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# The Investigation of Factors Potentially Affecting the Susceptibility of Native *Plutella xylostella* Populations to *Bacillus thuringiensis* Cry Toxins

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***Abstract* – Despite the prominent and worldwide use of *Bacillus thuringiensis* (Bt) insecticidal toxins in agriculture, knowledge of the mechanism by which they kill pests remains incomplete, therefore the aim of this research was to investigate on recently identify factors that affect the susceptibility of insects to Bt (protein levels, midgut bacteria and mutations on the ABCC2 transporter) in five brazilian populations of *Plutella xylostella* larvae and to overcome the mechanism of resistance. The analyses were made in about 60 guts extracts in 1 ml of 50% glycerol solution using the following process: gut analysis (total protein, total proteas, toxin digestion and esterase activities); gut bacteria analysis; and ABCC2 gene analysis. There were not differences in the amount of esterases between the populations, therefore we cannot associate esterase level with resistance. Differences in composition of the larval gut microbiota were not correlated with resistance. It is unclear whether similar interactions occur in the field where the intestinal micrbiotas of lepidopteran larvae may be more complex than the apparent mono-associations described here. However it is possible that gut bacteria may present a novel target for the management of *P. xylostella*. The nature of the protective effect of gut bacteria is also unclear. Previous studies suggested that lactic acid bacteria may acidify the gut thereby decreasing host susceptibility to alkali-soluble Cry toxins such as CrylAc. There are no mutation in the ABCC2, accordingly there is no association of ABCC2 with Bt resistance.**

***Keywords* – Diamondback Moth, Esterase, Gut Bacteria, ABCC2.**

## I. INTRODUCTION

*Plutella xylostella* (L.) (Lep.: Plutellidae) is a major insect pest of cruciferous crops both in Brazil and worldwide [1][2]. Although it can be controlled with both synthetic and biological insecticides *P. xylostella* can rapidly evolve resistance to a variety of insecticides [3]. Furthermore different populations of *P. xylostella* can arise due to geographic isolation, resulting in reproductive isolation and in physiologically distinct populations with different susceptibilities to various control tactics. Such a variation requires management systems tailored to particular populations. The most common biopesticides used to control *P. xylostella* are based on the entomopathogenic bacterium *Bacillus thuringiensis* Berliner (Bt) (Bacillaceae) [4] [5]. A number of studies have observed susceptibility variations to Bt in different populations of *P. xylostella*, and another pest *Spodoptera frugiperda* (J.E. Smith) (Lep.: Noctuidae), from countries in Latin America, Europe and various Brazilian states [6]-[8]. Roux [9] used an ISSR-PCR technique to differentiate 19 *P. xylostella* populations from several countries. The authors emphasized that in the tropics the high number of generations per year may favor the emergence of mutations and thus increases the divergence between individuals, and the high selection pressure caused by the indiscriminate application of pesticides favors the emergence of tolerant or resistant populations of this insect. *P. xylostella* was the first insect in which field resistance to Bt was identified [10]. This increased the interest of Bt researchers about this pest [11]-[21].

*Bacillus thuringiensis* is a rod-shaped, spore-forming bacterium that produces crystalline inclusions during sporulation [22][23][5]. These crystals contain protein toxins, known as delta-endotoxins, which are responsible Bt's toxic activity against Coleoptera, Diptera, Hemiptera, Hymenoptera, Isoptera, Lepidoptera and Orthoptera [24][25][4][26].

Although many different insects are susceptible to Cry toxins, the Bt mode of action is not clear cut. After ingestion of spores/crystals by the insect, the crystals are solubilized and then to a greater or lesser extent are cleaved by the digestive proteases down to a protease-resistant core (the so-called active toxin). These toxins can pass through the peritrophic membrane, bind to specific receptors located on the apical membrane of the midgut columnar cells and form pores in the membrane. These pores interfere with cell physiology by abolishing transmembrane ionic gradients and can lead to colloid-osmotic lysis of the cells due to the massive influx of solutes from the midgut lumen. In turn, destruction of the cells results in extensive damage to the midgut epithelial tissue and death of the intoxicated larvae. The insect can also suffer from starvation, since shortly after ingestion of the toxin the insect ceases feeding [27][22][5][28].

In recent years, an elaborate model involving the sequential binding of the toxins to different membrane receptors has been proposed to describe the events leading

to membrane insertion and pore formation. However, it has also been proposed that, in contradiction to mechanism, Bt toxins function by activating intracellular signaling pathways which lead to the necrotic death of their target cells without the need for pore formation [29]. Vachon et al. [28] pointed out that the available information still supports the notion that Bt Cry toxins act by forming pores, but most events leading to their formation, following binding of the activated toxins to their receptors, remain relatively poorly understood.

Our understanding of the mechanism of action of Bt toxins has been complicated recently by the discovery that mutations in the gene encoding an ABCC2 transporter are responsible for resistance to Bt toxins in four different insect species [30][31]. The Bt Cry1Ac resistance locus (BtR-1) in the NO-QA strain of *P. xylostella* from Hawaii was originally identified using anonymous AFLP markers [32]. A sequenced AFLP marker linked to BtR-1 contained coding sequence for the predicted gene Thyroid Hormone Receptor Interactor 12 (GenBank JN030496), which has an ortholog located on chromosome 15 of *Bombyx mori* L. (Lep.: Bombycidae) conserved chromosomal synteny, predicted proteins from *B. mori* chromosome 15 were compared using BLAST against a *P. xylostella* transcriptome (454-ESTs) to design specific primers for linkage mapping. Sixteen genes were mapped in most progeny in backcrosses to NO-QA (3 families, 184 bioassay survivors, 46 controls), and a linkage map was generated to identify the resistance locus. As ABCC2 was correlated with Cry1Ac resistance in *Heliothis virescens* (Fabricius) (Lep.: Noctuidae), the ortholog from *P. xylostella* was cloned and sequenced using a genomic BAC library constructed from a susceptible strain Geneva88 [33]. The gene contains 26 exons, and these were verified through PCR amplification from Bt-susceptible (Geneva88) and *Bt*-resistant (NO-QAGE) midgut cDNA. The resistant strain NO-QAGE contained a 30-bp deletion in exon 20, which is predicted to remove the 12$^{th}$ and final transmembrane domain and aberrantly position the carboxyl-terminal outside the cell. If this gene is translated and inserted into the midgut membrane, a core ATP-binding loop is expected to be nonfunctional.

