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Effect of Two *Bacillus thuringiensis*¹ Proteins on Development of the Fall Armyworm² after Seven-Day Exposure

Efecto en el Desarrollo del Gusano Cogollero² Después de Ser Expuesto por Siete Días a Dos Proteínas de *Bacillus thuringiensis*¹

Maribel Portilla^{3*}, Carlos A. Blanco⁴, Renéé Arias⁵, and Yu Cheng Zhu³

Abstract. Field-evolved resistance of fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), to the bacterium *Bacillus thuringiensis* (Bt) has been reported worldwide as one of the most serious threats to the sustainability of Bt maize crop. Therefore, it is important to assess the magnitude of adult survival and the possibility of cross-resistance of fall armyworm neonates exposed to Bt proteins. In this study, bioassays were used to examine susceptibility of two field-collected Cry1Fa-resistant strains of fall armyworm from Puerto Rico (456RR, 512RR) and their crosses with a susceptible strain (Monsanto SS) (456SR-RS, 512SR-RS). LC₅₀ values varied in both Cry1Fa-resistant strains and in their backcrosses with the susceptible strain. The two RR strains were more tolerant to Cry1Fa and Cry1Ac proteins in earlier instars of development than were their crosses. Greater survival to the adult stage was obtained in the 512 RR strain and their RS-SR crosses when exposed to all concentrations of Cry1Ac and 1Fa. Survival to adult in the 456 RR was much greater when exposed to Cry1Fa than to Cry1Ac. Adults of 456 RR and their crosses survived only when exposed to the lowest concentrations of Cry1Ac. Our data confirmed great resistance to Cry1Fa and Cry1Ac in *S. frugiperda* larvae from Puerto Rico. However, based on the larvae that survived the 7-day diet bioassay and developed to pupae and adult maturity on regular diet, their LC₅₀ values were less for both resistant strains and their crosses.

Resumen. La resistencia desarrollada en campo en el gusano cogollero del maíz, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), ha sido reportada a nivel mundial, la cual es una grave amenaza para la sostenibilidad del cultivo de maíz Bt. Por lo tanto, es importante evaluar la magnitud de la supervivencia de los adultos y la posibilidad del cruce de resistencia de los neonatos del gusano cogollero del maíz expuestos a proteínas de Bt. En este estudio, se utilizaron bioensayos para

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examinar la susceptibilidad de dos cepas de campo del gusano cogollero del maíz colectada en Puerto Rico (456RR, 512RR) resistentes a la proteína Cry1FA junto con sus cruzas con una cepa susceptible (Monsanto SS) (456SR-RS, 512SR-RS). Los valores LC_{50} variaron tanto para las cepas resistentes a Cry1Fa como para sus retro-cruzas con la cepa susceptible. Las dos cepas RR tuvieron mayor tolerancia a las proteínas Cry1Fa y Cry1Ac en sus primeros estadios de desarrollo, que sus cruzas. Se obtuvo una mayor supervivencia en estado adulto con la cepa 512RR y sus cruzas RS-SR cuando se expusieron a todas las concentraciones de Cry1Ac y 1Fa. La supervivencia de adultos en la cepa 456RR fue mucho mayor cuando se expuso a Cry1Fa que cuando se expuso a Cry1Ac. Nuestros datos confirmaron resistencia a Cry1Fa y Cry1Ac en larvas de *S. frugiperda* de Puerto Rico. Sin embargo, según la población de larvas que sobrevivieron al bioensayo de dieta de 7-días y alcanzaron el estado de pupas y adultos en dieta regular, sus valores de LC_{50} fueron menores tanto para las cepas resistentes como para sus cruzas.

Introduction

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), is a destructive pest that attacks 186 host plants belonging to 42 families: Poaceae (35.5%), Fabaceae (11.3%), Solanaceae and Asteraceae (4.3%), Rosaceae and Chenopodiaceae (3.7%), and Brassicae and Cyperaceae (3.2%) (Casmuz et al. 2010). Fall armyworm is native to the tropical region from the United States to Argentina of the Western Hemisphere. The insect annually disperses long distances during the summer months. It can be found in all the U.S. states east of the Rocky Mountains but is a regular and serious pest in the southeastern states (Blanco et al. 2010). Its resistance to diverse insecticides amplifies its impact worldwide. Transgenic maize plants that contain genes from *Bacillus thuringiensis* Berliner (Bt) that express microbial insecticidal proteins have become an integral component of crop management systems to control the pest.

Crops developed through biotechnology offered enormous benefits to the environment, health of humans and animals, and improvement of socioeconomic conditions of farmers and the public (ISAAA Brief 2017). Hectares planted with Bt crops worldwide increased from 1.1 million in 1996 to 98.5 million in 2016, with a cumulative total of more than 830 million; Bt corn (*Zea mays* L.), cotton (*Hirsutum gossypium* L.), and soybean (*Glycine max* L.) accounted for >99% of the total. The benefits of Bt crops, however, are threatened by selection for pest resistance. From the first commercial planting of genetically modified crops until today, 36 cases of resistance to Bt proteins have been reported. Of the 36, four cases were reported for fall armyworm: 1) in Puerto Rico to Cry1F maize marketed in 2003 (low doses of Cry1F) (Blanco et al. 2010, Storer et al. 2010, Jurat-Fuentes et al. 2011, Huang et al. 2014, Arias et al. 2015, Zhu et al. 2015); 2) in Brazil to Cry1Ab maize marketed in 2008 (low doses of Cry1Ab) (Farias et al. 2014, Omoto et al. 2015); 3) in Brazil to Cry1F maize marketed in 2009 (low doses of Cry1F) (Farias et al. 2015); and 4) in Brazil to Vip3Aa maize marketed in 2010 (high doses of Vip3Aa) (Bernardi et al. 2014). Because of development of this resistance in the field, it is limiting the economy and environmental benefits of using transgenic plants for control.

