APPLIED ENTOMOLOGY AND INNOVATION (AEI)

VOLUME 1 ISSUE 1 (2023)

PUBLISHED BY
E-PALLI PUBLISHERS, DELAWARE, USA

Applied Entomology and Innovation (AEI)

Volume 1 Issue 1, Year 2023
https://journals.e-palli.com/home/index.php/aei

Para Sodium Channel Expression Profiling of the pink bollworm *Pectinophora Gossypiella* (Saunders) (Lepidoptera : Gelichidae) Exposed to Some Insecticide Using Real-Time PCR

Hanan Salah El-Din Taha[1*]

Article Information

Received: October 14, 2023
Accepted: November 10, 2023
Published: November 17, 2023

Keywords

Malathion, Chlorpyrifos, Cypermethrin, Deltamethrin, PARA Voltage-Gated Sodium Channel, Real-Time PCR, Reference Gene, Stability Level, Insecticide Treatments and Pectinophora Gossypiella

ABSTRACT

Pyrethroid insecticides are widely used to control many agricultural pests, its neurotoxic mode of action works by modifying the normal function of voltage-gated sodium channels dynamic leading to insect paralysis and death. The Pink Bollworm *Pectinophora gossypiella* (Saunders) is a serious pest in attacking cotton in Egyptian fields. Insecticide control facing problems because of its biological features of hiding inside bolls and being protected from insecticide applications. Thus, a large number of bolls infested with larvae collected from fields of Sharkia and Benysuef governorate, and exposed to insecticide of pyrethroid and organophosphorus sub-lethal concentrations equal LC_{50} defined from laboratory bioassay toxicity features. Thus, the PARA voltage-gated sodium channel domain ll relative expression was investigated using the molecular marker 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 was extracted. Expression profiling, stability, ranking of reference gene, and validation of the optimum reference genes were calculated using the three optimal gene finders. Results showed that chlorpyrifos and malathion treated genes having lower mean weights and were considered transcriptionally stable and considered ideal reference genes. statistical analysis results were Hierarchical clustering, Heat map, and principle component analysis based on various clustering systems and algorithms as distance measures were completed and gene copy number was about 10 copies. Data showed the most expressed ratio of samples was chlorpyrifos followed by malathion compared with the control, cypermethrin, and deltamethrin. The most effective insecticide was cypermethrin followed by malathion and the least the other two insecticides according to CT values.

INTRODUCTION

Pyrethroid (Py) insecticides are widely used to control a wide range of agricultural pests, due neurotoxic mode of action, its structure is a synthetic derivative of the natural pyrethrin esters of chrysanthemic acid (Elliot *et al.* 1978). It works by modifying the normal function of voltage-gated sodium channels dynamic leading to insect paralysis and death. The exact function is inducing activation and inactivation deliberation; prolonging the Na` currents related to membrane depolarization (Warmke *et al.* 1997). The possible insect mechanism of resistance is knockdown resistance mechanisms (kdr and super kdr) and the PARA voltage-gated sodium channel (PSC) that exact genes encoded (Loughney *et al.* 1989). Mutations are produced through association with locus specified through alterations in the PSC-protein and amino acids of point alteration in the S6 segment of domain II or III of the sodium channel gene resulting in a leucine to phenylalanine substitution leads to a reduction in sensitivity to pyrethroids (Ffrench-Constant *et al.* 1998). The voltage-sensitive sodium channel role is the generation and propagation of action potentials in vertebrates and invertebrates neurons, its structure is about glycosylated a-subunit polypeptide folds in the membrane to form the main body of the channel pore, with association of two smaller subunits in mammalian brain isoforms (Catterall 1988). The sodium channel is the primary physiological target of the pyrethroid insecticides, in insects encoding specific genes and mutations (Wu and Ganetzky 1980; Loughney *et al.* 1989). One gene was detected off the PSC and knockdown resistant (M918T mutation of kdr) in houseflies (*Musca domestica*) but more than one type of sodium channel gene was detected in mammals and, two in *Drosophila*, (Williamson *et al.* 1996 and Miyazaki *et al.* 1996), and mutations in cockroach discovered by (Dong, 1997). Also, one gene was detected in horn fly (Guerrero *et al.* 1997), in diamondback moth, *Plutella xylostella* (Sonoda etal. 2012), *Anopheles gambiae* (Torres *et al.* 1999), and in cockroaches (Dong 1997). The cotton plant is the major economic fiber crop in Egypt, suffer seasonal infestation by such arthropod group of bollworm complex as the Pink Bollworm *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) the most destructive pest in cotton production and others (Moustafa *et al.* 2015). Frequently at cultivation seasons cotton crops demand an urgent need of many pesticide classes of application on cotton growth season (El-Saadany *et al.* 1975 and El-Shaarawy *et al.* 1975). Certainly Larvae fed on buds, causing fruit shedding and the high percentage seed loss, represent a commercial problem due to larval stage frequently

