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Authors: Niu, Ying, Meagher, Robert L., Yang, Fei, and Huang, Fangneng

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# 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

YING NIU<sup>1</sup>, ROBERT L. MEAGHER JR.<sup>2</sup>, FEI YANG<sup>1</sup> AND FANGNENG HUANG<sup>1\*</sup>

<sup>1</sup>Department of Entomology, Louisiana State University Agricultural Center, Baton Rouge, LA 70803-1710, USA

<sup>2</sup>Insect Behavior and Biocontrol Unit, CMAVE USDA-ARS, Gainesville, FL 32608, USA

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

## ABSTRACT

Larval survival of Cry1F-susceptible (FL), -resistant (PR and Cry1F-RR), and -heterozygous (FL × PR and Cry1F-RS) populations of the fall armyworm, *Spodoptera frugiperda* (J. E. Smith) to purified Cry1F protein and corn leaf tissue of 7 *Bacillus thuringiensis* (Bt) corn hybrids and 5 non-Bt corn hybrids was evaluated in the laboratory. The 7 Bt corn hybrids represent 5 Bt corn traits: Herculex® I, which expresses a single Bt protein (Cry1F), and Genuity® VT Double Pro™, VT Triple Pro™, SmartStax™, and Agrisure® Viptera™ 3111, which contain ≥ 2 pyramided Bt genes. The original FL and PR populations were collected from corn fields in 2011 in Florida and Puerto Rico, respectively. Diet-incorporation bioassays showed that FL was susceptible to Cry1F protein with a LC<sub>50</sub> value of 0.13-0.23 µg/g, while PR was highly resistant to Cry1F protein (> 137-fold). FL was also susceptible to all 7 Bt corn hybrids with a 7-day mortality of > 95%, while PR and a backcrossed and reselected population, Cry1F-RR, were highly resistant to Cry1F corn leaf tissue. The resistance was recessive or incompletely recessive. None of the 5 populations of *S. frugiperda* could survive on Viptera™ 3111, suggesting this Bt corn trait can completely overcome the resistance and thus should provide a means of managing Cry1F resistance in *S. frugiperda*. However, Cry1F-RR exhibited a significant cross-resistance to the leaf tissue of the other 3 pyramided Bt corn traits. The possible cross-resistance between single-gene and pyramided Bt corn products suggest that careful selection of Bt genes is essential in use of gene pyramiding strategy for resistance management.

**Key Words:** *Spodoptera frugiperda*; Bt susceptibility; Cry1F; multiple genes; resistance management; Bt corn

## RESUMEN

Se evaluó en el laboratorio la sobrevivencia de las larvas en poblaciones del gusano cogollero del maíz, *Spodoptera frugiperda* (J. E. Smith) (FL), susceptibles a la proteína Cry1F (FL), -resistentes (PR y Cry1F-RR) y heterocigotos (FL × PR y Cry1F-RS) a la proteína purificada Cry1F del tejido de la hoja de 7 híbridos de maíz con *Bacillus thuringiensis* (Bt) y 5 híbridos de maíz sin Bt. Los 7 híbridos de maíz con Bt representan 5 características de maíz con Bt: Herculex® I, que expresa una sola proteína Bt (Cry1F), y de Genuity® VT Doble Pro™, VT Triple Pro™, SmartStax™ y Agrisure® Viptera™ 3111, que contiene genes de ≥ 2 piramidales Bt. Las poblaciones originales de FL y PR fueron recogidas de los campos de maíz en el 2011 en Florida y Puerto Rico, respectivamente. Los bioensayos de líneas de incorporación-Dieta mostraron que la FL fue susceptible a la proteína Cry1F con un valor CL<sub>50</sub> de 0.13 a 0.23 g / g, mientras que la PR fue altamente resistente a la proteína Cry1F (> 137 veces). La FL también fue susceptible a los 7 híbridos de maíz con Bt, con una mortalidad de 7 días de > 95%, mientras que la PR y una población retrocruzada y re-seleccionada, Cry1F-RR, fue altamente resistente al tejido de la hoja de maíz Cry1F. La resistencia fue recesiva o incompletamente recesiva. Ninguna de las 5 poblaciones de *S. frugiperda* no podría sobrevivir sobre Viptera™ 3111, lo que sugiere que esta característica de maíz Bt puede superar por completo la resistencia y por lo tanto debe proveer un medio para manejar la resistencia Cry1F en *S. frugiperda*. Sin embargo, Cry1F-RR exhibió una resistencia cruzada significativa con el tejido de la hoja de las otras 3 características de maíz Bt piramidales. La posible resistencia cruzada entre los productos de maíz Bt de un solo gen y piramidal sugiere que la

selección cuidadosa de los genes Bt es esencial en el uso de la estrategia de pirámide de genes para el manejo de resistencia.

Palabras Clave: *Spodoptera frugiperda*; susceptibilidad Bt; Cry1F; genes múltiples, manejo de resistencia, maíz Bt

Transgenic corn hybrids expressing *Bacillus thuringiensis* (Bt) proteins were initially developed to reduce injury from corn stalk borers such as the European corn borer, *Ostrinia nubilalis* (Hübner) and southwestern corn borer, *Diatraea grandiosella* (Dyar). Fall armyworm, *Spodoptera frugiperda* (J. E. Smith), is an important pest of corn in both North and South America (Pashley et al. 1985; Pashley 1986; Buntin et al. 2004; Chilcutt et al. 2007). Several studies have evaluated the field efficacy of first generation single gene Bt corn products (e.g. YieldGard® Corn Borer, Herculex®I) against *S. frugiperda* (Buntin et al. 2000; 2004; Buntin 2008; Siebert et al. 2008). Results of these studies showed that the single gene Bt corn also could suppress *S. frugiperda* but the suppression levels were usually not high enough to qualify as "high dose". For this reason, *S. frugiperda* is not listed as a target species of the first generation Bt corn technologies except Herculex®I expressing the Cry1F protein (US EPA 2001a).

