



Performance of Cry1A.105-selected fall armyworm (Lepidoptera: Noctuidae) on transgenic maize plants containing single or pyramided Bt genes[☆]



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ABSTRACT

Cry1A.105 is a Cry protein expressed in some transgenic *Bacillus thuringiensis* (Bt) maize products. In this study, performance of five populations of fall armyworm, *Spodoptera frugiperda* (J.E. Smith), were evaluated on four non-Bt and eight commercial and experimental Bt maize hybrids/lines (hereafter referred as maize products). The five insect populations included one Cry1A.105-susceptible strain, two Cry1A.105-resistant strains, and two F₁ heterozygous genotypes. The eight Bt maize hybrids/lines consisted of five single-gene Bt maize products containing Cry1A.105, Cry2Ab2, Cry1F, or Cry1Ab protein, and three pyramided Bt maize products expressing Cry1A.105/Cry2Ab2, Cry1A.105/Cry2Ab2/Cry1F, or Cry1Ab/Vip3A for targeting aboveground lepidopteran maize pests. In the study, neonates of each population were tested on leaf tissues in the laboratory and whole plants in the greenhouse. Cry1A.105 and Cry1F maize killed 92.2–100% susceptible larvae in both test methods, while resistant larvae survived well on these two maize products. Performance of the two F₁ populations on Cry1A.105 and Cry1F maize varied between the two test methods. In leaf tissue bioassay, Cry1Ab maize was marginally effective against the susceptible population. In contrast, few live larvae and little leaf injury from any of the five populations were observed on Cry2Ab2 and the three pyramided Bt maize products. The results of this study showed evidence of cross resistance of the Cry1A.105-resistant *S. frugiperda* to Cry1F and Cry1Ab maize, but not to the Bt maize products containing Cry2Ab2 or Vip3A. Data generated from this study will be useful in developing resistance management strategies for the sustainable use of Bt maize technology.

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1. Introduction

Transgenic crops (e.g. maize, cotton, and soybean) containing *Bacillus thuringiensis* (Bt) genes have been widely planted for controlling some major insect pests (James, 2014). As with many other pest management tools, evolution of resistance in the pest populations is a threat to the sustainable use of Bt crop technology. Since the first Bt crops were commercialized in 1996, great efforts in implementation of resistance management plans have been

made for the sustainable use of Bt crop technology (Ostlie et al., 1997; Huang et al., 2011; Matten et al., 2012; Tabashnik et al., 2013). However, due to the intensive use of Bt crops over the last 20 years, field resistance resulting in insect control problems has occurred in at least four major target species in several countries (van Rensburg, 2007; Storer et al., 2010; Dhurua and Gujar, 2011; Gassmann et al., 2011; Farias et al., 2014a, 2014b; Huang et al., 2014).

Fall armyworm, *Spodoptera frugiperda* (J.E. Smith), is a target pest of both Bt maize and Bt cotton in North and South America, as well as a target pest of Bt soybean in Brazil (Farias et al., 2014a; Yang et al., 2016). Up to now, *S. frugiperda* is the first and only target insect that has developed field resistance to Bt crops at multiple locations across different countries and continents (Storer et al., 2010; Farias et al., 2014a, 2014b; Huang et al., 2014). In Puerto Rico, Cry1F maize (event TC1507) was commercially planted to

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control *S. frugiperda* in 2003, while field control problems occurred three years later (Storer et al., 2010). Similarly, in Brazil, Cry1F maize was first commercially available in the 2009/2010 season for controlling *S. frugiperda* and other lepidopteran pests. Field resistance in *S. frugiperda* was documented in 2011, and currently the resistance has spread throughout the Western Bahia region of the country (Farias et al., 2014a, 2014b). In addition, field resistance of *S. frugiperda* to Cry1F maize has also been documented in some areas of the southern United States (Huang et al., 2014).

To slow the development of resistance, maize hybrids containing two or more pyramided Bt genes have been commercialized in the United States and several other countries (Ghimire et al., 2011; Matten et al., 2012; Buntin and Flanders, 2015). Relative to the single-gene Bt maize, these pyramided Bt maize products are usually more effective against some target pests, especially Noctuidae species such as the corn earworm (*Helicoverpa zea* [Boddie]) and *S. frugiperda* (Burkness et al., 2010; Niu et al., 2014; Yang et al., 2013, 2015). The widespread Cry1F resistance in *S. frugiperda* has sparked concerns about the durability of the pyramided Bt crops (Huang et al., 2014; Bernardi et al., 2015; Santos-Amaya et al., 2015; Yang et al., 2016). One of the Bt proteins expressed in some pyramided Bt maize is Cry1A.105. This Bt toxin is a chimeric protein incorporating domains I and II from Cry1Ab or Cry1Ac, domain III from Cry1F, and the C-terminal domain from Cry1Ac (Biosafety Clearing-House, 2014). During 2011, two Cry1A.105-resistant strains of *S. frugiperda* were isolated from field populations collected in Florida (Huang et al., 2016). In this study, we evaluated the survival and plant injury of these two Cry1A.105-resistant populations, along with a susceptible population and two F₁ heterozygous genotypes, on commercial and experimental Bt maize hybrids/lines containing single or pyramided Bt genes (hereafter, 'maize products' refers to both commercial hybrids and non-commercially experimental lines). Information generated from this study should be useful in understanding the cross-resistance among the commonly used Bt maize traits and developing effective resistant management strategies for the sustainable use of Bt maize technology.

2. Materials and methods

2.1. Insect sources

Three populations of *S. frugiperda* including a known Cry1A.105-susceptible strain (SS) and two Cry1A.105-resistant (FL32 and FL67) strains were used as the original insect sources in the study. SS was collected from maize fields near Weslaco, Texas, in 2013. SS was susceptible to purified proteins of Cry1A.105, Cry2Ab2, and Cry1F, as well as to maize leaf tissues and whole plants expressing Cry1A.105, Cry2Ab2, Vip3A, and Cry1F proteins (Huang et al., 2014, 2016). FL32 and FL67 were isolated from two single-pairing families collected from maize fields in Collier County, Florida, in 2011 (Huang et al., 2016). Both FL32 and FL67 have been shown to possess major resistance alleles to Cry1A.105 maize plants by using an F₂ screen and have demonstrated a significant level of resistance (>116-fold) to the Cry1A.105 protein. The two resistant populations also survived and developed well on whole plants of Cry1A.105 maize in the greenhouse (Huang et al., 2016). In the laboratory, larvae of the three populations were reared individually on maize leaf tissues or a meridic diet (Ward's Stonefly *Heliothis* diet, Rochester, NY) as described in Niu et al. (2013). Before FL32 and FL67 were used in the current study, they had been backcrossed with SS twice and reselected for resistance with Cry1A.105 maize leaf tissues, as described in Dangal and Huang (2015).

