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

Rabab, AD Allam1*, Sobeiha AK2, Khidr AA1, Neima K Al-Senosy3
1- Bollworms Research Department Plant Protection Research Institute, Agricultural Research Center, Dokki, Giza
2- Plant Protection Dept, Fac Agric, Ain Shams Univ, P.O. Box 68, Hadyek Shoubra 11241, Cairo, Egypt
3- Genetic Department, Faculty of Agric, Ain Shams Univ, P.O. Box 68, Hadyek Shoubra 11241, Cairo, Egypt

* Corresponding author: rbaballam22@gmail.com
DOI:10.21608/ajs.2021.92821.1412
Received 5 September, 2021 ; Accepted 18 December, 2021

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 (Ct) value of 25 for the ribosomal protein subunit7 (rps7). For the BtR gene, the Ct values of analyzed biological groups ranged from 20 for laboratory strain to 24 for Fayoum group. For Cad1, the Ct 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-
titation 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 *Cad1* 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 *Cad1*) based on the $2^{-\Delta\Delta Ct}$ method by Livak and Schmittgen (2001).

The method proposed the normalization of each target gene (*BtR* and *Cad1*) by subtracting from the reference gene (i.e., *rps7*) for each biological sampling group, including the control, as follows:

- 1st to calculate the $\Delta Ct$ for the biological laboratory group (G5):

\[
\Delta Ct_{G5} = Ct_{\text{Target gene (G5)}} - Ct_{rps7}
\]

### Table 1. Forward and reverse primers sequence used in qPCR

| Gene  | Forward (5'-3')                     | Reverse (5'-3')           | Band size (bp) |
|-------|-------------------------------------|---------------------------|----------------|
| *BtR* | GAACCAGACATTGCACCAT                | CGGTCCGTGCTATTACCCTT      | 99             |
| *Cad1*| GTGGTACGCGAAGAGATCCCG              | TGTGAAAGTCCCGAAGATCCCG   | 91             |
| *rps7*| CCGTGAGTCTGAGAAGAAGA               | AGGATACGCGTACACTGA        | 150            |
2 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±1°C and 70±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 Fayoum, 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 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):

\[ \Delta C_{T \, \text{G#}} = C_{T \, \text{Target gene (G#)}} - C_{T \, \text{rps7}} \]

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 ΔΔCt for each sampling group (G1 to G4):

\[ \Delta \Delta C_{T \, \text{G#}} = C_{T \, \text{Target gene (G#)}} - C_{T \, \text{Target gene (G5)}} \]

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 = \(2^{-\Delta \Delta C_{T}}\)

### 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 |
|------------------|----------------------------------|----------------|
| Fayouab          | 104.7a                          | 1.44           |
| Bihera           | 97.83b                          | 1.34           |
| Qalubia          | 76.77b                          | 1.05           |
| Kafr-Sheikh      | 94.63c                          | 1.30           |
| Laboratory       | 72.83c                          | 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-esters 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 El Sheikh field colony strain.

Aforementioned results are supported by Hung and Ottea (2004) reported that esterases activity related with resistance to organophosphorous as well as pyrethroid insecticides a larvae of Heliothis viresrens 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 esterases’s activity in the fourth instar larvae as well as the adult stage of the filed colony strains of Pectinophora gossypiella collected from, Menoufia, Gharbia, Sharkia and Assiut governorate and chlorpyrifos resistant strains from, Menoufia, Gharbia, Sharkia and Assiut governorate and chlorpyrifos resistant strains displayed higher levels 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               | 1.51           |
| Bihera          | 312.00               | 1.17           |
| Qalubia         | 282.33               | 1.06           |
| Kafrel-Sheikh   | 278.67               | 1.05           |
| Laboratory      | 265.67               | 1.00           |
| F               | 36.50                |                |
| p               | 0.0001               |                |

Enzymatic activity was expressed as µg 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 (Liu 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  | ΔCt | ΔΔCt | Fold-change |
|-------|---------------|-----|-----|------|-------------|
| BtR   | 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           |
| Cad1  | 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, G3, and G4; 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.

![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](image)

**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.

References

Abdel-Baset TT (2009) Comparative toxicological and molecular studies on the pink bollworm, Pectinophora gossypiella and the mosquito, Culex pipiens. Ph.D. Thesis, Faculty of Science, Ain-Shams University 85-93.