[1] Central Agricultural Pesticide Laboratory, Agricultural Research Center, Dokki Giza, Egypt
[*] Corresponding author's e-mail: Hanansalah412@yahoo.com

enters diapause for a time in seed capsules and emerging after long lived for about more than 50 days at different climate conditions, and survived for many months till pupation emergence (Moustafa *et al.* 2021). For these reasons, insecticide application can control only the eggs and first instar remain outside bolls considered the main problem. In addition to the development of resistance toward most classes of insecticides, represent difficult problem facing the effective control (Saad *et al.* 2015 and Sayed and El-Ghobary 2019). Moreover, many trials of synergism get insufficient results of efficiency (El-Refai *et al.* 2009). The most recommended insecticide to control this pest were organophosphates (OP) and Pyrethroids (Py) as deltamethrin is a cyclopropane-carboxylate ester obtained by formal condensation between 3-(2, 2-dibromovinyl) -2, 2-dimethylcyclo propane carboxylic acid and cyano (3-phenoxy phenyl) methanol work as phosphoprotein phosphatase inhibitor, a calcium channel agonist and an antifeedant (Prusty *et al.* 2015). And cypermethrin is a carboxylic ester resulting from the formal condensation between 3-(2, 2-dichlorovinyl)-2, 2-dimethylcyclo propane carboxylic acid and the alcoholic hydroxy group of hydroxyl (3-phenoxyphenyl) acetonitrile (Leake *et al.* 1985). By comparing pyrethroids with OP as Malathion is O, O-dimethyl Di thiophosphate of diethyl mercapto succinate or parasympathomimetic that can bind to (AChE) enzyme at nerve endings, its metabolite malaoxon, push ACh the neurotransmitter to accumulates at the nerve junction and results in overstimulation of the nervous system. In addition, c hlorpyrifos 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 (Reigart and Roberts 1999). After subsequent development of the molecular method of the Real-time PCR by Bustin (2002) and others, that accurately quantify the nucleic acids of cells or tissues, and define the biological differences of malformed tissues and quantify molecular changes due to infection diseases or natures disorders or artificial stress. As well as genotypes differentiation through single nucleotide polymorphisms and gene expression level of samples. Several strategies of PCR processes especially Real-time PCR working through thermal cycles, is the linear ground, early exponential, log-linear, and plateau phases to attain normalising data including reference, gene selection, similarity of sample size and quality of RNA, by means of definite primers and templates (Huggett *et al.* 2005). The important step of real-time PCR process is the cDNA synthesis that can greatly affect the overall results, in addition to the reverse transcriptase enzyme and dithiothreitol (DTT) are PCR inhibitors during RNA isolation and characterization for quantity and integrity. Data gathered from fluorescent emission at each reaction cycles of the four PCR phases are important for calculating background signal, cycle threshold (Ct), and amplification efficiency. These results processed by some method of data analysis to obtain the relative quantity of target mRNA and six techniques are currently used but ΔΔCt method were chosen (Wong and Medrano 2005). Although the numerous steps with experimental error, it gives high efficiency on data normalization for quantification of target gene expression (Pfaffl *et al.* 2002 and Bustin, 2002). In this study, the gene of PSC-domain ll after *P. gossypiella* insecticide treated with Sublethal concentration of two Py and two OP, samples plus control results attained the relative expression profiles using the molecular marker real-time PCR in the presence of specific primer and templates were completed, then some statistical analysis were completed.

MATERIALS AND METHODS

Insect Sources and Maintenance

Extremely much quantity of cotton plant bolls collected from Egyptian cotton cultivated lands of Sharkia and Benisuef governorates, heavily invested by *P. gossypiella*, in cotton cultivation mid-season, transferred to laboratory, larvae emerged from bolls, placed in petri dishes provided with piece of cotton for maintenance and used directly for the insecticide bioassay and the molecular biology.

Insecticides used:

Selected insecticides 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, common name structure, mode of actions and active ingredient information offered in Table (1).

Table 1: Insecticide formulation information and 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. Type ll	C22H19Cl2NO3	Neurotoxic
10%EC	Deltamethrin	Deltamethrin	Py. Type ll	C22H19Br2NO3	Neurotoxic

Insecticide Bioassay and LC_{50} Lethal Concentration Treatments

Bioassay procedure was completed according to Paramasivam and Selvi (2017) and Cetin *et al.* (2006). 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 replicates were 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. Data statistically analyzed using Polo, LeOra Software (1989), to attain LC_{50}, 90 according to Finney (1971), and correction of mortality were included. After LC_{50} values determination, a much number of larvae were treated with LC_{50} concentration value of the four tested insecticides individually. Then larvae died were rejected and the survived larvae were collected after 24h of treatment to investigate the effect of this lethal concentration on the gene expression level called delta ct values, and send to molecular research laboratory for PCR molecular procedure accomplishment.

Molecular Procedures and Steps

Primer Design and Quantitative Real-Time PCR

In this study, primers designed for detecting PSC-domain ll -changes in *P. gossypiella* domain II gene was as follows; Forward primer: GGCCGACGTTTAATTTACTC and Reverse primer: CCATCGTGGTA'GGTCTCCAT. The designed primer size was 20 nucleotides, length was 177 pb, and GC content between was about 35%- 42% to give product size of 100-200 bp, and melting temperatures was 60–65 °C (Table 2), it was suitable for successful amplification (Wang *et al.* 2006).

