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# Diagnostic expression of the cotton pink bollworm *Pectinophora gossypiella* (Saunders) *Ace1* exposed to some insecticide by the real-time PCR

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## *ABSTRACT*

The Pink Bollworm *Pectinophora gossypiella* (Saunders) is a devastating pest in Egyptian cotton field. Insecticide control failure may be happen due to its biological features of hide inside bolls and rarely exposed to insecticide application. Thus, a bulk of bolls infested with larvae collected from fields of Sharkia and Benysuef governorate. Moreover, exposed to py, Op sub lethal concentrations in laboratory bioassay. Toxicity features as $LC_{50}$, $LC_{90}$ and the slope, and RR was completed. Whereas the common target site of OP and pyrethroid is the acetylcholinesterase enzyme. Thus, the gene *Ace1* encoding *AChE* relative expression was investigated using molecular marker of real-time PCR procedures via the steps of generating data was RNA isolation and characterization, *cDNA* synthesis, then generating normalization factors, and *Ct* data attained and analyzed using $2^{-\Delta\Delta}$CT method to quantify the gene expression quantitatively of insecticide treated larvae tissue extracted. Data showed the most expressed ratio of samples was cypermethrin followed by deltamethrin compared with the control and the least was malathion and chlorpyrifos, thus, the most effective insecticide was malathion and the least were cypermethrin according to *ct* values. Hierarchical clustering combined with Heat map and principle component analysis based on various clustering systems and algorithm as distance measures were completed. Validation of reference gene in addition to gene expression stability analyzed by the three optimal gene finders and showed that deltamethrin and cypermethrin treated genes having lower mean weights and considered transcriptionally stable and ideal reference genes.

**Contribution/Originality:** The great and highly efficiency real-time PCR tool properties for detecting changes after insecticide treatment to agriculture pest tissue and ability to distinguishing between insecticide efficiency against the proper pest.

## 1. INTRODUCTION

The Egyptian cotton crop suffer seasonally infestation by a such arthropod group of bollworm complex as *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) besides *Helicoverpa armigera* (Hubner), *Earias vittella* (Fab.), *E. insulana* (Boisduval), *Spodoptera litura* (Fab.) and other insects Khidr, et al. [1]; Rani, et al. [2] and Sarwar [3]. Pink bollworm larvae fed on buds, causing fruit shedding, seed loss and moths represent a commercial problem because its larval stage frequently enters diapause while in seed capsules, emerging after long lived for about than 50 days at different climate conditions and survived for many months Abd-Elhady and Abd El-Aal [4] and

Darwish, et al. [5]. Insecticide application can control only the eggs and first instar remain outside bolls because larval stage enters bools and remain inside till pupal emergence in addition to the development of resistance toward most classes of insecticides represent difficult problem fronting the effective control Tanani and Ghoneim [6] and Salama, et al. [7]. The insect target active site of organophosphorus (Op) and pyrethroids (Py) insecticides efficiency is the Acetylcholinesterase (*AChE*), it represent as significant enzyme at cholinergic synapses in insect central nervous system, encoding locus, *Ace* degrades the neurotransmitter acetylcholine in the insect synapse [8, 9]. There are two *AChE* (ace1 or ace2) genes were discovered in many insect species such as *Helicoverpa assulta* and *Plutella xylostellas*, where *ace1* gene encodes the predominant synaptic and catalytic enzyme site that is the main target of OP and carbamate pesticides, based on its higher expression level and frequently detected point mutations associated with insecticide resistance Kim and Lee [10] and Revuelta, et al. [11].

It has impact on the ace gene evolution, cloned from Drosophila and discovered via isolation of a range of different mutants as amino-acid replacements producing insensitivity to a range of different insecticide inhibitors against Colorado potato beetle *Leptinotarsa decemlineata* [12-14]. Several and different types of polymerase chain reaction (PCR) have been invented and rapidly improved within some years after discovery by Mullis [15]. PCR is easy and quick in vitro method to amplify any target DNA fragment using DNA polymerase for the amplification during three steps: denaturation, primer hybridization or annealing and extension. Two common enzymes used which are Pfu DNA (DeoxyRiboneuclic acid) polymerase, Taq DNA polymerase that isolated from bacteria [16] to synthesize new strand using template in the presence of primers, deoxyribonucleotide triphosphates (dNTPs), Mg2+ and proper buffer system [17-20]. The real-time PCR (RT)steps performed from RNA (*Ribonucleic acid)* isolation to data analysis for measuring gene expression of amplification and detection data during the process of fluorescent chemistries intensity reaction in correlation of time named PCR cycles, resolved by 4 phase is linear, early exponential, log-linear or exponential, and plateau phase. The amount of fluorescence has reached a threshold is higher 10 times than the baseline levels, and PCR reaches its optimal amplification period reflects the true reaction kinetics of the sample [21]. The cycle occurs recognizes as cycle threshold (*Ct*) that refer to the time when fluorescence intensity is greater than background fluorescence. Whereas the greater the quantity of target DNA, the faster increase in fluorescent signal will generate that yielding a lower *Ct* value. Absolute quantitation of data requires serially diluted standards of known concentrations to generate a standard curve that produces a linear relationship between *Ct* and initial amounts of total RNA or cDNA and determines the input copy number, usually by relating the PCR signal to a standard curve. Relative Quantitation data is the changes in sample gene expression based on the external standard or a reference sample (calibrator) ratio for comparing results. Normalization of gene expression data to correct sample-to-sample variation is important step. The $2^{-\Delta\Delta CT}$ method is a convenient way to analyse the relative changes in gene expression from real-time quantitative PCR experiments [22]. It is necessary to validate the expression stability of a control gene by uses of multiple housekeeping genes (mRNA), which is the most conservative method of data normalization. It is genes thought to have stable expression and employed as controls in gene expression assays. The most recommended insecticide to control this pest were deltamethrin is a cyclopropanecarboxylate ester obtained by formal condensation between 3-(2, 2-dibromovinyl) -2, 2-dimethylcyclo propane carboxylic acid and cyano (3-phenoxyphenyl) methanol work as phosphoprotein phosphatase inhibitor and a calcium channel agonist and an antifeedant [23]. Cypermethrin is a carboxylic ester resulting from the formal condensation between 3-(2, 2-dichlorovinyl)-2, 2-dimethylcyclo propanecarboxylic acid and the alcoholic hydroxy group of hydroxyl (3-phenoxyphenyl) acetonitrile [8, 24]. Malathion is O, O-dimethyl dithiophosphate of diethyl mercaptosuccinate or parasympathomimetic OP that can bind to the enzyme (*AChE*) at nerve endings, its metabolite malaoxon, push ACh to accumulates at the nerve junction and results in overstimulation of the nervous system. Chlorpyrifos is a chlorinated OP insecticide 0, 0-diethyl 0-(3, 5, 6-trichloro-2-pyridinyl)-phosphorothioate, affecting the normal function of the nervous system by inhibiting the breakdown of acetylcholine (ACh), a neurotransmitter [25]. In this study, the gene *Ace1* encoding *AChE* relative expression

