Arab Universities Journal of Agricultural Sciences
(2021) 29 (3) 925-931
Website: http://ajs.journals.ekb.eg

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

Rabab AD Allam[1*], Sobeiha A Kamel[2], Khidr A Aboelala[1], Neima K Al-Senosy[3]
1. Bollworms Research Dept, 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 Dept, Fac of Agric, Ain Shams Univ, P.O. Box 68, Hadyek Shoubra 11241, Cairo, Egypt

* Corresponding author: rbaballam22@gmail.com

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, Glutathione-S-transferase, β–esterases

Abstract: A study was conducted to assess two enzyme detoxifying activities and insecticide-resistance gene expression quantitation using real-time (PCR). Four populations of pink bollworms were compared with the 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 (*rps*7). For the *BtR* gene, the *Ct* values of analyzed biological groups ranged from 20 for the laboratory strain to 24 for the 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 requires, according to the 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 important since it infests cotton bolls and causes great damage resulting in a magnitude loss of yield production. *P. gossypiella* is considered a key insect infesting cotton plants, as indicated by the fact that 75% of insecticide applications used on cotton are directed against pink bollworms (Salama 1983).

Rapid biochemical determination is a great important method for detecting resistance in field populations (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 a remarkably high level of the enzymatic esterase activity that metabolite this kind of insecticide sequesters pyrethroid insecticide. Zidan et al (2012) showed chlorpyrifos and profenophos-treated strains of pink and spiny bollworm 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 to many researchers (Lü et al 2018).

The investigation aimed to measure enzyme 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

The rearing technique in this study was based on an artificial diet of kidney beans according to Miller et al (1996).

2.2 Determination of enzyme activity

2.2.1 Determination of non-specific esterase's activity

Beta-naphthyl acetate as a substrate was used to determine the beta-esterase 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 a reference. The target genes were amplified by newly planned specific primers**.** The primers were designed using the Primer 3 web-tool based on gene templates of *Pectinophora gossypiella* retrieved from the NCBI database. Primer 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 ΔCt for the biological laboratory group (G5):

$$\mathbf{\Delta Ct_{G5} = Ct_{Target\ gene\ (G5)} - Ct_{rps7}}$$

Table 1. Forward and reverse primers sequence used in qPCR

Gene	Forward (′5-′3)	Reverse (′3-′5)	Band size (bp)
BtR	GAACCAGACATTCGCCAT	CGGTCCGTTGCTATTACCTT	99
Cad1	GTGGTAGCGAAGGAGATCCG	TGTGCAAGTCCCGAACTCTC	91
rps7	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):

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

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):

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

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 a field population of *Pectinophora gossypiella* collected from different Governorates

The activities of the determined hydrolyzing enzymes, namely gutathion-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 of any contamination of insecticides under constant conditions in the laboratory at 27±1ºC and 70±5% relative humidity. Determination of the enzyme activity may shed light on the insecticide effect used on a large scale under field conditions related to induction effects on the enzyme activity in these strains.

3.1.1 Glutathione-S-transferases Activity (GSTs)

The enzymatic activity was measured as m mole substrate conjugated/min./ g.b.wt. The obtained results in **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 enzyme 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 of different field groups of *P. gossypiella* ranged between 1.05 and 1.44.

Table 3. Glutathione-S-transferase in the 4th instar larvae of *P. gossypiella* homogenates of different field populations 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.bwt.

3.1.2 Nonspecific Beta esterase activity

Results represented in **Table 4** showed a 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 in 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 the aforementioned Governorates were 1.51, 1.17, 1.06 and 1.05; respectively as compared with the baseline laboratory strain. It was

obvious 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 the Kafr-Elsheikh field colony strain.

The aforementioned results are supported by Hung and Ottea (2004) reported that esterases activity related to resistance to organophosphorus as well as pyrethroid insecticides a larvae of *Heliothis viresens* were developed and determined. The results are in accordance with those published by Zhou 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 the field group of *H. armigera*, to pyrethroid, is due to the high level of esterase isoenzymes that metabolite the toxicants. Abdel-Baset (2009) revealed that non-specific esterase 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 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 bollworm 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 remarkably 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 µg naphthol released/ min./ g. b.wt.

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

BtR and Cad1gene 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 real-time PCR (RT-PCR). The results manifested an amplification curve that was converted to specific cycle threshold values (*Ct*; **Fig 1**). RT-PCR was repeatedly reported as a reliable molecular technique as a 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 (*rps*7). 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 the 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 relationship 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.

