Detoxifying Enzymatic Activity and Insecticide-Resistance Gene Expression in Field Populations of Pink Bollworm, (*Pectinophora gossypiella* (Saund.))

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DOI: [10.21608/ajs.2021.92821.1412](https://doi.org/10.21608/ajs.2021.92821.1412)

Received 5 September, 2021 ; Accepted 18 December, 2021

Keywords:

Detoxifying enzymes,
Insecticide-resistance,
Gene expression
real-time (PCR),
Pink bollworm

Abstract: Study was conducted to assess two enzyme detoxifying activities and insecticide-resistance gene expression quantitation using real-time (PCR). Four populations of pink bollworm were compared with baseline laboratory strain. Field populations showed higher levels of enzymatic activity, glutathione-S-transferase and β -esterases, than those of the laboratory strain. The amplification curves scored a cycle threshold (C_t) value of 25 for the ribosomal protein subunit7 (*rps7*). For the *BtR* gene, the C_t values of analyzed biological groups ranged from 20 for laboratory strain to 24 for Fayoum group. For *Cad1*, the C_t values ranged from 19 for laboratory strain to 23 for Fayoum and Qalubia populations. Therefore, the present work introduces a method for the challenge of monitoring resistance to Bt toxins in crops which require, according to IPM (Integrated pest management) program, a wise insecticide application.

1 Introduction

In 1913, the pest spread to such a degree that it became a real danger to the cotton crop in Egypt. It's importance since it infests cotton bolls and causes great damage resulting in magnitude loss of the yield production. The *P. gossypiella* is considered key insect infesting cotton plants, as indicated by the fact that 75% of insecticide applications used on cotton are directed against pink bollworm (Khidr et al 2003).

Rapid biochemical determination is a great important method for detecting resistance in field population (Abdel-Baset 2009). The esterases hydrolyzing β -naphthyl acetate in the fenitrothion resistant strain of *Spodoptera littoralis* play an important role in insecticide resistance (Saleh

1981). Young et al (2005) revealed that resistance in field groups of the cotton bollworm, *Helicoverpa armigera* to pyrethroid toxicity is due to remarkable high level of the enzymatic esterase activity that metabolite this kind of insecticides sequesters pyrethroid insecticide. Zidan et al (2012) showed chlorpyrifos and profenophos-treated strains of pink and spiny bollworms larvae had increased glutathione-S-transferase (GST) activity than the baseline laboratory group.

Real-time (PCR) was repeatedly reported as a reliable molecular quantifying gene expression for a gene of interest in many researches (Lü et al 2018).

Investigation aimed to measure enzymes activity in whole homogenate for different field populations compared with the baseline laboratory strain, and to study the insecticide-resistance gene expression quan-

titration using real-time PCR of field collected *P. gossypiella*, 4th instar larvae from different Egyptian Governorates compared with the baseline laboratory strain.

2 Materials and Methods

2.1 Rearing technique

Rearing technique in this study was based on artificial diet of kidney bean according to Abd El-Hafez et al (1982).

2.2 Determination of enzymes activity

2.2.1 Determination of non-specific esterase's activity

Beta naphthyl acetate as a substrate was used to determine the beta esterases activity (Van Asperen, 1962).

2.2.2 Determination of glutathione -S- transferase

Glutathion-S-transferase (GST) catalyzes the conjugation of reduced glutathione (GSH) with 1-chloro 2,4-dinitrobenzene (CDNB) via the -SH group of glutathione. The conjugate, S-(2,4-dinitro-phenyl)-L-glutathione could be detected as described by the method of Habig et al (1974).

2.3 Real-time PCR was used to quantify insecticide resistance gene expression

2.3.1 Ribose Nucleic Acid (RNA) extraction

RNA was prepared by RNA Kit (Thermo Scientific, Fermentas, #K0731).

2.3.2 Real-time PCR

The *BtR* and *CadI* gene expression were quantified and normalized using the ribosomal protein subunit 7 (*rps7*) gene as reference. The target genes were amplified by newly planned specific primers. The primers were designed using Primer3 webtool based on gene templates of *Pectinophora gossypiella* retrieved from the NCBI database. Primers specificity was tested using the BLAST tool.