The superfamily of ABC proteins takes its name from the ATP binding cassette, an intracellular domain that binds and hydrolyzes ATP in a cycle that drives transport of molecules across a lipid bilayer membrane. The functional transporter consists of two cytosolic nucleotide-binding domains (NBDs) that bind and hydrolyze ATP and two integral transmembrane domains [31]. The biological function of ABCC2 is unknown, but its similarity to multidrug resistance proteins suggests that it could export small hydrophobic toxins from midgut epithelial cells for eventual elimination in the feces. Heckel [31] speculates that ABCC2 could function as one of the toxin binding proteins in the sequential binding model discussed by Bravo et al. [29] and further proposes that binding of the toxin to ABCC2 only occurs when the transporter is in its open configuration. When the transporter closes then the toxin is pushed into the membrane. This transient interaction between the toxin and ABCC2 may explain why no one has yet identified ABCC2 as a toxin binding protein. Hernandez-Martinez et al. [30] suggested an alternative model in which ABCC2 is indirectly affecting the mechanism of action of the Bt toxins. Mutations in the gene encoding ABBC2 affect its function which then affects the physiology of the cell in some way that then affects the ability of the Bt toxin to form a pore. Both papers conclude that the functional role of the ABCC2 protein remains to be established, but that mutations in it clearly have significant consequences on the susceptibility of insects to Bt.

The bacterial microflora of insects, confined to the intestine, is rich, diverse and comprises both Gram positive and negative bacteria. Many of these help in the digestion of food, but others are pathogenic or potentially pathogenic [34]. In two controversial studies Broderick et al. [35][36] suggested out that *Bt* is unable to kill *Lymantria* dispar (L.) (Lep.: Erebidae), *Manduca sexta* (L.) (Lep.: Sphingidae), *Pieris rapae* (L.) (Lep.: Pieridae) and *Vanessa cardui* (L.) (Lep.: Nymphalidae), in the absence of gut bacteria. Prior exposure of larvae of to a combination of four antibiotics to remove the gut bacteria severely reduced the toxicity of a commercial preparation of Bt. Reinfection of larvae with an isolate of *Enterobacter* sp. rescued the toxicity of Dipel®. A subsequent study [45] established that the loss of activity of Dipel® was due to a direct effect of residual antibiotics in the larvae to the live bacteria present in the Dipel® product. They not only concluded that the native gut bacteria were not required for the toxicity of Bt but in fact showed that the presence of native gut bacteria helped protect the insect against Bt. In summarizing the above data Raymond et al. [37] concluded that Bt is primarily an insect pathogen, that its primary means of reproduction is in an insect cadaver, and that it does not require the assistance of other microbes for its pathogenicity.

Despite the prominent and worldwide use of Bt insecticidal toxins in agriculture, knowledge of the mechanism by which they kill pests remains incomplete, therefore the aim of this research was to investigate on recently identify factors that affect the susceptibility of insects to Bt (protein levels, midgut bacteria and mutations on the ABCC2 transporter) in five Brazilian populations of *P. xylostella* larvae and to overcome the mechanism of resistance.

## II. Materials and Methods

### *A. Insect populations*

All *P. xylostella* populations were collected in areas with no history of insecticide application. For insect rearing the methodology described by Barros & Vendramim [38] has been used and adapted to the conditions of the Laboratory of Insect Rearing and Biology, UNESP, Jaboticabal, Brazil. Larvae from four native, one selected Brazilian populations and one UK population were used in this project as follows:

Population 1 (PC) - collection date: May 19, 2008 - collection in cabbage plants in the city of Alegre-ES (Brazil) with 68 generations in the laboratory.

Population 2 (PA) - collection date: July 22, 2008 - collection in cabbage plants in the city of Alegre-ES (Brazil) with 65 generations in the laboratory.

Population 3 (Px) - collection date: January 15, 2007 - collection in cabbage plants in the city of Recife-PE (Brazil) with 98 generations in the laboratory.

Population 4 (SBT) - collection date: July 5, 2010 - collection in cabbage plants in the city of Jaboticabal-SP (Brazil) with 25 generations in the laboratory.

Population 5 (Bt) - collection date: May 19, 2008 - collection in cabbage plants in the city of Alegre-ES (Brazil) with 58 generations in the laboratory; selected each generation with Bt HD-1. For this current study we tested the ratio of resistance of this population, and we verified that it was 78 fold resistance to Cry1Ac toxin (Unpublished data).

Population 6 (UK) – The population is known as NO-QA, it is from Germany, collected around 3-4 years back with about 50 generations in the lab. It's a highly resistant population; selected with insecticidal Cry1Ac.

About 300 guts were removed from third instar larvae of each population, these guts were divided and stored frozen in 50% glycerol solution in small eppendorfs® tubes.

*B. Preparation of the gut extract*

About 60 guts extracts in 1 ml of 50% glycerol solution were smashed until I got a homogeneous solution; the mixture was centrifuged for 5 min at 14000rpm. The supernatant was separated from the pellet and stored frozen for the enzymes assays, and the pellet was resuspended in 1ml of sterile water and stored frozen for the DNA assays. This process was repeated for each population according of guts amount (1- UK, 3- SBT, Px, PC and PA or 4- Bt).