Herculex®I Cry1F corn was first registered in 2001 in the United States and later became commercially available in the United States and Puerto Rico in 2003 to control stalk borers and some Noctuidae moths including *S. frugiperda*. This insect has been reported as the most important corn pest in Puerto Rico (US EPA 2007; Storer et al. 2010). Unfortunately, field resistance to Cry1F corn was observed in *S. frugiperda* populations in Puerto Rico in 2006 (Storer et al. 2010). This became the first example of field resistance to commercial Bt crops in the world (US EPA 2007; Matten et al. 2008; Storer et al. 2010). Besides intensive plantings of Cry1F corn during the 3 yr in Puerto Rico, several other factors might have contributed to the development of field resistance (US EPA 2007; Storer et al. 2010; Huang et al. 2011). To delay resistance development and broaden the target spectrum, a gene-pyramiding strategy has been utilized to develop transgenic plants that express multiple Bt proteins with dissimilar modes of action for targeting a same group of insect pests (Ghimire et al. 2011). The first commercialized pyramided Bt corn technologies for managing lepidopteran pests in the United States included Genuity®VT Double Pro™, Genuity®VT Triple Pro™, Genuity® SmartStax™, and Agrisure® Viptera™ 3111. Compared to the first generation single-gene Bt corn, the pyramided Bt corn products are more effective for controlling some Noctuidae species including *S. frugiperda* (Burkness et al. 2010)

and thus *S. frugiperda* is listed as a target in all pyramided Bt corn traits that have been commercialized for managing above-ground lepidopteran corn pests in the United States (US EPA 2009; 2010; Monsanto 2012; Syngenta 2012).

During 2011, 2 field populations of *S. frugiperda* were established from larvae collected from corn fields in Florida and Puerto Rico, respectively. Preliminary studies showed that the Puerto Rico population was highly resistant to Cry1F corn plants, while the Florida population was still susceptible to the Cry1F corn. Therefore, these 2 populations of *S. frugiperda* should provide great value for analyzing cross-resistance to other Bt corn technologies, especially to the recently commercialized pyramided Bt corn. The objectives of this study were to 1) document the resistance of the field population of *S. frugiperda* from Puerto Rico to purified Cry1F protein and commercial Cry1F corn and 2) to determine the cross-resistance of this population to pyramided corn products.

## MATERIALS AND METHODS

### Insect Sources

Two field populations of *S. frugiperda* were established from larvae collected from corn fields in Florida and Puerto Rico, respectively, in 2011. The Florida population (FL) was initiated from 96 larvae sampled from Hendry County in south Florida and the Puerto Rico population (PR) was developed from >300 larvae collected in southern Puerto Rico. Field-collected larvae were reared individually on a meridic diet (Ward's Stonefly Heliothis diet, Rochester, NY) in 30-ml plastic cups (Fill-Rite, Newark, NJ) until the pupal stage. The larval-rearing cups were held in 30-well trays (Bio-Serv, Frenchtown, NJ) and placed under room conditions until pupation. Pupae of each population were placed in 3.8-L paper containers (Huhtamaki Foodservice, De Soto, KS) containing ~100g of vermiculite (Sun Gro, Pine Bluff, AR) for adult emergence, mating, and oviposition. Insect populations had been maintained in the laboratory for 2 generations for FL and 3 generations for PR when this study was initiated.

Susceptibility of *S. frugiperda* was evaluated using 2 approaches: 1) a diet incorporating bioassay with purified Cry1F protein and 2) testing on leaf tissue of Bt and non-Bt corn hybrids. There were 2 independent trials for each test approach. For the diet incorporation bioassays, the first trial

used the original 2 populations (FL and PR) that were established from larvae collected from fields without further selection in the laboratory. In the first trial with leaf tissue bioassays, larval mortality was evaluated for 3 insect populations including FL, PR, and an  $F_1$  population (FL  $\times$  PR) that was generated by crossing FL and PR. Results of the first trial showed that compared to FL, the PR population was highly resistant to both the purified Cry1F protein and Cry1F corn leaf tissue (see below). After the first trial with Cry1F protein and corn leaf tissue, the original PR larvae were selected on Cry1F corn (Pioneer 31D59) leaf tissue for 2 generations. In the selection process, 2-3 pieces of leaf tissue were placed in each well of 32-well C-D International trays (Bio-Ba-32, C-D International, Pitman, NJ). Approximately 5-10 newly hatched larvae were released in each well. For each generation, >1000 neonates were selected on Cry1F corn leaf tissue. After 7 days, the survivors were transferred into the diet. After each selection, approximately 120-180 survivors were reared until the next generation. If the number of survivors was more than enough (e.g. >180 larvae), only the survivors with a relatively bigger body size were used to develop the next generation. The Cry1F corn leaf tissue selected-PR populations were then backcrossed with the FL population and reselected for Cry1F resistance in  $F_2$  generations on Cry1F corn leaf tissue. The procedures of the reselections for Cry1F resistance in the  $F_2$  generations of the backcrosses were the same as described above. Thereafter, the backcrossed and reselected population was referred as Cry1F-RR. The Cry1F-RR population had been continuously selected on Cry1F corn leaf tissue for at least 2 more generations before it was used for this study. In addition, another  $F_1$  population (Cry1F-RS) was developed by crossing individuals from FL and Cry1F-RR. In the second trial, susceptibility of *S. frugiperda* was evaluated for all 3 populations including FL, Cry1F-RR, and Cry1F-RS in both diet incorporation and leaf tissue bioassays.

#### Source of Cry1F Protein and Corn Leaf Tissue

In the diet incorporation bioassays, purified trypsin-activated (99.9%) Cry1F protein was obtained from Case Western Reserve University, Cleveland, OH. The Cry proteins were produced using recombinant *Escherichia coli* culture and were subsequently activated with trypsin. The activated Cry proteins were lyophilized before they were used in the bioassays. The purity of Cry1F proteins was determined using high-performance liquid chromatography and sodium dodecyl sulfate polyacrylamide gel electrophoresis (Pusztai-Carey et al. 1995; Masson et al. 1998).