investigation performed using the molecular marker real-time PCR via the steps of generating data is RNA isolation and characterization, *cDNA* synthesis, generating normalization factors, and *Ct* data, analysis to the most common of Egyptian cotton boll pest is *P.gossypiella* samples treated with sublethal concentration of the tested insecticides plus case of control and treated samples and explain the differences.

## 2. MATERIALS AND METHODS

### *2.1. Insect Sources and Maintenance*

The cotton bollworm pest collected because of highly field infestation during mid-season of cotton field. Therefore, a big bulk of cotton plant bolls collected from Egyptian cotton cultivated lands of Sharkia and Benisuef governorates, heavily invested heavily by *P. gossypiella*, transferred to laboratory then, larvae emerged from bolls, placed in petri dishes provided with piece of cotton for maintenance and larvae used directly for insecticide bioassay.

### *2.2. Insecticides Used*

Selected insecticides were obtained from the imported chemicals of the central agriculture pesticide laboratory manufactured by china agrochemical companies, belongs to two groups of chemicals (Py. and OP), and insecticide information were found in Table 1.

**Table 1.** Insecticide information and its site of action.

| AI% | Common name | Trade name | Chemical class | Structure | Site of action |
|---|---|---|---|---|---|
| 48%EC | Chlorpyrifos | Tannker | OP. | C9H11Cl3NO3PS | Neurotoxic |
| 57%EC | Malathion | Malathon | OP. | C10H19O6PS2 | Neurotoxic |
| 10%EC | Cypermethrin | Biomethrin | Py. typell | C22H19Cl2NO3 | Neurotoxic |
| 10%EC | Deltamethrin | Deltamethrin | Py. typell | C22H19Br2NO3 | Neurotoxic |

**Note:** EC is the insecticide type of formulation (Emulsifiable concentrate).

### *2.3. Insecticide Bioassay and Sub Lethal Concentration Treatments*

Bioassay procedure was completed according to Paramasivam and Selvi [26] and Cetin, et al. [27]. Using Watmann paper saturated with insecticide serial concentrations dissolved in acetone and left to dry placed on petri dish and provided with ten larvae of *P. gossypiella* for insecticide exposure. Four replicate completed and control treated by acetone only. Dishes maintained under laboratory conditions 25:28 ℃, 12:12 h light: dark and 75 humidity and mortality count at 24 hours after treatments and data statistically analyzed using LeOra Software [28] used for attain $LC_{50, 90}$ according to Finney [29] and correction of mortality were included. After $LC_{50}$ determination, a much number of larvae were treated with $LC_{50}$ concentration value of the individual tested insecticides to investigate the effect of this sub lethal concentration on the gene expression level and *delta ct* values, larvae died were rejected and survived larvae were collected after 24h of treatment and send to biochemical laboratory for PCR molecular procedure accomplishment.

### *2.4. Molecular Procedures and Steps*

#### *2.4.1. Primer Design and Quantitative Real-Time PCR*

In this study the primers designed for detecting acetylcholine changes in *P. gossypiella ace1* gene was as follows, Forward primer: CCCAACACAGATATGCAGGA and Reverse primer: TATTATCTTTCACCCATTGC. The design was about product size was 20 nucleotides and length 292 pb, GC content between 35%- 42% give product size of 100-200 bp**,** melting temperatures was 60–65 °C suitable for successful PCR amplification [30].

#### *2.4.2. Insect Tissue Preparation and Total RNA Purification Protocol*

RNA purification protocols was using Gene JET™ RNA Purification Kit as at site www.thermoscientific.com/onebio: where it contain protein-kinase, lysis and wash buffers. The amount of Lysis

Buffer, 20 µL of 14.3 M β-mercaptoethanol (biological antioxidant), to each 1 mL volume added. The amount of Proteinase K solution, 10 µL of Proteinase K (included) to 590 µL of TE buffer (10 mM Tris HCl (Hydrochloric acid), pH 8.0, 1 mM EDTA (ethylenediaminetetraacetic acid)) diluted. A total RNA molecules longer than 200 nucleotides obtained from *P. gossypiella* larvae cultured cells to use in Real-Time polymerase chain reaction (RT-PCR). Samples homogenized in Lysis Buffer contained guanidine thiocyanate, and a chaotropic salt capable of protecting RNA from endogenous RNases and cause RNA to bind to the silica membrane while the lysate spun through the column. The lysate mixed with ethanol and loaded on a purification column. Silica-based membrane technology in the form of the spin column provided. The column washed with wash buffers to remove impurities. Pure RNA is then eluted under low ionic strength conditions with nuclease-free water [31].

### *2.4.3. Synthesis of cDNA from RNA Reactions Procedures Was as Follows*

11ul RNA in a 0.2ml tube that placed in ice and 1ul oligo (dt) primer was added. The mix in 65oC for 5 min incubated with total volume was 12ul. Then placeed the tube back on ice, add 8ulto 12ul, 4ul (5x) reaction buffer, 1ulRiboLockRNase Inhibitor, 2ul10 mMdNTP Mix, and 1ulRevertAid M-MuLV Reverse Transcriptase. Then the total volume was 20ul. Then the reaction mixed gently and fixed into the PCR machine 42oC for 60 min or 70oC for 5 min.

