4) fitted to a probit model (2003–2012 SAS Institute, Cary, North Carolina).

Results

MORTALITY ASSAYS

Results of Cry1F bioassays for fall armyworm field populations collected in 2012 are presented in Table 2. LC50 values and the respective confidence intervals ranged from 8.32 (6.86–9.95) (Muleshoe, Texas) to 14.53 (11.48–18.12) ng/cm² (Lubbock, Texas) in 2012. The LC50 of the susceptible laboratory population in 2012 was 2.89 (2.39–2.45) ng/cm². For 2013 collections, the LC50 values ranged from 3.61 (2.73–4.65) (Bradenton, Florida) to 22.11 (13.02–36.84) ng/cm² (Palm Beach, Florida), whereas the LC50 of the susceptible laboratory population was similar to the results of 2012, with 2.79 (2.39–3.26) ng/cm². Therefore, differences in Cry1F susceptibility between the most susceptible and the most tolerant field populations were 2- and 6- fold for 2012 and 2013, respectively. The slopes of the mortality regressions were similar between field-collected populations in both years, but slightly higher in laboratory colonies. Sensitivity ratio values were close to 1 for most populations tested. In 2013, 1 population from Palm Beach, Florida, showed a sensitivity ratio of 6.12 (5.02–7.46).

GROWTH INHIBITION ASSAYS

Results based on growth inhibition of *S. frugiperda* treated with Cry1F are presented in Figs. 1 and 2. EC50 values and their respective 95% confidence intervals ranged from 0.10 (0.07–0.14) (Muleshoe, Texas) to 0.48 (0.37–0.6) ng/cm² (Lubbock, Texas), whereas the EC50 of the susceptible laboratory population was 0.33 (0.32–0.34) ng/cm². EC50 values ranged from 0.10 (0.08–0.12) (Johnston, Iowa) to 0.29 (0.23–0.34) ng/cm² (Palm Beach, Florida) in 2013 studies, whereas the EC50 of the susceptible laboratory population was 0.41 (0.4–0.42) ng/cm². The range of variation in susceptibility indicated by growth inhibi-

Table 2. Probit analysis of mortality and sensitivity ratios of *Spodoptera frugiperda* neonates exposed to the Cry1F protein from *Bacillus thuringiensis*.

Population	Year of collection	n	Slope ± SE	LC50 (95% CI) ^a	LC90 (95% CI) ^a	χ ²	df	SR50 ^b	95% CI
Bradenton, FL	2012	767	1.68 ± 0.10	13.04 (10.84–15.69)	75.46 (57.95–104.04)	2.18	5	1.57*	1.20–2.04
Lubbock, TX	2012	1,275	2.08 ± 0.13	14.53 (11.48–18.12)	60.22 (45.37–87.54)	7.47	5	1.75*	1.38–2.21
Muleshoe, TX	2012	767	2.17 ± 0.18	8.32 (6.86–9.95)	32.35 (25.72–43.33)	1.60	5	—	—
Altoona, FL	2012	510	1.83 ± 0.15	10.10 (6.77–14.90)	50.56 (31.33–105.03)	8.80	5	1.21	0.91–1.62
Bradenton II, FL	2012	763	1.51 ± 0.12	13.08 (10.35–16.27)	92.03 (68.53–133.26)	4.97	5	1.57*	1.17–2.11
Colhoun, TX	2012	767	1.38 ± 0.09	10.23 (8.11–12.78)	60.22 (45.37–87.54)	1.99	5	1.23	0.91–1.65
UNL ^c	2012	768	2.25 ± 0.20	2.89 (2.39–2.45)	10.72 (8.50–14.51)	0.07	5	—	—
Bradenton, FL	2013	1,518	1.95 ± 0.11	3.61 (2.73–4.65)	16.46 (12.02–25.26)	10.29	5	—	—
Palm Beach, FL	2013	1,572	1.39 ± 0.06	22.11 (13.02–36.84)	183.83 (96.00–535.96)	38.45	5	6.12*	5.02–7.46
Cameron, TX	2013	509	2.25 ± 0.23	13.86 (11.03–17.13)	51.50 (39.26–74.28)	2.44	5	3.84*	2.96–4.98
Lubbock, TX	2013	1,020	2.11 ± 0.13	5.19 (4.14–6.47)	20.99 (15.74–30.73)	6.58	5	1.44*	1.17–1.76
Johnston, IA	2013	764	1.47 ± 0.09	6.97 (5.07–9.53)	51.74 (33.84–91.86)	7.08	5	1.93*	1.79–3.23
UNL ^c	2013	767	2.44 ± 0.18	2.79 (2.39–3.26)	9.37 (7.57–12.25)	2.36	5	—	—