In addition, two F₁ heterozygous genotypes, FL32-RS and FL67-RS, were developed by reciprocal crosses of the two resistant

strains with SS. FL32-RS was a mixture of the two F₁ heterozygous genotypes produced from the reciprocal crosses of FL32 with SS, while FL67-RS was a mixture of the two F₁ heterozygous genotypes produced from the reciprocal crosses of FL-67 with SS.

2.2. Maize products

Performance of the five insect populations (SS, FL32, FL67, FL32-RS, and FL67-RS) described above was examined against 12 maize products, which consisted of four non-Bt and eight Bt maize products (Table 1). The eight Bt maize products included five single-Bt and three pyramided Bt maize hybrids/lines. The five single-Bt maize products were Cry1AP, Herculex[®] I (abbreviated product ID, HX1), YieldGard[®] (YG), Cry2AP, and Cry2APH; and the three pyramided products were Genuity[®] VT Double Pro[™] (VT2P), Genuity[®] SmartStax[™] (SMT), and Agrisure[®] Viptera[™] 3111 (VIP3). Cry1AP, Cry2AP, and Cry2APH were three non-commercially experimental lines provided by Monsanto Company (St. Louis, MO). Cry1AP contains a single Bt gene encoding Cry1A.105, which targets aboveground lepidopteran pests including *S. frugiperda* (Huang et al., 2014). Both Cry2AP and Cry2APH contain a single Bt gene encoding Cry2Ab2, but Cry2APH expresses a higher level of the Cry2Ab2 protein than does Cry2AP (Huang et al., 2014; Niu et al., 2016). HX1 expresses the Cry1F protein (event TC1507) and YG contains the Cry1Ab gene for controlling lepidopteran pests. VT2P expresses Cry1A.105 and Cry2Ab2, SMT expresses these two proteins plus the Cry1F protein, and VIP3 expresses both Cry1Ab and Vip3A; all of which target aboveground maize lepidopteran pests (Buntin and Flanders, 2015). In addition, SMT also produces Cry3Bb1 and Cry34/35Ab1, and Vip3 also expresses mCry3A. These four Bt proteins target the belowground maize rootworms *Diabrotica* spp (Coleoptera: Chrysomelidae), with no activity for moth pests. Each of the four non-Bt maize products tested in this study was closely related to one or two of the Bt maize products (Table 1).

Two seeds of a maize product were planted in each 18.9-L plastic pot containing ~5 kg of standard potting soil mixture (Perfect Mix[™], Expert Gardener products, St. Louis, MO) in a greenhouse located in Baton Rouge, LA, as described in Wangila et al. (2012). The non-expression for each non-Bt maize product or expression of the expected Bt protein(s) for each Bt maize product was confirmed using ELISA-based assays (EnviroLogix, Quantiplate[™] kits, Portland, ME). In this study, performance of the five populations of *S. frugiperda* on the non-Bt and Bt maize products was evaluated by two methods: leaf tissue bioassay in the laboratory and whole-plant test in the greenhouse.

2.3. Leaf tissue bioassay in the laboratory

Two independent trials were performed with the leaf tissue bioassay in the laboratory. Trial-I evaluated SS on nine maize products, and FL32 and FL67 on all 12 maize products listed in Table 1. Owing to limited insect supply, SS was not evaluated on the three pyramided maize products in Trial-I. Trial-II examined all five insect populations on 11 of the 12 maize products (Cry2AP was not included due to limited seed supply). In the leaf tissue bioassay, fully-expanded leaves from maize plants at the V5–V8 stages were removed from greenhouse-grown plants and used in the leaf tissue bioassay described in Niu et al. (2013). The leaves were cut into pieces of approximately 3–4 cm in length. Two to three pieces of leaf tissues were then placed in each well of 32-well C-D International trays (Bio-Ba-32, C-D International, Pitman, NJ). Four neonates (<24 h old) of each population were placed on the surface of the leaf tissues in each well (Niu et al., 2013). Bioassay trays containing leaf tissues and neonates were placed in growth chambers maintained at 28 °C, 50% RH, and a 16-h:8-h (L:D) photoperiod.

Table 1
Non-Bt and Bt maize products evaluated in this study.

Maize product ID	Bt gene	Maize product	Traits ^a	Event
NBt	None	Pioneer 31P40	Non-Bt	Closely related to Pioneer 31D59
	None	DKC 61-22	Non-Bt	Closely related to DKC 61-49 and DKC 61-21
	None	N78N-GT	Non-Bt	Closely related to N78N-3111
	None	Expl	Non-Bt experimental line	Closely related to Cry1A.105Ln and Cry2Ab2Ln
Cry1AP HX1	Cry1A.105	Cry1A.105Ln	Experimental line	Not available
	Cry1F	Pioneer 31D59	Herculex [®] I	TC1507
YG Cry2AP	Cry1Ab	DKC 69-70	YieldGard [®]	MON 810
	Cry2Ab2	Cry2Ab2Ln	Experimental line with low expression of Cry2Ab2 protein	Not available
Cry2APH	Cry2Ab2	Cry2Ab2Hn	Experimental line with high expression of Cry2Ab2 protein	Not available
VT2P SMT	Cry1A.105, Cry2Ab2	DKC 64-04	Genuity [®] VT Double Pro [™]	MON 89034
	Cry1A.105, Cry2Ab2, Cry1F, Cry3Bb1, Cry34/35Ab	DKC 62-08	Genuity [®] SmartStax [®]	MON 89034 + TC1507 + MON 88017+DAS-59112-7
VIP3	Vip3A, Cry1Ab, mCry3A	N78N-3111	Agrisure [®] Viptera [™] 3111	Bt11 + MIR162 + MIR604

^a Maize products that are not labeled as an experimental line are commercial hybrids.

Larval mortality was recorded on the 7th day after release of the neonates. In each trial, there were four replications for each combination of maize product and insect population, and each replication contained 32 neonates in eight wells ($n = 32 \times 4 = 128$).

2.4. Whole-plant test in the greenhouse

As in the leaf tissue bioassay, two independent trials were conducted in the greenhouse tests: each trial evaluated the performance of all five insect populations on 11 of the 12 maize products listed in Table 1 (Cry2AP was not included in the greenhouse tests owing to limited seed supply.) In each trial, four neonates (<24 h old) of an insect population were manually placed into the whorl of a plant at the V5–V9 stage. Treatments in each trial were replicated four times in a randomized complete block design with one pot (2 plants) per replication. Maize leaf injury ratings were determined using the Davis scale of 1 (no damage or few pinholes) to 9 (most leaves with long lesions) (Davis et al., 1992) on the 14th day after larval inoculation. Plants containing live larvae were recorded immediately after rating the leaf injury as described in Niu et al. (2014).