In the leaf tissue tests, susceptibility of *S. frugiperda* was evaluated on leaf tissue of 5 non-Bt

and 7 Bt corn hybrids (Table 1). The 7 Bt corn hybrids represent 5 Bt corn traits, which include 1 single-gene Bt corn product, Herculex®I and 4 pyramided Bt corn products, Genuity®VT Double Pro™, Genuity®VT Triple Pro™, Genuity® SmartStax™, and Agrisure® Viptera™ 3111. Herculex®I contains a single Bt gene, Cry1F (Event TC1507), effective for above-ground lepidopteran insects. VT Double Pro™ expresses 2 Cry proteins, Cry1A.105 and Cry2Ab2 (Event MON89034) and both proteins are effective against above-ground lepidopteran species including *S. frugiperda* (Monsanto 2012). VT Triple Pro™ contains the same 2 Cry proteins in VT-2P plus Cry3Bb1 (MON88017) which is effective against below-ground corn rootworms *Diabrotica* spp. (Coleoptera: Chrysomelidae) (Monsanto 2012). SmartStax™ produces 6 Bt proteins including the 3 Bt proteins of VT Triple Pro™ plus Cry1F (Event TC1507) targeting lepidopteran species and Cry34/35Ab1 (Event DAS-59122) against rootworms (Monsanto 2012). Viptera™ 3111 expresses 3 Bt proteins including Vip3A (Event MIR162) and Cry1Ab (Event Bt11) for controlling lepidopteran species and mCry3A (Event MIR604) for managing rootworms (DiFonzo & Collen 2012). The 5 non-Bt corn hybrids were genetically closely related to 1 or 2 of the 7 Bt corn hybrids. In each of the 2 trials, larval mortality of *S. frugiperda* was evaluated on corn leaf tissue of 5 Bt corn hybrids representing 5 Bt corn technologies along with 2 (1<sup>st</sup> trial) or 3 (2<sup>nd</sup> trial) non-Bt corn hybrids (Table 1). Expression (or not expression) of Bt proteins in plants was confirmed using ELISA-based assays (EnviroLogix, Quantiplate™ kits, Portland, ME).

#### Diet Incorporation Assays

Larval susceptibility of *S. frugiperda* to purified Cry1F protein was individually assayed using a diet incorporation procedure in 128-cell trays (C-D International, Pitman, NJ). In each bioassay, 6-8 Cry1F concentrations were used. Cry1F concentrations used in each bioassay were slightly different depending on the insect population and amount of Cry1F protein available. In the first trial, Cry1F concentrations of 0, 0.1, 0.316, 1, 3.16, 10, and 31.6 µg/g were used to assay both FL and PR populations. Based on the results of the first trial, Cry1F concentrations used in the 2<sup>nd</sup> trial were modified to 0, 0.0316, 0.1, 0.316, 1, 3.16, and 10 µg/g in assaying FL. In addition, concentrations of 31.6 (for both PR and Cry1F-RS) and 100 µg/g (for PR only) were also included in the second bioassays. To prepare the appropriate concentrations of Bt diet, purified Cry1F protein was first suspended in distilled water at the room temperature and stirred completely using an iron stick to ensure that the protein was uniformly distributed in the solution. The Cry1F solutions were then mixed with a meridic diet (WARD'S

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

Traits	Corn hybrid	Event	Used in	Abbreviation in the figures	Bt genes	Major target pests
Non-Bt	DKC 61-22	—	Trial 1	NonBt-1	closely related to DKC 61-21	—
Non-Bt	DKC 67-86	—	Trial 1	NonBt-2	closely related to DKC 67-88	—
Non-Bt	DKC 63-45	—	Trial 2	NonBt-1	closely related to DKC 61-21	—
Non-Bt	N78N-GT	—	Trial 2	NonBt-2	closely related to N78N-3111	—
Non-Bt	Pioneer 31G66	—	Trial 2	NonBt-3	closely related to Pioneer 31D59	—
Herculex <sup>®</sup> I	Pioneer 31D59	TC1507	Trial 1 & 2	HX1	Cry1F	lepidoptera
Genuity <sup>®</sup> VT Double Pro <sup>™</sup>	DKC 64-04	MON89034	Trial 1	VT-2P	Cry1A.105, Cry2Ab2	lepidoptera
	DKC 63-87	MON89034	Trial 2	VT-2P	Cry1A.105, Cry2Ab2	lepidoptera
Genuity <sup>®</sup> VT Triple Pro <sup>™</sup>	DKC 67-88	MON89034+ MON 88017	Trial 1	VT-3P	Cry1A.105, Cry2Ab2, Cry3Bb1	lepidoptera & rootworms
	DKC 62-97	MON89034+ MON 86017	Trial 2	VT-3P	Cry1A.105, Cry2Ab2, Cry3Bb1	lepidoptera & rootworms
Genuity <sup>®</sup> SmartStax <sup>®</sup>	DKC 61-21	MON89034+ MON88017+TC1507+ DAS-59122	Trial 1 & 2	SmartStax	Cry1A.105, Cry2Ab, Cry1F, Cry3Bb1, Cry34/35Ab	lepidoptera & rootworms
Agrisure <sup>®</sup> Vipitera <sup>™</sup> 3111	N78N-3111	MIR162+Bt11+MIR604	Trial 1 & 2	VIP	Vip3A, Cry1Ab, mCry3A	lepidoptera & rootworms



Stonefly *Heliothis* diet) just prior to placing the diet into individual cells of the 128-cell trays. In the bioassay, approximately 1 g of treated diet was placed into each cell. One neonate (<24 h) was released on the diet in each cell. After larval inoculation, cells were covered with vented lids (C-D International, Pitman, NJ). The bioassay trays were placed in environmental chambers maintained at 28 °C, 50% RH, and a 16:8 (L:D) h photoperiod. Larval mortality was recorded on the 7<sup>th</sup> day after inoculation. Larvae were considered dead if they did not respond after being touched with a camel hair brush. In a bioassay, each combination of insect population by Cry1F concentration was replicated 4 times with 16-32 larvae in each replicate.

#### Leaf Tissue Test

Fully expanded leaf tissues of Bt and non-Bt corn hybrids were removed from greenhouse grown V5-V8 stage plants. In the bioassays, 2-3 pieces of leaf tissue were placed in each well of a 32-well C-D International tray (Bio-Ba-32, C-D International, Pitman, NJ). In each of the 2 trials, 4 neonates (<24 h old) of each of 3 populations were placed on the surface of the leaf tissue in each well. Bioassay trays containing leaf tissues and neonates were placed in growth chambers maintained at the same conditions as for the diet incorporation bioassays. Larval mortality was recorded on the 7<sup>th</sup> day after release of neonates. As mentioned above, larvae were considered dead if they did not respond after being touched with a camel hair brush. In each trial, there were 4 replications for each combination of corn hybrid and insect population and each replication included 32 neonates in 8 wells ( $n = 128$ ).