Selection of resistance by fall armyworm worldwide to the proteins expressed in Bt maize technology is a concern mostly in tropical regions because of intense agricultural production with multiple cropping seasons (Bernardi et al. 2015), due in

part to lack of compliance with non-Bt structure refuge recommendations (Farias et al. 2014).

The goal of this study elaborated the need for information on the impact of exposure to Bt toxins (Cry1Fa and Cry1Ac) by larvae after bioassays were used to determine the effect of Bt toxin on adult survival and possible large amounts of cross-resistance. The information is important for assessment of the effectiveness of host plant refuges for resistance management practices.

Materials and Methods

The study was done at the Southern Insect Management Research Unit (SIMRU), USDA, ARS, at Stoneville, MS. *S. frugiperda* strains resistant to Cry1Fa (456 and 512) were established from individual larvae collected from field populations in maize at Puerto Rico (Santa Isabela) (Blanco et al. 2010). The strains were cultured separately for several generations and group-mated or pair-mated for rearing according to the method described by Blanco et al. (2009). Surviving adults from third and fourth generations of 456 and 512 strains were used. A Bt-susceptible laboratory strain of fall armyworm used for the crosses and check was obtained from a colony by Monsanto at Union City, TN.

Single pairs of females (♀) and males (♂) from the generations of resistant strains F₄ 456RR and F₃ 512RR, with the Bt-susceptible laboratory strain designated F₁ susceptible Monsanto generation (SS) and heterozygous (R♀S♂-S♀R♂) were confined. Each pair was maintained separately for mating and oviposition using the method described by Blanco et al. (2009). Eggs from each single resistant pair (456 and 512), susceptible pair, and reciprocal crosses (F₅ 456♀ x F₁ Monsanto♂; F₅ 456♂ x F₁ Monsanto♀; F₄ 512♀ x F₁ Monsanto♂; and F₄ 512♂ x F₁ Monsanto♀) were collected between the 3rd and 4th day after mating. Neonates from each single pair that emerged on the same day were used for bioassays and followed throughout the entire life cycle until adults were obtained. The standard assay method (Blanco et al. 2010) was used by overlaying Cry1Fa and Cry1Ac purified proteins produced in *Pseudomonas fluorescens* (Flüggel) on lepidopteran artificial diet (Shaver and Raulston 1971). Fifteen concentrations (ng/cm²) of Cry1Fa (0, 0.00007, 0.0007, 0.007, 0.07, 0.78, 7.8, 15.6, 31.2, 62.5, 125, 250, 500, 1000, 2000, 3000), and Cry1Ac (0, 0.00002, 0.0002, 0.002, 0.026, 0.26, 2.6, 5.2, 10.4, 20.8, 41.6, 83.3, 166, 333, 666, 1333) were added per single well in a plastic tray (BAW128, Bio-Assay Tray-128 Cells, Newark, DE) with dispensed, cooled solidified diet (1.5 ml of diet per cell). A check (nontreated diet) was used in bioassays of both Cry proteins. A neonate larva was put into each well and the tray was covered with a ventilated plastic top to prevent diet dehydration (BACV16, Bio-Assay Lid-16 Cells, Newark, DE) or larvae from escaping. Each concentration and check were replicated at four, eight, and 16 times depending on availability, and one larva was used per concentration/toxin/susceptible strain, resistant strain, and their reciprocal crosses. Seven days after exposure to toxin, the surviving larvae were transferred individually into 37-ml plastic cups (T-125, Solo Cup Company) that contained 5 ml of artificial diet without Bt protein (Shaver and Raulston 1971), where they remained until the adult stage. Numbers that died were recorded every day for the first 7 days after neonates were exposed to the toxins, and 3 days thereafter until insects fed regular artificial diet reached adulthood (individuals with inhibition growth were counted as being alive until their death). Larval, pupal, and adult mortality was evaluated.

Mortality data for each insect strain and reciprocal crosses were analyzed by PROBIT procedure using common logarithm (log to base 10) of the concentration (SAS, 2012). χ^2 goodness-of-fit was used to determine if response data followed the linear Probit model LC_{50} . Differences in LC_{50} were considered significant if 95% confidence limits did not overlap. Mortality for each bioassay was corrected for check effects by using Abbott's formula (1925). Resistance ratio (RR_{50}) and confidence intervals were calculated using the method by Robertson and Priesler (1992).