2.5. Data analysis

In both leaf tissue bioassay and whole-plant test, the performance of each insect population was similar among the four non-Bt maize products in each trial (see Results); thus, data on larval survival in both tests, as well as the leaf injury ratings in the whole-plant test, were pooled across the four non-Bt maize products. To normalize treatment variances for data analysis, the raw data of larval survivorship rate (recorded from the leaf tissue bioassays) and percentages of plants with live larvae (recorded in the whole-plant tests) (Niu et al., 2014) were transformed using the arcsine of ($x^{0.5}$), while data on the leaf injury rating were transformed to the log ($x+1$) scale (Zar, 1984). The transformed data were analyzed with a two-way analysis of variance for each of the two leaf tissue bioassays and greenhouse tests (SAS Institute, 2010), with maize product and insect population as the two main factors. In addition, because the overall results between the two greenhouse trials were generally consistent, data for each variable measured in the greenhouse trials were pooled across the two trials. The pooled data were then analyzed using mixed models with trial as a random

factor (SAS Institute, 2010). Analysis with the mixed models was not performed for leaf tissue bioassays due to the differences in the insect populations and maize products evaluated between the two trials. For each trial and the combined data, treatment means were separated using LSMEANS tests at $\alpha = 0.05$ level. Untransformed means were presented in the figures.

The effective dominance levels (D_{ML}) of the two Cry1A.105-resistant populations on the leaf tissues and whole plants of Cry1AP and HX1 were estimated by using the method described in Roush and McKenzie (1987). To calculate the dominance levels, the observed larval survival data of an insect population on a Bt maize product were first corrected to the survival on the non-Bt maize products using the method described in Abbott (1925). The corrected-survivorship rates were then used to calculate the dominance levels for each of the two test methods. D_{ML} for the leaf tissue bioassay was estimated based only on Trial-II because F₁ heterozygous insect populations were not included in Trial-I, whereas for the whole-plant tests, D_{ML} was based on pooled data from the two trials.

3. Results

3.1. Larval survival of *S. frugiperda* on leaf tissues of non-Bt and Bt maize products containing single or pyramided genes

The effects of insect population, maize product, and their interaction on larval survivorship were all significant for both trials of the leaf tissue bioassay (Table 2). The overall performance of each of the three insect populations (SS, FL32, and FL67) that were evaluated in both trials was consistent between the two trials across all the maize products, with few exceptions. In general, larvae of the three populations survived well on leaf tissues of the non-Bt maize products with a survivorship rate of 57.6–73.0% in Trial-I and 44.1–72.3% in Trial-II after 7 days (Fig. 1). SS on leaf tissues of Cry1AP (Cry1A.105) maize showed a survivorship rate of <1% in both trials. FL32 on Cry1AP maize leaf tissues showed a survivorship of 70.3% in Trial-I and 53.9% in Trial-II, while the survivorship of FL67 was lower, 26.6–32.0% in the two trials (Fig. 1). In Trial-II, which included the two F₁ heterozygous populations, leaf tissues of Cry1AP was effective against both RS populations, with a zero survivorship after 7 days for FL32-RS and 7% for FL67-RS (Fig. 1). The effective dominance level, D_{ML} , based on the leaf

Table 2
Analysis of variance on the data from the leaf tissue bioassay in the laboratory and whole-plant test in the greenhouse.

Parameter & trial	Source of variation	df ₁ , df ₂ ^a	F-value	P-value
Leaf tissue bioassay				
Larval survival in trial-I ^b	Insect	2,100	67.31	<0.0001
	Maize	8,100	281.26	<0.0001
	Insect × maize	13,100	38.63	<0.0001
Larval survival in trial-II	Insect	4,120	41.33	<0.0001
	Maize	7,120	195.71	<0.0001
	Insect × maize	28,120	9.59	<0.0001
Whole-plant test				
Leaf injury rating in trial-I	Insect	4,161	5.70	0.0003
	Maize	7,161	140.04	<0.0001
	Insect × maize	28,161	3.61	<0.0001
Leaf injury rating in trial-II	Insect	4,171	7.95	<0.0001
	Maize	7,171	148.91	<0.0001
	Insect × maize	28,171	2.67	<0.0001
Leaf injury rating-pooled	Insect	4,374	13.03	<0.0001
	Maize	7,374	284.8	<0.0001
	Insect × maize	28,374	5.33	<0.0001
Larval survival in trial-I	Insect	4,164	0.71	0.5852
	Maize	7,164	22.12	<0.0001
	Insect × maize	28,164	0.95	0.5450
Larval survival in trial-II	Insect	4,171	1.37	0.2478
	Maize	7,171	28.78	<0.0001
	Insect × maize	28,171	0.91	0.6027
Larval survival-pooled	Insect	4,374	2.00	0.0933
	Maize	7,374	54.00	<0.0001
	Insect × maize	28,374	1.53	0.0438

^a Df₁ is the degree of freedom of the nominator and df₂ is the degree of freedom for the denominator (error).

^b In trial-I of the leaf tissue bioassay, survival of the SS population on the three pyramided Bt maize products (VT2P, SMT, and VIP3) was not evaluated.

tissue bioassay was 0 for FL32 and 0.21 for FL67, indicating recessive or incompletely recessive resistance on the Cry1AP leaf tissues (Table 3).

FL32 and FL67 also exhibited significant cross-resistance to HX1 (Cry1F) maize. HX1 maize leaf tissues killed 92.2–96.1% SS larvae in the 7-day assays in both trials, while FL32 and FL67 showed a survivorship rate of 70.3–73.4% and 20.3–31.3%, respectively (Fig. 1). In Trial-II, the larval survivorship of FL32-RS (14.8%) was not significantly ($P > 0.05$) different from that of SS (7.8%), but significantly ($P \leq 0.05$) lower than that of FL67-RS (37.1%). The survivorship of FL67-RS was similar ($P > 0.05$) to that of FL67 (Fig. 1). The calculated D_{ML} based on the leaf tissue bioassay with the HX1 maize was 0.27 for FL32 and 1 for FL67, suggesting that the resistance of the two populations was more dominant on HX1 (Cry1F) leaf tissues than on Cry1AP (Cry1A.105) leaf tissues (Table 3). Leaf tissues of Cry1Ab maize showed marginally effectiveness against the susceptible population, but, in general, were ineffective against the two resistant populations. SS on Cry1Ab leaf tissues exhibited an average survivorship rate of 40.1% in the two trials, while the two resistant and the two F₁ heterozygous populations showed a survivorship of 52.2% and 49.9%, respectively (Fig. 1), suggesting that both Cry1A.105-resistant populations were also cross-resistant to the Cry1Ab maize leaf tissues.

However, neither of the Cry1A.105-resistant populations showed any cross-resistance to the maize products containing the Cry2Ab2 protein (Cry2AP and Cry2APH). Larval survivorship of SS on leaf tissues of the low-expressing Cry2Ab2 line (Cry2AP) was 16.4% in Trial-I, and the corresponding survivorship of FL32 and FL67 was even lower, $\leq 5.5\%$. On the high-expressing Cry2Ab2 maize line (Cry2APH), survivorship of the five insect populations was 0–0.8% in the two trials (Fig. 1). The three pyramided Bt maize products, VT2P, SMT, and VIP3 were effective against both Cry1A.105-susceptible and -resistant *S. frugiperda*. Leaf tissues of

these three Bt maize products killed 100% of SS in Trial-II (the only trial where SS was evaluated on pyramided products), and 75.0–100% of the other four populations in the two trials (Fig. 1).