*C. Total protein concentration estimation*

The BIO–RAD Protein Assay reagent was used according to the instructions of the manufacturer for the estimation of the protein concentration. Aliquots (10µl) of the gut extract were added to 1 ml of the dye (1 in 4 dilution in water); after 5 min incubation at room temperature, absorbance was measured at 595nm.

*D. Esterase gel*

Gut extracts were mixed 1:1 with 2x sample buffer (no SDS), boiled for 4min and loaded onto a PAGE Mini-Gel. Electrophoresis was performed at 200V for 40 min in a Mini-PROTEAN® II Electrophoresis Cell (Bio-Rad) using a tris-glycine buffer (25 mM tris, 190 mM glycine) followed by staining in the dark for 10 min with 10 ml of a solution containing 50mg Fast Blue RR salt in 25 ml 200mM phosphate buffer (pH 6.0) and with 200µl of 18,6 mg of 1-naphthyl acetate in 1ml acetone. Afterwards the gel was fixed with 5% acetic acid. The results were visualized and analyzed through Gel Doc™ EZ Imager (BIO-RAD®) coupled to a software image analysis (Image Lab™ Software – version 4.0).

*E. Toxin digestion*

5µl of a 4 µl/ml suspension of crystals (Cry1Ac) were centrifuged and the supernatant removed. The pellet was resuspended in 20µl carbonate-bicarbonate buffer (pH 10.5), 1 µl of 0.1M DTT and 1 µl 1:10 diluted gut extract was added. The samples were incubated for 1 hour at 37°C. 5µl of the samples were mixed with 5µl of loading buffer (95 µl 2x SDS sample buffer and 5 µl of 2-Mercaptoethanol), boiled for 4 min and loaded onto a SDS PAGE Mini-Gel. Electrophoresis was performed at 200V for 40 min in a Mini-PROTEAN® II Electrophoresis Cell (Bio-Rad) using a tris-glycine buffer (25 mM tris, 190 mM glycine, 3.5 mM SDS) followed by coomasie blue staining (45% [v/v] methanol, 10% [v/v] acetic acid, 0,25% [w/v] coomasie brilliant blue R) for 30 min. Afterwards, gels were distained with 30ml of destain solution (45% [v/v] methanol, 10% [v/v] acetic acid, 0.25% [w/v]). The results were visualized and analyzed through Gel Doc™ EZ Imager (BIO-RAD®) coupled to a software image analysis (Image Lab™ Software – version 4.0).

*F. Total protease activity assay*

Total proteolytic activity was assayed using 1% (w/v) azocasein as a substrate in 50mM $Na_2CO_3$, pH 9.8, according to a method modified from García-Carreño et al. [47]. 10µl of enzyme solution was mixed with 250µl of substrate solution and incubated for 30 min at 37°C. The reaction was stopped by adding 1.2ml of 10% (w/v) TCA (Trichloroacetic acid). The mixture was centrifuged for 5 min at 12000rpm. The supernatant was separated from the undigested substrate (pellet) and 1.4ml of 1M NaOH was added to the supernatant. The absorbance at 440nm for the released dye was recorded. The assay included blanks that were prepared following the condition described but without adding gut extracts.

*G. DNA extraction*

The purification of Total DNA from Animal Tissues (Spin-Column Protocol- on Gram-Positive bacteria) was performed with the DNeasy Blood & Tissue Kit (Qiagen®), according to the manufacturer's recommendations.

*H. Polymerase chain reaction (PCR): based on the bacterial 16S rRNA DNA gene*

Bacterial 16S rRNA genes were amplified using the universal primers 27f (5'-GTGCTGCAGAGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CACGGATCCTACGGGTACCTTGTTACGACTT-3') [48]. PCR contents for a 50µl volume were 0.5µl of 100pmol/µl forward (27F) and reverse (1492R) primers, 25 µl of Go Taq® Green Master Mix (Promega®), 0.5µl of bacterial DNA-sample and sufficient quantity of ultra-pure sterile water to 50µl. The thermal sequence and amplification timing were: an initial denaturation at 94 °C for 2 min followed by 35 cycles of: denaturation at 94 °C for 30 sec.; annealing at 58 °C for 30 sec. and elongation at 72 °C for 45 sec., followed by final extension at 72 °C for 5 min.

*I. Polymerase chain reaction (PCR): based on the ABCC2 gene - Exon*

The ABCC2 gene were amplified using designed primers ExonF (5'- GGACGTGATCCCGGTGG-3') and ExonR (5'-CGTGCGGCAGCTTAGTGTAC-3'). PCR contents for a 50µl volume were 0.5µl of 100pmol/µl forward (ExonF) and reverse (ExonR) primers, 25 µl of Go Taq® Green Master Mix (Promega®), 0.5µl of bacterial DNA-sample and sufficient quantity of ultra-pure

sterile water to 50µL. The thermal sequence and amplification timing were: an initial denaturation at 94 °C for 2 min followed by 35 cycles of: denaturation at 94 °C for 30 sec.; annealing at 58 °C for 30 sec. and elongation at 72 °C for 45 sec., followed by final extension at 72 °C for 5 min.

*J. Polymerase chain reaction (PCR): based on the ABCC2 gene - Intron*

Another attempt to ABCC2 gene was amplified using other designed primers IntronF (5'-GACGTAGCAACAAGGGGGCAACAT-3') and IntronR (5'-GCTCCACGGCGGTCATCTG-3'). PCR contents for a 50µl volume were 0.5µl of 100pmol/µl forward (ExonF) and reverse (ExonR) primers, 25 µl of Go Taq® Green Master Mix (Promega®), 0.5µl of bacterial DNA-sample and sufficient quantity of ultra-pure sterile water to 50µL. The thermal sequence and amplification timing were: an initial denaturation at 94 °C for 2 min followed by 35 cycles of: denaturation at 94 °C for 30 sec.; annealing at 58 °C for 30 sec. and elongation at 72 °C for 45 sec., followed by final extension at 72 °C for 5 min.

