Susceptibility of Louisiana and Florida Populations of Spodoptera frugiperda (Lepidoptera: Noctuidae) to Pyramided Bt Corn Containing Genuity®Vt Double Pro™ and Smartstax™ Traits

Authors: Yang, Fei, Qureshi, Jawwad A, Leonard, B. Rogers, Head, Graham P., Niu, Ying, et al.

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SUSCEPTIBILITY OF LOUISIANA AND FLORIDA POPULATIONS OF *SPODOPTERA FRUGIPERDA* (LEPIDOPTERA: NOCTUIDAE) TO PYRAMIDED BT CORN CONTAINING GENUITY®VT DOUBLE PRO™ AND SMARTSTAX™ TRAITS

FEI YANG[1], JAWWAD A QURESHI[2], B. ROGERS LEONARD[1], GRAHAM P. HEAD[3], YING NIU[1] AND FANGNENG HUANG[1,*]
[1]Department of Entomology, Louisiana State University Agricultural Center, Baton Rouge, LA; 70803-1710, USA

[2]Department of Entomology and Nematology, University of Florida, IFAS SWFREC, Immokalee, FL 34142; USA

[3]Monsanto Company, St. Louis, MO 63167, USA

*Corresponding author; E-mail: fhuang@agcenter.lsu.edu

ABSTRACT

The fall armyworm, *Spodoptera frugiperda* (J. E. Smith), is a target species of transgenic corn containing pyramided Bt genes in the United States. During 2011, a total of 149 F_2 two-parental family lines were established using single-pairing of *S. frugiperda* collected from 3 locations in Louisiana and Florida. This study examined the susceptibility of these F_2 two-parental family lines to 2 commonly used pyramided Bt corn traits, Genuity®VT Double Pro™ and Genuity®SmartStax™. Nine out of the 149 family lines showed less susceptibility to the leaf tissue of Genuity®VT Double Pro™ or Genuity®SmartStax™ plants. Larvae of these 9 family lines exhibited significant survivorship and growth on leaf tissue of the Bt corn plants. Two laboratory colonies were established from the F_2 survivors of 2 of the 9 family lines. However, larvae from both colonies could not survive on whole plants of their corresponding Bt corn products in the greenhouse, suggesting these families were not resistant to the pyramided Bt corn traits. The results suggest that the pyramided Bt corn products containing Genuity®VT Double Pro™ or Genuity®SmartStax™ corn traits are effective in protecting against *S. frugiperda.*

Key Words: fall armyworm; pyramided Bt corn, susceptibility; *Bacillus thuringiensis*

RESUMEN

El gusano cogollero, *Spodoptera frugiperda* (JE Smith), es la especie objetivo del maíz transgénico que contiene genes Bt piramidales en los Estados Unidos. Durante el 2011, se establecieron un total de 149 F_2 de dos líneas paternas utilizando el apareamiento de individuos de S. *frugiperda* obtenidas de 3 ubicaciones en los estados de Louisiana y la Florida. Este estudio examinó la susceptibilidad de estas F_2 de dos líneas familiares paternas a 2 rasgos de maíz con Bt piramidal utilizadas comúnmente, Genuity® VT Doble Pro™ y Genuity® SmartStax™. Nueve de las 149 líneas familiares mostraron una menor susceptibilidad al tejido de la hoja de plantas de Genuity® VT Doble Pro™ o Genuity® SmartStax™. Las larvas de estas 9 líneas familiares mostraron una sobrevivencia significativa y el crecimiento sobre el tejido de las hojas de las plantas de maíz con Bt. Se establecieron dos colonias de laboratorio de los sobrevivientes F_2 en 2 de las 9 líneas familiares. Sin embargo, las larvas de ambas colonias no podrían sobrevivir en plantas enteras de sus productos de maíz Bt correspondientes en el invernadero, lo que sugiere que estas familias no fueron resistentes a los rasgos de maíz Bt piramidales. Los resultados sugieren que los productos de maíz con Bt piramidal que contienen rasgos de maíz de Genuity® VT Doble Pro™ o Genuity® SmartStax™ son eficaces en la protección contra *S. frugiperda*.