3.2. Leaf injury ratings of *S. frugiperda* to non-Bt and Bt maize containing single or pyramided genes in the whole-plant tests

Leaf injury ratings caused by the five populations of *S. frugiperda* were generally consistent between the two trials in the greenhouse. The effects of maize product, insect population, and their interaction on leaf injury ratings were all significant for each of the two trials and for the pooled data analysis (Table 2). There were no significant ($P > 0.05$) differences in the leaf injury ratings of non-Bt maize plants among the five insect populations for each trial and for the pooled data. After 14 days, when the trials were terminated, all five populations caused heavy leaf injuries to the non-Bt maize plants, with an overall average leaf injury rating of 7.4 for the pooled data (Fig. 2).

SS caused little damage to Cry1A.105 (Cry1AP) maize plants, with a leaf injury rating of 1.6 for the pooled data (Fig. 2). Compared to SS, both resistant populations caused significantly ($P \leq 0.05$) greater injury to Cry1AP. The leaf injury ratings of Cry1AP caused by FL67 (4.9 in Trial-I and 5.8 in Trial-II) were somewhat greater than those caused by FL32 (3.0 in Trial-I and 3.7 in Trial-II). The difference between the two resistant populations was not significant ($P > 0.05$) for the analysis based on each of the individual trials, but significant ($P \leq 0.05$) for the pooled data analysis (Fig. 2). The leaf injury rating to Cry1AP caused by the two heterozygous populations (FL32-RS and FL67-RS) was significantly ($P \leq 0.05$) greater than that caused by SS. In general, FL32-RS and FL32 caused similar ($P > 0.05$) injury to Cry1AP, while the leaf injury caused by FL67-RS was somewhat lower than that caused by FL67. The difference between FL67-RS and FL67 was not significant ($P > 0.05$) for Trial-II, but significant ($P \leq 0.05$) for Trial-I and the pooled data analysis (Fig. 2).

As observed in the leaf tissue bioassay, both FL32 and FL67 showed cross-resistance to Cry1F (HX1) maize in the whole-plant test. HX1 was effective against SS and had little leaf injury. Compared to SS, both FL32 and FL67 caused significantly greater ($P \leq 0.05$) leaf injury to HX1. The leaf injury to HX1 caused by the two resistant populations was similar ($P > 0.05$) for both trials, with an average leaf injury rating of 5.4 for the pooled data (Fig. 2). The leaf injury levels of HX1 in each trial were similar ($P > 0.05$) between the two RS populations, but overall the injury in Trial-II was greater than in Trial-I. Based on the pooled data analysis, the leaf injury rating (an average of 2.9) on HX1 plants infested with FL32-RS or FL67-RS was significantly greater ($P \leq 0.05$) than that caused by SS, but significantly less ($P \leq 0.05$) than on plants infested with the resistant populations (Fig. 2).

Cry1Ab maize (YG) was marginally effective in reducing the damage by the susceptible and FL32, but ineffective for the other three populations. Leaf injury rating of the pooled data was 4.8 for SS and 4.2 for F32, while it was 6.8–7.0 for the other three populations (Fig. 2). In contrast, little or no leaf injury was observed on plants of Cry2APH and the three pyramided Bt maize hybrids infested with any of the five populations. In the pooled analysis of the two trials, the two resistant populations caused an average leaf injury rating of 1.2 to Cry2APH, 1.3 to VT2P, 1.3 to SMT, and 1.1 to VIP3; with few exceptions, none of these values were significantly different from those infested with SS (1.0–1.5) or RS (1.2–2.5) across the four maize products (Fig. 2).

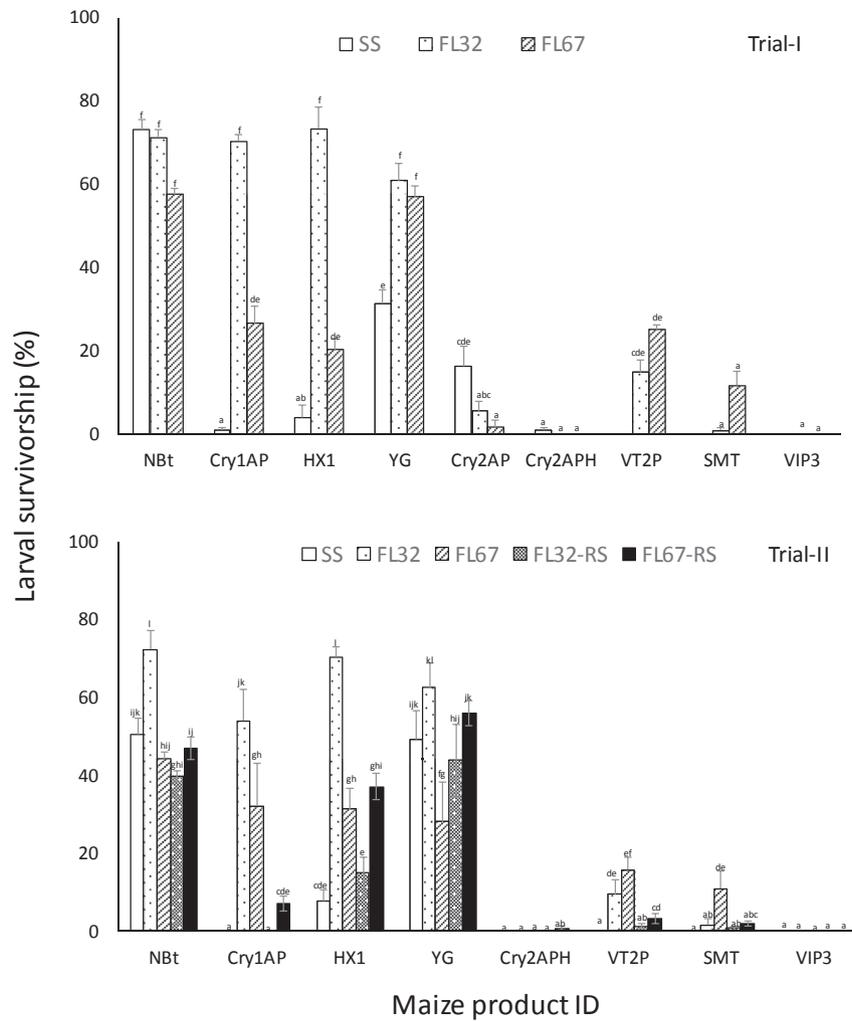


Fig. 1. Larval survivorship (mean ± sem %) of Cry1A.105-susceptible (SS), -heterozygous (FL32-RS, FL67-RS), and -resistant populations (FL32 and FL67) of *Spodoptera frugiperda* after 7 days of feeding on leaf tissues of non-Bt and Bt maize products expressing single or multiple Bt proteins. Mean values followed by a same letter are not significantly different ($\alpha = 0.05$; LSMEANS test).