Abd El-Hafez A Metwally AG, Saleh MRA (1982) Rearing pink bollworm Pectinophora gossypiella (Saunders) on kidney bean diet in Egypt (Lepidoptera: Glechiidae). Research, Bulletin., Faculty of Agriculture, Zagazig University 576, 1-10.
Badr FAA (2016) Monitoring resistance in different field strains of the Mediterranean fruit fly, *Ceratitis capitata* (wied.) to toxicity of the organophosphorus insecticide malatox in relation to enzymes activity. *Egyptian Journal of Plant Protection Research* 4, 39-57.

Bansal R, Mittapelly P, Chen Y, Mamidala P, Zhao C and Michel A (2016) Quantitative RT-PCR Gene Evaluation and RNA Interference in the Brown Marmorated Stink Bug. PLOS ONE 11 (5), e0152730. https://doi.org/10.1371/journal.pone.0152730

Fabrick JA, Lolita GM, Dannialle ML, Joe H, Gopalan CU, Alex JY, Yves C, Xianchun L and Bruce E T (2020) Reduced cadherin expression associated with resistance to *Bt* toxin Cry1Ac in pink bollworm. *Pest Management Science* 76, 67-74. https://doi.org/10.1002/ps.5496

Gassmann AJ, Carrière Y and Tabashnik BE (2009) Fitness Costs of Insect Resistance to Bacillus thuringiensis. *Annual Review of Entomology* 54, 147-163. https://doi.org/10.1146/annurev.ento.54.110807.090518

William HH, Mighael JP and William BJ (1974) Glutathione S-Transferase. the first enzymatic step in mercapturic acid Formation. *Journal of Biological Chemistry* 249, 7130-7139. https://doi.org/10.1016/S0022-1910(62)90074-4

Heckel DG (2021) The Essential and Enigmatic Role of ABC Transporters in Bt Resistance of Noctuids and Other Insect Pests of Agriculture Insects 12, 389. https://doi.org/10.3390/insects12050389

Hung H, Ottea JA (2004) Development of pyrethroid substrates for esterase associated with pyrethroid resistance in the tobacco bodywork, *Heliothis virescens* (F). *Journal of Agricultural and Food Chemistry* 52, 21, 6539-6545. https://doi.org/10.1021/jf0493472

Khidr AA, El-Heneidy AH, Abdel-Halim A, Eissa MA, Matar AM (2003) Comparative studies between the efficiency of the egg parasitoid, Trichogramma evanescens West. and the insecticidal applications against the cotton bollworms in Egyptian cotton fields. The First Int. Egyptian-Romanian conference of Zaga-zig University 6-8 December, 455-464.

Livak KJ, Schmittgen TD (2001) Analysis of a relative gene expression data using real-time quantitative PCR and the 2^{ΔΔT} method. Methods 25, 402-440. https://doi.org/10.1006/meth.2001.1262

Lü J, Yang C, Zhang Y, Pan H (2018) Selection of Reference Genes for the Normalization of RT-qPCR Data in Gene Expression Studies in Insects: A Systematic Review. Frontiers in Physiology https://doi.org/10.3389/fphys.2018.01560

Saleh WS (1981) Comparative biochemical studies on resistance and susceptible strains of the Egyptian cotton leaf worm, Spodoptera littoralis (Boisd.). Ph.D., Thesis Faculty of Agriculture, Al-Azhar University Egypt

Van Asperen, K (1962) A study of housefly esterase by means of sensitive colorimetric method. *Journal of Insect Physiology* 8, 401-414. https://doi.org/10.1016/0022-1910(62)90074-4

Young SJ, Gunning RV, Moores GD (2005) The effect of piperonyl butoxide on Pyrethroid-resistance-associated esterases in *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae). *Pest Management Science* 61, 397-401. https://doi.org/10.1002/ps.996

Xuguo Z, Michael ES, Lance JM, Laurence DC, Blair DS (2003) Characterization of general esterases from Methyl parathion- Resistant and susceptible populations of western corn rootworm. *Journal of Economic Entomology* 96,6, 1855-1863. https://doi.org/10.1093/jee/96.6.1855

Zidan NEA, El-Naggar JB, Aref SA, El-Dewy ME (2012) Field evaluation of different pesticides against cotton bollworms and sucking insects and their side effects. *The Journal of American Science* 8,2, 128-136. http://www.jofamericanscience.org/.../