### *2.4.4. Real Time PCR Cycling Protocol Was Two-Step Completed Concluded*

The thermal cycler were programmed according to the references as follows: Initial denaturation 95°C, 10 s and 1 cycle, Denaturation 95°C, 15 s and 40 cycle and Annealing/Extension 60°C, 60 s and 40 cycle. All solutions centrifuged after melting. Master mix reactions were prepared by adding to a each tube (except template DNA) 20 µl reaction: 10 µl Maxima SYBR Green (cyanine dye used as nucleic acid stain )/ROX dye qPCR Master Mix (2X), Forward Primer is 0.25 µl, Reverse Primer is 0.25 µl, Template DNA is 2 µl, Water, nuclease-free is 7.5 µl and total volume is 20 µl adjusted. ROX dye used as a passive reference dye to normalize the fluorescent signal and therefore improves the precision of PCR results. Then Mixed thoroughly and appropriate volumes distributed into PCR tubes or plates, the reactions were gently mixed without creating bubbles and without vortex. Samples placed in the cycler and the program started. The nucleotide sequence of 18S determined using a dye terminator cycle sequencing kit analyzed. Table 2 the primer used in Rt-PCR method for Ace1 gene expression analysis of P.gossypiella treated larvae by sublethal concentration of tested OP and Py. insecticides.

**Table 2.** Primer used in Rt-PCR of *Ace1* gene expression analysis.

| Gene name | Forward and reverse Primer sequence (5'-3') | Molecular function | GC content | Product size | Length-bp |
|---|---|---|---|---|---|
| Ace1 | For:CCCAACACAGATATGCAGGA<br>Rev:TATTATCTTTCACCCATTGC | Cholinergic synapses | 50%<br>35% | 20<br>20 | 292 |
| RT-18S | For:GGCCTTCGGGATCGGAGTAA<br>Rev:GCAAATGCTTTCGCAGTTGTT | 18S ribosomal RNA protein | 60%<br>42.8% | 20<br>21 | 113 |

### *2.4.5. Statistical Data Analysis*

Data of *ace1* normalization with multiple housekeeping genes in the experimental samples were calculated. In addition the statistical analysis performed individually by dividing the fluorescent data by its normalization factor, and comparative *Ct* ($2^{-\Delta\Delta Ct}$) attained the changes in gene expression as a relative fold difference between experimental and calibrator samples, using the Livak method Livak and Schmittgen [22] and Andersen, et al. [32], constructed by GoldBio trade mark . The data of gene up and down regulations calculated according to Peredo and Cardon [33] of statistical hypothesis testing (The null hypothesis H0 means there is no difference and the alternative hypothesis H1means there is difference), this data obtained via z test and its common formula calculations. One-way analysis of variance (ANOVA) using SPSS software for detecting significant difference in amplification efficiencies between sample groups were included.

**Table 3.** Toxicity response of *P. gossypiella* field collections treated by tested insecticides.

| Sharkia collection | | | | | | Benisuef collection | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Pesticide** | **Slope** | **Intercept** | **$LC_{50}$ (95 % CI).** | **$LC_{90}$(95 % CI)** | **$\chi^2$** | **Slope** | **Intercept** | **$LC_{50}$ (95 % CI confidence intervals).** | **$LC_{90}$(95 % CI)** | **$\chi^2$** | **RR** |
| Chlorpyrifos | 2.48±0.087 | 2.75 | 8.1(5.5-12.0) | 28(19-41.6) | 0.34 | 1.94±0.105 | 3.4 | 6.5(4-10.4) | 29.6(18.4-48) | 0.81 | 1.;2 |
| Malathion | 2.0±0.097 | 2.83 | 10.9(7.0-16.9) | 44.7(28.8-69.3) | 0.82 | 1.6±0.12 | 3.57 | 7.5(4.3-13) | 46(26.5-79) | 0.70 | 1.4 |
| Cypermethrin | 2.6±0.38 | 4.2 | 1.98(1.37-2.87) | 6.25(4.3-9) | 0.53 | 2.3±0.09 | 5.4 | 0.66(0.43-1.0) | 2.4(1.6-3.6) | 0.96 | 3 |
| Deltamethrin | 2.72±0.36 | 4.3 | 1.75(1.24-2.55) | 5.4(3.7-7.8) | 0.44 | 1.9±0.1 | 6.0 | 0.27(0.16-0.45 | 1.2(0.7-2.1) | 0.96 | 6.4 |

## 3. RESULTS AND DISCUSSIONS

### 3.1. Toxicity Outline of the Selected Insecticides in Bioassay

Insecticide responses parameters of *P. gossypiella* larvae that collected from Sharkia and Benysuef governorate exposed to py, Op concentrations in laboratory bioassay. Results represented by $LC_{50}$, $LC_{90}$ and the slope, data found in (Table 3). Results achieved that Sharkia $LC_{50}$ data were less than Benisuef $LC_{50}$ data then it considered the most susceptible and as reference population to determine the resistance ratio (RR). The toxicity values $LC_{50}$ was 8.1, 10.9, 1.98 and 1.75 ppm of Sharkia and 6.5,7.5,0.66, and 0.27 ppm of Benisuef, this for chlorpyrifos, malathion, cypermethrin and deltamethrin respectively. The detected RR for comparing results was 1.2, 1.4, 3 and 6.4 fold of resistance for the same arrangement respectively. Generally, in Egypt *P. gossypiella* is serious pest was easy to control by insecticide alone or in rotation and by other different control methods. It was detected in previous field experiments searches exhibited good efficacy as was carried out by Darwish, et al. [5] and treatment of pheromones and/ with parasitoid *Trichogramma evenecens* by El-Bassouiny [34] the biocide using bacteria or viruses formulations revealed effective control carried out by El-Lebody, et al. [35].