Palabras Clave: gusano cogollero; piramidal maíz Bt, susceptibilidad, *Bacillus thuringiensis*

Since its first commercialization in 1996, transgenic corn expressing *Bacillus thuringiensis* (Bt) proteins has been widely planted worldwide, especially in the United States and Canada (James 2011). In general, these Bt corn hybrids are effective in suppressing 2 classes of corn pests: above-ground Lepidoptera such as the European corn borer, *Ostrinia nubilalis*

(Hübner) (Crambidae), and southwestern corn borer, *Diatraea grandiosella* Dyar (Crambidae); and below-ground Coleoptera such as the western corn rootworm *Diabrotica virgifera virgifera* Leconte (Chrysomelidae) and northern corn rootworm *Diabrotica barberi* Smith & Lawrence. Until now, transgenic Bt technology for corn can be classified into 2 generations (Buntin & Flanders 2012; Huang et al. 2012). First generation transgenic corn hybrids express a single Bt gene for controlling a target species. These single-gene Bt corn products are very efficient in controlling the major stalk borer species, but most have only limited efficacy for suppressing *Spodoptera frugiperda* (J. E. Smith) (Noctuidae) (Adamczyk & Mahaffey 2008; Huang et al. 2011). *S. frugiperda* is an important corn pest in the Western Hemisphere from the United States to Argentina (Pashley et al. 1985; Pashley 1986, 1988). Studies have shown that all first generation single-gene Bt corn products do not produce a "high dose" for *S. frugiperda* (Chilcutt et al. 2007; Adamczyk & Mahaffey 2008; Hardke et al. 2011; Huang et al. 2011). As a result, *S. frugiperda* is excluded from the target list for single-gene Bt corn products except for Herculex®I which expresses the Cry1F protein (US-EPA 2001, 2005). Unfortunately, with the extensive use of Herculex®I corn in Puerto Rico, field resistance of *S. frugiperda* to Cry1F corn was observed in 2006 (Matten et al. 2008; Storer et al. 2010). The Cry1F resistance in *S. frugiperda* was shown to be autosomally inherited and recessive (Storer et al. 2010, 2012a). To broaden the target spectrum and delay resistance development, a gene pyramiding strategy has been used to develop transgenic Bt corn containing 2 or more Bt proteins that are dissimilar in mode of action but effective against the same target pests (Monsanto 2012). This strategy relies on the expression of Bt proteins with different modes of action in a pyramided product. Therefore, the likelihood for evolution of resistance to a pyramided product is expected to be lower than against single trait products (Monsanto 2012). These second generation pyramided Bt corn hybrids are more effective in controlling *S. frugiperda* (Burkness et al. 2010; Niu et al. 2013). Consequently, *S. frugiperda* is listed as a target species for all currently commercialized pyramided Bt corn products in the United States (Monsanto 2012; Syngenta 2012).

Pyramided Bt corn products also require certain necessary actions to maintain their durability in the marketplace. In this regard, a major potential threat is the evolution of resistance in target pests to pyramided Bt corn hybrids. In 2011, we established 149 two-parental family lines using single-pair matings from 3 field populations of *S. frugiperda* collected from Louisiana and Florida. The objective of this study was to examine the susceptibility of these family lines to 2 major pyramided Bt corn traits, Genuity®VT Double Pro™ and Genuity®SmartStax™.

Materials and Methods

Insect Collection and Rearing

Feral larvae of *S. frugiperda* were collected during 2011 from sorghum fields in Franklin and Rapides parishes in Louisiana and from sweet corn fields in Collier County in Florida. In each location, larvae were sampled at 2 different times with a 1- to 2-wk interval between the 2 samplings in each location. All field-collected larvae were reared individually on a meridic diet (WARD'S Stonefly Heliothis diet, Rochester, New York) until pupal stage as described in Niu et al. (2013).

Use of Single Pairs to Establish Two-Parental Iso-Family Lines

Newly emerged virgin male and female moths of *S. frugiperda* were paired in 2- or 3.8-L paper containers (Huhtamaki Foodservice, De Soto, Kansas) containing ~100g of vermiculite (Sun Gro, Pine Bluff, Arkansas). Adult containers were placed in environmental chambers maintained at 28 °C, > 90% RH, and 14:10 h L: D for adult emergence, mating, and oviposition. Progeny (F_1) produced from each pair were considered as a two-parental family line and was reared individually in the 30-mL cups containing meridic diet as mentioned above (Niu et al. 2013). Fifty-to-eighty F_1 adults of each line were sib-mated in 3.8 L cardboard cartons (Neptune Paper Products, Newark, New Jersey) to produce F_2 generation for each two-parental family line.

