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# Evidence of multiple/cross resistance to Bt and organophosphate insecticides in Puerto Rico population of the fall armyworm, *Spodoptera frugiperda*

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ABSTRACT

Fall armyworm (FAW) is a damaging pest of many economic crops. Long-term use of chemical control prompted resistance development to many insecticide classes. Many populations were found to be significantly less susceptible to major Bt toxins expressed in transgenic crops. In this study, a FAW strain collected from Puerto Rico (PR) with 7717-fold Cry1F-resistance was examined to determine if it had also developed multiple/cross resistance to non-Bt insecticides. Dose response assays showed that the PR strain developed 19-fold resistance to acephate. Besides having a slightly smaller larval body weight and length, PR also evolved a deep (2.8%) molecular divergence in mitochondrial oxidase subunit II. Further examination of enzyme activities in the midgut of PR larvae exhibited substantial decreases of alkaline phosphatase (ALP), aminopeptidase (APN), 1-NA- and 2-NA-specific esterase, trypsin, and chymotrypsin activities, and significant increases of PNPA-specific esterase and glutathione S-transferase (GST) activities. When enzyme preparations from the whole larval body were examined, all three esterase, GST, trypsin, and chymotrypsin activities were significantly elevated in the PR strain, while ALP and APN activities were not significantly different from those of susceptible strain. Data indicated that multiple/cross resistances may have developed in the PR strain to both Bt toxins and conventional insecticides. Consistently reduced ALP provided evidence to support an ALP-mediated Bt resistance mechanism. Esterases and GSTs may be associated with acephate resistance through elevated metabolic detoxification. Further studies are needed to clarify whether and how esterases, GSTs, and other enzymes (such as P450s) are involved in cross resistance development to Bt and other insecticide classes.

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## 1. Introduction

The fall armyworm (FAW), *Spodoptera frugiperda* (J. E. Smith), is one of the most serious pests of corn, cotton, soybean, and other economically important crops across the western hemisphere on the American continent [1–4]. Larval damages to corn usually include extensive defoliation, damage to the corn husk, burrowing and destroying the growth potential of plants [5,6]. FAW is an occasional pest of cotton; however, the pest status of FAW in cotton in Brazil has increased with the recent changes in cotton crop systems, such as double cropping and the use of cover or winter crops with non-tillage cropping systems [7]. Bt cotton may suppress certain FAW infestations, but it does not provide complete control of this insect pest. On cotton, FAW larvae feed on both blooms and bolls, and they have the potential to damage cotton fruiting structures at rates comparable to those of the cotton bollworm, *Helicoverpa zea*, and tobacco budworm, *Heliothis virescens* [8]. On soybean, FAW larvae feed on both leaves and pods and may cut the stems and reduce the crop population below the optimal level, and their infestation will occasionally reach a level high enough to cause damage [9,10].

Control of the FAW has depended exclusively on insecticides, and extensive uses of chemicals prompted resistance development to major classes of insecticides in many areas [11] 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% [12]. Since the late 1990s, genetically engineered maize and cotton that express *Bacillus thuringiensis* Berliner (Bt) protein toxins have been used for

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controlling various lepidopteran larvae, including FAW [13–16]. However, because of less susceptibility of FAW to the bacterial toxins expressed in these crops [16–18], fewer FAW larvae are effectively suppressed [14]. The constant exposure to sublethal dosages of toxins in target populations may present strong pressure for rapid selection of resistant individuals [19]. Consequently, the FAW was found to be highly resistant to transgenic maize expressing Cry1Fa in Puerto Rico [18,20].

In our previous study, FAWs were collected from corn fields in Puerto Rico (PR), and were subjected to Bt toxicity assays with Cry1Ac and Cry1Fa. Results indicated that PR isolines had high tolerance to Cry1Fa (7717-fold) and Cry1Ac (>42-fold) when compared to reference (susceptible) strains [21]. Because FAW have had a longer history of exposure to chemical insecticides than exposure to Bt toxins, we hypothesize that resistance evolution to insecticides may facilitate or correlate to Bt resistance development in the same population, because many defense-related genes and enzymes, such as P450 oxidases, esterases, and glutathione S-transferases, are involved in metabolic pathways for detoxifying xenobiotics. In this study, we examined a FAW strain from Puerto Rico for its susceptibility to a commonly used organophosphate insecticide acephate, and compare physiological and molecular parameters between PR and a susceptible strain.

## 2. Materials and methods

### 2.1. FAW populations

The PR FAW strain was originally collected from maize in Puerto Rico, and isoline 456 (PR or R) was subjected to biological and physiological comparisons with a laboratory susceptible strain (Monsanto or S). The PR isoline had a 7717-fold Cry1F-resistance ratio and 42-fold Cry1Ac-resistance ratio compared to the laboratory susceptible strain. Details of the establishment of the PR and susceptible colonies were described in Blanco et al. [21]. All of the FAW larvae were maintained on nutri-soy wheat germ artificial diet (1 gallon diet contains 156 g nutri-soy flour, 133 g wheat germ, 36 g Wesson salt, 156 g sugar, 36 g vitamin, 85 g agar, 3.8 g methyl paraben, 3.8 g sorbic acid, 3.8 g aureomycin, and 10 ml acid mix [540 ml distilled water, 418 ml propionic acid, and 42 ml phosphoric acid].