*K. DNA electrophoresis on agarose gel*

The amplified products were submitted to horizontal electrophoresis on 1% agarose gels stained with GelRed™ Nucleic Acid (Biotium®) (0.5 µl/30ml) in running buffer TBE 1X pH 8,0 (44.58 M Tris; 0.44 M Boric Acid; 12.49 mM EDTA). The electrophoresis was performed in 120V/400mA during 30 min. For the determination of the amplified products, we used a molecular weight marker (1 Kb DNA Ladder, Invitrogen®. The results were visualized and analyzed through an ultraviolet transilluminator Gel Doc™ EZ Imager (BIO-RAD®) coupled to a software image analysis (Image Lab™ Software – version 4.0).

*L. Amplicons extraction from agarose gel*

After the PCR reaction, the band relative to the amplicon was cut from the agarose gel with a scalpel blade, and placed in eppendorfs® tubes properly identified. Further, the extraction of the PCR product from the agarose gel was performed using the QIAquick® Gel Extraction Kit (250) (Qiagen®), according to the manufacturer's recommendations.

*M. Sequencing*

Samples were sent for sequencing to Eurofins MWG Operon-Europe.

## III. RESULTS

*A. Analysis of gut extracts*

*Total Protein*

To determine the total protein and to establish if its amount were linear, gut extracts of the 5 populations were compared and analyzed. First we set up different amounts (2.5µl, 5.0 µl, 7.5 µl and 10 µl) for random samples of the extracts to identify a possible linear range. The curves obtained are showed in fig. 1.

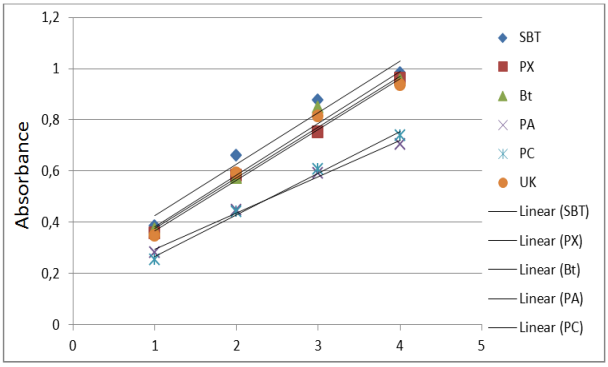


Fig.1. Comparison of the total protein absorbance between the gut extracts from samples of SBT, PX, Bt, PA, PC, and UK populations in different amounts (2.5µl, 5.0 µl, 7.5 µl and 10 µl) establishing a linear range.

The curves obtained for all populations showed linear range, meaning that increasing concentration of gut extracts, increases the absorbance, consequently major quantity of total protein. Despite the small variation it's possible to assume that SBT population has bigger amount of total protein than PC population, for example.

Once the total protein linear range was made, it was decided that a 0.5 absorbance would be chosen as baseline concentration for the guts extracts to do the others enzyme assays. Then, adjustment of all samples to 0.5 was made along with its confirmation by repeating the total protein assay 3 times for each population and each replicate (Table 1). Therefore, all the concentrations of the 6 populations were adjusted to 0.5 total protein absorbance.

Table 1: Total protein absorbance (repeated 3 times) and the average from the gut extracts of SBT, PX, Bt, PA, PC and UK populations and their replicates.

| Population | Absorbance-1 | Absorbance-2 | Absorbance-3 | Average |
|---|---|---|---|---|
| **SBT-1** | 0.503 | 0.475 | 0.497 | 0.492 |
| **SBT-2** | 0.591 | 0.537 | 0.539 | 0.556 |
| **SBT-3** | 0.489 | 0.520 | 0.568 | 0.526 |
| **PA-1** | 0.500 | 0.500 | 0.508 | 0.503 |
| **PA-2** | 0.575 | 0.590 | 0.458 | 0.541 |
| **PA-3** | 0.506 | 0.491 | 0.481 | 0.493 |
| **PX-1** | 0.616 | 0.777 | 0.597 | 0.663 |
| **PX-2** | 0.511 | 0.487 | 0.460 | 0.486 |
| **PX-3** | 0.506 | 0.491 | 0.494 | 0.497 |
| **PC-1** | 0.512 | 0.560 | 0.480 | 0.517 |

| | | | | |
|---|---|---|---|---|
| **PC-2** | 0.576 | 0.517 | 0.509 | 0.534 |
| **PC-3** | 0.501 | 0.515 | 0.503 | 0.506 |
| **Bt-1** | 0.543 | 0.587 | 0.613 | 0.581 |
| **Bt-2** | 0.487 | 0.543 | 0.568 | 0.533 |
| **Bt-3** | 0.531 | 0.568 | 0.533 | 0.544 |
| **Bt-4** | 0.555 | 0.498 | 0.452 | 0.502 |
| **UK** | 0.521 | 0.564 | 0.546 | 0.544 |

*Total Protease*

Because resistance has been associated with differences in gut protease activities [49], total protease was measured giving us the following results. The table 2 showed that exist variation in the average between populations, being 0.053, 0.047, 0.052, 0.028, 0.057 and 0.032 respectively for SBT, PA, Px, PC, Bt and UK populations. Also, table 2 showed variation in each population, with broader range in SBT and Bt populations (0.060 for both) and narrow range for PA (0.014) and UK (0.015) populations. This results lead's us to assume that there are no differences in the protease activities related to Bt resistance between these populations, once each replicate, and each population showed random numbers.