Table 3
Effective dominance level (D_{ML}) of two Cry1A.105-resistant populations (FL32 and FL67) of *Spodoptera frugiperda* on leaf tissues and whole plants of Cry1A.105 (Cry1AP) and Cry1F (HX1) maize products.

Test method	Maize product ID	Insect	Effective dominance D_{ML} ^a
Leaf tissue bioassay	Cry1AP	FL32	0.00
		FL67	0.21
	HX1	FL32	0.27
		FL67	1.00 ^b
Whole-plant test	Cry1AP	FL32	0.75
		FL67	0.50
	HX1	FL32	0.40
		FL67	0.86

^a D_{ML} ranges between 0 and 1; $D_{ML} = 0$ means that the resistance is completely recessive, while $D_{ML} = 1$ indicates completely dominant resistance.

^b The calculated value based on the survival data observed in the leaf tissue bioassay was 1.15.

3.3. Larval survival of *S. frugiperda* on non-Bt and Bt maize containing single or pyramided genes in the whole-plant tests

In the whole-plant test, the effect of insect population and the interaction of insect population and maize product on larval survival of *S. frugiperda* was not significant in the analysis for either of

the two single trials, but the effect of maize product was significant for each trial and the pooled data analysis (Table 2). In addition, the interaction effect was also significant in the pooled data analysis. Larvae of the five populations survived well on non-Bt maize plants in both trials, and there were no significant ($P > 0.05$) differences in the larval survival rates among the five populations for either of the trials or for the pooled data analysis. Across the five populations and two trials, live larvae were observed on 52.4–72.9% of the non-Bt plants after 14 days of larval release (Fig. 3). In contrast, no live larvae were observed from Cry1AP plants infested with SS in either trial, while 41.7–62.5% of the Cry1AP plants infested with FL32 or FL67 contained live larvae. The larval survival rate on Cry1AP plants was not significantly ($P > 0.05$) different between the two resistant populations, suggesting that both FL32 and FL67 were highly resistant to these plants (Fig. 3). The survival rate of RS on Cry1AP was similar ($P > 0.05$) between FL32-RS and FL67-RS in each of the two trials and in the pooled data analysis. In general, the survival rate of the two RS populations on Cry1AP was numerically greater than that of SS, but lower than that of the two resistant populations. In the pooled data analysis, the difference in the larval survival relative to SS was significant ($P \leq 0.05$) for both RS populations, while, relative to resistant populations, it was significant for FL67-RS, but not for FL32-RS. The calculated D_{ML} based on the pooled

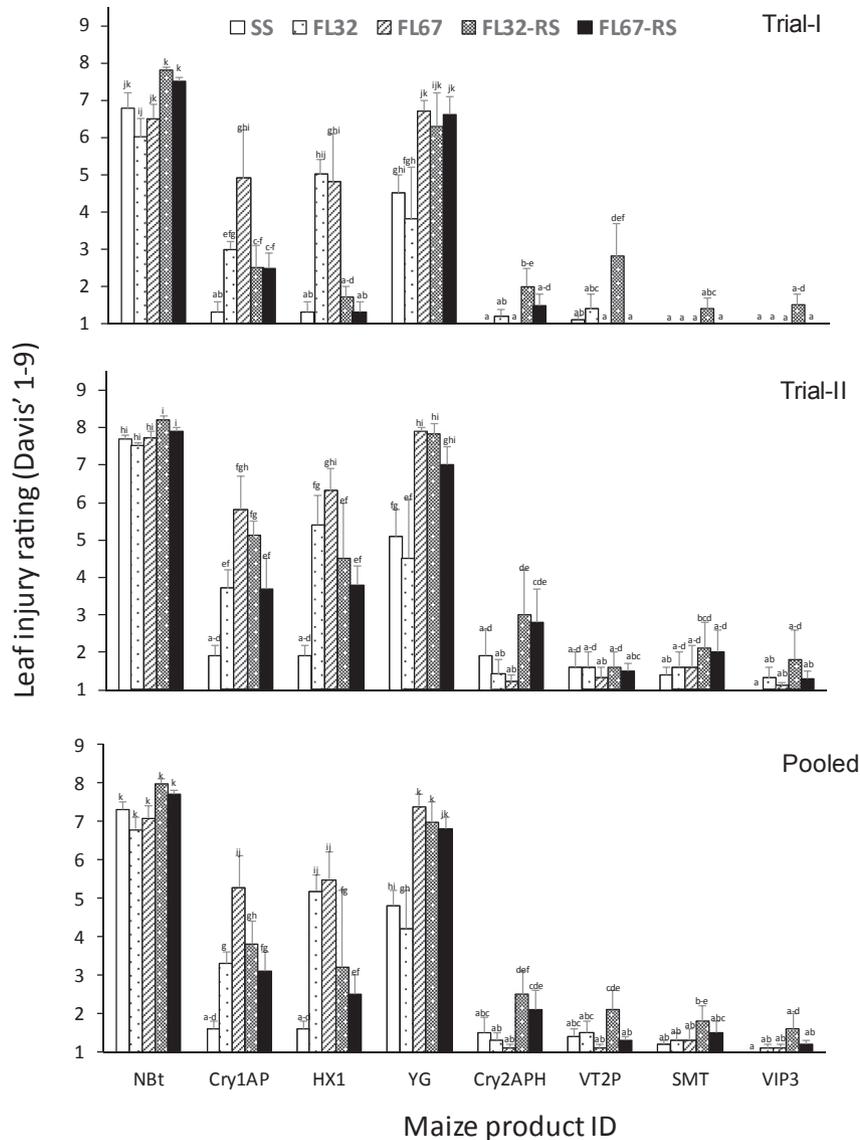


Fig. 2. Leaf injury ratings (mean \pm sem) of Bt-susceptible (SS), -heterozygous (FL32-RS, FL67-RS), and -resistant (FL32, FL67) populations of *Spodoptera frugiperda* on non-Bt and Bt maize plants expressing single or multiple Bt proteins. Higher values indicate greater injury. Mean values followed by a same letter are not significantly different ($\alpha = 0.05$; LSMEANS test). Only the first and the last letters are presented over the bar if four or more letters were needed for the LSMEANS tests. For example, the label “c-f” over the bar representing the survivorship of FL32-RS on Cry1AP in Trial-I means “cdef”.

data was 0.75 for FL32 and 0.50 for FL67, suggesting that the resistance in the two populations was intermediate to incompletely dominant when it was measured on whole Cry1AP (Cry1A.105) maize plants in the greenhouse (Table 3).