Insect Tissue Preparation and Total RNA Purification Protocol

The Gene JET™ RNA Purification Kit as at site www.thermoscientific.com/onebio: contain protein-kinase, lysis and wash buffers was used. The amount of Lysis Buffer adjusted by 20 µl of 14.3 M β-mercaptoethanol to each 1 mL volume added. The amount of Proteinase K solution was 10 µl of Proteinase K (included) to 590 µl of TE buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA) diluted. A total RNA molecule purification longer than 200 nucleotides obtained from *P. gossypiella* larvae cultured cells to use in real-time PCR. Insect body samples homogenized and lysed in Lysis Buffer, which contains 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 rolled 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 were provided. Column washed with buffers to remove impurities. Pure RNA was then eluted under low ionic strength conditions with nuclease-free water (Boom *et al.* 1990).

Synthesis of cDNA from RNA Reactions Procedures was as Follows

11µl RNA in a 0.2ml tube that placed on ice and 1µl oligo (dt) primer was added. The mix in 65 °C for 5 min incubated and total volume was 12µl. Then 8 µl to 12µl, 4µl (5x) reaction buffer, 1µl RiboLockRNase Inhibitor, 2µl10 mMdNTP Mix, and 1µl Revert Aid M-MuLV Reverse Transcriptase was added, and total volume was 20µl. Then the reaction was mixed gently, and entered the PCR machine at 42 °C for 60 min or at 70 °C for 5 min.

Real-Time PCR Protocol was as Follows

The master mix reaction was prepared after melting all solutions were centrifuged. By adding; 20 µl reaction to each tube (except template DNA), 10 Maxima SYBR Green (Cyanine dye used as nucleic acid stain)/ROX dye qPCR, Master Mix (2X) was 10 µl. forward primer was 0.25 µl, reverse primer was 0.25 µl, DNA template was 2 µl, water, nuclease-free was 7.5 µl, and total volume was 20 µl. ROX dye is used as a passive reference dye to normalize the fluorescent signal and therefore improves the precision of PCR results. Reactions mixed without creating bubbles and the volumes were dispensed into PCR tubes gently. The samples were placed in the cycler, the program started, the thermal cycler protocols in Two-step cycling were: Initial denaturation at 95°C for10 s and 1 cycle, Denaturation at 95°C for 15 s and 40 cycle and Annealing/Extension at 60°C for 60 s and 40 cycle. The nucleotide sequence of 18S was determined using a dye terminator cycle sequencing kit analyzed. The primer used in real-PCR method for PSC- domain II of gene expression analysis of *P. gossypiella* treated larvae by sublethal concentration of tested OP and Py. insecticides were found in table 2.

Table 2: Information of Real-time-PCR used a primer in PSC-domain ll gene expression check

Primers	Forward and reverse Primer Sequence (5'-3')	Molecular Function	GC content	Product size	length-bp
Para	For: GGCCGACGTTTAATTTACTC	Cholinergic synapses	45%	20	177
	Rev: CCATCGTGGTAGGTCTCCAT		55%	20	
RT-18S	For: GGCCTTCGGGATCGGAGTAA	18S ribosomal RNA protein	60%	20	113
	Rev: GCAAATGCTTTCGCAGTTGTT		42%	21	

Statistical Data Analysis

Data of PSC-domain ll normalization with multiple housekeeping genes was calculated for each individual samples by dividing the fluorescent data by its normalization factor produces the normalized data. Then performed the statistical analysis of comparative Ct (2-ΔΔCt) and calculated changes in gene expression as a relative fold difference between experi¬mental and calibrator samples, that not require a standard curve, using the Livak method (Livak and Schmittgen 2001) over the common site https://goldbio.com/qpcr-and-rtqpcr-analysis-tool. The data of gene up and down regulations calculated according to Peredoa and Cardon (2020) of statistical hypothesis testing (The null hypothesis H_0

means there is no difference and the alternative hypothesis H_1 means 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 between sample groups were included.