Table 2: Total protease absorbance (repeated 3 times) and the average from the gut extracts of SBT, PX, Bt, PA, PC and UK populations and its replicates.

| Population | Absorbance-1 | Absorbance-2 | Absorbance-3 | Average |
|---|---|---|---|---|
| **SBT-1** | 0.014 | 0.014 | 0.032 | 0.020 |
| **SBT-2** | 0.098 | 0.090 | 0.072 | 0.087 |
| **SBT-3** | 0.049 | 0.053 | 0.057 | 0.053 |
| **PA-1** | 0.050 | 0.055 | 0.042 | 0.049 |
| **PA-2** | 0.052 | 0.047 | 0.061 | 0.053 |
| **PA-3** | 0.044 | 0.030 | 0.043 | 0.039 |
| **PX-1** | 0.081 | 0.075 | 0.059 | 0.072 |
| **PX-2** | 0.047 | 0.043 | 0.052 | 0.047 |
| **PX-3** | 0.035 | 0.061 | 0.020 | 0.039 |
| **PC-1** | 0.009 | 0.014 | 0.017 | 0.013 |
| **PC-2** | 0.064 | 0.054 | 0.060 | 0.059 |
| **PC-3** | 0.014 | 0.001 | 0.028 | 0.014 |
| **Bt-1** | 0.096 | 0.070 | 0.088 | 0.085 |
| **Bt-2** | 0.026 | 0.048 | 0.021 | 0.032 |
| **Bt-3** | 0.100 | 0.069 | 0.089 | 0.086 |
| **Bt-4** | 0.014 | 0.042 | 0.021 | 0.026 |
| **UK** | 0.039 | 0.024 | 0.033 | 0.032 |

In function of differences in total protein between the gut extract we decided to compare specific activities that is calculated dividing the average of the total protease activity by the average of the total protein activity (Table 3). The results showed data that varies between 0.055 (PC population) until 0.103 (Bt population) for specific proteases activities.

Table 3: Enzymes specific activity from the gut extracts of SBT, PX, Bt, PA, PC and UK populations and their replicates.

| Population | Activity |
|---|---|
| **SBT-1** | 0.041 |
| **SBT-2** | 0.156 |
| **SBT-3** | 0.101 |
| **PA-1** | 0.097 |
| **PA-2** | 0.099 |
| **PA-3** | 0.079 |
| **PX-1** | 0.108 |
| **PX-2** | 0.097 |
| **PX-3** | 0.078 |
| **PC-1** | 0.026 |
| **PC-2** | 0.111 |
| **PC-3** | 0.028 |
| **Bt-1** | 0.146 |
| **Bt-2** | 0.059 |
| **Bt-3** | 0.158 |
| **Bt-4** | 0.051 |
| **UK** | 0.059 |

*Toxin Digestion*

The association between protease activity and resistance is believed to be due to defective activation of the toxin [17]. To test this directly we digested the protein with the enzymes of the guts. The results are showed in the figures 2 and 3, in which all of the bands, from all of the 6 population, showed the same pattern. The bands that are fainter, as in populations SBT, PA and replicates 1 and 2 of Px, indicates that are less amount of enzymes.

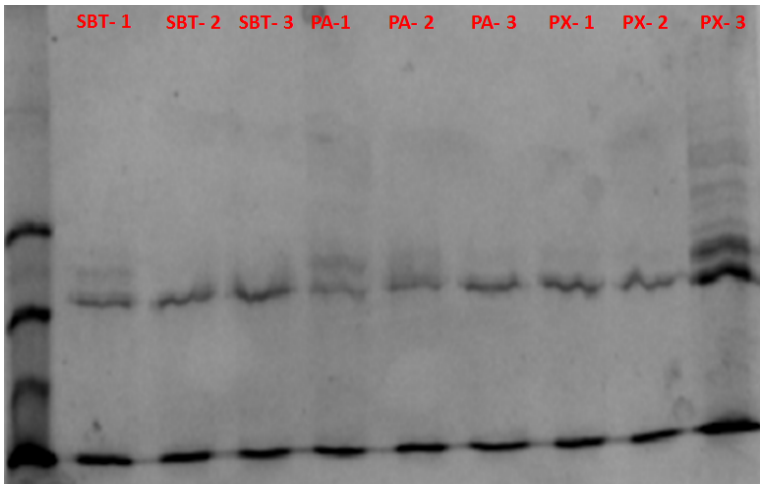


Fig.2. Photo of PAGE Mini-Gel representing the toxin (Cry1Ac) digestion to the guts enzymes of the following population: SBT, PA, PX. The numbers associated with population represents a particular of replicate.

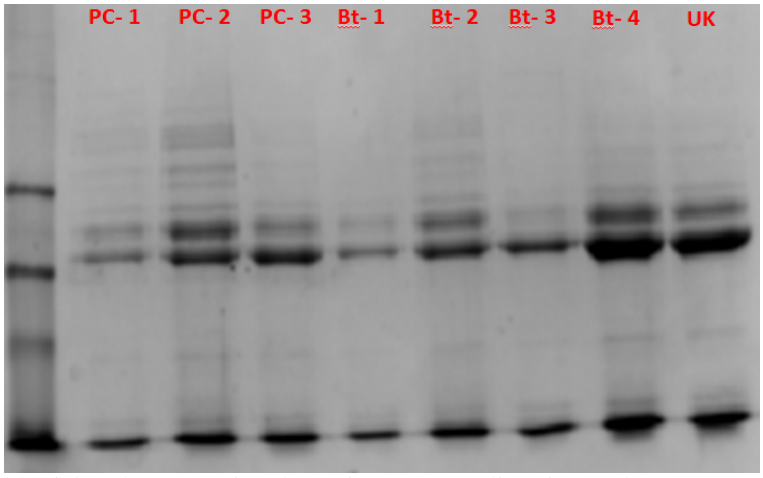


Fig.3. Photo of PAGE Mini-Gel representing the toxin (Cry1Ac) digestion to the guts enzymes of the following population: PC, Bt, UK. The numbers associated with each population represents a particular replicate.