### 3.2. Determination of Relative Expression

Quantification of ΔΔ *Ct* to all insecticide treatment samples successively calculated to compare between target and reference treatment and control. Where Ct values for each genes averaged and calculated using delta-delta Ct method and relative quantification determined using the Livak method [22]. In two way normalized and non-normalized expression (*ΔCt* and *ΔΔCt* method) were completed assuming that PCR efficiencies of insecticide treatment and control of *Ace1* gene and the housekeeping gene were similar and equal 100% and no need to correct. Subsequently Expression Ratio= $2^{-\Delta\Delta Ct}$. Comparison of the expression levels of reference genes data were found in Table 4, expressed as fold change were found in Figure 1 and 2. Data showed the most expressed ratio of samples was cypermethrin equal 4.1 times as control and the least was malathion 0.138 fold change and 0.563 for chlorpyrifos, where average *ct* values ranged from 39.35, 33.96 to 34.735 for cypermethrin, malathion and chlorpyrifos, respectively. Thus, the most effective insecticide was malathion and the least was cypermethrin according to *ct* values. According to Skern, et al. [36] the fold change indicates whether a gene is up-regulated or down-regulated, where, If Fold Change > 0 it means that gene is more expressed or up-regulated, but if it less than < 0 it means that this gene is less expressed or down-regulated. Results of gene up and down regulations Table 4 showed differential expression results between insecticide refers to the most up regulated insecticide treatment was cypermethrin and the least was malathion respectively. Statistical z test calculation of up and down regulation showed data not supported the null hypothesis H0 and the alternative hypothesis H1. Where the assumption of treatments was not equal, but hypothetically accepted by (two-tailed z test distribution 1.9600) in lower and upper mean (37.13-38.26), H1 suggestion (mean Less than) was rejected and H1 suggestion (Greater than) was accepted (One-tailed distribution 1.6449). Rendering results attained by Revuelta, et al. [11] investigate that RNAi of *ace1* and *ace2* in *Blattella germanica* reveals their differential contribution to *AChE* activity and sensitivity to insecticide chlorpyrifos. From literature, it seems there is a big difference between *ace1* and 2. of *AChE* insensitivity fluctuation that responsible for insect resistance mechanism and mutations in the ace gene as identified in *Drosophila melanogaster*, *Musca domestica* and mosquitos. However, other insects, the ace gene cloned by homology with Drosophila does not code for the insensitive *AChE* in resistant individuals, indicating the existence of a second ace locus Weill, et al. [37] and Weill, et al. [38]. Other facts illustrated was the relative transcript abundances estimated by quantitative real-time PCR from a salmon as Skern, et al. [36] found results can vary intensely depending on the method chosen for data analysis. The same was supposed by Cikos, et al. [39] where cytokine expression normalized to three determined reference genes and analysed by six methods was different.

**Table 4.** Gene expression profiling of *P.gossypiella* treated by sublethal concentration of py. and Op insecticides and relative quantification calculated of Rt-PCR using Livak method.

| Insecticide | Average ref (HK) | Average target (*Ace1*) | Δ ct-treatment | Δ ct-control | ΔΔ ct | Expression raio | Fold change | Up & down regulation | Gene-Copy number |
|---|---|---|---|---|---|---|---|---|---|
| Control Sharkia | 39.42 | 34.415 | -- | -- | -- | -- | -- | -- | 9.9 |
| Chlorpyrifos | 38.9 | 34.735 | -4.17 | -5.0 | 0.83 | 0.563 | 0.574 | 1.03 | 10 |
| Malathion | 36.14 | 33.96 | -2.14 | -5.0 | 2.86 | 0.138 | 0.141 | -1.20 | 10.01 |
| Control Benisuef | 37.31 | 40.46 | -- | -- | -- | -- | -- | -- | 99.9 |
| Cypermethrin | 38.295 | 39.35 | 1.05 | 3.1 | -2.0 | 4.1 | 4.257 | -1.05 | 10.0 |
| Daltamethrin | 36.13 | 39.05 | 0.92 | 3.1 | -2.18 | 4.53 | 1.17 | -1.06 | 10.0 |

Expression ratio = ΔCtreatment / ΔCtcontrol and Fold change = $2^{-}$ ΔΔCt treatment

ΔΔCt = - [ΔCt treatment –ΔCt control]

ΔCt(treatment)= Ct(target treatment)–Ct(reference treatment),

ΔCt(control)= Ct(target control)–Ct(reference control)

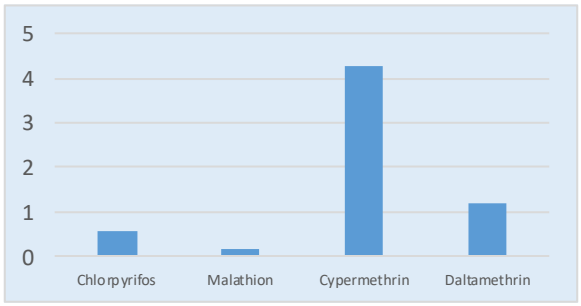



**Figure 1.** Fold change.

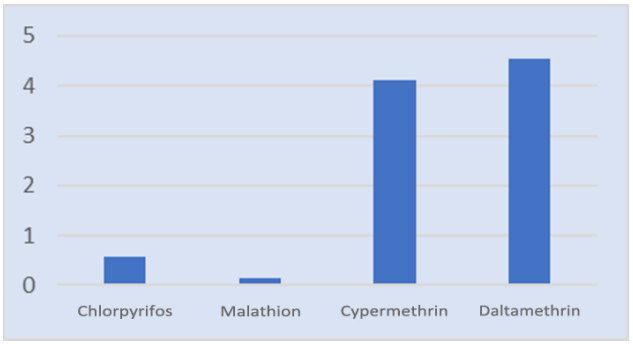



**Figure 2.** Expression raio.

### *3.3. Expression Profiling*

Interpretation of *P.gossypiella Ace1*expression profiling using cluster analysis including Hierarchical clustering combined with Heat map based on various clustering systems and algorithm as distance measures like Euclidian distance, similarity or gene expression pattern method plus Pearson correlation, also get data of up and down-regulation, data visualization appeared by a dendrogram generated from GenEx software, illustrated in Figure 2 and 3. Dendograms showing the representative nodes data of Heat map displayed the clustering and classification methods to identify treatments based on the sample or genes distribution group together represent gene expression and regulation. Data in Principal component analysis groups of samples/genes analyzed based on correlated expression (Figure 5).

Figure 4 Heat map visualization of cluster analysis for composition of the Rt-PCR ace1 relative expression of the insecticides treated P. gossypiella larvae, the target, control and housekeeping values data displayed in heat map

showed a grid of each row represents a gene (treatment, control of target or housekeeping gene) and each column represents a sample. The color and intensity used to represent changes of gene expression. The red one represents up-regulated genes, the green represents down-regulated genes and black represents unchanged expression.