RESULTS AND DISCUSSIONS

Toxicity Response of the Selected Insecticides Used in Bioassay

Insecticide responses of field collected *P. gossypiella* larvae exposed to two candidate classes of insecticide (PY and OP) represented by LC_{50}, LC_{90} and the slope, data found in (Table 3). Larvae used in insecticide bioassay were collected from Sharkia and Benysuef governorate of *P. gossypiella* infested cotton in Egyptian fields. The toxicity values appear at very small values refer to susceptibility levels to the tested insecticides against this pest. Benisuef collected larvae used in toxicity bioassay was the most susceptible larvae, was considered the reference population and used to calculate resistance ratio (RR) according to that essential for comparing results. Thus, RR values detected were ranged from 1.2 to 6.4-fold of resistance to chlorpyrifos and deltamethrin respectively. Data also refer to deltamethrin was the most efficient insecticide to kill this pest followed by cypermethrin then chlorpyrifos then malathion. Insecticide efficiency could be affected by toxicity conditions as temperature, humidity and insect species natures that under investigations. Consequently, the toxicity order in some search reviews explained toxicity differences of the same insecticides at many tests, as Abdullahi *et al.* (2020), give a benefit of the toxicity arrangement of the insecticides from most-to-least toxic are chlorpyrifos > cypermethrin + dimethoate > deltamethrin, when tested as residual toxicity against the fruit fly *Bactrocera invadens*. Either Hafez (2021), study efficiency against house fly *Musca domestica*, results provide toxicity arrangement was alpha-cypermethrin, deltamethrin, bifenthrin, cypermethrin, cyfluthrin, fenitrothion, chlorpyrifos, and malathion. In addition, Mansee and Montasser (2003), study the toxicity under temperature and light exposure against flour beetles *Tribolium castaneum* using the residual film method, mortality increased with temperature and light. Descending order was chlorpyrifos-methyl >deltamethrin > malathion. Additional searches touched on these insecticide resistance and reports about many insect species by (Horowitz *et al.* 1998). Different means of insecticide applications may give better results, whereas Salama *et al.* (2013), found that sequence of insecticide application against *P. gossypiella* gave good effect more than individual applications which being more than 38.02% reduction. Moreover, El-Bakry *et al.* (2014), said that reduction of pink bollworm on cotton treated with chlorpyrifos and cyhalothrin formulations alone or mixed with adjuvants provided high mortality percentage.

Table 3: Toxicity response of *P. gossypiella* exposed to selected OP. and Py.insecticides

	Sharkia collection					Benisuef collection					RR
Pesticide	**Slope**	**Intercept**	**LC_{50} (95 % CI)**	**LC_{90}(95 % CI)**	**χ^2**	**Slope**	**Intercept**	**LC_{50} (95 % CI)**	**LC_{90}(95 % CI)**	**χ^2**	
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(29-69.3)	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

Determination of Relative Expression

Quantification of ΔΔ Ct to all insecticide treated samples successively calculated after normalization with the required reference gene to compare between target, reference treatment and control, where Ct values all averaged and calculated using delta-delta Ct method, and relative quantification determined using the Livak method (Livak and Schmittgen 2001). The normalized and non-normalized expression (ΔCt and ΔΔCt method) analyzed by assuming that PCR efficiencies of insecticide treatment and control of PSC-domain ll gene and the housekeeping gene were similar and equal 100% and no need correct by standard curve, subsequently Expression Ratio = ΔCtreatment / ΔCt control but fold change= $2^{-\Delta\Delta Ct}$ were attained. The comparison for expression levels of reference genes expressed as (fold change and expression ratio), data found in table (4) and Fig (1 and 2). Data showed the most expressed sample 652.57 was cypermethrin fold change then 7.34 for deltamethrin,1.10 for malathion, and 0.692 for chlorpyrifos, where Ct values

were 32.43 to 36.8 to 33.37 to 36.82 respectively, for PSC-domain ll sample gene. Then the most effective insecticide was cypermethrin and the least was deltamethrin followed by malathion and chlorpyrifos. Data of gene status up and down regulations showed the down-regulated insecticide treatment was cypermethrin recorded negative values -7.4 then deltamerhrin was -1.6 and the up-regulated insecticide was chlorpyrifos and malathion recorded positive values 4.54 and 7 respectively. Data showed that cypermethrin and deltamethrin are the functional genes against PSC-domain ll in *P. gossypiella* samples. The data attained by applying different curve fitting techniques to achieve expression patterns over time, according to some researchers like Cook *et al.* (2007) shows such as scatter plots, volcano plots, and p value versus fold change plots. Those plots were in Fig 3,4,5,6 and Fig7 was important. The algorithm for the p-value calculation of Student's t-test a univariate technique of analysis (Table 5), selected to screening properties of the expression function and similarities as found by (Fig 3 to 7). Using z test to test if samples Ct and housekeeping Ct are equally by formula was Z Test = $(\bar{x}-\mu)/(\sigma/\sqrt{n})$. Where $\bar{x}$ = Mean of sample, μ = Mean of population, σ = Standard deviation of population and n = Number of observations. Using stat plus software, results of statistical z test calculation showed data not supported the null hypothesis H0 and the alternative hypothesis H1 was accepted. Where the assumption of treatments was not equal, but hypothetically accepted by (two-tailed z test distribution 1.9600), mean differences was 73.7 and lower and upper mean (36.6-38.77), H1 suggestion (mean Less than) was rejected and H1 suggestion (Greater than) was accepted in (One-tailed distribution). The reasons of the variations in Ct values at time points and differences because of changes in the mRNA quantity levels of the reference genes during the PCR process (Zhao and Fernald 2005). According to Skern *et al.* (2005), fold change indicates whether a gene is up or down-regulated, where, if fold Change > 0 it means that gene is more expressed or up-regulated, but if it is less than < 0 it means that this gene is less expressed or down-regulated (Fig 2). From the literature, some factors affect Ct values were illustrated by Skern *et al.* (2005) found relative transcript abundances estimated by quantitative Real-time PCR of salmon results could vary intensely depending on the method chosen for data analysis. The same results were supposed by Zhou *et al.* (2019) which found 13 and 7 of *Sogatella furcifera* genes were up-regulated after thiamethoxam and abamectin, treatment respectively, at LC_{25} treatment. Therefore, VanGuilder *et al.* (2008) carried out numerous molecular biology applications especially gene expression analysis by reverse-transcription quantitative PCR to elucidate these analysis techniques, and found that future of qPCR remains bright.