Results

Differences in susceptibility between strains (RR and SS) and their reciprocal crosses ($R\phi S\sigma$ and $R\sigma S\phi$) were evident when exposed to Cry1Fa (Figs. 1ABC, 2ABC) than to Cry1Ac (Figs. 1DEF, 2DEF) where survival of both resistant strains was greater at all concentrations, while in reciprocal crosses, larvae survived only at low concentrations of Bt proteins. The 456 strain to Cry1AC responded similarly but only at the larval stage (7 days after exposure).

After exposed for 7 days to diet containing Bt proteins, larvae were transferred to regular diet to complete the cycle. Growth was inhibited at prepupal and pupal stages, thus affecting emergence and survival of adults. The effect was more evident in larvae exposed to large concentrations of Bt proteins (Fig. 1F). The response differed from the 512 strain, in which concentration-response curves for the four genotypes indicated variability among concentrations after exposure, showing similar tolerance between resistant strains and reciprocal crosses SR-RS (Fig. 2DEF).

Despite much tolerance, survival of the four genotypes exposed to purified Cry1Fa and Cry1Ac decreased through time for both resistant strains and their crosses, resulting in lower LC_{50} values and resistant ratios (RR_{50}) based on adult survival, as those based only on survival of larvae after 7 days. However, no resistant strain or its cross was nearly as susceptible (more mortality and growth inhibition) than the check (SS) (Tables 1-4).

Resistance ratios were significantly greater for resistant 456 and 512 strains than their crosses (Figs. 1AD, 2AD). Offspring of inbred F_4 in 456 and 512 strains had RR_{50} from 790,526 to $4.3E+22$ ng/cm², respectively, for Cry1Fa at 7 days after exposure, and 59 to 876,745 for the reciprocal crosses (Table 1). This pattern (resistance ratios > 100,000) remained in both resistant strains in adults that developed to maturity after 7-day-old larvae were transferred to regular diet. The estimated RR_{50} based on that adult survival decreased 2.9-fold in the 456 strain, and $7.75E+14$ in the 512 strain, compared with LC_{50} and RR_{50} obtained only on larval survival 7 days after initiation of the bioassay. The RR_{50} s (adult survival) for the reciprocal crosses significantly decreased, ranging from 15 to 1,472. LC_{50} for the 456 and 512 strains was significantly less when exposed to Cry1Ac than Cry1Fa, with LC_{50} value of 203 and 284 ng/cm² 7 after exposure. LC_{50} values based on adult survival for reciprocal crosses ranged from 1.71 to 45 ng/cm². Tolerance to Cry1Ac was greater based on LC_{50} adult mortality (41ng/cm²) only for reciprocal cross $F_3 512\phi \times F_1 Mon\sigma$. LC_{50} of the other crosses decreased to <1.0, ranging from 0.002 to 0.123 (Table 4). LC_{50} for resistant strain Cry1Ac decreased 13-fold for 456 strain and 18-fold for 512 strain.