Source of Plant Materials

Leaf tissue of two pyramided corn hybrids, DKC 64-04 containing Genuity®VT Double Pro™ traits and DKC 61-21 containing Genuity®SmartStax™ traits (Monsanto, St Louis, Missouri), was used in examination of the susceptibility of *S. frugiperda* (Table 1). Genuity®VT Double Pro™ (MON89034) corn contains the Cry1A.105 and Cry2Ab2 proteins for controlling above-ground lepidopteran species including *S. frugiperda.* Genuity®SmartStax™ expresses 6 Bt proteins including the 2 Bt proteins in Genuity®VT Double Pro™ together with Cry1F (TC1507) targeting lepidopteran pests and Cry3Bb1 (MON88017) and Cry34/35Ab1 (DAS-59122) for controlling underground rootworms, *Diabrotica* spp. In addition, a genetically closely related non-Bt corn hybrid, DKC 61-22 (Monsanto, St Louis, Missouri), was used to establish the baseline survival of *S. frugiperda*. Both Bt and non-Bt corn hybrids were planted in 18.9 L pots each containing ap-

TABLE 1. HYBRIDS USED IN EVALUATION OF SUSCEPTIBILITY OF *SPODOPTERA FRUGIPERDA* FAMILY LINES TO BT CORN.

Corn trait	Corn hybrid	Event	Bt genes	Major target pest
Non-Bt	DKC 61-22	—	—	—
Genuity®VT Double Pro™	DKC 64-04	MON89034	Cry1A.105, Cry2Ab2	Lepidoptera
Genuity®SmartStax®	DKC 61-21	MON89034 + MON88017 + TC1507 + DAS-59122	Cry1A.105, Cry2Ab, Cry1F, Cry3Bb1, Cry34/35Ab	Lepidoptera & *Diabrotica* spp. rootworms

proximately 5 kg of a standard potting soil mixture in a greenhouse located at the Louisiana State University Agricultural Center in Baton Rouge, Louisiana as described in Wu et al. (2007). Expression of Cry1A.105, Cry2Ab2, and Cry1F proteins in plants was also confirmed using ELISA-based assays (EnviroLogix, Quantiplate™ kits, Portland, Maine).

Leaf Tissue Bioassays

Bioassays for examining susceptibility to Genuity®VTDoublePro™andGenuity®SmartStax™ corn in *S. frugiperda* were performed in 32-well trays (Bio-Smart-32, C-D International, Pitman, New Jersey) containing corn leaf tissues of V4-V10 stages of greenhouse grown corn plants. Based on our preliminary study involved in consideration of both larval cannibalism effect and labor intensity needed in the bioassay, 4 individuals of *S. frugiperda* in each well were considered to be an appropriate number used in the leaf tissue bioassay to minimize larval cannibalism. For each insect family line and Bt corn hybrid, a total of 96 F_2 neonates were assayed in 24 wells (4 neonates/well) each containing 2-4 pieces of approximately 3-cm fresh leaf tissue as described in Niu et al. (2013). The bioassay trays were placed in growth chambers maintained at 28 °C, ~50% RH and 16:8 h L:D. Leaf tissue was replaced every 3 days. The number of live larvae, larval stages (small larvae: ≤ 2nd instar and large larvae: ≥ 3rd instar), and larval body mass of small and large larvae were recorded at 7 d after inoculation.

Baseline Survival

Baseline survival of a Bt-susceptible strain (Bt-SS) of *S. frugiperda* on leaf tissue of non-Bt and the 2 Bt corn hybrids was determined using the same method of the leaf tissue bioassays described above. Bt-SS was established from larvae collected from corn fields in Hendry County, Florida, USA during 2011, and it has been documented to be susceptible to both Genuity®VT Double Pro™ and Genuity®SmartStax™ (Niu et al. 2013). In addition, larval survival of 7 F_2 family lines of *S. frugiperda* established from the field collections in Louisiana and Florida was also examined on corn leaf tissue of 4 non-Bt corn hybrids [Agrisure® NK N78N-GT (Syngenta, Minnetonka, Minnesota), Pioneer 31G66 (Pioneer Hi-Bred, Johnston, Iowa), DKC 67-86 (Monsanto, St. Louis, Missouri), as well as DKC 61-22] using the same method as used for assaying the Bt-SS strain. For statistical analysis, mortality data of the F_2 family lines collected from a state were combined across the non-Bt corn hybrids. Mortality data were transformed with arcsine $(x)^{0.5}$, and then subjected to a one-way analysis of variance (ANOVA). Differences in larval mortality among

the 3 sources (Louisiana, Florida, and Bt-SS) of *S. frugiperda* were separated with LSD tests at α = 0.05 level (SAS Institute, 2010).