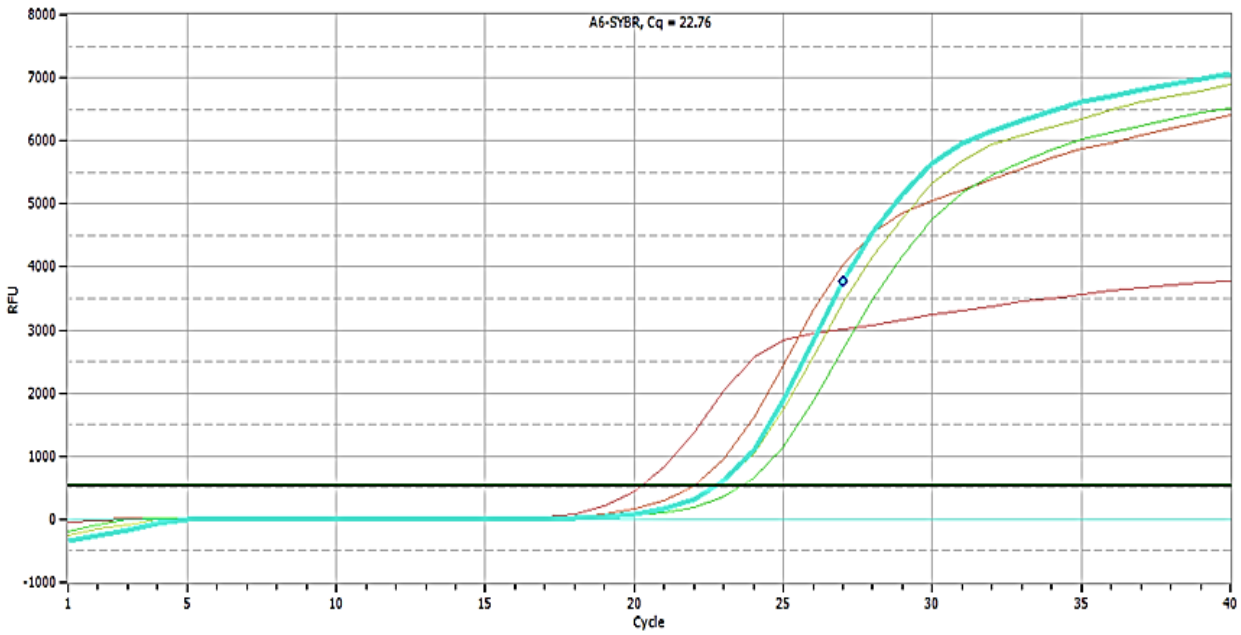

Fig 1. Amplification curves converted to specific cycle thresholds for quantifying gene expression levels relative to insecticidal resistance in a field population of *P. gossypiella,* 4th instar larvae compared to laboratory strain

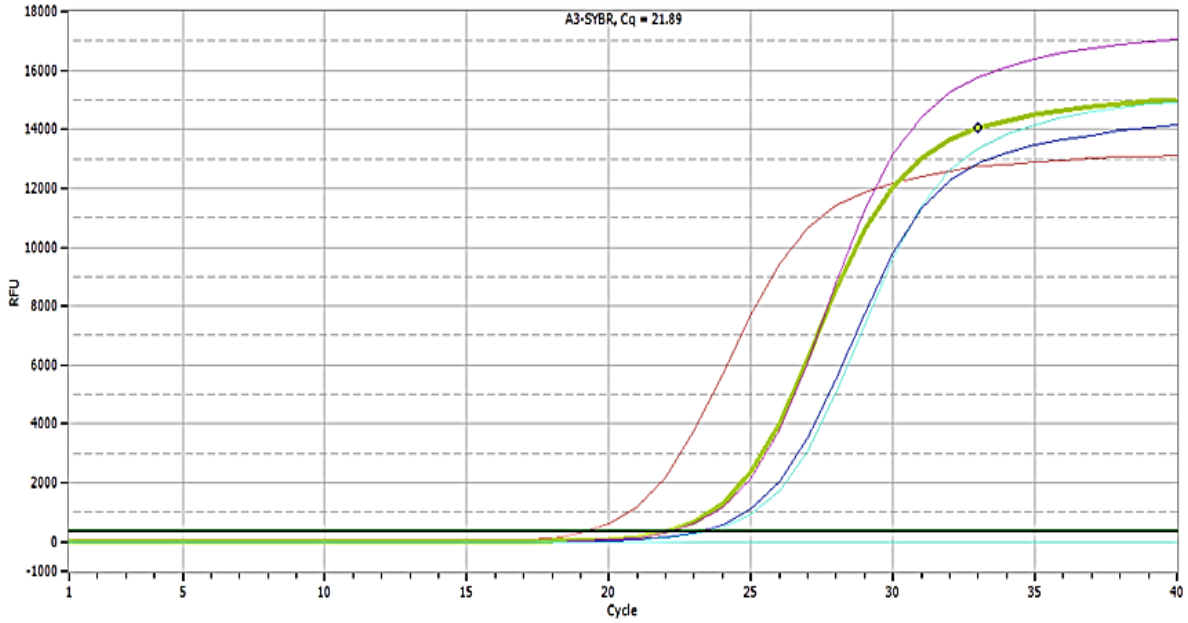

Fig 2. Exponential amplification curves of the RT-PCR assay. The above and below plots represent the *BtR* and *Cad1* genes, respectively. The lines represent the fluorescence rate (RFU) absorbed for the amplified DNA quantity each cycle. CT values are determined based on the threshold (green horizontal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