*Esterase activities*

Variations in esterase levels can affect susceptibility to certain chemical insecticides and there has been one report claiming that an increase in esterase can cause resistance to Bt toxins [42]. Therefore we measured the esterase activities and the results are shown in figures 4 and 5. Each band represents different kinds of esterases, due to the differences in the pattern of the bands, we can see that population UK has at least one different band, indicating the existence of a different kind of esterase.

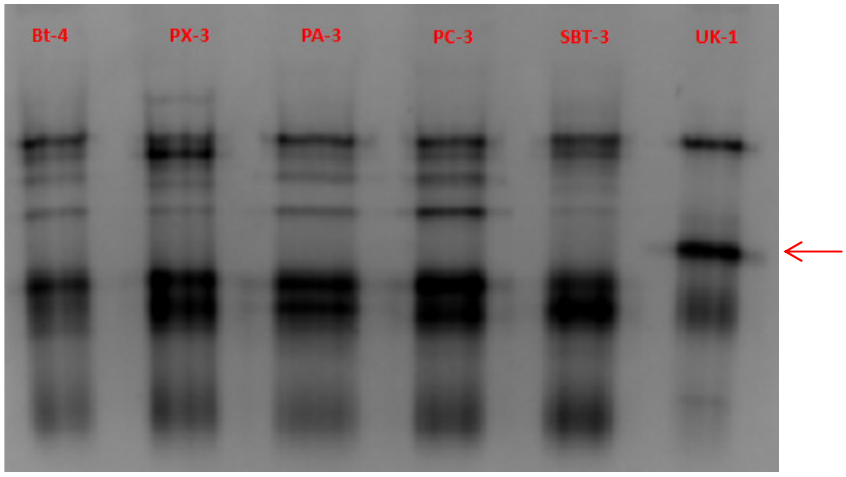


Fig.4. Photo of a native PAGE Mini-Gel of gut esterase activity from the following populations: Bt, PX, PA, PC, SBT and UK.

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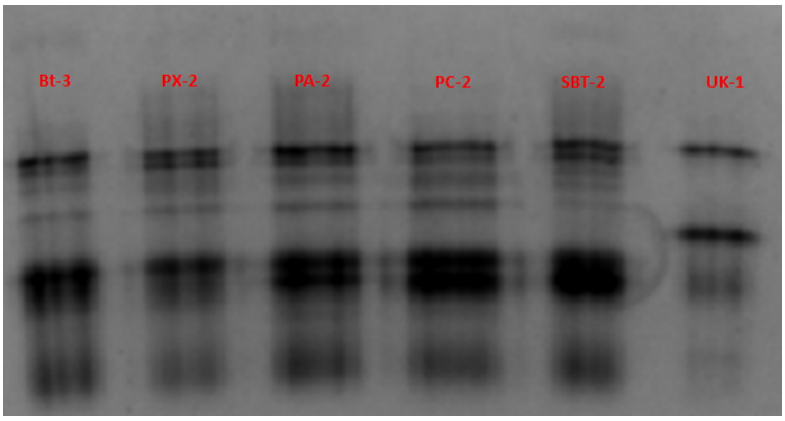


Fig.5. Photo of a native PAGE Mini-Gel of gut esterase activity from the duplicates of the following populations: Bt, PX, PA, PC, SBT and UK.

*B. Analysis of gut bacteria*

There are publications indicating that gut bacteria can influence susceptibility of insects to Bt [45], thus in this work we analyzed the guts bacteria by extracting and sequencing the 16S rRNA gene. The BLAST program was used to analyze the nucleotide sequences (BLASTN), aiming to search and compare similar sequences in the database International (GenBank) with those obtained. The sequences obtained are transcribed below:

UK bacteria
GGCGGCGTGCCTAATACATGCAAGTCGAACGCTT
CTTTTCCCACCGGAGCTTGCTCCACCGGGAAAAG
AGGAGTGGCGAACGGGTGAGTAACACGTGGGTA
ACCTGCCCATCAGAAGGGGATAACACTTGGAAAC
AGGTGCTAATACCGTATAACAATCGAAACCGCAT
GGTTTCGTTTTGAAAGGCGCTTTACGGTGCCGCTG
ATGGATGGACCCGCGGTGCATTAGCTAGTTGGTG
AGGTAACGGCTCACCAAGGCCACGATGCATAGCC
GACCTGAGAGGGTGATCGGCCACATTGGGACTGA
GACACGGCCCAAACTCCTACGGGAGGCAGCAGTA
GGGAATCTTCGGCAATGGACGAAAGTCTGACCGA
GCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATC
GTAAAACTCTGTTGTTAGAGAAGAACAAGGGTGA
GAGTAACTGTTCACCCCTTGACGGTATCTAACCA
GAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGCGGCAAGC

Bt Bacteria
GGCGGCGTGCCTAATACATGCAAGTCGAACGCTT
CTTTTCCCACCGGAGCTTGCTCCACCGGGAAAAG
AGGAGTGGCGAACGGGTGAGTAACACGTGGGTA
ACCTGCCCATCAGAAGGGGATAACACTTGGAAAC
AGGTGCTAATACCGTATAACAATCGAAACCGCAT
GGTTTCGTTTTGAAAGGCGCTTTACGGTGCCGCTG
ATGGATGGACCCGCGGTGCATTAGCTAGTTGGTG
AGGTAACGGCTCACCAAGGCCACGATGCATAGCC
GACCTGAGAGGGTGATCGGCCACATTGGGACTGA
GACACGGCCCAAACTCCTACGGGAGGCAGCAGTA
GGGAATCTTCGGCAATGGACGAAAGTCTGACCGA
GCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATC
GTAAAACTCTGTTGTTAGAGAAGAACAAGGGTGA
GAGTAACTGTTCACCCCTTGACGGTATCTAACCA
GAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGTGGCAAGCGTTGTCCGGATTTA
TTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAG
TCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGG
GTCATTGGAAACTGGGAGACTTGAGTGCAGAAGA
GGAGAGTGGAATTCCATGTGTAGCGGTGAAATGC
GTAGATATATGGAGGAACACCAGTGGCGAAGGC
GGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGA
AAGCGTGGGGAGC