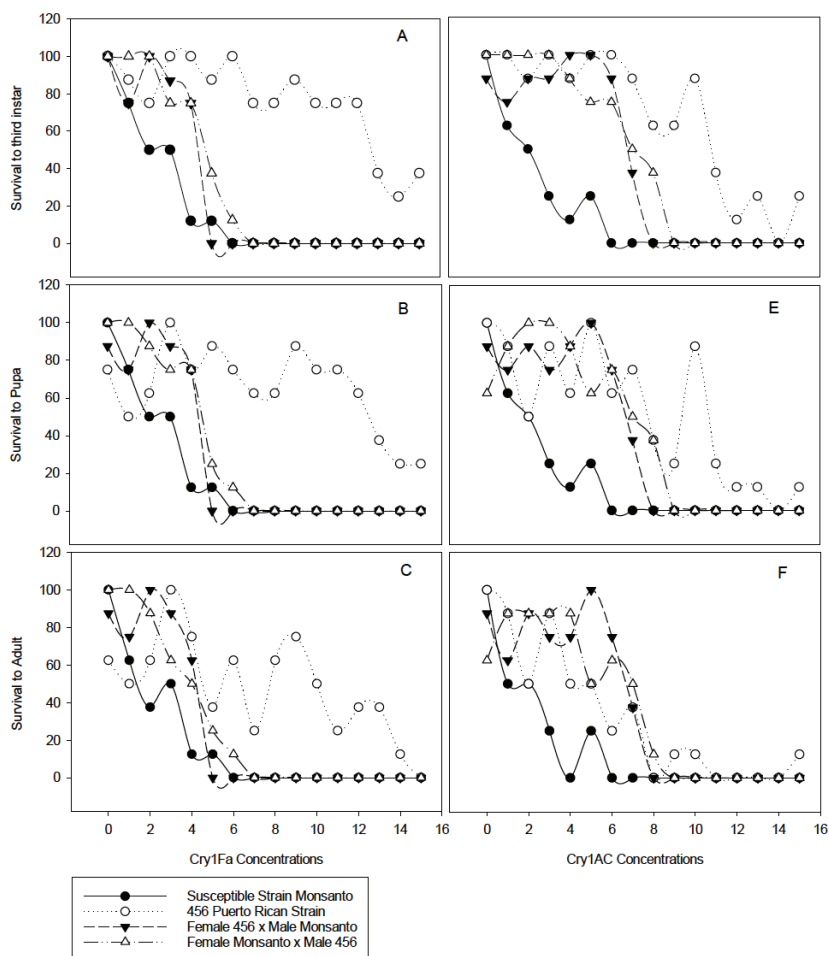


Fig 1. Concentration-response of *Spodoptera frugiperda* neonate 456 resistant strain and its reciprocal crosses in 7-day diet-overlay bioassays with purified Cry1Fa and Cry1Ac (A,D) transferred to regular diet for pupa (B,E) and adult (C,F) maturity. Concentrations Cry1F (ng/cm²): 0: check, 1: 0.00007, 2: 0.0007, 3: 0.007, 4: 0.07, 5: 7.8, 6: 7.8, 7: 15.6, 8: 31.2, 9: 62.5, 10: 125, 11: 250, 12: 500, 13: 1000, 14: 2000, and 15: 3000. Concentrations Cry1Ac (ng/cm²): 0: check, 1: 0.00002, 2: 0.0002, 3: 0.002, 4: 0.026, 5: 0.26, 6: 2.6, 7: 5.2, 8: 10.4, 9: 20.8, 10: 41.6, 11: 83.3, 12: 166, 13: 333, 14: 666, and 15: 1333.

Fig. 1. Respuesta de neonatos de *Spodoptera frugiperda* cepa 456 y de sus cruces reciprocos a concentraciones de proteínas purificadas Cry1Fa and Cry1Ac inoculadas en dieta artificial en bioensayos de 7-d despues de su exposicion (A,D), transferidas a dieta regular para obtener el desarrollo de pupa (B, E) y adulto (C,F).

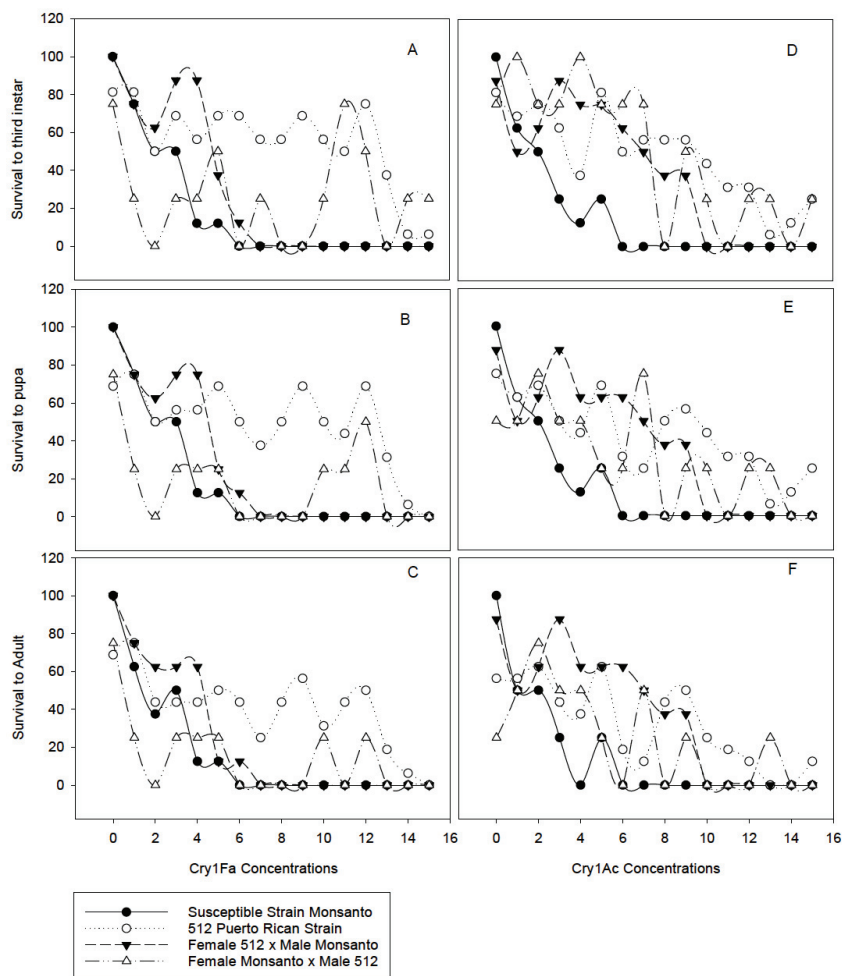


Fig. 2. Concentration-response of *Spodoptera frugiperda* neonates 512 resistant strain and its reciprocal crosses in 7-day diet-overlay bioassays with purified Cry1Fa and Cry1Ac (A,D), transferred to regular diet for pupa (B,E) and adult (C,F) maturity. Concentrations Cry1F (ng/cm²): 0: check, 1: 0.00007, 2: 0.0007, 3: 0.007, 4: 0.07, 5: 7.8, 6: 7.8, 7: 15.6, 8: 31.2, 9: 62.5, 10: 125, 11: 250, 12: 500, 13: 1000, 14: 2000, and 15: 3000. Concentrations Cry1Ac (ng/cm²): 0: check, 1: 0.00002, 2: 0.0002, 3: 0.002, 4: 0.026, 5: 0.26, 6: 2.6, 7: 5.2, 8: 10.4, 9: 20.8, 10: 41.6, 11: 83.3, 12: 166, 13: 333, 14: 666, and 15: 1333

Fig. 2. Respuesta de neonatos de *Spodoptera frugiperda* cepa 512 y de sus cruces reciprocos a concentraciones de proteínas purificadas Cry1Fa and Cry1Ac inoculadas en dieta artificial en bioensayos de 7-d despues de su exposicion (A,D), transferidas a dieta regular para obtener el desarrollo de pupa (B, E) y adulto (C,F).