Data on larval survival in the whole-plant tests also showed that both FL32 and FL67 were cross-resistant to Cry1F (HX1) maize plants. After 14 days of larval release, live larvae were found in 12.5% and 0% of HX1 plants infested with SS in Trial-I and Trial-II, respectively, while these values were 50.0–54.2% for FL32 and 37.5–66.7% for FL67. The difference between SS and the two resistant populations was, in most cases, significant ($P \leq 0.05$) for each trial and for the pooled data analysis (Fig. 3). The survivorship of the two RS populations on HX1 was somewhat greater than that of SS, ranging from 16.7% to 54.2% in the two trials. Relative to SS, the difference was significant ($P \leq 0.05$) for FL67-RS in Trial-I and the pooled analysis, while the difference relative to resistant populations was not significant ($P > 0.05$) in the pooled analysis for either FL32-RS or FL67-RS (Fig. 3). The calculated D_{ML} based on the

pooled data from the two trials was 0.40 for FL32 and 0.86 for FL67, suggesting that the resistance to Cry1F maize was intermediate to incompletely dominant when measured on whole plants of HX1 maize (Table 3).

The larval survival data from the greenhouse whole-plant tests suggested that the single-gene Cry1Ab maize (YG) was ineffective against any of the five insect populations. For the two trials, an average of 37.5–75% of the YG plants contained live larvae after 14 days, which was not much lower than the survivorship rates observed on the non-Bt maize plants (Fig. 3). In contrast, whole plants of Cry2APH and the three pyramided Bt maize products were effective against all five populations. Across these four maize products and both whole-plant trials, live larvae were observed from an average of 3.6% of the plants infested with the two resistant and two F_1 heterozygous populations (Fig. 3).

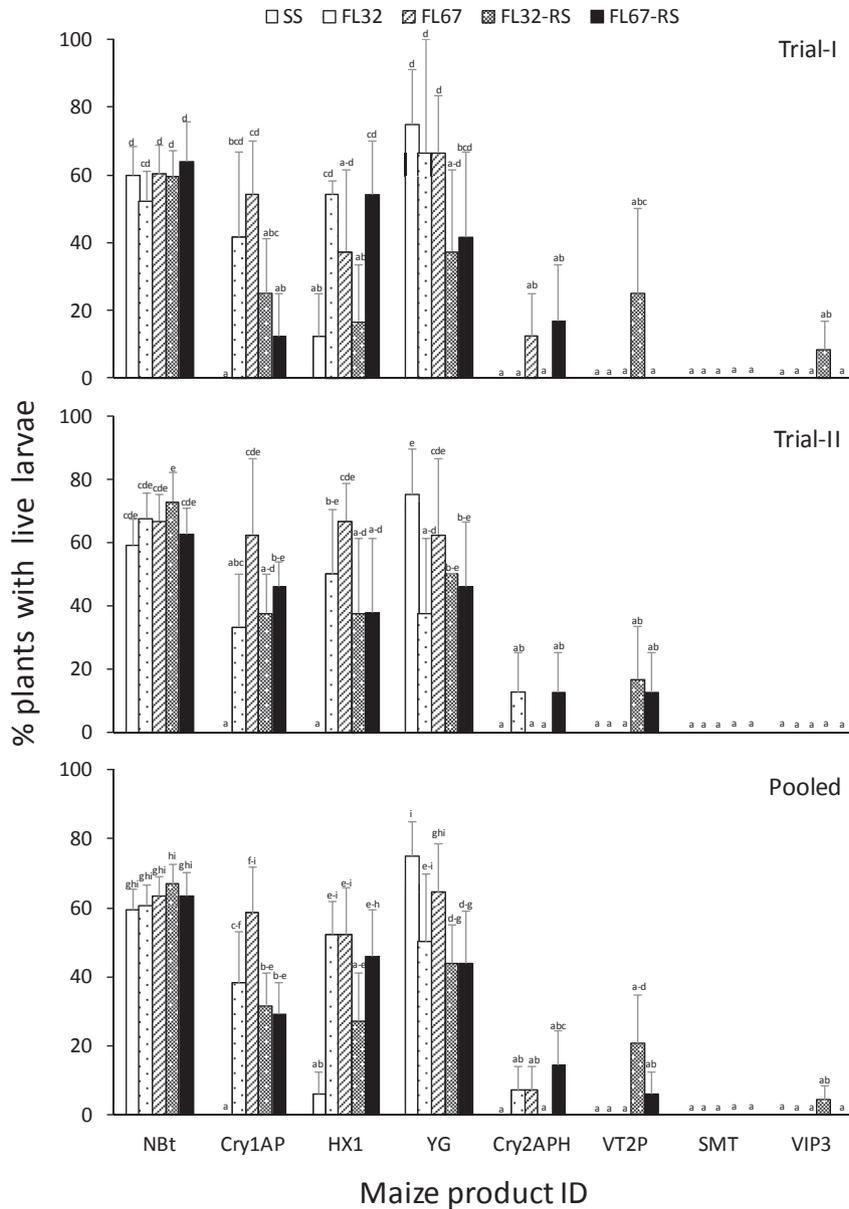


Fig. 3. Percent plants (mean ± sem) containing live larvae of Bt-susceptible (SS), -heterozygous (FL32-RS, FL67-RS), and -resistant (FL32, FL67) populations of *Spodoptera frugiperda* on non-Bt and Bt maize plants expressing single or multiple Bt proteins. Mean values followed by a same letter are not significantly different ($\alpha = 0.05$; LSMEANS test). Only the first and the last letters are presented over the bar if four or more letters were needed for the LSMEANS tests. For example, the label “a-d” over the bar representing the survivorship of FL67 on HX1 in Trial-I means “abcd”.

4. Discussion

A previous study demonstrated that FL32 and FL67 were resistant to the Cry1A.105 protein, allowing the larvae to survive and develop on whole Cry1A.105 maize plants (Huang et al., 2016). In the present study, these two populations also survived well on the Cry1A.105 maize product in the leaf tissue bioassay and whole-plant test. The results further validate that both FL32 and FL67 are highly resistant to Cry1A.105 maize.

Understanding the functional dominance level of resistance is important in resistance management. This study showed that the effective dominance level, D_{ML} , of the Cry1A.105 resistance in *S. frugiperda* appeared to vary depending on the insect population, Bt maize product, and test method. Resistance in FL32 and FL67 on leaf tissues of Cry1A.105 maize was recessive to incompletely

recessive, while on whole Cry1A.105 plants it was moderate to incompletely dominant. Several possible reasons might explain the observed variation. First, the genetic basis of the Cry1A.105 resistance in the two populations might not be the same, resulting in different dominance levels in the two populations. Second, the level of Cry1A.105 protein can vary in different plant tissues and plant growth stages (Monsanto, 2009; US-EPA, 2010), which could cause differences in survival of the RS larvae between feeding on whole plants in the greenhouse and on leaf tissues in containers in the laboratory. The variation in the dominance levels observed on different test plant materials suggests that careful experimental designs are needed for evaluating the ‘high-dose’ qualification of Bt maize against *S. frugiperda*.