#### Data Analysis

In the diet incorporation bioassay, larval mortality of *S. frugiperda* at a Cry1F concentration was corrected with mortality on the control diet using the method as described in Abbott (1925). The corrected concentration/mortality data were then subjected to a probit analysis to determine the Cry1F concentration that produced a 50% mortality value ( $LC_{50}$ ) and the corresponding 95% confidence interval (CI) (Finney 1971; SAS Institute 2010). For each bioassay, the Cry1F concentrations used in the probit analysis included the highest concentration that produced zero mortality, the lowest concentration that resulted in 100% mortality, and all results between those extremes (Huang et al. 2007). In the bioassays with PR and Cry1F-RR, no significant larval mortality was observed even at the highest Cry1F concentrations tested, and thus the  $LC_{50}$  values for these 2 populations were considered to be greater than the

highest Cry1F concentrations used in the bioassays. Resistance ratios for each Cry protein were calculated using the  $LC_{50}$  value of PR, Cry1F-RR, or Cry1F-RS divided by the  $LC_{50}$  of the FL population.

Because the  $LC_{50}$  values of the PR and Cry1F-RR populations couldn't be calculated with the probit analysis, larval mortality data, after transformed by arcsine ( $x$ )<sup>0.5</sup>, were also subjected to a two-way analysis of various (ANOVA) with Cry1F concentration and insect population as the 2 main factors. Similarly, in the 2 trials using corn leaf tissue, percent larval mortalities were first transformed by arcsine ( $x$ )<sup>0.5</sup> and then analyzed using a 2 way ANOVA with corn hybrid and insect population as the 2 main factors (SAS Institute, 2010). Treatment means in all ANOVAs were separated with LSMEANS test at  $\alpha = 0.05$  level (SAS Institute, 2010).

In addition, the dominance level of Cry1F resistance in *S. frugiperda* was estimated using 2 approaches. The first approach involved the use of the Stone's dominance "D" value. The  $LC_{50}$  values estimated in the 2<sup>nd</sup> diet incorporation bioassays were used to calculate the dominance "D" value using the formula described in Stone (1968). The "D" value ranges from -1 to 1: a value of -1 indicating resistance is completely recessive; a value of 0 suggesting resistance is additive; and a value of 1 implying resistance is completely dominant. The dominance level of Cry1F resistance in *S. frugiperda* was also estimated as "effective dominance",  $D_{ML}$ , using the method as described in Bourguet et al. (2000).  $D_{ML}$  ranges between 0 and 1.  $D_{ML} = 0$  refers to a completely recessive resistance and  $D_{ML} = 1$  means the resistance is completely dominant. In this study,  $D_{ML}$  was estimated using the mortality data of the 3 insect populations recorded in each of the 2 trials on corn leaf tissue of 4 Bt corn technologies.  $D_{ML}$  for Viptera<sup>TM</sup>3111 couldn't be calculated because all insect populations exhibited 100% mortality on the Bt corn leaf tissue in both trials.

## RESULTS

#### Susceptibility of Field Populations from Florida and Puerto Rico to Purified Cry1F Protein: Trial # 1

The FL population was susceptible to the purified Cry1F protein with a  $LC_{50}$  of 0.23 $\mu$ g/g and a 95% CI of 0.11-0.37 $\mu$ g/g (Table 2). Relative to FL, PR was highly resistant to the Cry1F protein. No significant larval mortality ( $\leq 13.7\%$ , corrected mortality) of PR was observed across all the Cry1F concentrations assayed and thus the  $LC_{50}$  value of this population was estimated to be > 31.6  $\mu$ g/g, which corresponded a resistance ratio of >137-fold.

Two-way ANOVA showed that the main effects of both Cry1F concentration and insect popula-

TABLE 2. LC<sub>50</sub>S AND 95% CONFIDENCE INTERVALS (CI) BASED ON LARVAL MORTALITY OF *SPODOPTERA FRUGIPERDA* NEONATES ON DIET TREATED WITH PURIFIED CRY1F *BACILLUS THURINGIENSIS* PROTEIN.\*

Population	N <sup>a</sup>	Slope ± SE	LC <sub>50</sub> (95%CI) (µg/g)	χ <sup>2</sup>	df	Resistance ratio
Trial 1						
FL	503	1.13 ± 0.18	0.23(0.11-0.37)	45.87	18	—
PR	—	—	>31.6	—	—	>137
Trial 2						
FL	553	1.38 ± 0.22	0.13(0.07-0.20)	71.62	18	—
Cry1F-RS	675	1.47 ± 0.16	1.07(0.76-1.50)	64.08	22	8.2
Cry1F-RR	—	—	>100	—	—	>769

<sup>a</sup>Total number of neonates assayed.  
<sup>b</sup>Resistance ratio of an insect population was calculated by dividing the LC<sub>50</sub> value of the population by that of the FL population.

tions on the 7-day larval mortality were significant ( $F = 31.31$ ;  $df = 6, 41$ ;  $p < 0.0001$  and  $F = 308.16$ ;  $df = 1, 41$ ;  $p < 0.0001$ , respectively). The effect of the interaction of the 2 factors was also significant ( $F = 26.82$ ;  $df = 6, 41$ ;  $p < 0.0001$ ). Significant larval mortality (50.2%) of the FL population occurred at 0.1 µg/g, the lowest concentration assayed, and the mortality reached 95.4% at 3.16 µg/g and 100% at 10 µg/g (Fig. 1). In contrast, Cry1F at all tested concentrations did not cause any significantly greater mortality to PR than those observed on non-Bt control diet.

Susceptibility of FL, Cry1F-RR, and Cry1F-RS Populations to Purified Cry1F Protein: Trial # 2

Similarly as observed in the first trial, the FL population was still susceptible to the purified Cry1F protein with a LC<sub>50</sub> of 0.13µg/g and a 95% CI of 0.07-0.20µg/g, which was not significantly different compared to the LC<sub>50</sub> value calculated in the first trial based on the overlapping of the 95% confident intervals (Table 2). The backcrossed and reselected population, Cry1F-RR, was also highly resistant to the purified Cry1F protein. Again, no significant mortality (≤8.7%, corrected mortality) of Cry1F-RR was recorded at the tested concentration range (up to 100 µg/g) and thus the LC<sub>50</sub> value was estimated to be > 100 µg/g, which was at least 769-fold greater than the LC<sub>50</sub> of FL. The LC<sub>50</sub> value of Cry1F-RS, the F<sub>1</sub> population of the cross between FL and Cry1F-RR, was 1.07µg/g with a 95% CI of 0.76-1.50µg/g, which was significantly greater than the LC<sub>50</sub> of FL based on the non-overlapping of the 95% confident intervals. However, the value of 1.07µg/g was considerably less than the LC<sub>50</sub> of Cry1F-RR.