Furthermore, larval mortality of the Bt-SS and 7 F_2 family lines was also individually assayed on a meridic diet (Ward's Stonefly Heliothis diet, Rochester, New York) as described in Niu et al. (2013). A total of 7 independent bioassays were conducted for Bt-SS strain during the period of this study, while there were 6 and 1 bioassays for the F_2 family lines collected from Florida and Louisiana, respectively. In each bioassay, approximately 1 g of diet was placed into each cell of the 128-cell trays (C-D International, Pitman, New Jersey). One neonate (<24 h) was released on the diet in each cell. The bioassay trays were placed in growth chambers maintained at 28 °C, ~50% RH, and 16:8 h L: D. Larval mortality was recorded on the 7th day after inoculation. In each bioassay, there were 4 replications with 32 larvae in each replication. As described in the leaf tissue bioassays, mortality data observed in the diet bioassays were transformed with arcsine $(x)^{0.5}$, and then subjected to a one-way ANOVA. Differences in larval mortality among the 3 insect sources were separated with LSD tests at α = 0.05 level (SAS Institute 2010).

Re-evaluation of Tolerant Strains

Nine F_2 family lines survived well in the leaf tissue bioassays (see results). Based on the baseline survivorship of the susceptible population of *S. frugiperda* on non-Bt plant leaf tissue, these family lines that had ≥ 3 large live larvae with a body mass of ≥ 30 mg of all large larvae after 7 days in the leaf tissue bioassays were considered to be potentially tolerant to the Bt corn traits. These same criteria were used to define the F_2 family lines possessing major resistance alleles to Cry1F corn plants (Huang et al. unpublished data). Laboratory strains of the potentially tolerant family lines were established from the survivors in the leaf tissue bioassays. These laboratory strains were then re-evaluated for larval survival on Bt leaf tissue in the laboratory and whole Bt corn plants in the greenhouse. A total of 2 leaf tissue tests and 2 whole plant trials were conducted for each family line. The leaf tissue tests were carried out in the same way as described in the bioassays with the F_2 family lines described above. In the whole plant tests, 5 neonates of a family line were inoculated into the whorl of each plant at V9-V10 stages of Bt and non-Bt corn hybrids. A total of 50 neonates of each tolerant family line were infested to 10 Bt plants within 5 pots in each test. Bt plants were confirmed for Bt protein expression with the ELISA-based assays (EnviroLogix, Quantiplate™ Kits, Portland, Maine). Similarly, for the test on non-Bt corn plants, a total of 60 neonates of the tolerant family lines were infested on 12 non-Bt plants (5 neonates/plant). Leaf injury ratings were recorded based on Davis' 1 (no injury) to 9 (heavy injury) scale (Davis et al. 1992) after 7 and 13 days, respectively, and the number of live larvae per plant was checked after 13 days. In addition, larval survival and leaf injury of Bt-SS strain were also evaluated on whole plants of the non-Bt (DKC 61-22) and the 2 Bt corn hybrids in the greenhouse to verify the insecticidal activity of the 2 Bt corn hybrids. In the tests with Bt-SS, a total of 48 neonates were infested in 16 plants (3 neonates/plant) of each hybrid at the V6-V8 plant stages. Insect survival and leaf injury ratings were reordered 15 days after release of neonates.

Results

Baseline Survivorship on Corn Leaf Tissue and Meridic Diet

Larval survivorship rate of the Bt susceptible population of *S. frugiperda* was 59.2 ± 1.8% on non-Bt corn leaf tissue after 7 days, while it was zero on leaf tissues of both Genuity®VT Double Pro™ and Genuity®SmartStax™ corn, suggesting leaf tissue of both pyramided Bt corn products expressed a sufficient level of Bt proteins to kill susceptible *S. frugiperda* and thus could be used for identifying individuals that were tolerant to the 2 Bt corn products. The effect of insect sources (Bt-SS, Louisiana and Florida) on larval survival on non-Bt corn leaf tissue was not significant (F = 0.82; df = 2, 69; P = 0.4464). The 7-day larval survivorship rates of the two-parental family lines collected from Louisiana and Florida were 57.3 ± 2.2% and 61.4 ± 2.4%, respectively, which were not significantly different ($P > 0.05$) compared to the mortality observed for Bt-SS. Effect of insect sources on larval mortality on meridic diet was also not significant (F = 1.39; df = 2, 53; P = 0.2585). Larvae of Bt-SS and the family lines derived from field collections survived well on the meridic diet with a 7-d mortality of 10.9 ± 1.7% for Bt-SS, 3.2± 1.3% for the family lines collected from Louisiana, and 9.7± 1.4% from Florida.