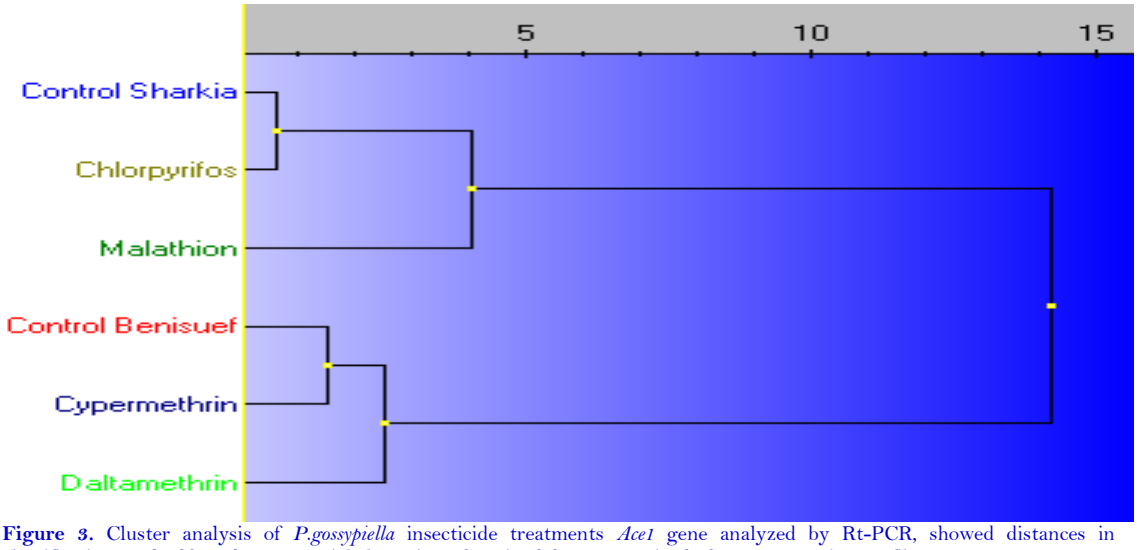



**Figure 3.** Cluster analysis of *P.gossypiella* insecticide treatments *Ace1* gene analyzed by Rt-PCR, showed distances in classification method based on Herarcial clustering of attained data categorized of gene expression profiles.

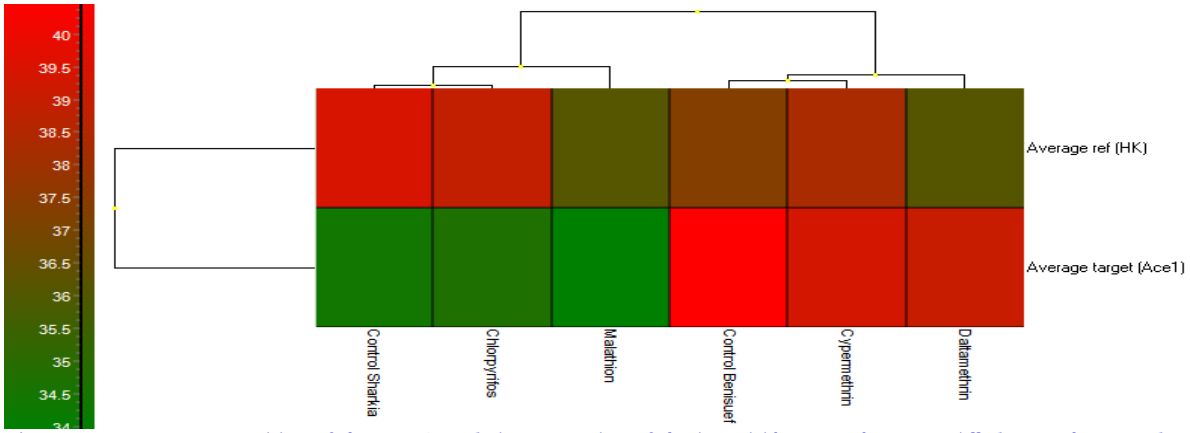



**Figure 4.** Heat map composition of the Rt-PCR relative expression of the insecticides treated *P. gossypiella* larvae, the control and housekeeping values Data displayed in heat map showed a grid of each row represents a gene (treatment, control of target or housekeeping gene and each column represents a sample. The color and intensity used to represent changes of gene expression. The red one represents up-regulated genes, green represents down-regulated genes and black represents unchanged expression.