Table 1. Mortality Response (LC₅₀) to Cry1Fa after Neonates of Resistant Strains of *Spodoptera frugiperda* from Puerto Rico and their Crosses with a Susceptible Strain (Mon) Were Exposed to Bt Toxin

Cuadro 1. Respuesta de Mortalidad (LC₅₀) a Cry1Fa después que los Neonatos de Cepas Resistentes de *Spodoptera frugiperda* de Puerto Rico y sus Cruzas con la Ceba Susceptible (Mon) fueron Expuestos a la Bt Toxina

Strain (RR, RS, SS)	Slope ± SE	LC ₅₀ (95% CI) ¹	Concentration response (ng/cm ²)						RR ₅₀ (95%CL) ⁴
			Probit trend						
			Test for slope ²			Test for GoF ³			
			X ²	P > X ²	X ²	P > X ²			
F ₁ Mon ♀ x F ₁ Mon ♂	0.234±0.062	0.0009 (1.7E-6 - 0.015)	14.273	0.0002	0.1951	0.9992	1		
F ₄ 456 ♀ x F ₄ 456 ♂	0.422±0.1798	1167 (290 - 18036)	5.497	0.019	0.9471	0.5022	970526 (21740 - 43326573)		
F ₃ 456 ♀ x F ₁ Mon ♂	n/a	0.0945 (-)	n/a	n/a	n/a	n/a	100 (-)		
F ₁ Mon ♀ x F ₃ 456 ♂	0.338±0.062	0.053 (0.008 - 0.231)	29.544	<.0001	0.5795	0.8726	59 (1.09 - 3122)		
F ₄ 512 ♀ x F ₄ 512 ♂	0.021±0.039	709391000 (-)	0.0214	0.0392	1.3701	0.1648	4.3E22 (9.3E-51 - 5.8E91)		
F ₃ 512 ♀ x F ₁ Mon ♂	1.851±0.546	1198 (676 -1657)	11.489	0.0007	0.9134	0.5379	876745 (22077 - 3.4E+08)		
F ₁ Mon ♀ x F ₃ 512 ♂	0.692±0.216	4.83 (0.00001 - 175)	9.163	0.0025	0.7127	0.7526	5331 (9 - 307505)		

¹LC₅₀ values are in ng/cm²; mortality was scored at 7 days.

²Test for slope significance indicated concentration affected mortality.

³Test for Goodness of Fit (GoF) significance indicated error from Probit trend was greater than expected for simple binomial response.

⁴RR₅₀ (Resistance Ratio) and 95% CI calculated using formula from Robertson and Priesler (1992). Differences among LC₅₀ values were significant if 95% CI did not include 1.0. RR₅₀ compare the LC₅₀ for Puerto Rican strains and crosses to the LC₅₀ for Monsanto strain as a check.

(-) Value for CI not calculated.

(n/a) Not available due to insufficient response.

Table 2. Mortality Response (LC₅₀) to Cry 1Ac after Neonates of *Spodoptera frugiperda*-Resistant Strains from Puerto Rico and their Crosses with a Susceptible Strain (Mon) Were Exposed to the Toxin

Cuadro 2. Respuesta de Mortalidad (LC₅₀) a Cry1Ac después que los Neonatos de Cepas Resistentes de *Spodoptera frugiperda* de Puerto Rico y sus Cruzas con la Cepa Susceptible (Mon) Fueron Expuestos a la Toxina

Species	Concentration response (ng/cm ²)					Probit trend				
	Slope ± SE	LD ₅₀ (95% CI) ¹	Test for slope ²			Test for GoF ³			RR ₅₀ (95%CL) ⁴	1
			X ²	P > X ²	X ²	P > X ²	X ²	P > X ²		
F ₁ Mon♀ x F ₁ Mon♂	0.197±0.054	0.0004 (3.41E-8 - 0.017)	13.257	0.0003	0.3174	0.9898				
F ₄ 456♀ x F ₄ 456♂	0.491±0.099	203 (901 - 431)	24.463	<.0001	1.2861	0.2124	425520 (2551 - 7.09E+8)			
F ₃ 456♀ x F ₁ Mon♂	5.998±155.329	15.08 (-)	0.002	0.969	0.3866	0.9746	34127 (91 - 12718267)			
F ₁ Mon♀ x F ₃ 456♂	0.341±0.062	1.71 (0.28 - 5.69)	30.024	<.0001	1.365	0.1674	3983 (21 - 762270)			
F ₄ 512♀ x F ₄ 512♂	0.359±0.106	284 (53 - 818)	11.554	0.0007	1.238	0.2438	105506 (491 - 22656681)			
F ₃ 512♀ x F ₁ Mon♂	0.304±0.106	42 (0.99 - 240)	8.191	0.0042	1.1253	0.3311	47269 (192 - 116363)			
F ₁ Mon♀ x F ₃ 512♂	1.061±0.473	45 (0.42 - 67)	5.038	0.025	0.569	0.8804	50379 (278 - 9103566)			

¹LC₅₀ values are in ng/cm²; mortality was scored at 7 days.