Susceptibility of Louisiana Populations of *S. frugiperda* to Genuity®VT Double Pro™ and Genuity®SmartStax™

A total of 48 F_2 family lines (or 96 feral individuals) and 29 F_2 family lines (or 58 feral individuals) of *S. frugiperda* were established from larvae collected from Franklin and Rapides parishes in Louisiana, respectively. All of these F_2 family lines were examined for susceptibility on leaf tissue of Genuity®VT Double Pro™ and Genuity®SmartStax™ corn in the laboratory. The F_2 leaf tissue bioassay showed that 4 of the 48 F_2 family lines from Rapides Parish and 1 of 29 lines from Franklin Parish had a portion of larvae that survived after 7 days on leaf tissue of Genuity®VT Double Pro™ with a total of 10 and 1 survivors, respectively (Table 2). Similar-

TABLE 2. LARVAL SURVIVAL OF TWO-PARENTAL FAMILY LINES OF LOUISIANA AND FLORIDA POPULATIONS OF *SPODOPTERA FRUGIPERDA* ON LEAF TISSUE OF GENUITY®VT DOUBLE PRO™ AND GENUITY®SMARTSTAX™ CORN.

Population	Location		No. F_2 lines assayed	No. lines survived	No. total survivors	No. lines with live large larvae	Total no. large larvae	Body mass of large larvae (mg/larva)
				Genuity®VT Double Pro™				
Louisiana	Rapides Parish		48	4	10	0	0	—
	Franklin Parish		29	1	1	0	0	—
	Subtotal		77	5	11	0	0	—
Florida	Collier County	1st sampling	43	20	93	9	30	9.0
		2nd sampling	29	11	50	4	12	11.3
	Subtotal		72	31	143	13	42	9.6
Total			149	36	154	13	42	9.6
				Genuity®SmartStax™				
Louisiana	Rapides Parish		48	6	13	2	3	5.7
	Franklin Parish		29	0	0	0	0	—
	Subtotal		77	6	13	2	3	5.7
Florida	Collier County	1st sampling	43	10	50	4	21	8.9
		2nd sampling	29	11	47	5	17	10.8
	Subtotal		72	21	97	9	38	9.7
Total			149	27	110	11	41	9.4

Note: Larvae that were ≥3rd instar were classified as large larvae.

ly, 6 lines of the Rapides Parish population had a portion of larval that survived after 7 days on Genuity®SmartStax™ leaf tissue with a total of 13 survivors, while none of the 29 lines of the Franklin population survived on leaf tissue of Genuity®SmartStax™. However, all live larvae of the 77 family lines on Genuity®VT Double Pro™ were small (≤ 2nd instars) and no large larvae (≥ 3rd instars) survived for 7 days in the leaf tissue bioassay (Table 2). A total of 3 large larvae from 2 family lines with an average of body mass of 5.7 mg were recovered on Genuity®SmartStax™ leaf tissue. Therefore, based on the criteria of Bt tolerant lines described above, none of the 77 F_2 family lines in the Louisiana populations of *S. frugiperda* qualified as potentially tolerant lines. The results of the F_2 leaf tissue bioassay suggested all of the 77 family lines were susceptible to the Genuity®VT Double Pro™ and Genuity®SmartStax™ hybrids.

Susceptibility of Florida Populations of *S. frugiperda* to Genuity®VT Double Pro™ and Genuity®SmartStax™

A total of 72 F_2 two-parental family lines (or 144 feral individuals) of *S. frugiperda* were established from Collier County, Florida during 2011 (Table 1). Among these family lines, 43 and 29 lines were developed from the first and second field collections, respectively. All 72 family lines were assayed for susceptibility on leaf tissue of Genuity®VT Double Pro™ and Genuity®SmartStax™ in the laboratory. For the 43 family lines derived from the first collection, 20 lines had a proportion of larvae that survived after 7 days on Genuity®VT Double Pro™ with a total of 93 survivors, and 10 lines had a proportion of larvae survived after 7 days on Genuity®SmartStax™ with a total of 50 survivors (Table 2). Among these survivors, a total of 30 large larvae with an average body mass of 9.0 mg/larva were recovered from 9 families on Genuity®VT Double Pro™ and 21 large larvae with an average body mass of 8.9 mg/larva were found from 4 family lines on Genuity®SmartStax™ (Table 2). For the 29 lines developed from the second collection, 11 lines survived on Genuity®VT Double Pro™ with a total of 50 live larvae and 11 families survived on Genuity®SmartStax™ with a total of 47 survivors (Table 2). Among these survivors, 12 large larvae with an average body mass of 11.3 mg/larva were obtained from 4 family lines on Genuity®VT Double Pro™ and 17 large larvae with an average body mass of 10.8 mg/larva were observed from 5 family lines on Genuity®SmartStax™ (Table 2).