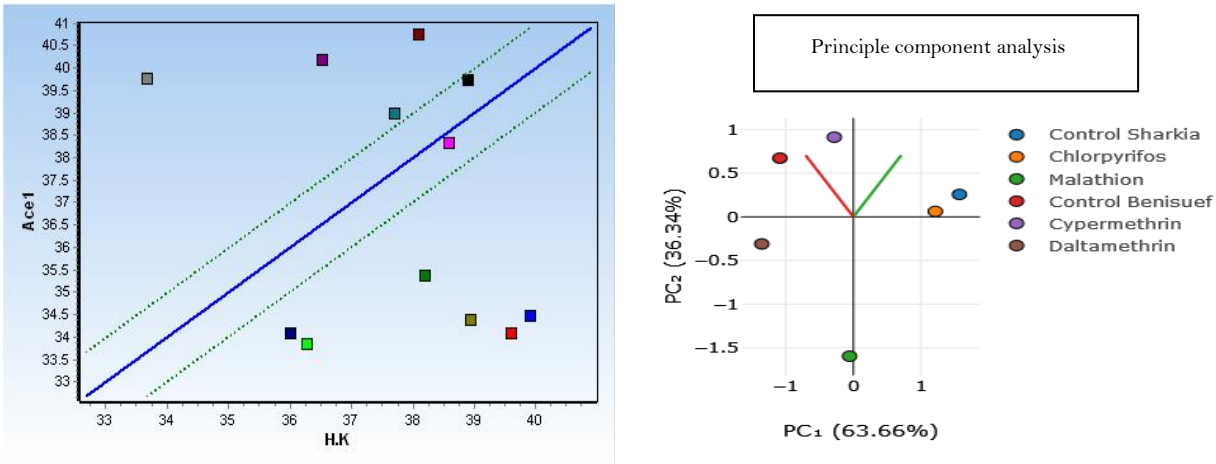
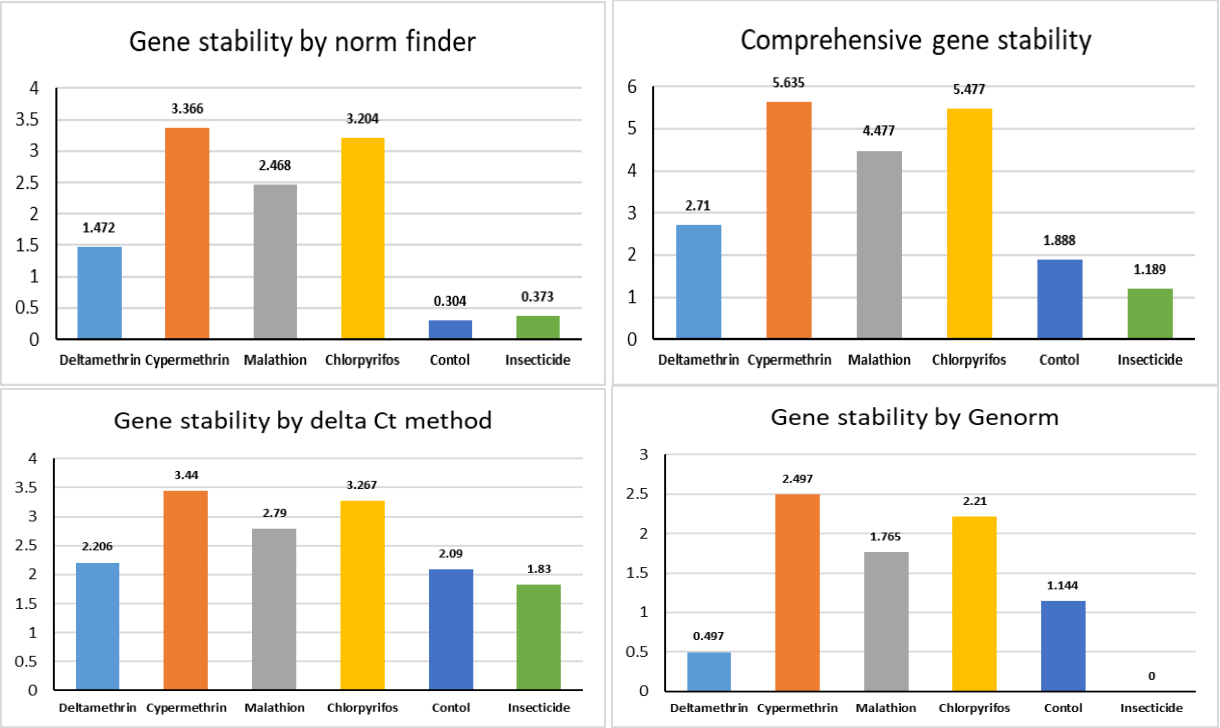



**Figure 5.** Principal component analysis of the *P. gossypiella* insecticide treatments showing classification of insecticide treatments gene (*Ace1*-RtPCR) expressed based on hierarchical clustering and distance similarity. Scatter plot between control group and test group data plotted of confident limit of primer efficiency (the blue line) with the slope and the intercept mirrored by (two dashed lines).

### *3.4. Gene Expression Stability and Ranking of Reference Genes*

Evaluation of reference gene expression stability was by using the internet site RefFinder, that a web tool for analysis, integrates four algorithms comparison was: GeNorm [40] NormFinder [41] BestKeeper [42] and delta-Ct method [22]. The RefFinder mirror site [40, 43, 44]. Results of the geomean of rankings generated by each algorithm, and geometric means of the gene weights calculated for a comprehensive final ranking order and stability values data were in (Tables 5, 6 and Figure 6). In general, expression stability data analyzed by the three optimal gene finders showed that deltamethrin and cypermethrin treated genes having lower mean weights and considered transcriptionally stable and as ideal reference genes.




**<-----Most stable gene --- Last stable gene----->**

**Figure 6.** Selection and validation of reference genes for RT-PCR-based analysis of *P. gossypiella* treated by Op and py. Insecticide using Gnorm, Normfinder, and Delta Ct method.

GeNorm calculated the stability value M was (3.57 for together) but for individual insecticide values recorded was, 1 for each means it less than 1.5 and the coefficient of variation value (CV) was (3.7) and the number of optimal reference genes is determined, data in (Table 5 and 6). Data of Gnorm showed ranking result suggested that the top two stable genes was cypermethrin and deltamethrin and the most suitable for normalization in insecticide efficiency detection, where the reference gene that have the lowest M value were considered the most stable gene. NormFinder data ranked the individual candidate genes according to their stability value, and suggested that cypermethrin and deltamethrin was the most stable and largely in agreement with the other three algorithms used. The comparative delta-Ct method estimated the most stable reference gene using the SD (standard deviations) means by pairwise comparison of two reference genes (Table 5 and Figure 6). The transcription data via the stability of gene expressions including CV, which detects the small amount of variability in relation to the mean of the expression levels in addition to the standard deviations (SD), where SD=1.4 and CV=3.71 which reflect the variability of gene expressions, based on the medians attained from Normality test. The p-value measured by the Shapiro–Wilks hypothesis indicated the good fit of the normal distribution, and p-value < 0.05 means a significant departure from normality. Bestkeeper calculates the SD value greater than 1 indicates high variation of the expression of a gene and, consequently, its instability and calculate the coefficient of variation of each candidate

gene. Comprehensive gene expression ranking and recommended reference genes revealed average gene stability values M ranged from 0.5 to 1 indicate high reference gene stability.

Figure 7 Expression stability analysis plotted by best keeper excel sheet that compared the SD and CV, and the Ct values, however the lower CV and SD is the higher stability. Dataset plotted to validate the specificity of the optimal reference genes. The lower and higher stability characterized by the divergence and convergence between point of HK (pink point) and Ace1 samples (dark blue points). If the two colored points closed to each other, this mean high efficiency of primer used or high stability of expression level. This study showed most pink point meet others dark blue points.