^aLethal concentrations in ng/cm².

^bSensitivity ratios calculated based on LC50 of most the tolerant field populations relative to the most susceptible in each year of study.

^cLC values are significantly different from the most susceptible field population in each year of study.

^dSusceptible strain maintained at the University of Nebraska.

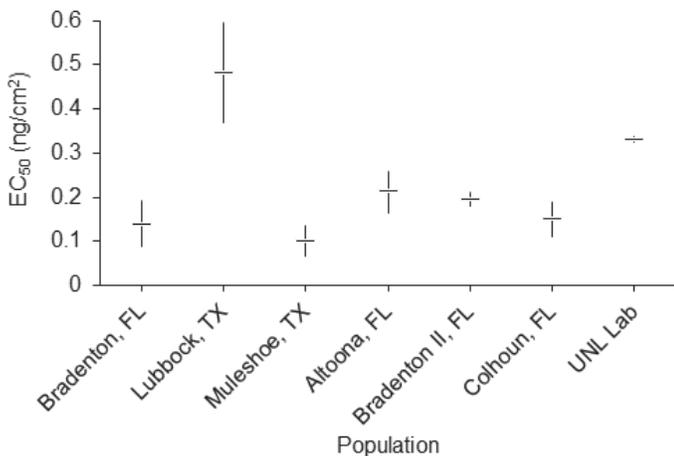


Fig. 1. EC₅₀s estimated by nonlinear regression of growth inhibition fitted to a probit model and the 95% confidence intervals of *Spodoptera frugiperda* neonates field collected in 2012 and exposed to the Cry1F *Bacillus thuringiensis* toxin.

tion between the most susceptible and the most tolerant populations was approximately 5- and 3- fold for the 2012 and 2013 studies, respectively.

Discussion

Estimates of lethal concentrations based on mortality and effective concentrations based on growth inhibition observed among *S. frugiperda* populations exposed to Cry1F toxin showed similar variation, ranging from 2- and 6- fold among field populations. The pooled data for mortality and growth inhibition for each year illustrate that growth inhibition provides a more sensitive estimate of susceptibility than mortality for *S. frugiperda* field populations (Fig. 3), indicating that larvae are responding to concentrations of Cry1F that do not cause mortality. However, given the labor and time involved with weighing larvae, mortality as described in these bioassays represents a less costly measure of susceptibility that provides similar measures of variation. The variation in *S. frugiperda* susceptibility to insecticidal protein Vip3A derived from *B. thuringiensis* was reported by Bernardi et al. (2014), indicating a 7-fold variation in susceptibility. Additionally, similar variation (between 3- to 8- fold) in susceptibility studies to other