²Test for slope significance indicates concentrations affects mortality

³Test for Goodness of Fit (GoF) significance indicated error from Probit trend was greater than expected for simple binomial response.

⁴RR₅₀ (Resistance Ratio) and 95% CI calculated using formula from Robertson and Priesler (1992). Differences among LC₅₀ values were significant if 95% CI did not include 1.0. RR50 compare the LC₅₀ for Puerto Rican strains and crosses to the LC₅₀ for Monsanto strain as a check.
(-) Value for CI not calculated.

Table 3. Mortality Response (LC₅₀) to Cry 1Fa Based on Adult Survival after Neonates of *Spodoptera frugiperda*-Resistant Strains from Puerto Rico and their Crosses with a Susceptible Strain (Mon) Were Exposed to Toxin for 7 Days and Transferred to Regular Artificial Diet until Adults Emerged

Cuadro 3. Respuesta de mortalidad (LC₅₀) a Cry1Fa Basada en la Supervivencia de Adultos Después que los Neonatos de Cepas Resistentes de *Spodoptera frugiperda* de Puerto Rico y sus Cruzas con la Cepa Susceptible (Mon) Fueron Expuestos a la Toxina

Concentration response (ng/cm ²)							
Strain (RR, RS, SS)	Slope ± SE	LC ₅₀ (95% CI) ¹	Probit trend				
			Test for slope ²		Test for GoF ³		
			X ²	P > X ²	X ²	P > X ²	RR ₅₀ (95%CL) ⁴
F ₁ Mon ♀ x F ₁ Mon ♂	0.212±0.064	0.0005 (9.2E-9 - 0.019)	11.0037	0.0009	0.2641	0.9959	1
F ₄ 456 ♀ x F ₄ 456 ♂	0.730±0.379	603 (-)	3.704	0.0543	1.5231	0.1003	340106 (1257 - 91951103)
F ₃ 456 ♀ x F ₁ Mon ♂	n/a	0.0842 (-)	n/a	n/a	n/a	n/a	160 (-)
F ₁ Mon ♀ x F ₃ 456 ♂	0.285±0.054	0.017 (0.001 - 0.103)	28.0507	<.0001	0.3605	0.9815	35 (0.132 - 9025)
F ₄ 512 ♀ x F ₄ 512 ♂	1.744±0.709	927 (169 - 1509)	6.0549	0.0139	0.5969	0.8589	55502300 (2.3E-11 - 1.4E26)
F ₃ 512 ♀ x F ₁ Mon ♂	0.086±0.027	12 (0.005 - 768)	9.769	0.0018	0.1677	0.9996	1472 (1.656 - 1309490)
F ₁ Mon ♀ x F ₃ 512 ♂	0.059±0.045	0.00147 (-)	1.7047	0.1917	0.7868	0.6751	15 (0.00001 - 19069928)

¹LC₅₀ values are in ng/cm2; mortality was scored based on adult survival.

²Test for slope significance indicates concentrations affects mortality.

³Test for Goodness of Fit (GoF) significance indicated error from Probit trend was greater than expected for simple binomial response.

⁴RR₅₀ (Resistance Ratio) and 95% CI calculated using formula from Robertson and Priesler (1992). Differences among LC₅₀ values are significant if 95% CI do not include 1.0. RR₅₀ compare the LC₅₀ for Puerto Rican strains and crosses to the LC₅₀ for Monsanto strain as a check.

(-) Value for CI not calculated.

(n/a) Not available due to insufficient response.

Table 4. Mortality Response (LC₅₀) to Cry1Ac Based on Adult Survival after Neonates of *Spodoptera frugiperda*-Resistant Strains from Puerto Rico and their Crosses with a Susceptible Strain (Mon) Were Exposed to the Toxin for 7 Days and Transferred to Regular Artificial Diet until the Adult Emerged

Cuadro 4. Respuesta de Mortalidad (LC₅₀) a Cry1Ac Basada en la Supervivencia de Adultos Después que los Neonatos de Cepas Resistentes de *Spodoptera frugiperda* de Puerto Rico y sus Cruzas con la Ceba Susceptible (Mon) fueron Expuestos a la Toxina

Species	Slope ± SE	LD ₅₀ (95% CI) ¹	Concentration response (ng/cm ²)				
			Probit trend				
			Test for slope ²		Test for GoF ³		
			X ²	P > X ²	X ²	P > X ²	RR ₅₀ (95%CL) ⁴
F ₁ Mon♀ x F ₁ Mon♂	0.195±0.055	0.00014 (1.041E-8 - 0.0054)	12.316	0.0004	0.5541	0.8914	1
F ₄ 456♀ x F ₄ 456♂	7.082±406.616	15.41 (-)	0.0003	0.9861	0.4502	0.9514	99881 (30 - 331659411)
F ₃ 456♀ x F ₁ Mon♂	0.159±0.064	0.06 (1.1E-17 - 5.146)	6.021	0.0141	1.2042	0.2683	380 (0.035 - 4131458)
F ₁ Mon♀ x F ₃ 456♂	0.103±0.035	0.123 (4.1E-11 - 12.21)	8.443	0.0037	2.1531	0.0091	99 (0.013 - 732947)
F ₄ 512♀ x F ₄ 512♂	1.591±0.719	16 (0.98 - 26.81)	4.902	0.0268	0.4437	0.9542	91230 (607 - 13700655)
F ₃ 512♀ x F ₁ Mon♂	1.217±0.668	41 (-)	3.319	0.0685	0.5788	0.8731	162245 (939 - 28008221)
F ₁ Mon♀ x F ₃ 512♂	0.112±0.043	0.002 (8.9E-21 - 1.72)	6.358	0.0117	0.9152	0.5361	13 (0.00011 - 1560250)