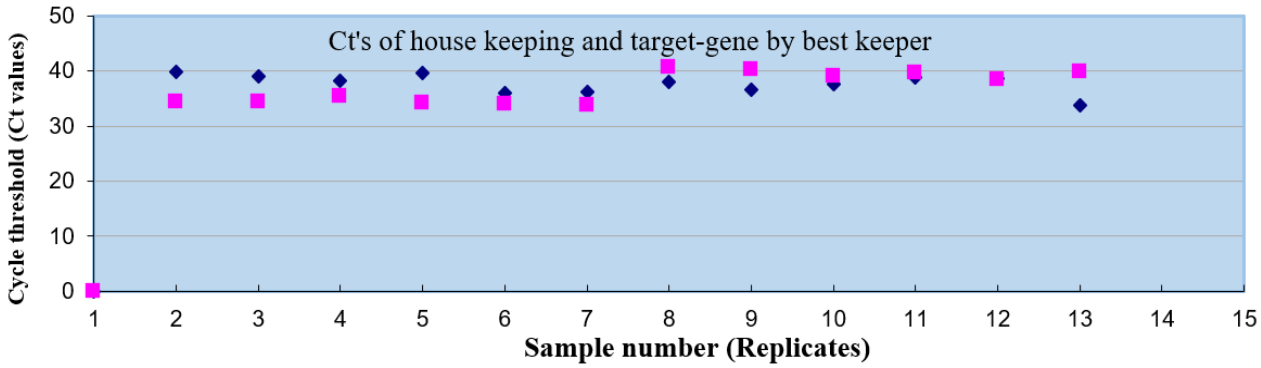



**Figure 7.** Expression stability analysis plotted by best keeper excel sheet that compared the SD and CV, and the *Ct* values, however the lower CV and SD is the higher stability. Dataset plotted to validate the specificity of the optimal reference genes, and the lower and higher stability characterized by the divergence and convergence between point of HK (pink point) and Ace 1 samples (dark blue points).

**Table 5.** Stability values of *Ace1* gene in *P.gossypiella* treated by insecticides, performed by RefFinder, Delta Ct, BestKeeper, NormFinder, and geNorm and coefficient of variation (CV analysis).

| Method | Insecticide | Control | Chlorpyrifos | Malathion | Cypermethrin | Deltamethrin |
|---|---|---|---|---|---|---|
| Delta CT | 1.83 | 2.09 | 3.29 | 2.79 | 3.44 | 2.2 |
| Normfinder | 0.37 | 0.30 | 3.2 | 2.46 | 3.36 | 1.47 |
| Genorm | 0 | 1.14 | 2.12 | 1.78 | 2.49 | 0.497 |

**Table 6.** Ranking order: Control genes ranked in order of their expression stability.

| Method | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Delta CT | Insecticide | Control | Daltamethrin | Malathion | Chlorpyrifos | Cypermethrin | -- |
| BestKeeper | Insecticide | Daltamethrin | Control | Cypermethrin | Malathion | Chlorpyrifos | -- |
| Normfinder | Control | Insecticide | Daltamethrin | Malathion | Chlorpyrifos | Cypermethrin | -- |
| Genorm | Insecticide | -- | Daltamethrin | Control | Malathion | Chlorpyrifos | Cypermethrin |

### *3.5. Validation of the Optimal Reference Genes*

The relative expression level of the target gene *P. gossypiella Ace1* treated by insecticides compared with control and normalization with reference genes values Table 4 showed similar expression trends except for cypermethrin, However, the variation in *Ace1* gene expression was larger in association with the unstable reference gene compared to the stable reference gene group. In Figure 8, illustrate the pair wise variations calculated by Gnorm for finding optimal number of reference gene for precise normalization in tissue-specific transcriptome of *P. gossypiella ace1* type using the comparative Δ-Ct method. Also Figure 8 showed deltamethrin and cypermethrin were the most stable reference gene and chlopyrifos and malathion were least, as well as the combination of the two best reference genes, were used respectively to normalize the expression profile. In addition, expression levels of chlorpyrifos compared with others *Ace1* were not varied significantly using the simple one way ANOVA (F=0.74, df= 11 and p=0.618) (Table 7). In addition, T-test assuming no correlation between samples and insecticide treatment and the statistical t test methods showed in (Table 8). There is one mentioned attained by Zhang, et al. [42] *Bemisia tabaci* expression profiles after exposure to 11 insecticides, selected reference was different in genes were determined according to the experimental conditions where he focused on the many microRNA and the stability of all the selected candidates in

relation to other variables including sex, tissue type, and developmental stage was different. In general, the pink bollworm were reported in many searches of molecular biology, specially resistance to insecticides or to Bt toxins, as Agrawal, et al. [45] study about 31,764 unique genes of assembled samples, found about 1,741 were differentially expressed and about 1,024 genes were down-regulated and 717 were up-regulated. Moreover, the metabolic resistance such as cytochrome P450, GST (Glutathion S-transferase) and carboxylesterase up regulated in resistant to *Bacillus thuriengensis* strains when compared with susceptible.

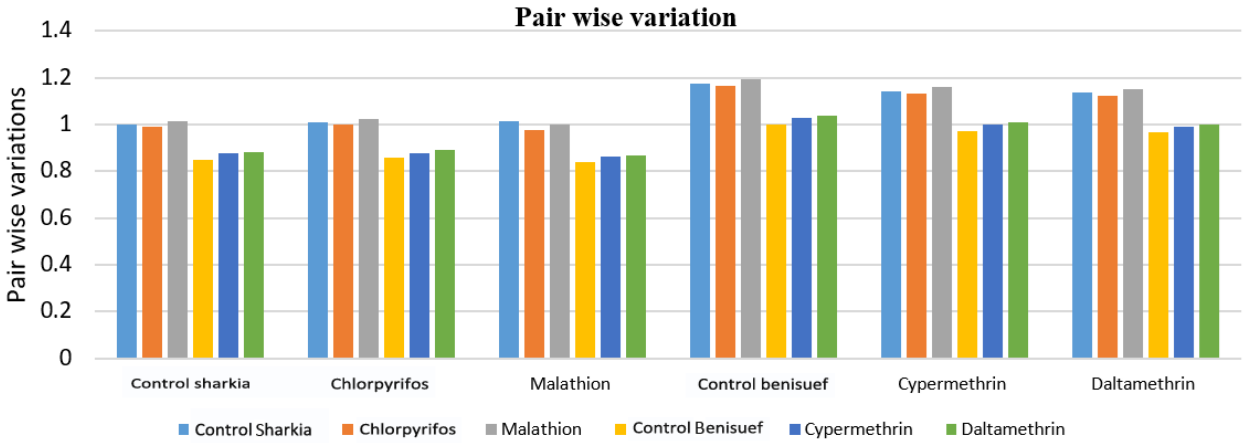



**Figure 8.** Pair wise variations calculated by Gnorm for finding optimal number of reference gene for precise normalization in *P. gossypiella ace1* type using the comparative Δ-Ct method).