The current study also showed that both Cry1A.105-resistant populations of *S. frugiperda* were highly cross-resistant to Cry1F

maize. The cross-resistance was incompletely recessive for FL32 but dominant for FL67 in leaf tissue bioassay, while it was incompletely dominant in the whole-plant tests for both populations. The non-recessive resistance could be one of the reasons that led to the rapid development of resistance to the Cry1F maize in some field populations of *S. frugiperda*. Cross-resistance of Cry1F-resistant *S. frugiperda* to Cry1A.105 protein or Cry1A.105 maize has also been reported in two previous studies (Huang et al., 2014; Bernardi et al., 2015); Cry1F and Cry1A.105 have similar structures and thus selection for resistance to one is expected to confer resistance to the other. A previous study also showed that Cry1A.105 resistance alleles in the field populations of *S. frugiperda* collected in 2011 from Florida, U.S. was relatively abundant, reached 0.056 with a 95% credibility interval of 0.032–0.087 (Huang et al., 2016). It was suspected that the relatively high level of Cry1A.105-resistance allele frequency detected in *S. frugiperda* populations in Florida might be a result of the selection of Cry1F resistance, together with the cross-resistance between Cry1F and Cry1A.105 (Huang et al., 2016). Both FL32 and FL67 used in this study were isolated from field populations in which Cry1F-resistance alleles were already abundant (Huang et al., 2014, 2016). The high level of 'cross-resistance' of FL32 and FL67 to Cry1F maize documented in the current study supports this interpretation. Both Cry1F and Cry1A.105 proteins are expressed in some current pyramided Bt maize products (Buntin and Flanders, 2015). The significant cross-resistance between Cry1F and Cry1A.105 in *S. frugiperda* plus the non-recessive nature of the resistance could diminish the durability of some pyramided Bt maize technology if effective resistance management plans are not implemented, especially in areas where Cry1F resistance has already widely occurred (Bernardi et al., 2015). In addition, the two Cry1A.105-resistant populations of *S. frugiperda* in the leaf tissue bioassay also showed cross-resistance to Cry1Ab maize. However, the cross-resistance to Cry1Ab maize became insignificant on whole plants because all five populations survived well in the greenhouse tests.

As reported for Cry1F resistance (Niu et al., 2013; Huang et al., 2014), the Cry1A.105-resistant populations of *S. frugiperda* are not cross-resistant to Cry2Ab2 or Vip3A; thus, both FL32 and FL67 are susceptible to the pyramided Bt maize products containing one of these two Bt genes. In the current Bt maize market, five Bt proteins (Cry1Ab, Cry1A.105, Cry1F, Cry2Ab2, and Vip3A) are available for controlling aboveground lepidopteran targets, including *S. frugiperda* (Huang et al., 2016). Up to now, all commercial pyramided Bt maize products targeting lepidopteran pests contain one or two of the three Cry1 proteins plus either Cry2Ab2 or Vip3A (Buntin and Flanders, 2015). As shown in the current study, the cross-resistance among Cry1A.105, Cry1F, and Cry1Ab essentially makes these pyramided maize products function as a single-gene Bt maize product against Cry1F/Cry1A.105-resistant *S. frugiperda*. Currently, university and industry scientists are evaluating new Bt maize products with triple modes of action, i.e., containing one of the three Cry1 proteins plus both Cry2Ab2 and Vip3A. It is expected that these new Bt maize products will soon become commercially available. Results of the current study support the use of these new triple-Bt maize products to manage the Cry1F/Cry1A.105-resistant populations of *S. frugiperda*. More importantly, we believe that integrated pest management tactics with diversified mortality factors must be implemented along with Bt crops to ensure the long-term success of Bt crop technology (Hutchison, 2015; Yang et al., 2016).