### 2.2. Chemicals

The Pierce Coomassie Plus Protein Assay Kit (23238) was purchased from ThermoFisher Sci. (Pittsburgh, PA, USA). The chemicals 1-chloro-2,4-dinitrobenzene (CDNB) (23,732-9), L-glutathione (GSH) reduced (G6529), alpha-naphthyl acetate (1-NA) (N8505), beta-naphthyl acetate (2-NA) (N6875), ρ-nitrophenyl acetate (PNPA) (N8130), fast blue salt (D9805), N-benzoyl-D,L-arginine4-nitroanilide hydrochloride (BApNA) (B3133) and N-succinyl-Ala-Ala-Pro-Phe P-Nitroanilide (SAAPFpNA) (S7388) leucine-p-nitroanilide (LpNA) (L9125), p-nitrophenyl phosphate disodium (pNPP) (N4645) were purchased from Sigma Chemical Co. (St. Louis, MO). Acephate (PS-738, technical grade) was purchased from Chem Service (West Chester, PA, USA).

### 2.3. Examination of morphological and molecular differences between PR and S strains

Larval length and weight were periodically measured after hatching. Ten larvae were individually measured and their average length and weight were treated as a replication. Three replications were included for each strain.

To detect any molecular differences between the PR and S strains, cytochrome oxidase II (COII) DNA fragment was amplified using a forward primer FAWCO2F (5′-CAACATGATCTAATCTTAATTTACAA AATAGAGC-3′) and a reverse primer FAWCO2R (5′-CAATTGGTATAAAACTATGATTAGCTCCAC-3′). The PCR conditions were optimized to produce a single band with expected size (c.a. 624 bp). COII DNA was sequenced from both directions using an automated sequencer (ABI Prism 3730XL). DNA extraction, PCR amplification, and sequencing were repeated with different larvae at least 10 times for each strain. The DNA sequence was confirmed as partial mitochondrial cytochrome oxidase subunit II gene through Blast similarity search of GenBank nucleotide databases of the National Center for Biotechnology Information (NCBI http://www.ncbi.nlm.nih.gov/). Clustal-W procedure [22] was used to perform sequence alignment including Internet based sequence analysis tool (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalwan.html) and MegAlign module of DNAStar Ver. 10. Details of other procedures were described in Zhu et al. [23].

### 2.4. Bioassay

For each concentration of acephate tested, three reps of ten larvae were treated for a total of thirty larvae per concentration. A stock concentration of 128 mg/ml of acephate dissolved in acetone was serially diluted to make the experimental concentrations. Ten 6-d old larvae were used for each replication, and three replications were included for each acephate concentration. To each larva, an application of 0.5 µl of each acephate concentration was administered to the junction of the thorax and abdomen on the back of the larva (after the third segment past the head). The larvae were then placed on their regular diet and mortality data were collected 24 hours after treatment. $LD_{50}$ values were calculated as dose response to acephate toxicity by using SAS Probit analysis [24]. Resistance ratios were obtained by dividing the $LD_{50}$ of a susceptible strain by the $LD_{50}$ of the PR strain.

### 2.5. Enzyme preparation

Midguts of 9-d old larvae were dissected in ice-cold 0.1 M Tris-HCl, pH 8.0. Midguts were then placed in a 1.5 ml centrifuge tube with 120 µl of cold Tris-HCl, pH 8.0. A plastic pestle was used to homogenize guts in buffer. Samples were centrifuged at 10,000× g for 5 minutes at 4 °C. Supernatant was taken and placed in a –80 °C freezer until assayed. Protein concentrations were determined using the Pierce protein assay kit which utilizes the Bradford method [25]. Bovine serum albumin was used to obtain the standard curve. For each of the following enzyme assays, the mean kinetic velocity (Mean V) was calculated as increase of OD405 per min in the linear portion of initial increasing phase of the enzymatic reaction using the KC4 program.

#### 2.5.1. Esterase assays

To determine esterase activity, micro-titer plate assays were conducted using PNPA (ρ-nitrophenyl acetate), alpha- and beta-naphthyl acetate as substrates (1-NA, 2-NA). For the assays with PNPA, PNPA was added to 10 µl of diluted enzyme homogenate for a final concentration of 0.5 mM PNPA within a final volume of 200 µl. A Bio-Tek $EL_x808_{iu}$ was used to monitor the assay at 400 nm for 10 minutes with readings taken every 15 seconds [26]. For assays with 1- and 2-NA, the following substrate solution was made fresh before each assay: 9 mg Fast Blue Salt, 300 µl of 0.113 M 1-NA or 2-NA, and 15 ml of 0.1 M sodium phosphate buffer, pH 7.0. Substrate solutions were vacuum filtered twice through Whatman 52 filter paper. Two-hundred and forty micro-liters of substrate solution were added to 10 µl of diluted enzyme homogenate to start the reaction. The assay was monitored at 450 nm for 10 minutes with measurements taken every 15 seconds [27].