(Fig 5) Scatter plot of the test group and control group, where every point in the plot describes expression

Table 4: Gene expression profiling of selected Py and Op insecticides treatment against P.gossypiella PSC-domain ll gene, relative quantification calculated using Livak Method

Insecticide	Aver.H.K	Aver.Para	Δ ct-treat.	Δ ct-control	ΔΔ ct	Exp.ratio	Fold Change	CV	STDV	KD%
Control	39.42	36.795	--	-2.63				1.3	2.48	
Chlorpyrifos	38.9	36.825	-2.1	-2.63	0.53	0.79	0.692	1.04	2.05	47
Malathion	36.14	33.37	-2.77	-2.63	-0.14	1.05	1.1	1.39	2.6	78
Control	37.31	40.86	--	3.55				1.78	3.42	
Cypermethrin	38.295	32.43	-5.8	3.55	-9.35	-1.652	652.57	2.93	7.63	-142
Daltamethrin	36.13	36.805	0.675	3.55	-2.87	0.191	7.33	0.34	1.26	77

⊿⊿Ct = - [⊿Ct treatment –⊿Ct control]

⊿Ct(treatment)= Ct(target treatment)−Ct(reference treatment), and ⊿Ct(control)= Ct(target control)−Ct(reference control)

% KD = (1 – ΔΔCq) × 100, Bass et al., 2007.

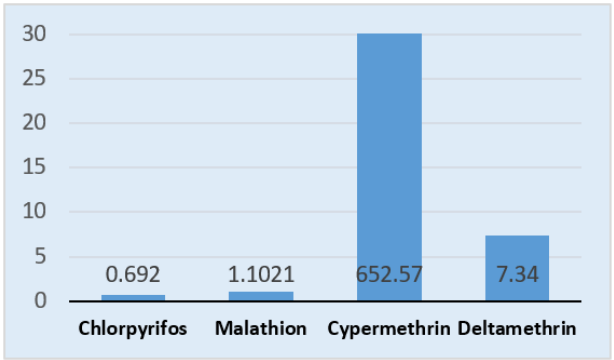

Figure 1: Fold change or transcription levels and expression ratio of real-time PCR analysed using the 2-ΔΔCt method of *P. gossypiella* PSC-domain ll gene after treatment by sublethal LC_{50} concentration.

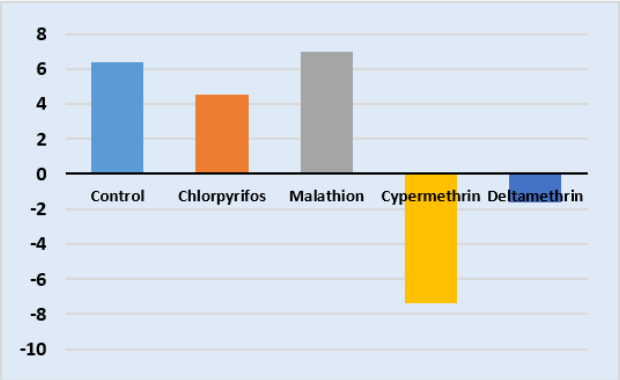

Figure 2: Up and down regulation of *P. gossypiella* insecticide treatment executed by real-time PCR

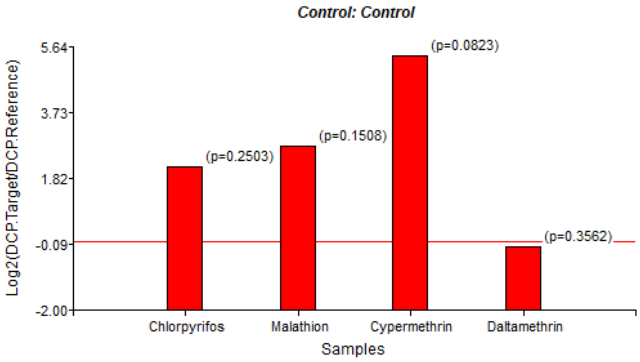

Figure 3: P-values versus Log2 fold change of the target genes (Insecticide treatments)

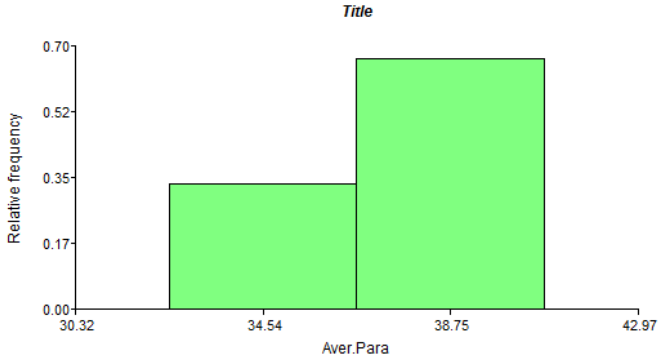

Figure 4: Average of para domain ll and housekeeping genes versus relative frequencies

values in transcriptome results in two group (This figure Produced by excel worksheet). Points in right of the orange line represent down-regulated genes of samples, but the point in the left of the blue line represent up-regulated genes and point in the middle of both lines considered unchanged.