Based on the criteria for a tolerant line defined above, 5 out of the 72 Florida family lines were considered to be tolerate leaf tissue of Genuity®VT Double Pro™. Three of these lines were from the first sampling including lines 13, 30, and 32 and two lines were from the second collection, i.e., lines 17 and 22 (Table 3). For Genuity®SmartStax™, a

TABLE 3. FAMILY LINES OF *SPODOPTERA FRUGIPERDA* THAT WERE CONSIDERED POTENTIALLY TOLERANT TO GENUITY®VT DOUBLE PRO™ OR GENUITY®SMARTSTAX™ CORN PRODUCTS.

Location	Bt corn used in leaf tissue bioassay	Family line	No. of live larvae		Larval body mass (mg/larva)	
			Small	Large	Small	Large
Collier-FL1	Genuity®VT Double Pro™	13	3	4	1.7	9.5
	Genuity®VT Double Pro™	30	19	7	1.8	15.0
	Genuity®VT Double Pro™	32	11	6	3.8	13.2
Collier-FL2	Genuity®VT Double Pro™	17	5	4	2.8	11.3
	Genuity®VT Double Pro™	22	15	5	1.6	13.6
Collier-FL1	Genuity®SmartStax™	30	3	4	1.3	8.3
	Genuity®SmartStax™	32	13	7	2.3	4.3
	Genuity®SmartStax™	45	2	9	1.0	8.0
Collier-FL2	Genuity®SmartStax™	25	3	7	3.7	12.0

Notes: FL1 and FL2 refer to the first sampling and second sampling, respectively, from Collier County in Florida.
Larvae that were ≤ 2nd instar were classified as small larvae, while larvae that were ≥3rd instar were classified as large larvae.

total of 4 family lines were identified as potentially tolerant lines which included 3 lines (lines 30, 32, and 45) from the first collection and 1 line (line 25) from the second sampling (Table 3). Attempts to establish laboratory colonies were made for all the 9 potentially tolerant family lines. To establish laboratory colonies of the potentially tolerant lines, survivors of each family line after the leaf tissue bioassay were transferred onto meridic diet and reared individually until the pupal stage. Pupae of each family line, if available, were then placed in paper containers for egg laying as described in Niu et al. (2013). Only 2 laboratory colonies of the 9 lines were successfully established from the survivors of the leaf tissue assays with F_2 generations. These 2 colonies were actually derived from the same F_2 family line (line 32) of the first sampling, one for Genuity®VT Double Pro™ (thereafter labeled as FL1-32VT) and one for Genuity®SmartStax™ (FL1-32SS) (Table 4). In most cases, the very limited number of survivors and varied larval developmental and adult emergence times within a family line resulted in the failure to establish laboratory colonies.

The 2 colonies of *S. frugiperda* that were considered to tolerate leaf tissue of Genuity®VT Double Pro™ or Genuity®SmartStax™ were re-evaluated twice for larval survival on leaf tissue of Genuity®VT Double Pro™ or Genuity®SmartStax™ leaf tissue. In the first re-evaluation, a total of 128 neonates of each colony were placed on the leaf tissue of their corresponding Bt corn products. After 7 days on Genuity®VT Double Pro™, only 3 small larvae (≤ 2nd instar) of FL1-32VT were recovered (Table 4), while 32 small (≤ 2nd instar) and 2 large (≥ 3rd instar) of FL1-32SS were found from Genuity®SmartStax™ leaf tissue (Table 4). In the second leaf tissue re-evaluation, a total of 640 neonates were tested on Genuity®VT Double Pro™. After 7 days, 58 small larvae and 2 large larvae were obtained (Table 4). Similarly, a total of 752 neonates were assayed against Genuity®SmartStax™ in the second re-evaluation and a total of 100 survivors were re-covered with 63 small and 27 large larvae after 7 days (Table 4).

Larvae of Bt-SS strain survived well on whole plants of the non-Bt corn hybrid with a survivorship of 37.5 ± 7.2% and a leaf injury rating of 9 (Davis' 1-9 scale) after 15 days, whereas no live larvae of Bt-SS were recovered from the 2 Bt corn hybrids with only little leaf injury (a leaf injury rating of 1.19 ± 0.19) to Genuity®VT Double Pro™ and no damage to Genuity®SmartStax™. The results validated the high level of insecticidal activity of the whole plants of the 2 Bt corn hybrids against *S. frugiperda* in the greenhouse. In the tests with the 2 potentially 'tolerant' insect families, both greenhouse tests showed no larvae of these 2 colonies could survive on the whole Bt plants of either Genuity®VT Double Pro™ or

TABLE 4. RE-EVALUATIONS OF SUSCEPTIBILITY OF TWO POTENTIALLY TOLERANT FAMILIES OF *SPODOPTERA FRUGIPERDA* AGAINST GENUITY®VT DOUBLE PRO™ AND GENUITY®SMARTSTAX™ CORN PRODUCTS.