### *3.6. Genotyping Analysis of P.gossypiella Samples*

The gene copy number variations data was calculated according to DNA amount [46] of *P.gossypiella* using Copy caller software of Multiplex Gene Expression Analysis of RT-qPCR for without need a standard curve, determining which wells have a true sample versus noise and carrying out replicate averaging, outlier detection, and statistical error analysis. Data were in Table 4 showed All copy number variations detected by *Rt-PCR* of all insecticide treated samples were between 8 and 10 copy gene that refer to there was an amino acid at just one positions were substituted. Similar results attained by some researchers as, Carvalho, et al. [47] in the chlorpyrifos resistant strains of red spider mite, *Tetranychus evansi*, gene copy number using qPCR showing there are around 8–10 copies of the gene in all samples that considered a mechanism responsible for fitness costs associated with the mutant alleles of the gene encoding the OP target (*Ace-1*). Haddi, et al. [48] found in leaf miner, *Tuta absoluta* five strains resistance to chlorpyrifos when sequencing the gene (*Ace-1*), the presence of alanine to serine substitution at a position linked. May some insects related to the same family were not varied in the point mutation detected in the gene encoding the enzyme *AChE*, As results found by Raboudi, et al. [49] in the potato aphid, *Macrosiphum euphorbiae*, and *Aphis gossypii* resistant to a carbamate found one point mutation produced in the amino acid sequence of *Ace1 gene*. Generally many searches cited that resistance to OP and py detected in insects showed *AChE* modification as single or multiple amino acid substitutions as drosophila and others [50].

Figure 9 Specificity and sensitivity plot of the primer amplification attained from total RNA and DNA extracted from P.gossypiella. This figures generated by Genex7, showed housekeeping genes and samples defined the optimal annealing temperatures at the beginning of PCR run and end of it by gradient PCR temperature was at 65°C that suitable for each gene. Additionally, the dissociation curves of the eight candidate reference genes showed a single peak and prediction score of sensitivity/specificity is 99 %.

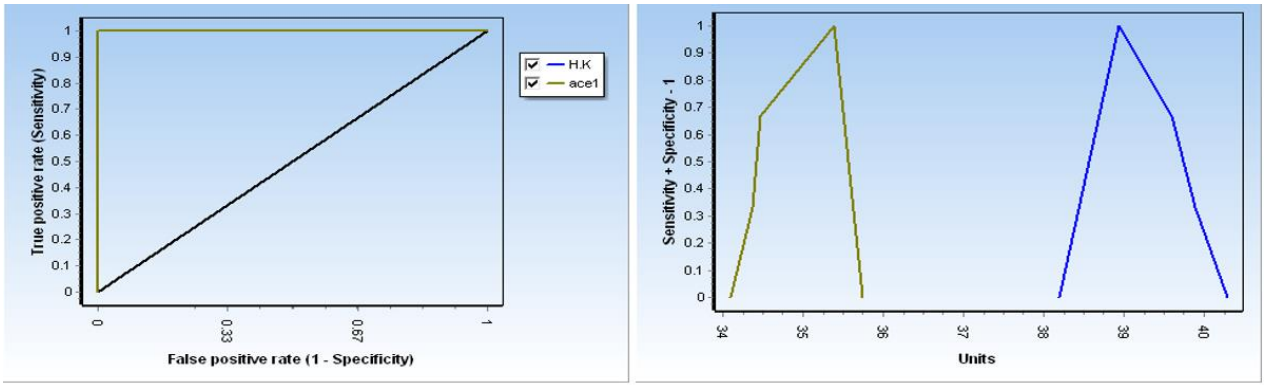



**Figure 9.** Amplification specificity and efficiency plot of the primer sets attained from Total RNA and DNA Extracted from *P.gossypiella* (figures generated by Genex7) showed housekeeping genes and samples define the optimal annealing temperatures of the candidate reference genes by gradient PCR was at 65°C that suitable for each gene. Additionally, the dissociation curves of the eight candidate reference genes showed a single peak and prediction score of sensitivity/specificity is 99 %.

**Table 7.** ANOVA results of the tested insecticides against *P.gossypiella* of Rt-PCR experiment.

| ANOVA | | | | | | | |
|---|---|---|---|---|---|---|---|
| Source of variation | D.f. | SS | MS | F | P-value | F crit | Omega sqr. |
| Between groups | 5 | 20.685 | 4.137 | 0.744 | 0.618 | 4.387 | -0.119 |
| Within groups | 6 | 33.355 | 5.559 | -- | -- | -- | -- |

**Note:** SS is summation of squares, MS is mean of summation of squares and ANOVA is significance differences between means.

**Table 8.** Regression coefficient and t test of the tested insecticides in Rt-PCR experiment.

| Variable | Coefficient | Std.err. | t-statistic | P-value | Lower95% | Upper95% | VIF | Std. coeff. |
|---|---|---|---|---|---|---|---|---|
| Constant | 58.497 | 37.888 | 1.543 | 0.197 | -46.697 | 163.691 | 0 | 0 |
| HK | -0.570 | 1.004 | -0.567 | 0.600 | -3.359 | 2.218 | 1 | -0.273 |

**Note:** HK is the housekeeping genes of the tested samples and VIF is the amount of multicollinearity in regression analysis.

## 4. CONCLUSIONS

The Real-time PCR has become the most widely used methods of gene quantitation because it claims great sensitivity and high efficiency for detecting differences between two or some samples take along great benefits. Moreover, the current developed relative quantitation, mathematical models and amplification efficiency calculations, types of normalization or data correction and detection chemistries that facilitate the whole subject of analysis. In this study detection of differences by real-time PCR between insecticide treatments of one agricultural pest were finished in laboratory procedures and statistical analysis results in few fold changes between samples. In contrary detection of differential gene expression by Rt-PCR is generally limited to detecting changes of twofold as example whereas detection of expression changes less than twofold may be required especially in field of toxicity by pesticides. The need reference gene, such as a housekeeping gene to express differential gene expression.

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