Two-way ANOVA also showed that effects of Cry1F concentration, insect population, and their interaction on 7-day larval mortality were all significant ( $F = 56.79$ ;  $df = 8, 72$ ;  $p < 0.0001$  for,  $F = 130.88$ ;  $df = 2, 72$ ;  $p < 0.0001$ , and  $F = 32.15$ ;  $df = 13, 72$ ;  $p < 0.0001$ , respectively). Compared to

the non-treated control diet, Cry1F at the tested concentrations did not result in any significant levels of mortality against the Cry1F-RR population, even at the highest concentration evaluated in the bioassays (100µg/g) (Fig. 1). In contrast, significant mortality (39.1%) was observed for the FL population at the lowest concentration tested (0.0316 µg/g). Mortality of FL reached 94.6% at 1µg/g and 100% at 3.16 µg/g. Mortality of the Cry1F-RS population at ≤1 µg/g was low, between 11.3 (0.0316 µg/g) and 30% (1.0 µg/g). The mortality values were significantly less ( $P < 0.05$ ) than those of the FL population, but in general were not significantly different compared to the mortalities observed for the Cry1F-RR population ( $P > 0.05$ ). At ≥3.16 µg/g, mortality of Cry1F-RS increased significantly as the Cry1F concentration increased and reached 80.7% at 3.16 µg/g and 96.6% at 10 µg/g. The mortality of Cry1F-RS at 10 µg/g was not significantly different from 100% that was observed for the FL population.

Susceptibility of FL, PR, and FL x PR Populations to Bt Corn Leaf Tissue: Trial # 1

The effect of corn hybrid, insect population, and their interaction on larval mortality at 7 days was significant ( $F = 82.42$ ;  $df = 6, 63$ ;  $p < 0.0001$ ,  $F = 35.91$ ;  $df = 2, 63$ ;  $p < 0.0001$ , and  $F = 4.22$ ;  $df = 12, 63$ ;  $p < 0.0001$ , respectively). Larval mortality of the 3 insect populations on leaf tissue of the 2 non-Bt corn hybrids after 7 days varied significantly, ranging from 9.4% for PR to 66.4% for FL on DKC 67-86 (Fig. 2). Except for these 2 extremes, there was generally no significant difference in larval mortality on the 2 non-Bt corn hybrids. A high mortality (96.9%) of FL larvae was observed on leaf tissue of Herculex®I expressing the Cry1F protein, which was significantly greater than the mortality observed on the non-Bt corn leaf tissue. In contrast, the PR larvae appeared to be highly resistant to Cry1F corn leaf tissue, with a 7-day mortality of only