(Fig 6) Volcano plot shows scatter point of Log 2-fold change expression versus P-value. To visualized and identification of genes that statistically significant. The most upregulated genes are towards the right, the most downregulated genes are towards the left, and the most statistically significant genes are towards the top according to Li etal., 2014.

(Fig 7) RNA-seq scatter plot display variations from two dimensions of insecticide treatment expression and modificated pathways using log^2Fold Change of treatments (hyper: >=0, hypo; <0), and the fold change. The methylated samples did not appear in the 4th quarter because the insect strain was almost susceptible and did not contain methylated product of log^2 FC, and this similar to bioassay results of insecticide according to the following site: Both figures plotted by https://www.bioinformatics.com.cn/en, a free online platform for data visualization.

Expression Profiling

Interpretation of relative expression level of the target gene *P. gossypiella* PSC-domain ll samples compared with control and housekeeping values to attain expression profiling of the insecticide treatments completed by using cluster analysis. Firstly: Hierarchical clustering

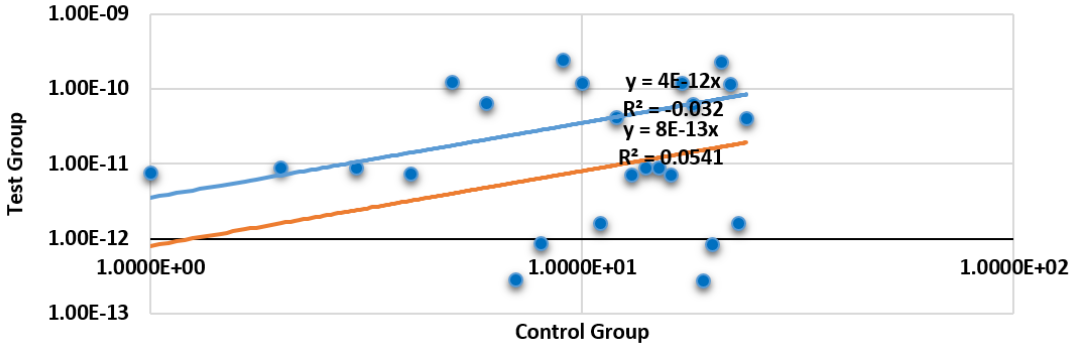

Figure 5: Scatterplot-Threshold for fold differences

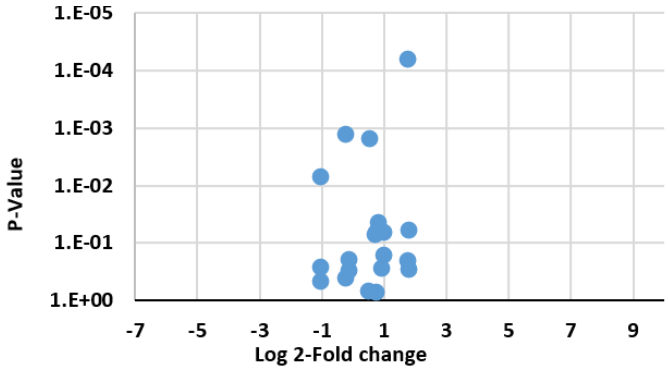

Figure 6: Volcano plot of fold differences threshold

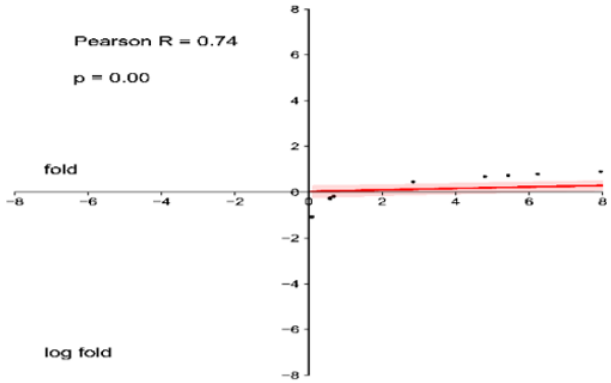

Figure 7: RNA-seq plot of fold differences threshold

combined with Heat map were included based on various clustering systems and algorithm aspects as distance measures like Euclidian distance and similarity method plus Pearson correlation completed. Data visualization appeared by a dendrogram generated from GenEx software, illustrated in Fig (8,9). Figure 8 shows the representative nodes of hierarchical clustering data of insecticide treatment group distributed together based on similarity in two main groups at point 4 and attached to one group at point 14 as distance of its gene expression pattern of chlorpyrifos, malathion, cypermethrin, and deltamethrin. Each group in the separated branch, Py and OP to identify the insecticide treatment individual samples that its genes regulated, or quantify responses to insecticide stress. In addition to, heat map in (Fig 9), that is a multivariate technique used to find structure in real-time PCR expression experiments required to point out all the connectivity among genes in a genome or group created colorfully. Each color represents the degree of gene expression, based on the functional characteristics of previously known genes. Likewise, discover how genes are related, dependent, and affect each other according to Zhao *et al.