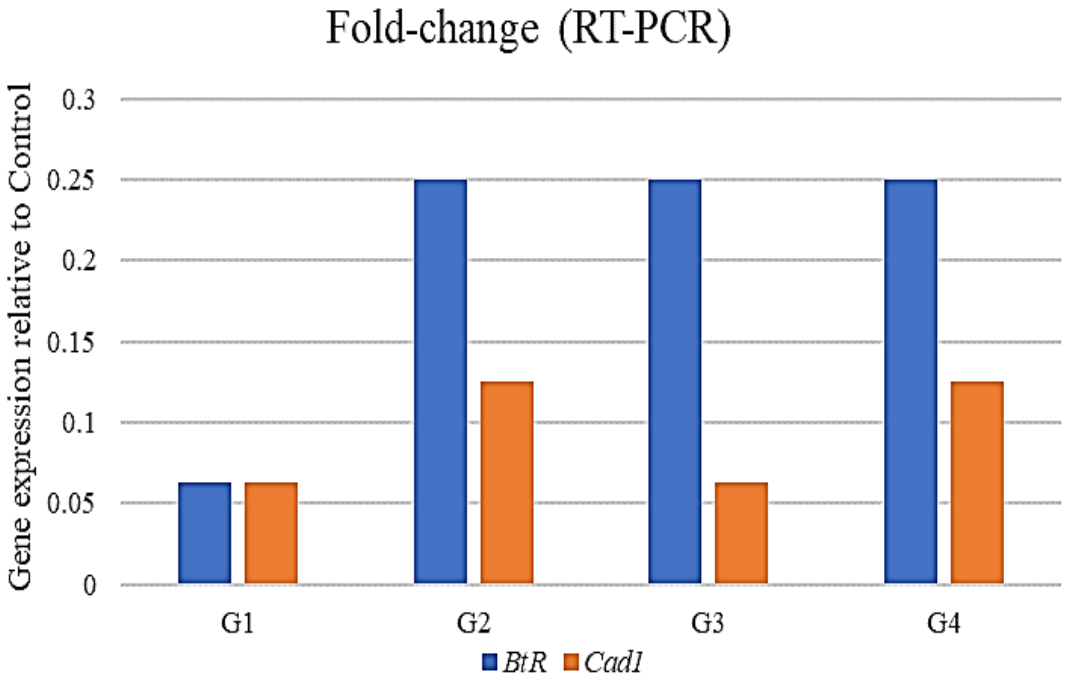

Fig 3. The fold-change values are based on RT-PCR estimated for two target genes (BtR and Cad1, normalized by rps7) for four biological sampling groups relative to the control group are shown

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.

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, et al (2016) Quantitative RT-PCR Gene Evaluation and RNA Interference in the Brown Marmorated Stink Bug. *PLOS ONE* 11, e0152730. https://doi.org/10.1371/journal.pone.0152730

Fabrick JA, Mathew Lolita G, Dannialle ML, et al (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, 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

Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione-S-transferase. The first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry* 249, 7130–7139. https://doi.org/10.1016/S0021-9258(19)42083-8

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, 6539–6545. https://doi.org/10.1021/jf0493472

Livak KJ, Schmittgen TD (2001) Analysis of a relative gene expression data using real–time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* 25, 402-408. https://doi.org/10.1006/meth.2001.1262

Lü J, Yang C, Zhang Y, et al (2018) Selection of reference genes for the normalization of RT-qPCR data in gene expression studies in insects: A Systematic Review. *Frontiers in Physiology* 9, 1560. https://doi.org/10.3389/fphys.2018.01560

Miller E, Stewart F, Lowe A, et al (1996) New method of processing diet for mass rearing pink bollworm, *Pectinophora gossypiella* (Saunders) (*Lepidoptera: Gelichiidae*). *Journal of Agricultural Entomology* 13, 129–137. https://api.semanticscholar.org/CorpusID:90618486

Salama HS (1983) Cotton-pest management in Egypt. *Crop Protection* 2, 183-191 https://doi.org/10.1016/0261-2194(83)90043-1.

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- 416. https://doi.org/10.1016/0022-1910(62)90074-4

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

Zhou X, Scharf ME, Meinke LJ, et al (2003) Characterization of general esterases from methyl parathion-resistant and -susceptible populations of western corn rootworm (Coleoptera: Chrysomelidae). *Journal of Economic Entomology* 96, 1855–1863. https://doi.org/10.1603/0022-0493-96.6.1855

Zidan NEA, El-Naggar JB, Aref SA, et al (2012) Field evaluation of different pesticides against cotton bollworms and sucking insects and their side effects. *The Journal of American Science* 8, 128-136. https://api.semanticscholar.org/CorpusID:212529463