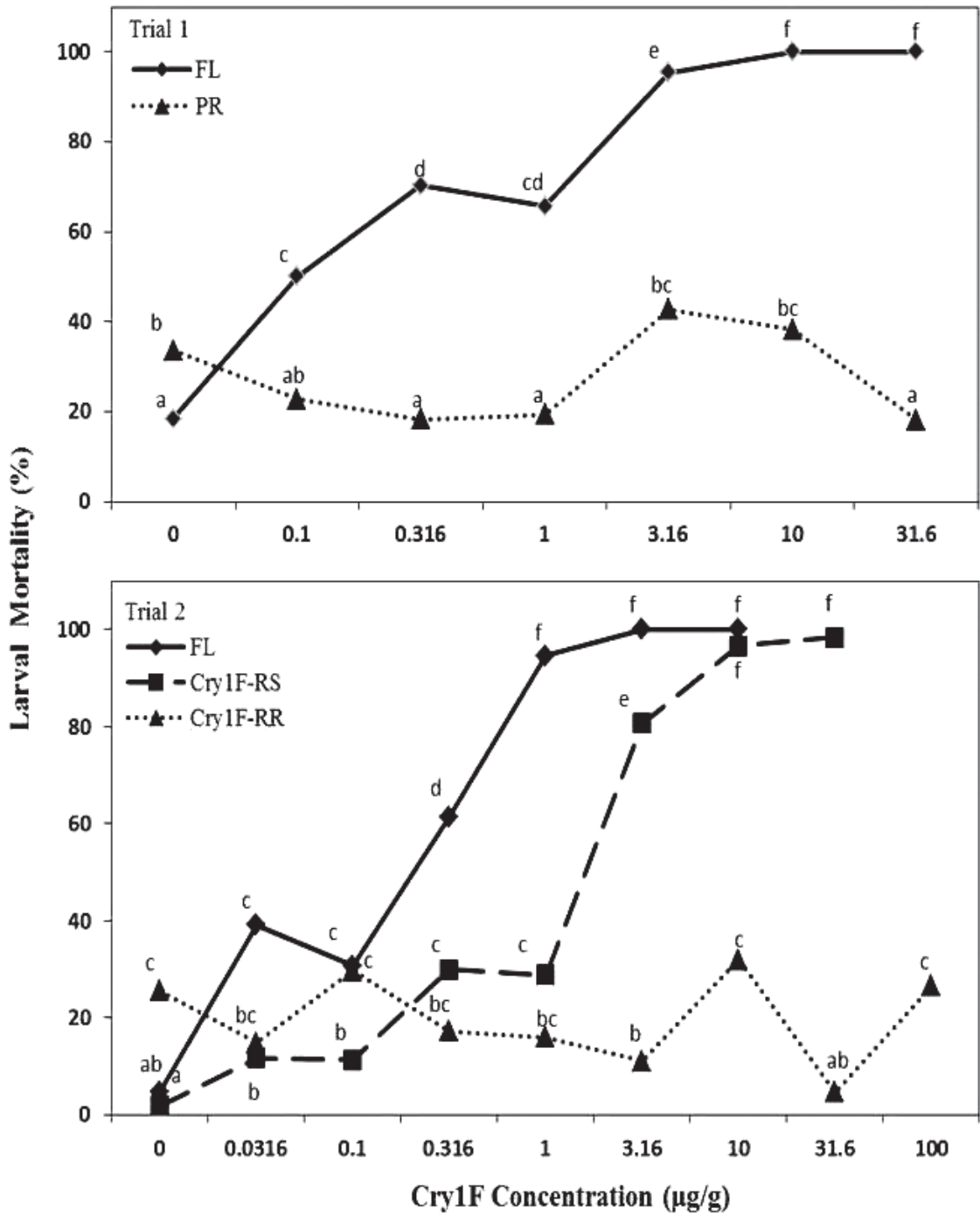


Fig. 1. Larval mortality of *Spodoptera frugiperda* after 7 days on diet treated with different concentrations of purified Cry1F protein. Mean values across all treatments in each figure followed by the same letter are not significantly different ( $P < 0.05$ ; LSMEANS test).

39.1%. This mortality level was similar to the average mortality (38.5%) of the 3 populations on the 2 non-Bt corn leaf tissue. The  $F_1$  population

of the cross between FL  $\times$  PR was susceptible to Cry1F leaf tissue, producing a 7-day mortality of 83.6% (Fig. 2). This was significantly greater



( $P < 0.05$ ) than that observed for PR but significantly less ( $P < 0.05$ ) than the mortality of FL. However, all 3 insect populations were susceptible to leaf tissue of the 4 pyramided Bt corn hybrids. No survivors of FL were observed after 7 days on the 4 pyramided Bt corn hybrids. Mortality of the FL x PR population was also high on these pyramided Bt corn products ( $>95\%$ ), which was not significantly different ( $P > 0.05$ ) than that observed for the FL population. PR larvae couldn't survive on Agrisure® Vipitera™ 3111 leaf tissue, while a few larvae (e.g. 6.2-14.1%) of PR survived on the other 3 pyramided Bt corn hybrids (Genuity®VT Double Pro™, VT Double Pro™, and SmartStax™).

#### Susceptibility of FL, Cry1F-RR, and Cry1F-RS Populations to Bt Corn Leaf Tissue: Trial # 2

As observed in the first trial, the effect of corn hybrid, insect population, and their interaction on larval mortality was all significant ( $F = 147.88$ ;  $df = 7, 71$ ;  $p < 0.0001$ ,  $F = 120.67$ ;  $df = 2, 71$ ;  $P < 0.0001$ , and  $F = 21.27$ ;  $df = 14, 71$ ;  $p < 0.0001$ , respectively). The 7-day larval mortality was in general similar ( $P > 0.05$ ) on leaf tissue of the 3 non-Bt corn hybrids across the 3 insect populations with an average mortality of 36.3% (Fig. 2). Again, larval mortality of the FL population was high and not significantly different ( $P > 0.05$ ) on the 5 Bt corn hybrids, ranging from 98.4-100%. The Cry1F-RS population was also susceptible to the 5 Bt corn hybrids with a mortality range of 93.0 to 100%. The mortality (93.0%) of Cry1F-RS on Herculex I was similar to that (94.5%) observed on the VT Double Pro™ but was significantly less than the mortalities (100%) on VT Triple Pro™, SmartStax, and Vipitera 3111. The backcrossed and reselected Cry1F-RR population was also not able to survive on the Vipitera 3111 hybrid. In contrast, larvae of the Cry1F-RR population survived well on the Cry1F corn hybrid with a 7-day mortality of 43.8%, which was not significantly different compared to the mortalities observed on the 3 non-Bt corn hybrids. However, unlike the performance of the PR population observed in the first trial, larvae of Cry1F-RR survived well on the other 3 pyramided Bt corn hybrids. The 7-day mortality of Cry1F-RR was only 34.4% on SmartStax™, which was not significantly different than the mortalities observed on the 3 non-Bt corn hybrids. The mortality (49.2%) of Cry1F-RR on VT Double Pro™ was also not significantly different compared to those values recorded on the non-Bt corn leaf tissue. Mortality (67.2%) of Cry1F-RR on the VT Triple Pro™ hybrid was significantly greater ( $P < 0.005$ ) than the mortality on non-Bt corn leaf tissue but significantly less ( $P < 0.001$ ) than that of FL and Cry1F-RS populations on Bt corn hybrids.

#### Dominance Level of Cry1F Resistance in *S. frugiperda*

Dominance level "D" measured using Stone's method (Stone 1968) based on the  $LC_{50}$  values of FL, Cry1F-RR, and Cry1F-RS populations was  $< -0.37$ , suggesting that Cry1F resistance in Cry1F-RR was recessive or incompletely recessive (Table 3). Because all 3 populations could not survive on leaf tissue of the Vipitera™ 3111 hybrid or N78N-3111 in both trials, effective dominance level,  $D_{ML}$ , couldn't be calculated for this Bt corn product.  $D_{ML}$  values measured based on larval mortality on leaf tissue of the other 6 Bt corn hybrids were consistent in the 2 trials, ranging from 0 to 0.33 in trial # 1 and from 0 to 0.22 in trial # 2. The results suggested that the Cry1F resistance in *S. frugiperda* was functionally recessive to incompletely recessive on leaf tissue of the 5 Bt corn hybrids representing 4 Bt corn traits, Herculex®I, Genuity®VT Double Pro™, Triple Pro™, and SmartStax™.