		Leaf tissue re-evaluations						Whole plant tests					
		Trial 1			Trial 2			Trial 1			Trial 2		
		Total no of larvae	No. of live larvae		Total no of larvae	No. of live larvae		Total no of larvae	No. of live larvae	Leaf injury ratings	Total no of larvae	No. of live larvae	Leaf injury ratings
Inset Line	Bt maize		Small	Large		Small	Large						
FL1-32VT	Genuity®VT Double Pro™	128	3	0	640	58	2	50	0	1.40 ± 0.22	50	0	1.25 ± 0.13
FL1-32SS	Genuity®SmartStax™	128	32	2	752	63	27	50	0	1.85 ± 0.11	50	0	1.35 ± 0.13

Notes: FL1 and FL2 refer to the first sampling and second sampling, respectively, from Collier County in Florida.
Larvae that were ≤ 2nd instar were classified as small larvae, while larvae that were ≥ 3rd instar were classified as large larvae.

Genuity®SmartStax™ and larvae of these 2 colonies caused no or little leaf injury to the 2 Bt corn hybrids (Table 4). In contrast, on non-Bt corn plants, the family line 32 caused an average of leaf injury rating of 5.42 ± 0.44 after 7 days. After 13 days, the leaf injury ratings increased to 7.67 ± 0.25 and an average of 2.3 ± 0.3 live larvae/plant were recovered from the non-Bt corn plants. The results of the greenhouse tests showed that the 2 potentially tolerant family lines were still susceptible to whole plants of the 2 pyramided Bt corn products. Greenhouse whole plant tests for the other 7 potentially tolerant lines were not performed because of the failure to establish colonies of these lines. Because of the similar performance of all of the 9 potentially tolerant lines in the leaf tissue bioassay (Table 3), and because all larvae of FL1-32VT and FL1-32SS were killed on whole plants of both Genuity®VT Double Pro™ and Genuity®SmartStax™ hybrids in both trials (Table 4), we concluded, without any additional information, that the other 7 potentially tolerant lines to Bt corn leaf tissue are most likely also susceptible to the lethal action of whole plants of the two pyramided Bt corn hybrids.

Discussion

Baseline line survival tests showed that the overall performance of *S. frugiperda* was consistent and similar between the Bt-SS and the family lines derived from field collections. Larvae of the Bt-SS strain and the family lines derived from the field collections exhibited a relatively high mortality (~40%) after 7 days on non-Bt corn leaf tissue. However, they survived well on the meridic diet with an overall 7-day survivorship of 92.1%. In addition, larvae of the family lines that were tested in the greenhouse also survived well on whole plants of non-Bt corn after 13-15 days and caused heavy leaf damage. The results of the baseline bioassays indicated that the Bt-SS strain and the two-parental family lines established from the field collections were healthy. Natural mortality of insects reared on fresh plant materials or intact plants are common (Gassmann et al. 2011; Ghimire et al. 2011; Wangila et al. 2012). We believe that the cannibalistic behavior of *S. frugiperda* larvae should not play a big role in the different mortality rates observed between the bioassays with meridic diet and fresh leaf tissue. Our preliminary bioassays showed that larval mortality of *S. frugiperda* reared on non-Bt corn leaf tissue from 1-to-4 insects per assay well did not increase significantly (Huang et al. unpublished data). Such differences in larval mortality observed between the diet bioassay and the leaf tissue test could be due to the existence of some natural resistance factors in the plants to insects. Similar results were also reported in other lepidopteran corn insect pests, such as *O. nubilalis*, *D. grandiosella*, and sugarcane borer, *Diatraea saccharalis* (F.) (Crambidae) (Huang et al. 2006).