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References

- Abbott, S., 1925. A method of computing the effectiveness of an insecticide. *J. Econ. Entomol.* 18, 265–267.
- Bernardi, D., Salmeron, E., Horikoshi, R.J., Bernardi, O., Dourado, P.M., Carvalho, R.A., Samuel, M., Head, G.P., Omoto, C., 2015. Cross-resistance between Cry1 proteins in fall armyworm (*Spodoptera frugiperda*) may affect the durability of current pyramided Bt maize hybrids in Brazil. *PLoS ONE* 10 (10), e0140130. <http://dx.doi.org/10.1371/journal.pone.0140130>.
- Buntin, D., Flanders, K., 2015. Bt Corn Products for the Southeastern United States, p. 2015. <http://www.caes.uga.edu/commodities/fieldcrops/gagrains/documents/2015SoutheastBtCorntraitstable.pdf>.
- Burkness, E.C., Dively, G., Patton, T., Morey, A.C., Hutchison, W.D., 2010. Novel Vip3A *Bacillus thuringiensis* (Bt) maize approaches high-dose efficacy against *Helicoverpa zea* (Lepidoptera: Noctuidae) under field conditions. *GM Crops* 1, 1–7.
- Biosafety Clearing-House, 2014. Gene and DNA sequence. Cry1A 105. <https://bch.cbd.int/database/record.shtml?documentid=43771>.
- Dangal, V., Huang, F., 2015. Fitness costs of Cry1F resistance in two populations of fall armyworm, *Spodoptera frugiperda* (J.E. Smith), collected from Puerto Rico and Florida. *J. Invertebr. Pathol.* 127, 81–86.
- Davis, F.M., Ng, S.S., Williams, W.P., 1992. Visual rating scales for screening whorl-stage corn for resistance to fall armyworm. *Miss. Agri. Fore. Exp. Sta. Tech. Bull.* 186.
- Dhurua, S., Gujar, G.T., 2011. Field-evolved resistance to Bt toxin Cry1Ac in the pink bollworm, *Pectinophora gossypiella* (saunders) (Lepidoptera: Gelechiidae), from India. *Pest Manag. Sci.* 67, 898–903.
- Farias, J.R., Andow, D.A., Horikoshi, R.J., Sorgatto, R.J., Fresia, P., Santos, A.C., Omoto, C., 2014. Field-evolved resistance to Cry1F maize by *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Brazil. *Crop Prot.* 64, 150–158.
- Farias, J.R., Horikoshi, R.J., Santos, A.C., Omoto, C., 2014. Geographical and temporal variability in susceptibility to Cry1F toxin from *Bacillus thuringiensis* in *Spodoptera frugiperda* (Lepidoptera: Noctuidae) populations in Brazil. *J. Econ. Entomol.* 107, 2182–2189.
- Gassmann, A.J., Petzold-Maxwell, J.L., Keweshan, R.S., Dunbar, M.W., 2011. Field-evolved resistance to Bt maize by western corn rootworm. *PLoS ONE* 6 (7), e22629. <http://dx.doi.org/10.1371/journal.pone.0022629>.
- Ghimire, M.N., Huang, F., Leonard, R.B., Head, G.P., Yang, Y., 2011. Susceptibility of Cry1Ab-susceptible and -resistant sugarcane borer to transgenic corn plants containing single or pyramided *Bacillus thuringiensis* genes. *Crop Prot.* 30, 74–81.
- Huang, F., Andow, D.A., Buschman, L.L., 2011. Success of the high dose/refuge resistance management strategy after 15 years of Bt crop use in North America. *Entom. Exp. App.* 140, 1–16.
- Huang, F., Qureshi, J.A., Meagher Jr., R.L., Reisig, D.D., Head, G.P., Andow, D.A., Ni, X., Kerns, D., Buntin, G.D., Niu, Y., Yang, F., Dangal, V., 2014. Cry1F resistance in fall armyworm *Spodoptera frugiperda*: single gene versus pyramided Bt maize. *PLoS ONE* 9 (11), e112958. <http://dx.doi.org/10.1371/journal.pone.0112958>.
- Huang, F., Qureshi, J.A., Head, G.P., Price, P.A., Levy, R., Yang, F., Niu, Y., 2016. Frequency of *Bacillus thuringiensis* Cry1A.105 resistance alleles in field populations of the fall armyworm, *Spodoptera frugiperda*, in Louisiana and Florida. *Crop Prot.* 83, 83–89.
- Hutchison, W.D., 2015. Insect resistance management and integrated pest management for Bt crops: prospects for an area-wide view. In: Soberón, M., Gao, A., Bravo, A. (Eds.), *Bt Resistance: Characterization and Strategies for GM Crops Producing Bacillus Thuringiensis Toxins*. CAB International, Wallingford, UK, pp. 186–201.
- James, C., 2014. Global Status of Commercialized Biotech/GM Crops: 2014. ISAAA Brief, No. 49. ISAAA, Ithaca, NY.
- Matten, S.R., Frederick, R.J., Reynolds, A.H., 2012. United States Environmental Protection Agency insect resistance management programs for plant-incorporated protectants and use of simulation modeling. In: Wozniak, C.A., McHughen, A. (Eds.), *Regulation of Agricultural Biotechnology: the United States and Canada*. Springer, Dordrecht, pp. 175–267.
- Monsanto, 2009. Safety Assessment of MON 89034. http://www.monsanto.com/products/documents/safety-summaries/mon89034_pss.pdf.
- Niu, Y., Meagher Jr., R.L., Yang, F., Huang, F., 2013. Susceptibility of field populations of the fall armyworm (Lepidoptera: Noctuidae) from Florida and Puerto Rico to purified Cry1F protein and corn leaf tissue containing single and pyramided Bt genes. *Fla. Entomol.* 96, 701–713.
- Niu, Y., Yang, F., Dangal, V., Huang, F., 2014. Larval survival and plant injury of Cry1F-susceptible, -resistant, and -heterozygous fall armyworm (Lepidoptera: Noctuidae) on non-Bt and Bt corn containing single or pyramided genes. *Crop Prot.* 59, 22–28.
- Niu, Y., Qureshi, J.A., Ni, X., Head, G.P., Price, P.A., Meagher Jr., R.L., Kerns, D., Levy, R., Yang, X., Huang, F., 2016. F₂ screen for resistance to *Bacillus thuringiensis* Cry2Ab2-maize in field populations of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) from the southern United States. *J. Invertebr. Pathol.* (in press).
- Ostlie, K.R., Hutchison, W.D., Hellmich, R.L., 1997. Bt Corn and European Corn Borer:

- Long Term Success through Resistance Management. North Central Region Extension Publication 602. University of Minnesota, St. Paul, MN.
- Roush, R.T., McKenzie, J.A., 1987. Economical genetics of insecticide and acaricide resistance. *Ann. Rev. Entomol.* 32, 361–380.
- Santos-Amaya, O.F., Rodrigues, J.V.C., Souza, T.C., Tavares, C.S., Campos, S.O., Guedes, R.N.C., Pereira, E.J.G., 2015. Resistance to dual-gene Bt maize in *Spodoptera frugiperda*: selection, inheritance, and cross-resistance to other transgenic events. *Sci. Rep.* 5, 18243. <http://www.nature.com/articles/srep18243>.
- SAS Institute Inc, 2010. SAS/STAT: 9.3 User's Guide, third ed. SAS Institute Inc., Cary, NC.
- Storer, N.P., Babcock, J.M., Schlenz, M., Meade, T., Thompson, G.D., Bing, J.W., Huckaba, R.M., 2010. Discovery and characterization of field resistance to Bt maize: *Spodoptera frugiperda* in Puerto Rico. *J. Econ. Entomol.* 103, 1031–1038.
- Tabashnik, B.E., Brévault, T., Carrière, Y., 2013. Insect resistance to Bt crops: lessons from the first billion acres. *Nat. Biotechnol.* 31, 510–521.
- US-EPA (U.S. Environmental Protection Agency), 2010. Biopesticides Registration Action Document: *Bacillus Thuringiensis* Cry1A.105 and Cry2Ab2 Insecticidal Proteins and the Genetic Material Necessary for Their Production in Corn. Biopesticides and Pollution Prevention Division, Washington, DC. http://www3.epa.gov/pesticides/chem_search/reg_actions/pip/mon-89034-brad.pdf.
- van Rensburg, J.B.J., 2007. First report of field resistance by the stem borer, *Busseola fusca* (Fuller) to Bt-transgenic maize. *S. Afr. J. Plant Soil* 24, 147–151.
- Wangila, D.S., Leonard, B.R., Bai, Y., Head, G.P., Huang, F., 2012. Larval survival and plant injury of Cry1Ab-susceptible, -resistant, and -heterozygous genotypes of the sugarcane borer on transgenic corn containing single or pyramided Bt genes. *Crop Prot.* 42, 108–115.
- Yang, F., Qureshi, J.A., Leonard, B.R., Head, G.P., Niu, Y., Hang, F., 2013. Susceptibility of Louisiana and Florida populations of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) to pyramided Bt corn containing Genuity®VT Double Pro™ and SmartStax™ traits. *Fla. Entomol.* 96, 714–723.
- Yang, F., Kerns, D.L., Leonard, B.R., Oyediran, I., Burd, T., Niu, Y., Huang, F., 2015. Performance of Agrisure®Viptera™ 3111 corn against *Helicoverpa zea* (Lepidoptera: Noctuidae) in seed mixed plantings. *Crop Prot.* 69, 77–82.
- Yang, F., Kerns, D.L., Brown, S., Kurtz, R., Dennehy, T., Braxton, B., Head, G., Huang, F., 2016. Performance and cross-crop resistance of Cry1F maize selected *Spodoptera frugiperda* on transgenic Bt cotton: implications for resistance management. *Sci. Rep.* 6 <http://dx.doi.org/10.1038/srep28059>, 28059.
- Zar, J.H., 1984. *Biostatistical Analysis*, second ed. Prentice-Hall, Englewood Cliffs, NJ.