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# Susceptibility of Isofamilies of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) to Cry1Ac and Cry1Fa Proteins of *Bacillus thuringiensis*

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**Susceptibilidad de Isofamilias de *Spodoptera frugiperda* (Lepidoptera: Noctuidae) a las Proteínas Cry1Ac y Cry1Fa de *Bacillus thuringiensis***

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**Abstract.** The fall armyworm, *Spodoptera frugiperda* (J. E. Smith), is one of the most important insect pests on the American continent. Its control has relied primarily on multiple applications of insecticides that can amount to 1,000 g of active ingredient per hectare on some of approximately 30 crops the insect damages. The use of genetically engineered crops that express *Bacillus thuringiensis* (Bt) Berliner toxins, Bt-corn, *Zea mays* L.; and Bt-cotton, *Gossypium hirsutum* L.; are other ways to control this insect. However, fall armyworm is one of the Lepidoptera species least susceptible to Bt proteins, and a case of high tolerance to Bt-corn has already being reported. We found the susceptibility to Cry1Ac and Cry1Fa proteins of Bt in 133 isofamilies from five regions of three countries was similar to the susceptibility of two Bt-susceptible laboratory colonies to these proteins. Four isofamilies from Puerto Rico were very tolerant to Cry1Fa and not so tolerant to Cry1Ac. Two of the four isofamilies were backcrossed with a Bt-susceptible laboratory colony and their progeny was as susceptible to both Bt proteins as was the Bt-susceptible colony, indicating that resistance to Bt is a recessive trait.

**Resumen.** El gusano cogollero, *Spodoptera frugiperda* (J. E. Smith), es una de las plagas más importantes del continente Americano. Su control se basa principalmente en múltiples aplicaciones de insecticidas que pueden llegar a acumular 1,000 gramos de ingrediente activo por hectárea en algunos de los 30 cultivos que este insecto ataca. El uso de cultivos genéticamente modificados que expresan toxinas de *Bacillus thuringiensis* Berliner (Bt), como el maíz Bt, *Zea mays* L. y el algodonero Bt *Gossypium hirsutum* L., son otra forma de controlar a esta

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plaga. Sin embargo, *S. frugiperda* es una de las especies con menor susceptibilidad a las proteínas Bt, y un caso de alta tolerancia a maíz Bt ya ha sido reportado. En este estudio encontramos que la susceptibilidad a las proteínas Cry1Ac y Cry1F en 133 isofamilias provenientes de cinco regiones de tres países, fue similar a la obtenida en dos colonias susceptibles de laboratorio. Cuatro de estas familias de Puerto Rico mostraron una elevada tolerancia a Cry1F y en menor grado a Cry1Ac. Dos de estas cuatro isofamilias se cruzaron con una familia de laboratorio susceptible y la progenie fue tan susceptible a ambas proteínas como lo fue la colonia susceptible, indicando que la resistencia en estas isofamilias es una condición genética recesiva.

## Introduction

The fall armyworm, *Spodoptera frugiperda* (J. E. Smith), is one of the most destructive insect pests on the American continent (Castro et al. 1988, Hruska and Gould 1997, Williams et al. 1997, Molina-Ochoa et al. 2001, Morillo and Notz 2001, Fernández 2002, Murúa and Virla 2004, de Melo et al. 2006, Zenner de Polanía et al. 2009). Larvae of this insect are polyphagous and among the most damaging pests of at least 30 crops (Ashley et al. 1989), including field and sweet maize, *Zea mays* L.; cotton, *Gossypium hirsutum* L.; sugarcane, *Saccharum officinarum* L.; soybeans, *Glycine max* (L.) Merr.; and grasses. The larvae are difficult to control with insecticide, and resistance to diverse insecticidal chemistries is common. Ineffective control of the pest can reduce maize yields as much as 73% (Hruska and Gladstone 1988). Chemical control of the fall armyworm in Mexico, on approximately 4.8 million hectares treated with at least one application of insecticide, ranges from 38 to 1,152 tons of active ingredient if pyrethroids are applied, or 2,304 to 2,692 tons of active ingredient if organophosphorous insecticides are utilized (from Terán-Vargas 2008), amounting to $40,000,000 (Dow AgroSciences internal market estimate).