PA Bacteria
GCAGTCGAACGCTTCTTTTCCCACCGGAGCTTGCT
CCACCGGGAAAAGAGGAGTGGCGAACGGGTGAG
TAACACGTGGGTAACCTGCCCATCAGAAGGGGAT
AACACTTGGAAACAGGTGCTAATACCGTATAACA
ATCGAAACCGCATGGTTTCGTTTTGAAAGGCGCT
TTACGGTGCCGCTGATGGATGGACCCGCGGTGCA
TTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGC
CACGATGCATAGCCGACCTGAGAGGGTGATCGGC
CACATTGGGACTGAGACACGGCCCAAACTCCTAC
GGGAGGCAGCAGTAGGGAATCTTCGGCAATGGA
CGAAAGTCTG

PC Bacteria
AAGTCGACGCTTCTTTTCCCACCGGAGCTTGCTCC
ACCGGGAAAAGAGGAGTGGCGAACGGGTGAGTA
ACACGTGGGTAACCTGCCCATCAGAAGGGGATAA
CACTTGGAAACAGGTGCTAATACCGTATAACAAT
CGAAACCGCATGGTTTCGTTTTGAAAGGCGCTTT
ACGGTGCCGCTGATGGATGGACCCGCGGTGCATT
AGCTAGTTGGTGAGGTAACGGCTCACCAAGGCCA
CGATGCATAGCCGACCTGAGAGGGTGATCGGCCA
CATTGGGACTGAGACACGGCCCAAACTCCTACGG
GAGGCAGCAGTAGGGAATCTTCGGCAATGGACG
AAAGTCTGACCGAGCAACGCCGCGTGAGTGAAG
AAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAG
AAGAACAAGGGTGAGAGTAACTGTTCACCCCTTG
ACGGTATCTAACCAGAAAGCCACGGCTAACTACG
TGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGC
GTTGTCCGGAT

PX Bacteria
GGCGGCGTGCCTATACATGCAAGTCGAACGCTTC
TTTTCCCACCGGAGCTTGCTCCACCGGGAAAAGA
GGAGTGGCGAACGGGTGAGTAACACGTGGGTAA

CCTGCCCATCAGAAGGGGATAACACTTGGAAACA
GGTGCTAATACCGTATAACAATCGAAACCGCATG
GTTTCGTTTTGAAAGGCGCTTTACGGTGCCGCTGA
TGGATGGACCCGCGGTGCATTAGCTAGTTGGTGA
GGTAACGGCTCACCAAGGCCACGATGCATAGCCG
ACCTGAGAGGGTGATCGGCCACATTGGGACTGAG
ACACGGCCCAAACTCCTACGGGAGGCAGCAGTAG
GGAATCTTCGGCAATGGACGAAAGTCTGACCGAG
CAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCG
TAAAACTCTGTTGTTAGAGAAGAACAAGGGTGAG
AGTAACTGTTCACCCCTTGACGGTATCTAACCAG
AAAGCCACGGCTAACTACGTGCCAGCAGCCGCGG
TAATACGTAGGTGGCAAGCGTTGTCCGGATTTAT
TGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGT
CTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGG
TCATTGGAAACTGGGAGACTTGAGTGCAGAAGAG
GAGAGTGGAATTCCATGTGTAGCGGTGATATGCG
TAGATATATGGAGGAACACCAGTGGCGAAGGCG
GCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAA
AGCGTGGGGAGCAAACAGGATTAGATACCCTGGT
AGTCCACGCCGTAAACGATGAGTGCTAAGTGTTG
GAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACG

SBT Bacteria
CGTGCCTAATACATGCAAGTCGAACGCTTCTTTTC
CCACCGGAGCTTGCTCCACCGGGAAAAGAGGAGT
GGCGAACGGGTGAGTAACACGTGGGTAACCTGCC
CATCAGAAGGGGATAACACTTGGAAACAGGTGCT
AATACCGTATAACAATCGAAACCGCATGGTTTCG
TTTTGAAAGGCGCTTTACGGTGCCGCTGATGGAT
GGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAA
CGGCTCACCAAGGCCACGATGCATAGCCGACCTG
AGAGGGTGATCGGCCACATTGGGACTGAGACACG
GCC

All of the sequences were over 99% similar to *Enterococcus mundtii* Collins gene for 16S rRNA

### *C. Analysis of the ABCC2 gene*

ABBC2 mutations in this gene have been shown to cause resistance to Bt in several insects and in particular a deletion was identified in a resistant population of *P. xyllostela* [33]. Therefore we decided to check whether or not these same deletions, or other mutations in this region, were present in our strains.

The exon which has the known mutation in the Bt-resistant (NO-QAGE) was sequenced.