Prior to RT-PCR, primers were prepared as follows:

- 1) At room temperature, the lyophilized primer was equilibrated.
- 2) Using the spin-centrifuge-vortex, the equilibrated primer was spun down for 3 sec.
- 3) The RNase-free water was used to dilute the lyophilized primer (both forward and reverse) and inverted two minutes at room condition.
- 4) A 5M stock primer was prepared by diluting the stock primer with RNase-free water buffer (pH 8.0) and storing it at -20°C until it was utilised. For each sample, the three genes were amplified in triplicates.

2.3.3 RT-PCR analysis

After calculating the cycle threshold (Ct) averages, the Ct value of the housekeeping gene (i.e., *rps7*) was selected to normalize and determine the relative gene expression or variation value of the target genes (*BtR* and *CadI*) based on the $2^{-\Delta\Delta\text{Ct}}$ method by Livak and Schmittgen (2001).

The method proposed the normalization of each target gene (*BtR* and *CadI*) by subtracting from the reference gene (i.e., *rps7*) for each biological sampling group, including the control, as follows:

- 1st to calculate the ΔCt for the biological laboratory group (G5):

$$\Delta\text{Ct}_{\text{G5}} = \text{Ct}_{\text{Target gene (G5)}} - \text{Ct}_{\text{rps7}}$$

Table 1. Forward and reverse primers sequence used in qPCR

Gene	Forward (5'-3')	Reverse (5'-3')	Band size (bp)
<i>BtR</i>	GAACCAGACATTCGCCAT	CGGTCCGTTGCTATTACCTT	99
<i>CadI</i>	GTGGTAGCGAAGGAGATCCG	TGTGCAAGTCCCGAACTCTC	91
<i>rps7</i>	CCGTGAGTTGGAGAAGAA	AGGATAGCGTCGTACACTGA	150

Table 2. The thermal cycler condition used during real-time PCR

Step	Temp/Duration	Cycles no.
Initial denaturation	95°C/ 10 min	1
Denaturation	95°C/ 15 sec	40
Annealing	60°C/ 30 sec	
Extension	72°C/ 30 sec	

- 2nd to calculate the ΔCt for each sampling field group (G1 to G4):

$$\Delta Ct_{G\#} = Ct_{\text{Target gene (G\#)}} - Ct_{\text{rps7}}$$

Then to normalize the biological sampling group by subtracting from the control one to estimate the gene expression of the target genes in the samples relative to the control as follows:

- 3rd to calculate the $\Delta\Delta Ct$ for each sampling group (G1 to G4):

$$\Delta\Delta Ct_{G\#} = Ct_{\text{Target gene (G\#)}} - Ct_{\text{Target gene (G5)}}$$

Then the final relative gene expression for each of the target genes in each sample relative to the control is known as fold-change and the following calculation:

- Fold change = $(2^{-\Delta\Delta Ct})$

3 Results and Discussions

3.1 Enzymes activity in field population of *Pectinophora gossypiella* collected from different Governorates

The activities of the determined hydrolyzing enzymes, namely glutathion-S-transferase and non-specific β -esterases were determined in the field strain of pink bollworm, *P. gossypiella* collected from different Governorates in comparison with the baseline laboratory strain reared free any contamination of insecticides under constant conditions in the laboratory at $27 \pm 1^\circ\text{C}$ and $70 \pm 5\%$ relative humidity. Determination of the enzymes activity may shed light on the insecticides effect used in large scale under field conditions related to induction effects on the enzymes activity in these strains.

3.1.1 Glutathion-S-transferases Activity (GSTs)

The enzymatic activity was measured as m mole substrate conjugated/min./ g.b.wt. The obtained results **Table 3** indicated that the 4th instar larvae of the field colony showed much higher levels of glutathione-S-transferase activity than

the laboratory strain. The mean enzymes activity levels in the 4th instar larvae of different field colony strains of *P. gossypiella* collected from Fayou, Bihera, Qalubia, and Kafr-Elsheikh Governorates as well as the laboratory strain were 104.7, 97.83, 76.77, 94.63 and 72.83 m mole substrate conjugated/min./g.b.wt; respectively. The enzymatic activity ratio different field groups of *P. gossypiella* ranged between 1.05 and 1.44.