Genetically engineered maize and cotton that express *Bacillus thuringiensis* Berliner (Bt) protein toxins control fall armyworm larvae (Williams et al. 1997; Lynch et al. 1999; Buntin et al. 2004 a, b; Sosa and Vitti-Scarel 2004; Zenner de Polanía et al. 2007; Siebert et al. 2008). However, because of less susceptibility of fall armyworm to the bacterial toxins expressed in these crops (Garczynski et al. 1991, Luo et al. 1999, Luttrell et al. 1999) or to Bt maize itself (Matten et al. 2008, but also see Siebert et al. 2008), fewer fall armyworm than other lepidopteran larvae are controlled (Lynch et al. 1999). Exposure to sublethal toxin levels represents much pressure for rapid selection of very resistant individuals (Tabashnik 1990). A recent report describes high levels of resistance to transgenic maize expressing Cry1Fa in Puerto Rico (Matten et al. 2008).

Evolution of field evolved resistance can be tested by quantifying the response of field insects to a pesticide in tests on 1) the progeny of mass-mated adults or 2) progeny of the mating of a single pair. The first method is commonly used and facilitates testing multiple alleles simultaneously. However, the parental genetic background in the tests can only be estimated, while using pair matings (isofamilies) or single-mated isofemales effectively segregates four parental genomes in the tested generations (Andow and Alstad 1998). Using this single-pair approach, the goal of this study was to document the susceptibility of fall armyworm larvae of isofamilies from five regions to the Cry1Ac and Cry1Fa Bt proteins currently expressed in transgenic cotton and maize.

## Materials and Methods

Fall armyworm larvae were obtained from maize in Puerto Rico (Santa Isabela), Mexico (Tamaulipas and Estado de México states), and the U.S. (Texas and Mississippi) and sent to the Agricultural Research Service laboratory of the U.S. Department of Agriculture in Stoneville, MS. When $P_0$ adults emerged, they were pair-mated following the methodology described in Blanco et al. (2009) to produce isofamilies. The second generations ($F_2$) were tested with serial dilutions of 16 concentrations of recombinant Cry1Fa or Cry1Ac purified proteins produced in *Pseudomonas fluorescens* (Flügge) Migula that were overlaid on insect artificial diet (Blanco et al. 2008a). Mortality was determined by counting severely stunted (did not reach second instar) and dead larvae 7 days after initiation. Specific mortality parameters ($EC_{50}$) were obtained using Proc Probit Log Normal analysis in the SAS software package (SAS Institute 2001). Differences in $EC_{50}$ values of isofamilies and a laboratory colony (Monsanto) were considered significant if the 95% confidence limits of the resistance ratio at the $EC_{50}$ level did not include 1.0 (Robertson and Priesler 1992). We also performed mass mating crosses of one gender of the $F_2$ or $F_3$ adults, from isofamilies that were very tolerant to Bt proteins, with moths of the opposite sex from the Monsanto colony. The matings served to 1) introgress different genetic material to isofamilies to delay inbreeding depression and 2) test for the mode of inheritance of tolerance to Bt. In 2009, we tested for Cry1Ac and Cry1Fa susceptibility in 16 isofamilies from Tamaulipas, six from Estado de México, 22 from Mississippi, and 13 from Texas.

## Results and Discussion

Large proportion of parasitism (approximately 50%) were observed in insects collected in Puerto Rico, compared with samples collected in Mexico or the U.S., which only showed parasitism of 0.5-1.0% of the larvae. As previously reported, most of the parasitoids belonged to the Tachinidae and Ichneumonidae families (Ashley 1979, Molina-Ochoa et al. 2001).

In bioassays of field-derived isofamilies, we did not detect differences in susceptibility to either Cry1Ac nor Cry1Fa compared to two reference laboratory colonies (Table 1). In contrast, when we tested 80 isofamilies from Puerto Rico, four revealed high tolerance to Cry1Fa in the initial $F_2$ and $F_3$ evaluations when compared to reference strains. All larvae of the four isofamilies survived the highest Cry1Fa concentration (11,018 ng/cm$^2$, representing more than 11,000-fold the $EC_{50}$ of susceptible larvae); therefore no $EC_{50}$ values could be calculated for the $F_2$ and $F_3$ generations. One of the four isofamilies was lost after the $F_2$ test because of lack of copulations between $F_2$ adults. No progeny was obtained when we attempted to backcross this isofamily with a laboratory colony, suggesting that decreased susceptibility to Cry1Fa had resulted in crucial reproductive fitness costs. The remaining three isofamilies that survived the initial bioassay were successfully tested for susceptibility to Cry1Fa and Cry1Ac on the $F_4$ generations.