¹LC₅₀ values are in ng/cm²; mortality was scored based on adult survival.

²Test for slope significance indicates concentrations affects mortality.

³Test for Goodness of Fit (GoF) significance indicates error from Probit trend is greater than expected for simple binomial response.

⁴RR₅₀ (Resistance Ratio) and 95% CI calculated using formula from Robertson and Priesler (1992). Differences among LC₅₀ values are significant if 95% CI do not include 1.0. RR₅₀ compare the LC₅₀ for Puerto Rican strains and crosses to the LC₅₀ for Monsanto strain as a check.

(-) Value for CI not calculated.

Discussion

Resistance enabled *S. frugiperda* from Puerto Rico to survive Cry1Ac and Cry1Fa Bt purified proteins in their diet during 7 days. However, development to pupal (Figs. 1B, 1E, 2B, 2E) and adult stages (Figs. 1C, 1F, 2C, 2F) was severely affected. Insects that survived toxin concentrations during bioassays were important for studying the genetic mechanism enabling them to survive. We showed that Cry1Fa rates impacted development to adulthood. Because of the “mosaic” expression of Bt proteins in maize kernels, and the influence of the environment and cultivar in production of proteins, fall armyworms could find sublethal doses that might or might not permanently affect their survival and passing of Bt-resistant genes to the next generation. Differences in susceptibility to either Bt protein were found among the resistant colonies and reciprocal outcrosses with a susceptible colony. Based on larval mortality through 7 days after emergence, our results and those of Blanco et al. (2010), Storer et al. (2010), Bernardi et al. (2014, 2015), Camargo et al. (2017), and Vassallo et al. (2019) indicated fall armyworms had developed resistance to Bt proteins. Populations from Puerto Rico were significantly less sensitive to Cry1Fa and Cry1Ac proteins than was susceptible Mon strain. Blanco et al. (2010) found four of 80 isofamilies from Puerto Rico (Santa Isabel) were tolerant to Cry1Fa in the initial F₂ and F₃ generations. The authors did not find variation in resistance for the four isofamilies that all survived the greatest Cry1Fa concentration representing more than 11,000-fold greater LC₅₀ than the susceptible population. Similar results were found by Storer et al. (2010) who bioassayed four populations from Puerto Rico, including one from Santa Isabel. They found the Santa Isabel colony did not differ in resistance from other colonies from different locations (LC₅₀ of >10000 ng/cm²) (34-fold each). The initial Cry1Fa-resistance frequency inferred by Blanco et al. (2009) has now been reported by Dangal and Huang (2015) who identified Cry1F resistance in two populations of fall armyworms from Puerto Rico and Florida. Farias et al. (2014, 2015) also reported Cry1Fa-resistance in a population of fall armyworms from Brazil. Velez et al. (2016) mentioned field resistance in fall armyworms from Puerto Rico and Brazil was recessive, autosomal, monogenic, and not linked to fitness costs. They also mentioned it remains to be determined if Cry1Fa resistance of fall armyworms from Puerto Rico, southern U.S., and Brazil are independent resistant events or the results of insect migration.

Our LC₅₀ for fall armyworms from Puerto Rico (456 and 512 strains collected from the same area of Santa Isabel) varied depending on the resistant parental strain and Cry protein to which they were exposed. Based on adults that survived and reached maturity after infected 7-day-old larvae were transferred to regular diet after the 7-day bioassay, the LC₅₀s clearly decreased for both resistant strains and their crosses; RR₅₀ remained high: 340,106-fold and 99,881-fold for 456 strain to Cry 1Fa and Cry 1Ac, respectively. Similarly, for 512 strain it was 55,502,300-fold and 91,230-fold when exposed to Cry 1Fa and Cry 1Ac, respectively. From the results, we assumed tolerance to large concentrations of Bt proteins by early instar *S. frugiperda* might determine much resistance but not real tolerance, with possible impact on reproductive ability. Probability of surviving to an adult and reproducing would be less than larvae that survive large doses of Bt proteins. For example, 43% of 456 strain larvae survived the greatest concentration of Cry1Fa (3,000 ng/cm²) and 30% survived the greatest concentration of Cry1Ac (1,333 ng/cm²). Thirty and 15% survived to the pupal stage, while none reached adulthood after exposure to Cry1Fa,

and only 8% of those exposed to Cry1Ac survived to the adult stage. Observations were similar for the 512 strain (Fig. 2ABCDEF). The insecticidal effects of Bt toxin in fall armyworm included less larval survival and pupation, as well as growth disruption. These were clear after the fifth instar. We noticed growth disruptions as inability of some larvae to successfully molt into the pupal stage or some pupae into the adult stage. However, detrimental effects in this study could be less severe under field conditions. Crespo et al. (2009) mentioned, that under normal circumstances, larvae feed on tissues with low expression of Bt toxin. Therefore, the caterpillar gut might not be as affected as it was in our investigation, and a larva might have a better chance to survive to the adult stage.