#### DISCUSSION

Since first being commercialized in 1996, Bt crops have gained an international attention and widely acceptance in the world, especially among corn and cotton producers in the United States (James 2011; NASS 2012). Cry1F expressed corn (event TC1507) was registered in 2001 in the United States to control above-ground lepidopteran pests including *S. frugiperda*. In 2003, Cry1F corn was first commercially cultivated in the United States and Puerto Rico to control *S. frugiperda*. This insect is the most important lepidopteran corn pest in Puerto Rico (US EPA 2007; Storer et al. 2012). Studies have revealed that field resistance to Cry1F corn in *S. frugiperda* occurred in late 2006 (US EPA 2007; Matten et al. 2008; Storer et al. 2010; Huang et al. 2011). In the current study, susceptibility of 2 field populations of *S. frugiperda* collected from Florida and Puerto Rico to purified Cry1F protein and corn leaf tissue of a commercial Cry1F corn hybrid was evaluated in the laboratory. Limited by the available amount of purified Bt protein, Cry1F susceptibility of *S. frugiperda* could be assayed up to only 100 µg/g in this study. No significant larval mortality of the PR and Cry1F-RR populations was observed even at the highest Cry1F concentrations examined in the bioassays. The  $LC_{50}$  values, therefore, could not be determined with the probit analysis for both populations (Table 2). Nevertheless, the results of this study clearly demonstrated that the population from Puerto Rico, compared to the Florida population, was highly resistant to both purified Cry1F protein and Cry1F corn leaf tissue. The Cry1F resistance was recessive or incompletely recessive as measured with both the Stone's dominance "D" value on Cry1F diet and the effective dominance level

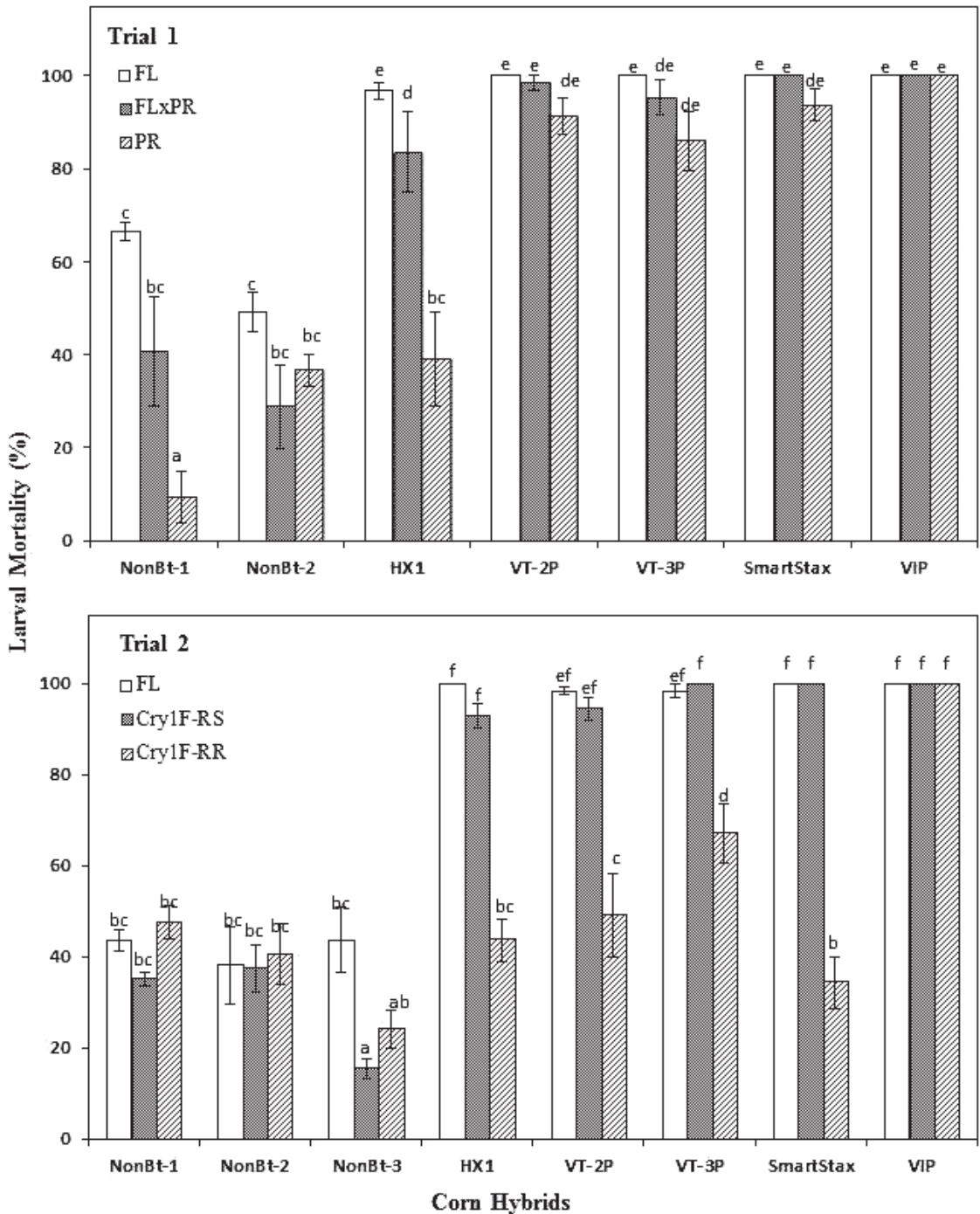


Fig. 2. Larval mortality of *Spodoptera frugiperda* after 7 days feeding on leaf tissue removed from non-Bt and Bt corn plants. Mean values across all treatments in each figure followed by the same letter are not significantly different ( $P < 0.05$ ; LSMEANS test). NonBt-1 in trial one = DKC 61-22, NonBt-1 in trial #2 = DKC 63-45, NonBt-2 in trial #1 = DKC 67-86, NonBt-2 in trial #2 = N78N-GT, NonBt-3 in trial #2 = Pioneer 31G66, HX1 = Pioneer 31D59, VT-2P in trial #1 = DKC 64-04, VT-2P in trial #2 = DKC 63-87, VT-3P in trial #1 = DKC 67-88 VT-3P in trial #2 = DKC 62-97, SmartStax = DKC 61-21, VIP = N78N-3111.

TABLE 3. DOMINANCE LEVEL OF CRY1F RESISTANCE IN *SPODOPTERA FRUGIPERDA* COMPUTED USING DATA FROM DIET INCORPORATING AND LEAF TISSUE BIOASSAYS.

Test material and trial	Dominance level*
Stone's dominance "D" value	
Diet incorporating, trial-2	-0.37
Effective dominance " $D_{ML}$ "	
Pioneer 31D59, leaf tissue-trial 1	0.23
DKC 64-04, leaf tissue, trial-1	0.19
DKC 67-88, leaf tissue, trial-1	0.33
DKC 61-21, leaf tissue, trial-1	0.00
N78N-3111, leaf tissue, trial-1	—
Pioneer 31D59, leaf tissue, trial-2	0.12
DKC 63-87, leaf tissue, trial-2	0.08
DKC 62-97, leaf tissue, trial-2	0.00
DKC 61-21, leaf tissue, trial-2	0.00
N78N-3111, leaf tissue, trial-2	—

\* Stone's dominance "D" value ranges from -1 to 1: a value of -1 indicates resistance is completely recessive; a value of 0 suggests resistance is additive; and a value of 1 implies resistance is completely dominant. Effective dominance " $D_{ML}$ " ranges between 0 and 1.  $D_{ML} = 0$  refers to a completely recessive resistance and  $D_{ML} = 1$  means the resistance is completely dominant.