The sequences aligned are transcribed below:

PC GT-
CATGGTGCTGACGGTGCTGCTGCAGCTGGCCGCG
CGGTTCACCAGCGACTTCCTGGC 59
PX GT-
CATGGTGCTGACGGTGCTGCTGCAGCTGGCCGCG
CGGTTCACCAGCGACTTCCTGGC 59
PA GT-
CATGGTGCTGACGGTGCTGCTGCAGCTGGCCGCG
CGGTTCACCAGCGACTTCCTGGC 59
Bt GT-
CATGGTGCTGACGGTGCTGCTGCAGCTGGCCGCG
CGGTTCACCAGCGACTTCCTGGC 59
SBT GT-
CATGGTGCTGACGGTGCTGCTGCAGCTGGCGGCG
CGGTTCACCAGCGACTTCCTGGC 59
ABCC2
GTCCATGGTGCTGACGGTGCTGCTGCAGCTGGCG
GCGCGGTTCACCAGCGACTTCCTGGC 60
********** **********

PC
GCAGATGACCGCCGTGGAGCGCGTGCTCGAGT
ACACTAAGCTGCCGCACGA--------- 110
PX
GCAGATGACCGCCGTGGAGCGCGTGCTCGAGTAC
ACTAAGCTGCCGCACGA--------- 110
PA
GCAGATGACCGCCGTGGAGCGCGTGCTCGAGTAC
ACTAAGCTGCCGCACGA--------- 110
Bt
GCAGATGACCGCCGTGGAGCGCGTGCTCGAGTAC
ACTAAGCTGCCGCACGA--------- 110
SBT
GCAGATGACCGCCGTGGAGCGCGTGCTCGAGTAC
ACTAAGCTGCCGCACGA--------- 110
ABCC2
GCAGATGACCGCCGTGGAGCGCGTGCTCGAGTAC
ACTAAGCTGCCGCACGAGGAGAATAT 120
**********

PC ----------
PX ----------
PA ----------
Bt ----------
SBt ----------
ABCC2 TAATGATGGCC 131

None of our population has that or other mutation on the exon.

## VI. Discussion

Resistance of insects to Bt toxins has been mainly observed in the laboratory, including insects such as *Plodia interpunctella* (Hübner) (Lep.: Pyralidae), *H. virescens*, *Spodoptera littoralis* Boisduval (Lep.: Noctuidae), *Ostrinia nubilalis* (Hübner) (Lep.: Crambidae), and *Culex quinquefasciatus* Say (Dip.: Culicidae). Until recently only *P. xylostella* has shown field resistance and resistant insects have been selected from fields in Hawaii, Florida, Pennsylvania, Indonesia, Malaysia, Central America, several states of USA, and Philippines [39].

Resistance development seems to involve various mechanisms, depending on the type of insect, the toxin, and the Bt strain. Reduced solubilization of the protoxin, alteration in the proteolytic processing, elimination of active toxin by precipitation and/or degradation, reduction of binding of the toxin to the membrane receptors in the midgut of insects, ABBC2 mutations in this gene, have been reported as mechanisms of resistance. Combination of these mechanisms might be involved in acquisition of resistance; however it is possible that other mechanisms may be involved as well.

A mechanism of resistance may involve gut proteinases that interact with Bt toxins. Enzymes from a strain of *H. virescens* resistant to Bt subspecie *kurstaki* (HD-73) were reported to process the protoxin more slowly and to degrade toxin faster than enzymes from a susceptible strain [40]. In *S. littoralis*, an increase in the specific activity of gut proteinases from fifth instar larvae was associated with a loss of sensitivity to Cry1C, possibly due to an increase in the degradation of toxin [41]. According to our results, the methodology used in this bioassay was not reliable, since every time the repetitions were done the results showed random numbers.

Sequestration is recognized as a potential *B. thuringiensis* delta-endotoxin resistance mechanism [17]. Findings concerning esterase binding to Cry1Ac are not significant; therefore this mechanism does not confer resistance in our population.

Esterase are known to have the ability to bind to the Cry toxin and thus detoxify this toxin, therefore esterase sequestration is a potential resistance mechanism. The potential for the insect esterase to bind to and detoxify *B. thuringiensis* toxins is of great concern because esterase-based resistance mechanisms in insects are not uncommon [42]. Nevertheless, there were no variations on the esterase activities levels of esterases between the populations indicating that the Bt population resitance is not linked to an increase of esterase levels.

It has been reported that Enterobacteriaceae are specifically required for CrylAc toxicity in several species of Lepidoptera and that CrylAc is non-lethal in insects that possess no gut bacteria [35][36]. According to this hypothesis elimination of certain types of bacteria from the insect intestine could result in resistance to CrylAc. Indeed, it would be expected that selection with CrylAc would favor those larvae which lack gut bacteria necessary for toxicity and/or which harbour bacteria that mitigate the toxic effects of CrylAc. In considering the latter possibility, it is noteworthy that in Lepidoptera gut bacteria may be transmitted from mother to offspring on the surface of eggs [43][44].

Since there are publications indicating that gut bacteria can influence susceptibility of insects to Bt [45], this work analyzed the guts bacteria by PCR and all sequences were sufficiently similar (99%) to be considered as a single relatedness group and clearly belonged to *E. mundtii*. So there were no differences in composition of the larval gut microbiota, wherefore the composition of the gut microbiota of the resistant population was also not correlated with resistance. It is unclear whether similar interactions occur in the field where the intestinal microbiotas of lepidopteran larvae may be more complex than the apparent mono-associations described here. However it is possible that gut bacteria may present a novel target for the management of *P. xylostella*. The nature of the protective effect of gut bacteria is also unclear. Previous studies suggested that lactic acid bacteria may acidify the gut thereby decreasing host susceptibility to alkali-soluble Cry toxins such as CrylAc.

As ABCC2 is correlated with Cry1Ac resistance in *H. virescens* [46], and ABBC2 mutations in this gene have been shown to cause resistance to Bt in several insects and in particular a deletion was identified in a resistant population of *P. xyllostela* [33]. Therefore we decided to check whether or not these same deletions, or other mutations in this region, were present in our strains. There are no mutations in the ABCC2, accordingly there is no association of ABCC2 with Bt resistance.

There are others mechanisms of action that are related and that confer resistance to a population, therefore, more studies is require in order to discover the reason for the resistance of this population.

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