Table 3. Glutathion S- transferase in the 4th instar larvae of *P. gossypiella* homogenates of different field population collected from different Governorates

Strains	glutathion S- transferase activity	Activity ratio
Fayoum	104.7 ^a	1.44
Bihera	97.83 ^{ab}	1.34
Qalubia	76.77 ^b	1.05
Kafrel-Sheikh	94.63 ^c	1.30
Laboratory	72.83 ^c	1.00
F	26.25	
p	0.0001	

Glutathion S- transferase activity was expressed as m mole sub. Conjugated/min./g.b.wt.

3.1.2 Nonspecific Beta esterase's activity

Results represented in **Table 4** showed comparison of non-specific esterase activity in the whole homogenates of the field colony strains compared with the baseline laboratory strain. Data showed some physiological differences were excited between the field colony strains and the baseline laboratory strain. It is clear that the levels of β - esterase's activity were much higher in all field instar larvae than the laboratory strain. The levels of β -esterases activity collected from different Governorates, namely Fayoum, Bihera, Qalubia, Kafr-Elsheikh and the baseline laboratory strains were 400, 312, 282.33, 278.67 and 265.67 μg β -naphthol released/min/g.b.wt; respectively. On the other hand, the corresponding activity ratios of the field colony strains of aforementioned Governorates were 1.51, 1.17, 1.06 and 1.05; respectively as compared with the baseline laboratory strain. It was obvi-

ous that the highest level of β -non-specific esterases was noticed in Fayoum field colony strain, whereas the lowest levels of β -esterases activity were exhibited in Kafr-Elsheikh field colony strain.

Aforementioned results are supported by Hung and Ottea (2004) reported that esterases activity related with resistance to organophosphorus as well as pyrethroid insecticides a larvae of *Heliothis virescens* were developed and determined. The results are accordance with those published by Zhao et al (2003), They found a link between higher esterase activity and methyl parathion resistance in *Diabrotica vigifera* (Lconte) populations in Nebraska. However, the results are in harmony with those published by Young et al (2005); they reported that resistance in field group of *H. armigera*, to pyrethroid is due to the high level on esterase isoenzymes that metabolite the toxicants. Abdel-Baset (2009) revealed that non-specific esterase's activity in the fourth instar larvae as well as the adult stage of the field colony strains of *Pectinophora gossypiella* collected from, Menoufia, Gharbia, Sharkia and Assiut governorate and chlorpyrifos resistant strains showed higher levels than those in the respective stage of the laboratory strain that was not subjected to any insecticide. Zidan et al (2012) showed that chlorpyrifos and profenophos-treated strains of both pink and spiny bollworms larvae had increased glutathione-S-transferase (GST) activity than the laboratory strain Badr (2016) determined hydrolases and glutathione S-transferase activities in different field colony strains of *Ceratitis capitata* flies. The author revealed that the different field strains showed remarkable higher levels of hydrolases and glutathione S-transferase activities than the laboratory strain.

Table 4. β -esterases activity in the 4th instar larvae homogenates of different field population collected from different Governorates

Strains	β -esterases activity	activity ratio
Fayoum	400.00 ^a	1.51
Bihera	312.00 ^b	1.17
Qalubia	282.33 ^{bc}	1.06
Kafrel-Sheikh	278.67 ^c	1.05
Laboratory	265.67 ^d	1.00
F	36.50	
p	0.0001	

Enzymatic activity was expressed as μ g naphthol released/ min./ g. b.wt.

3.2 Insecticide-resistance gene expression quantitation using real-time PCR of field populations

BtR and *Cad1* gene expression relative to insecticide treatments in pink bollworm 4th instar larvae were investigated. The quantification of both target genes (i.e., *BtR* and *Cad1*) was successful for the five biological samples using the real-time PCR (RT-PCR). The results manifested an amplification curves that were converted to specific cycle threshold values (*Ct*; **Fig 1**). RT-PCR was repeatedly reported as a reliable molecular technique as potential tool for quantifying gene expression levels for a gene of interest (Lü et al 2018).