Except for the four isofamilies from Santa Isabela, all the isofamilies generated from insects collected in Puerto Rico showed similar susceptibility to Cry1Ac and Cry1Fa compared to two laboratory colonies used as reference. Very elevated tolerance values for Cry1Fa (more than 7,000-fold higher $EC_{50}$) and not-so-elevated for Cry1Ac ($\geq$12-fold higher), were detected for isofamilies 456 and 512 in their $F_4$ generation bioassays compared to the response of the laboratory colonies.

Table 1. Severe Growth Inhibition and Death ($EC_{50}$) Obtained with Cry1Fa and Cry1Ac from *Bacillus thuringiensis* in *Spodoptera frugiperda* Isofamilies and Laboratory Colonies

| Sample / Isofamily | Slope ± SE | Significance of Slope | | $EC_{50}$ (ng / cm²) | | Goodness of Fit | | Resistance Ratio [a] |
|---|---|---|---|---|---|---|---|---|
| | | $X^2$ | Prob. | Dose | 95% FL | $X^2$ | Prob. | |
| Tests with Cry1F | | | | | | | | |
| $F_2$ Edo. México, MX [b] | 0.607 ± 0.729 | 69.4 | <0.0001 | 1.206 | 0.55 - 2.16 | 13.4 | 0.06 | 1.37 |
| $F_2$ Mississippi, US [b] | 0.498 ± 0.072 | 47.2 | <0.0001 | 1.750 | 0.63 - 3.62 | 9.15 | 0.01 | 2.27 |
| $F_2$ Santa Isabel, PR [c] | 0.295 ± 0.505 | 34.3 | <0.0001 | 0.857 | 0.11 - 2.90 | 82.5 | 0.001 | 1.84 |
| $F_2$ Tamaulipas, MX [b] | 0.496 ± 0.060 | 67.1 | <0.0001 | 1.165 | 0.45 - 2.33 | 19.2 | 0.03 | 1.52 |
| $F_2$ Texas, US [b] | 0.425 ± 0.505 | 70.9 | <0.0001 | 1.009 | 0.39 - 2.05 | 25.6 | 0.03 | 1.49 |
| $F_4$ of Isofamily 456 | 0.300 ± 0.153 | 4.9 | 0.02 | 3652 | ≥348 | 17.0 | 0.19 | 7717 [d] |
| $F_3$ 456♀♀ x Monsanto♂♂ | 0.779 ± 0.260 | 8.98 | 0.002 | 0.429 | 0.11 - 1.67 | 4.4 | 0.98 | 0.42 |
| Monsanto♀♀ x $F_3$ 456♂♂ | 0.213 ± 0.076 | 7.8 | 0.005 | 0.0001 | 0 - 0.001 | 0.9 | 0.96 | 0.0006 [d] |
| $F_4$ Isofamily 512 | 0.194 ± 0.058 | 10.8 | 0.001 | 2426 | ≥294 | 26.2 | 0.01 | 9999 [d] |
| $F_3$ 512♀♀ x Monsanto♂♂ | 0.344 ± 0.131 | 6.9 | 0.008 | 0.383 | 0 - 7.70 | 20.4 | 0.004 | 0.69 |
| Monsanto♀♀ x $F_3$ 512♂♂ | 0.163 ± 0.069 | 5.5 | 0.01 | 0.004 | 0 - 0.13 | 3.3 | 0.64 | 0.02 |
| $F_4$ Isofamily 519 | 0.700 ± 0.143 | 23.9 | <0.0001 | 546 | 239 - 1013 | 7.0 | 0.71 | 575 [d] |
| Benzon colony | 0.300 ± 0.040 | 56.2 | <0.0001 | 0.142 | 0.05 - 0.33 | 4.7 | 0.44 | 0.24 |
| Monsanto colony | 0.538 ± 0.130 | 17.0 | <0.0001 | 0.811 | 0.16 - 2.25 | 8.56 | 0.32 | 1.00 |
| Tests with Cry1Ac | | | | | | | | |
| $F_2$ Edo. México, MX [b] | 0.340 ± 0.072 | 22.2 | <0.0001 | 3.927 | 0.49 - 11.08 | 1.39 | 0.92 | 1.09 |
| $F_2$ Mississippi, US [b] | 0.376 ± 0.125 | 8.9 | 0.002 | 6.041 | 0 - 39.3 | 114.9 | 0.001 | 2.04 |
| $F_2$ Santa Isabel, PR [c] | 0.326 ± 0.041 | 61.6 | <0.0001 | 10.362 | 3.64 -21.23 | 2.17 | 0.90 | 3.00 |
| $F_2$ Tamaulipas, MX [b] | 0.277 ± 0.055 | 24.8 | <0.0001 | 4.853 | 0.47 - 15.87 | 4.33 | 0.66 | 1.72 |
| $F_2$ Texas, US [b] | 0.270 ± 0.050 | 28.3 | <0.0001 | 11.998 | 1.59 -35.96 | 2.42 | 0.48 | 3.51 |
| $F_4$ of Isofamily 456 | 0.516 ± 0.139 | 13.7 | 0.0002 | 219.7 | 64.7 - 904.4 | 9.9 | 0.70 | 42.24 [d] |
| $F_3$ 456♀♀ x Monsanto♂♂ | 0.454 ± 0.156 | 8.48 | 0.003 | 1.38 | 0.003 - 7.23 | 11.6 | 0.33 | 0.29 |
| Monsanto♀♀ x $F_3$ 456♂♂ | 0.161 ± 0.042 | 14.7 | <0.0001 | 0.00006 | 0 - 0.001 | 8.1 | 0.41 | 0.00005 [d] |
| $F_4$ Isofamily 512 | 0.162 ± 0.036 | 19.5 | <0.0001 | 13.43 | 1.49 - 93.8 | 22.7 | 0.04 | 12.20 [d] |
| $F_3$ 512♀♀ x Monsanto♂♂ | 0.268 ± 0.058 | 20.6 | <0.0001 | 3.74 | 0.33 - 15.71 | 23.2 | 0.04 | 1.39 |
| Monsanto♀♀ x $F_3$ 512♂♂ | 0.245 ± 0.065 | 14.0 | 0.0002 | 5.54 | 0.32 - 28.75 | 11.7 | 0.55 | 2.33 |
| $F_4$ Isofamily 519 | 0.257 ± 0.065 | 15.4 | <0.0001 | 227.0 | 47.6 - 2,020 | 8.99 | 0.53 | 88.98 [d] |
| Benzon colony | 0.373 ± 0.069 | 29.0 | <0.0001 | 6.099 | 1.21 - 15.40 | 8.2 | 0.21 | 1.53 |
| Monsanto colony | 0.421 ± 0.094 | 19.8 | <0.0001 | 4.431 | 0.75 - 10.69 | 9.9 | 0.70 | 1.00 |