Corn leaf tissues have been used in the F_2 screen for detecting Bt resistance alleles in *D. saccharalis* and *D. grandiosella* (Huang et al 2007a, 2007b, 2012). In those studies, larval survival on corn leaf tissue in the F_2 screen was found to be highly correlated with survival on whole Bt corn plants in the greenhouse. However, in the current study we found that both FL1-32VT and FL1-32SS had a high survivorship on Bt corn leaf tissue, but could not survive on whole Bt plants in the greenhouse. To confirm this observation, 2 independent trials were conducted with both Bt corn products in the greenhouse and the results were validated. The difference in performance of *S. frugiperda* on leaf tissue and whole plants suggests that any survivors observed on leaf tissue in laboratory bioassays should be re-examined carefully on the whole plants to confirm resistance.

A previous study had shown that both Genuity®VT Double Pro™ and Genuity®SmartStax™ corn products were excellent against a Bt susceptible strain of *S. frugiperda* and almost 100% mortality was observed on both leaf tissue tests in the laboratory and whole plant tests in the greenhouse (Niu et al. 2013). However, in the current study, a considerable number of larvae from many family lines survived in the leaf tissue bioassay, especially of the populations collected from Florida. All 9 potentially tolerant lines identified in the leaf tissue in this study were derived from the Florida populations. Such survival on leaf tissue of the two pyramided Bt corn products may be due to a major resistance allele to the Cry1F protein in Genuity®SmartStax™ and/or cross-resistance to Cry1A.105 protein in Genuity®VT Double Pro™ and Genuity®SmartStax™. In another study, the same F_2 family lines of *S. frugiperda* used in the current study were screened against a Cry1F corn hybrid (Huang et al. unpublished data). The F_2 screen on Cry1F corn leaf tissue showed that 46 out of these family lines possessed at least 1 major resistance allele to Cry1F corn plants, which included 11 family lines of the Louisiana populations and 35 lines from the Florida populations. These family lines of *S. frugiperda* were found to be highly resistant to both purified Cry1F protein and whole Cry1F plants. All of the 9 family lines that showed a high larval survivorship and significant larval growth on leaf tissue of the pyramided Bt corn plants in the current study were among the lines that were found to carry major resistance alleles to Cry1F corn plants (Huang et al. unpublished data). The results suggest that the Cry1F resistance alleles in these tolerant family lines could result in a significant growth and survival on the leaf tissue of the pyramided Bt plants. The Cry1A.105 in the pyramided Bt corn plants is a chimeric protein incorporat-

ing domains I and II from Cry1Ab and Cry1Ac and domain III from Cry1F (US-EPA 2010). It is, therefore, possible that some level of cross-resistance could exist between Cry1F and Cry1A.105 because of the associations in their gene structures and the relative tolerance of *S. frugiperda* to Cry1Ab/Cry1Ac proteins (US-EPA 2001; Chilcutt et al. 2007; Hardke et al. 2011).

If Cry1F resistance and/or cross-resistance to Cry1A.105 in *S. frugiperda* were present, it was not enough to allow larvae to survive on whole plants of Genuity®VT Double Pro™ or Genuity®SmartStax™, most likely because the activity of the Cry2Ab2 protein in the plants. Cry2Ab2 has a protein structures that differs from that of Cry1A.105 and these 2 proteins have different binding sites in the midguts of the target insects; thus Cry2Ab2 has a mode of action distinct from that of Cry1F or Cry1A.105 (Storer et al. 2012b). Several studies have shown that usually a Cry1A resistant insect is not cross-resistant to Cry2Ab2 (Wu et al. 2009; Sivasupramaniam et al. 2008; Brévault et al. 2009). Similarly, a recent study also showed that a highly Cry1F corn resistant population of *S. frugiperda* collected from Puerto Rico survived well on leaf tissue of Genuity® VT Double Pro™ and Genuity® SmartStax™ corn hybrids in a 7-day-bioassay (Niu et al. 2013) but could not survive on whole plants either of Genuity® VT Double Pro™ or Genuity®SmartStax™ in the greenhouse (Ying Niu unpublished data). The results of these studies showed that these two pyramided Bt corn traits could provide some value in managing the Cry1F resistance in *S. frugiperda*. However, once resistance occurs to one Bt protein in a target insect, a pyramided Bt corn plant may just function as a single-gene Bt trait and resistance management strategy in such situations should be evaluated in future studies.

In summary, the results of the leaf tissue bioassays in the laboratory and whole plant tests in the greenhouse showed that all 149 two-parent family lines of *S. frugiperda* collected from the 3 locations in Louisiana and Florida during 2011 were susceptible to either Genuity®VT Double Pro™ or Genuity®SmartStax™ corn products. The results suggest that the pyramided Bt corn products containing Genuity®VT Double Pro™ and Genuity®SmartStax™ corn traits are effective against *S. frugiperda* including those possessing resistance alleles to Cry1F corn.

Endnotes

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