" $D_{ML}$ " on Cry1F corn leaf tissue. In the calculation of dominance, the FL population was considered homozygous susceptible with no resistance alleles. The resistance could be more recessive than that measured in this study if the assumption was not true. The dominance levels of the Cry1F resistance estimated in this study appeared to be similar as reported in another population of *S. frugiperda* collected from Puerto Rico in 2007 (Storer et al. 2010). It was reported that upon an initial confirmation of the field resistance to Cry1F corn in Puerto Rico, the technology providers immediately stopped the commercial sale of Cry1F corn seeds to growers in this area (Matten et al. 2008; Storer et al. 2010). Although limited by the insect sampling in the current study, our results suggest that the field resistance to Cry1F corn was persistent in Puerto Rico even after several yr without planting of Cry1F corn. A recent study also reported that field populations of *S. frugiperda* collected from 2 other locations in 2011 were still highly resistant to Cry1F protein in diet (Storer et al. 2012).

In both leaf tissue tests, all 5 populations of *S. frugiperda* could not survive for 7 days on the Agrisure® Vipitera™ 3111 hybrid. The results suggested that Vipitera™ 3111 Bt corn could completely overcome the Cry1F resistance and thus should provide a means for managing Cry1F resistance in this important target pest of Bt corn.

In a previous study, survival of 14,400 neonates from 150 two-parental family lines of *S. frugiperda* collected from Florida and Louisiana was evaluated on Agrisure® Vipitera™ 3111 plants using an  $F_2$  screen (Yang et al. 2013). Results of that study showed that all larvae were killed within 7 days on Vipitera™ 3111 corn leaf tissue. Although both the current and previous studies were not designed to evaluate the high dose assumption, the results of these studies suggest that Vipitera™ 3111 corn is highly effective and likely produces a "high-dose" against *S. frugiperda*.

The Cry1F-susceptible population, FL, and the 2  $F_1$  populations of 2 crosses, FL x PR and Cry1F-RS, were also susceptible to the other 3 pyramided Bt corn products: Genuity®VT Double Pro™, Triple Pro™, and SmartStax™. The 7-day mortality on these Bt corn products was  $\geq 94.5\%$  in both trials (Fig. 2). The results demonstrated that the Cry1F resistance in *S. frugiperda* was functionally recessive or nearly completely recessive on corn leaf tissue of the 3 pyramided Bt corn traits as showed in the  $D_{ML}$  values in Table 3. However, performance of the Cry1F resistant populations (PR and Cry1F-RR) on corn leaf tissue of Genuity®VT Double Pro™, Triple Pro™, and SmartStax™ varied between the 2 trials. In the first trial, PR larvae appeared to be susceptible to the 3 pyramided Bt corn products with a 7-day mortality of 85.9-93.8% (Fig. 2). In contrast, larvae of the backcrossed and reselected population, Cry1F-RR, survived well on the 3 pyramided Bt corn products in the second trial with a 7-day mortality of only 34.4 to 67.2% (Fig. 2). The exact reasons causing the difference are unknown. One of the most likely reasons could be due to a result of the continued selections on Cry1F corn leaf tissue both before and after the backcross. For example, the original population (PR) collected from Puerto Rico might still not be homozygous for the Cry1F resistance and continued selection on Cry1F corn leaf tissue could eliminate the susceptible and probably heterozygous individuals in the population and thus could further elevate the resistance level in the Cry1F-RR population.

Additional studies are still needed to demonstrate if the Cry1F-RR population could survive on whole plants of these pyramided Bt corn products. Nevertheless, the results of the current study suggest that at least some levels of cross-resistance to the 3 pyramided Bt corn traits exist in Cry1F corn resistant *S. frugiperda*. Both VT Double Pro™ and Triple Pro™ contain Cry1A.105 and Cry2Ab2, while SmartStax™ expresses those proteins and Cry1F. Cry1A.105 is a chimeric gene comprised of domains I and II which are identical with the respective domains from Cry1Ab and Cry1Ac and domain III of Cry1F (Biosafety Clearing-House 2009). Thus



it should not be surprising that some levels of cross-resistance could exist between Cry1F and Cry1A.105 because of the association in the gene structures of the 2 proteins. Studies have shown that *S. frugiperda* was somewhat tolerant to the single gene Cry1Ab corn hybrids (US EPA 2001b; Chilcutt et al. 2007; Hardke et al. 2011; Huang et al. 2011). In addition, Cry1Ac usually shares similar binding sites with Cry1Ab in the insect midgut membranes (Ballester et al. 1999; Ferré & Van Rie 2002; Hua et al. 2001; Tan 2010) and thus Cry1Ab resistance is often found to be cross-resistant to Cry1Ac in many insect species (Tabashnik et al. 1994; Ferré & Van Rie, 2002; Rang et al. 2004; Siqueira et al. 2004; Wu et al. 2009; Pereira et al. 2010; Tan 2010; Crespo et al. 2011; Zhang et al. 2013). Cry2Ab2 is a different protein compared to Cry1A and studies have shown that a Cry1A resistant insect is usually not cross-resistant to Cry2Ab2 (Wu et al. 2009; Brévault et al. 2009; Sivasupramaniam et al. 2008). Thus, the survival of the Cry1F-RR population on the 3 pyramided Bt corn products, Genuity®VT Double Pro™, VT Triple Pro™, and SmartStax™, could be due to a combination factor of cross-resistance and the expression level of Cry2Ab2 protein that may be not high enough by it alone to kill the Cry1F resistant larvae in a 7-day period of bioassays. The possible cross-resistance between single-gene and pyramided Bt corn in *S. frugiperda* suggest that careful selection of different Bt genes is essential in use of gene pyramiding strategy for resistance management.

#### ENDNOTES

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