[a]Calculated based on Monsanto's colony response, [b]Response of all isofamilies together, [c]Response of all isofamilies that did not demonstrate high tolerance to a *Bacillus thuringiensis* protein, [d]Significantly different from the Monsanto colony.

In comparison, not-so-elevated levels of resistance to Cry1Fa were detected for isofamily 519 (about 500-fold greater $EC_{50}$), but this isofamily was most resistant to

Cry1Ac (>80-fold greater $EC_{50}$) (Table 1). Larvae from backcrossing moths from isofamilies 456 and 512 with a laboratory colony (Monsanto) were as susceptible as the reference strains to both Cry1Fa and Cry1Ac, supporting that *B. thuringiensis* resistance in the two isofamilies was transmitted as a recessive trait. Attempts to backcross isofamily 519 $F_2$ adults with adults from Monsanto produced no progeny because of lack of copulations. Similar results have been found with samples from four regions of Puerto Rico, which had high levels of tolerance to Cry1F and moderately tolerant to Cry1Ac. Tolerance conditions of these samples were also highly recessive and autosomally inherited (Storer et al. 2010).

Interestingly, different amounts of susceptibility were detected for backcrosses with the 456 isofamily depending on the gender of the susceptible parent, which may suggest potential contributions to resistance of sex-linked genes in this strain, as has been reported for tobacco budworm, *Heliothis virescens* (Fabricius) (Blanco et al. 2008b). Our data confirm and expand on a previous report (Matten et al. 2008, Storer et al. 2010), and support the existence of Cry1Fa-resistant insects in field populations of fall armyworm in Puerto Rico.

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