* (2021). The second interpretation analysis of expression results is the Principal Component Analysis (PCA) a multivariate technique where, groups of samples or genes are analyzed based on correlated expression (Fig 10), that construct axes to give insights into gene expression profiling and similarities that co-regulation patterns and compacted representation of large amounts of data. Here the two axes showed (Pc_1 = 63% and Pc_2 = 36% percentage). The simple one-way ANOVA test of significance assuming no correlation between samples

and showed no significant differences between treatments (Table 5).

Similar results about PSC-domain ll insecticide treatment were attained by Ingham *et al.* (2021), found sub-lethal exposure of Py against highly Py resistant Anopheles coluzzii population and identifies 44 transcription factors regulating insecticide transcripts explain keys of pesticide response complexity. Moreover, Bala *et al.* (2019), about sequencing of PCS-domain II reveals one kdr mutation exist in cyhalothrin (Py) resistant larvae of Tuta absoluta and chlorpyrifos-methyl (OP) get average mortalities 80% but expression was not similar.

(Fig 8) Hierarchical cluster analysis of real-time PCR of PSC-domain ll gene from P.gossypiella treatments showed distances in classification method based on Herarcial clustering of attained data categorized in two group of (PY and OP) insecticide gene expression profiles.

(Fig 9) Heat map composition of the relative expression of the insecticides treated *P. gossypiella* larvae, values displayed showed a grid of each row represents a gene PSC-domain ll 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 the black represents unchanged expression.

(Fig 10) Principle component analysis of the P.gossypiella insecticide treatments showing classification of insecticide treatments genes (PSC-domain ll gene of Real-time PCR) expression based on hierarchical clustering and distance similarity. Figure (8, 9, 10) produced by using Genex7 software.

Table 5: ANOVA results between and within of control and treatment group, multiple regression, and t-test

ANOVA-Source of Variation	**d.f.**	**SS**	**MS**	**F**	**p-value**	**F crit**	**Omega Sqr.**
Between Groups	1	13.848	13.848	2.201	0.152	4.301	0.048
Within Groups	22	138.409	6.291				
Total	23	152.256					
Multiple regression , T test							
	Coefficients	**Std Err**	**LCL**	**UCL**	**t Stat**	**p-value**	**H0 (5%)**
Intercept	32.043	6.442	17.690	46.397	4.974	0.001	Rejected
para	0.156	0.177	-0.239	0.552	0.881	0.399	Accepted
T (5%)	2.228						