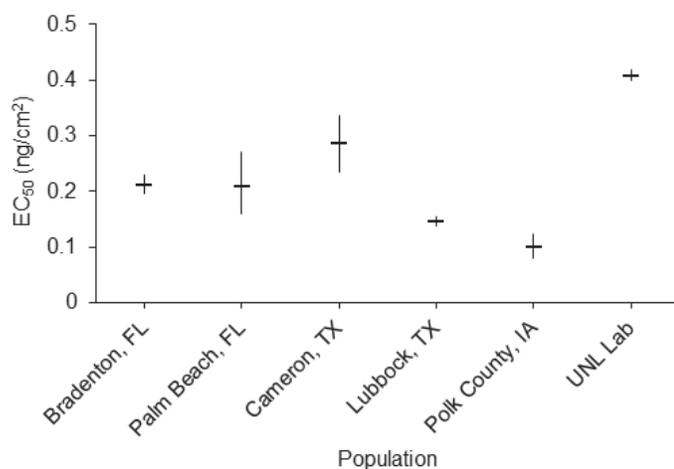


Fig. 2. EC₅₀s estimated by nonlinear regression of growth inhibition fitted to a probit model and the 95% confidence intervals of *Spodoptera frugiperda* neonates field collected in 2013 and exposed to the Cry1F *Bacillus thuringiensis* toxin.

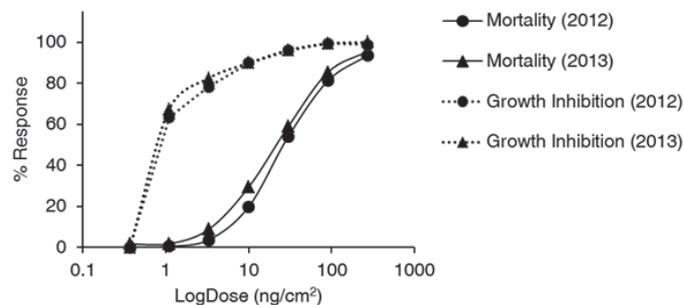


Fig. 3. Mean percentage of mortality and mean percentage of growth inhibition responses of *Spodoptera frugiperda* for 2012 and 2013 field-collected populations exposed to Cry1F *Bacillus thuringiensis* toxin.

Bt toxins has been observed (Marçon et al. 1999; Stone & Sims 1993; Blanco et al. 2008).

Previous studies that specifically measured *S. frugiperda* susceptibility to Cry1F showed high rates of survival on Cry1F-expressing corn hybrids in the field and relatively high frequency of resistance alleles in certain populations from southern Florida (Vélez et al. 2013; Huang et al. 2014). In addition to Florida, resistant alleles were detected in Louisiana and Texas (Vélez et al. 2013; Huang et al. 2014) although at lower frequencies. The higher frequency of resistance alleles observed in Florida is not uniform across all collections (Vélez et al. 2013) and may suggest that there is local selection from exposure to Cry1F-expressing hybrids. These reports are consistent with the general reduced susceptibility of the Palm Beach, Florida, population bioassayed in 2013, which was the least susceptible of all populations assayed. In contrast to the higher LC₅₀ in this population, our results did not show any general pattern of reduced susceptibility in other populations. The reduced susceptibility of the Palm Beach population may be the result of increased use of Cry1F corn and increased selective pressures in localized areas because other populations assayed from Florida were not different from the overall baseline.

In general, the overall variation of LC and EC estimates observed among overwintering fall armyworm populations indicates that the populations surveyed in this study are still susceptible to Cry1F. In addition, the susceptibility of the one migratory population from Iowa was similar to that of the other populations sampled and suggests that migratory populations remain susceptible to Cry1F. In contrast, Farias et al. (2014) reported field-evolved resistance of fall armyworm to Cry1F in populations from Brazil with resistance ratios >5000-fold based on diet overlay bioassays. However, Nagoshi et al. (2007) reported that corn-strain populations from Brazil identified as being resistant to Cry1F were different from corn-strain populations found in Florida based on comparisons of the frequency distribution of haplotypes using polymorphism in the mitochondrial *COI* gene. Moreover, Florida populations are more closely related to populations from Puerto Rico than Brazil populations (Nagoshi et al. 2010). It is possible that substantial gene exchanges with Caribbean island populations, especially Puerto Rico, where Cry1F resistance is widely distributed, in combination with localized selection pressure may contribute to increased tolerance of populations in the future.

Annual resistance monitoring programs for target pest species with laboratory bioassays are an important component of insect resistance management programs (Shelton & Zhao 2009). The regular assessment of susceptibility of target pest populations from areas where the risk of resistance evolution is high should be implemented in order to allow resistance to be detected before it becomes unmanageable (Yu 2008).

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