The amplification curves scored a cycle threshold (*Ct*) value of 25 for the ribosomal protein subunit 7 (*rps7*). This value is selected as a reference to the amplification of the tested genes **Fig 2**. For the *BtR* gene, the *Ct* values for the analyzed biological groups ranged from 20 for laboratory strain (G5) to 24 for Fayoum (G1). For *Cad1*, the *Ct* values for the analyzed biological groups ranged from 19 (G5) to 23 for Fayoum and Qalubia field groups (G1 and G3; **Table 5**).

Table 5. The *CT*, delta-*CT*, and Delta-delta-*Ct* values as estimated from the RT-PCR for each group. Those values were used to calculate the fold change for five biological sampling groups for *BtR* and *Cad1* genes

Genes	Samples	Ct	Δ Ct	$\Delta\Delta$ Ct	Fold-change
<i>BtR</i>	Fayoum	24	-1	4	0.0625
	Kafr El Sheikh	22	-3	2	0.25
	Qalubia	22	-3	2	0.25
	Behira	22	-3	2	0.25
	laboratory	20	-5	0	1
<i>Cad1</i>	Fayoum	23	-2	4	0.0625
	Kafr El Sheikh	22	-3	3	0.125
	Qalubia	23	-2	4	0.0625
	Behira	22	-3	3	0.125
	laboratory	19	-6	0	1

The housekeeping genes are a group of genes that are responsible for cell viability and important physiological events. In molecular biology, the measurement of a housekeeping gene as an internal reference for

each sample/gene is crucial for determining gene regulation, whether a gene is upregulated (induced) or downregulated (suppressed) due to a specific case of study (Bansal et al 2016). In the current analysis, the estimated rps7 Ct value was greater than all the other genes, as Ct is reversely proportioned with gene expression concentration; thus, rps7 expression is lower than the target genes, which indicates that both *BtR* and *Cad1* are upregulated due to the insecticide presence inducing the gene expression considered as gene resistance in the insect body of the tested field body groups of the pest. Bollworms, budworms, and armyworms all belong to the Noctuidae insect family, which comprises some of the most devastating agricultural pests. It is necessary to interpret the relation of *Bt* toxins of field research with their targets to assess the danger of resistance evolution (Heckel 2021).

After normalization, the fold change in *BtR* gene expression was 0.25 times the control for G2, 3, and 4; and 0.06 times the control for G1. The fold-change in the *Cad1* gene expression was 0.125 times the control for G2 and G4; and 0.0625 times the control for G1 and G3. Thus, after

normalization, comparing the relative expression values among the two target genes is possible, showing a higher expression level for *BtR* than *Cad1* (Fig 3). As anticipated, both genes are relatively equal in expression performance, where the two genes are positively correlated, as the reduced cadherin gene (*Cad1*) is in the pink bollworm, a receptor protein linked to *Bt* toxin resistance (Fabrick et al 2020).

Although both genes are positive indicators for the insect's ability to process and tolerate the insecticide, the collected samples showed a lower expression than the control group (i.e., laboratory untreated insect lines). In the field, In the absence of *Bt* toxins, insect resistance to *Bacillus thuringiensis* (*Bt*) toxins reduces the fitness of resistant individuals (Gassmann et al 2009). In every case, the pink bollworm's amazing flexibility is demonstrated by its ability to evolve resistance through both qualitative and quantitative alterations in receptor proteins. (Fabrick et al 2020). Therefore, it presents challenges for monitoring and managing resistance to *Bt* crops and requires a wise insecticide application according to the methods prescribed by the IPM (Integrated pest management) program.

Fig 1. Amplification curves converted to specific cycle threshold for quantifying gene expression levels relative to insecticidal resistance in field population of *P. gossypiella*, 4th instar larvae compared to laboratory strain

Fig 2. Exponential amplification curves of the RT-PCR assay. The above and below plots represent the *BtR* and *Cad1* genes, respectively. The lines represent the fluorescence rate (RFU) absorbed for the amplified DNA quantity each cycle. CT values are determined based on the threshold (green horizontal line)

Fig 3. The fold-change values are based on RT-PCR estimated for two target genes (*BtR* and *Cad1*, normalized by *rps7*) for four biological sampling groups relative to the control group are shown

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