Modeling studies predict that if resistant adults mate with susceptible adults, and Bt plants are sufficiently toxic to kill heterozygous progeny from such mating, evolution of resistance will be delayed (Georghiou and Taylor 1977). This theory could be applied to our investigation where low tolerance was observed in reciprocal crosses compared with Monsanto and resistant strains except the F_3 512♀ x F_1 Mon♂ cross whose hybrid neonates were very tolerant to Cry1Ac (Table 2) with a RR_{50} of 162,245-fold. The LC_{50} value for reciprocal crosses of 456: 456♀ x Mon♂ (0.0945 ng/cm²) and Mon♀ x 456♂ (0.053 ng/cm²) were 9,705- and 16,449-fold less tolerant to Cry1Fa than their resistant strain (456) with LC_{50} of 1,167 ng/cm² (970,526-fold) (Table 1). Similar patterns were observed for 456 to Cry 1Ac and 512 to both proteins (Table 2). Our results agreed with those of Storer et al. (2010) who found LC_{50} s >3333 ng/cm² (>18-fold, sensitivity ratio) for a resistant parental strain and LC_{50} s of 583 ng/cm² (3.2-fold) and 639 ng/cm² (3.5-fold) for its reciprocals 7 days after exposure to the Bt Cry1Fa protein. However, the magnitude of sensitivity ratio they found for fall armyworm in Cry1Ac proteins was much less than for Cry1Fa which suggested low cross-resistance to Cry1Ac proteins in Cry1F-resistant *S. frugiperda* from Puerto Rico. The population collected at Santa Isabel, Puerto Rico was characterized as recessive, autosomal inheritance of resistance to Cry1Fa. Bernardi et al. (2015) and Monnerat et al. (2015) found similar results in Brazil where fall armyworms were very tolerant to Cry1Fa protein. Bernardi et al. (2015) mentioned that fall armyworms also conferred much cross resistance to Cry1A and Cry1Ab and low cross resistance to Cry2Ab2 based on 7-day bioassays on diet and leaf tissue.

Resistance of Puerto Rican colonies to Bt toxins was studied by Blanco et al. (2010) (456, 512, and 519 colonies); Jurat-Fuentes et al. (2011) (456 and 512 colonies); and Zhu et al. (2015), Arias et al. (2015), and Jakka et al. (2016) (456 colony). However, to our knowledge, the present study was first to examine resistance of the colonies to Cry1Ac and Cry1Fa, and the effect of exposing L_1 larvae in 7-day bioassays and development of the insect throughout its life cycle. The trade-off between Bt resistance and fitness was caused by severe growth disruption after the 5th instar as well as low population growth in surviving pupal and adult stages. This demonstrated that resistance of *S. frugiperda* was incompletely recessive to both Cry1Ac and Cry1Fa toxins. Autosomal inheritance of resistance is questionable, but resistance might be inherited from the mother, as opposed to a previous report by Blanco et al. (2008). Our results should be interpreted with caution, because neonates that survived the bioassay and reached adulthood were obtained from low concentration (0.007 to 1000 ng/cm² of Cry1Fa and 0.002 to 20.8 ng/cm² of Cry1Ac) (Figs. 1-2) of Bt toxins that insects might not normally find. However, our findings illustrated that if *S. frugiperda* responded to Bt toxins in the field as it did in the laboratory, mating resistant and susceptible insects would produce offspring with low tolerance, easily controlled by GE crops. When fall armyworm

acquires resistance to *B. thuringiensis*, the insects are not as 'fit' as their susceptible counterparts, are less competitive in the environment, produce fewer progeny, and might be eliminated. Niu et al. (2013) mentioned that of 150 F₂ two-parent families that survived purified Cry1A and Cry2Ab2 in 7-day diet bioassays, 149 survived a 7-day leaf-tissue bioassay, but no larva survived on whole plants of commercial maize hybrids containing the proteins in a greenhouse. Yang et al. (2015) suggested that although resistant alleles to the single gene Cry1A.105 are found in Louisiana, Texas, Georgia, and Florida; the joint frequency of the resistance alleles to both Cry1A.105 and Cry2Ab2 in fall armyworm still might be rare in the populations. Thus, commercial pyramiding maize hybrids could be considered a good strategy for managing the pest in the U.S. In general, data generated in this study expanded on previous studies and confirmed that Cry 1-based hybrid crops are facing challenges in managing fall armyworms mainly in tropical regions because of longer growing seasons, absence of freezing winter temperatures, extensive biodiversity, and most importantly low compliance with non-Bt structure refuge recommendations. Velez et al. (2016) emphasized the importance of meeting assumptions of the high-dose/refuge strategy for successful delay of resistance.

Conflicts of Interest

The authors declare no conflicts of interest.

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