LCL – Lower and upper limit of the 95% confidence interval

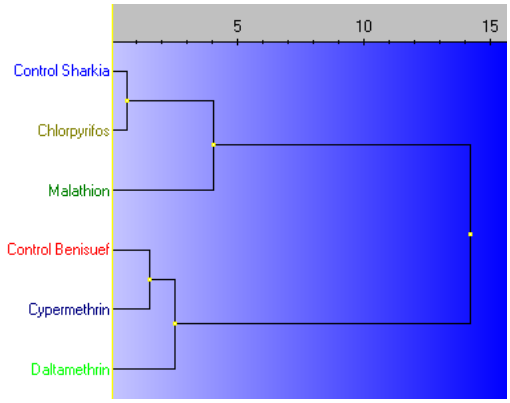

Figure 8: Hierarchical cluster analysis

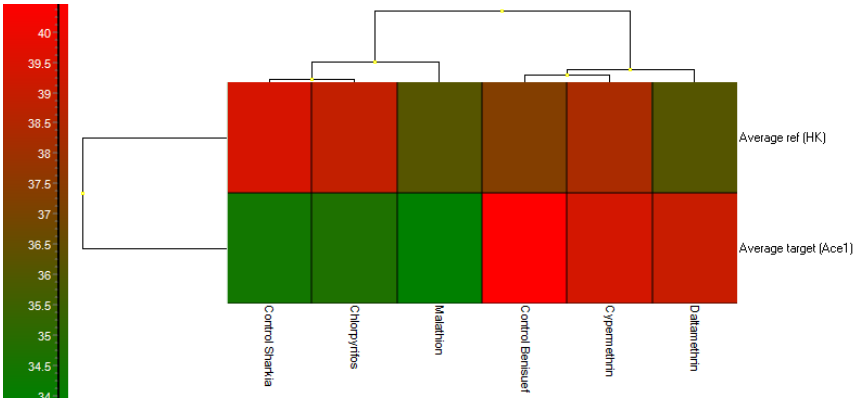

Figure 9: Heat map composition

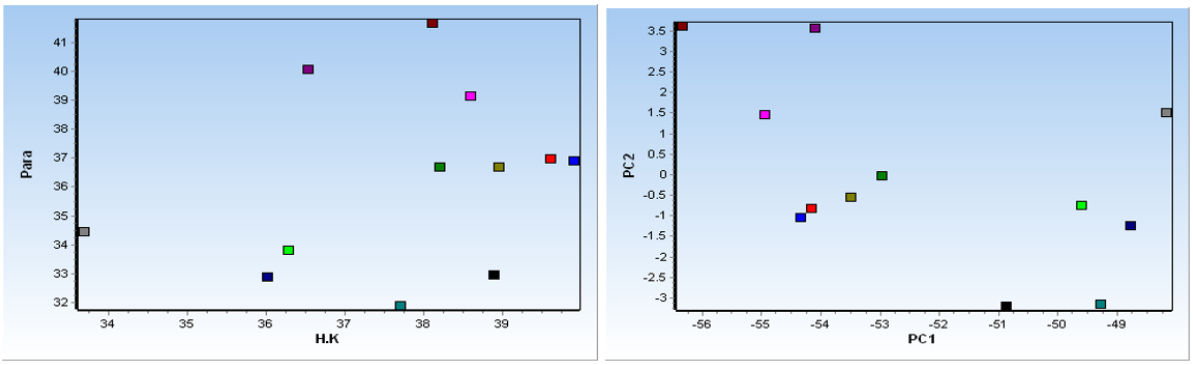

Figure 10: Principle component analysis

Gene Expression Stability and Ranking of Reference Genes

Evaluation of reference gene and quantification of expression stability completed using the internet site RefFinder, a web tool prepared for analysis and integrates the common four algorithms as comparison was GeNorm (Vandesompele, *et al.* 2002), NormFinder (Mao *et al.* 2021), BestKeeper (Zhang *et al.* 2022), and delta-Ct method according to Livak and Schmittgen (2001) and Kozera and Rapacz (2013). The RefFinder mirror site (http://blooge.cn/RefFinder/). Or http://blooge.cn/RefFinder/?type=reference#. It calculates the geometric mean of the gene weights to obtain an overall ranking and the comprehensive final ranking order and stability values. Results are found in tables (6 and 7) and illustrated by fig (11, 12). Expression stability accomplished by the three finders showed that chlorpyrifos and malathion-treated genes having lower mean weights, considered transcriptionally stable, that used as ideal reference genes. From the literatures, many insect or pest expression experiments proved that the candidate reference genes could help to improve the precision of the detection of gene expression at different developmental stages of insects such as bumblebee species (Qin *et al.* 2021) and Bombyx mori (Teng *et al.* 2012). Factors affecting the expression level of control genes (housekeeping genes), firstly: may vary among tissues or cells and may change under certain conditions and are critical for genes that are often used to normalize mRNA levels between different samples (Silver *et al.* 2006 and Spiegelaere, *et al.* 2015). Interpretation of stability results of the three tools concluded that GeNorm stability values calculated, proved that there are top two stable genes was chlorpyrifos and malathion have the lower mean weight values and considered the most suitable for normalization in insecticide efficiency detection and search. The reference gene with the lowest M value (Genex7 software calculations) should be the most stable gene, where M was (3.69 for together) but for individual insecticide was, 1 for each means of insecticide-treated samples it less than 1.5 refer to stability level. Lower M values indicate greater stability; good reference genes have an $M < 0.5$, whereas M values up to 1 are acceptable for heterogeneous samples according to Wang *et al.* (2017). Norm Finder stability results explained that individual candidate genes ranked according to their stability value, and suggested that chlorpyrifos and malathion were the most stable and largely in agreement with the other three algorithms used. The comparative delta-Ct method assesses the transcription data via 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 SD of the most stable reference gene by pairwise comparison of two reference genes. Moreover, CV values was 4.73 and 8.47 for housekeeping and target gene respectively and SD was 1.78 and 3.06 for the same arrangement respectively, which reflect the variability of gene expressions, based on medians attained from Normality test. The p-value measured by the Shapiro–Wilks hypothesis indicated the good fit of the normal distribution, it was > 0.1 and means a significant departure from normality. In fact Wang *et al.* (2017) said that housekeeping genes are commonly used as reference genes to normalize expression values of target genes, and good selection helps to more sensitively reflect gene expression changes, by use of geNorm and NormFinder software, to calculate variations of 13 commonly used housekeeping genes of peripheral nerve injury.

(Fig 11) Selection and validation of reference genes for PSC-domain ll sample gene changes detected by real-time PCR-based analysis of *P. gossypiella* treated by Py and Op insecticide using Genorm, Normfinder, and Delta Ct method in addition to the comprehensive ranking.

(Fig 12) Expression Stability examination of BestKeeper by comparing SD and CV, and the ct values, of samples genes and housekeeping points plotted adjacent to each other and outlier excluded. However, the lower CV and SD is the higher stability.

Similarly, Bestkeeper determines the stability of candidate reference genes based on SD and CV of their raw Ct values, if SD value greater than 1 indicates high variation of the expression of the sample gene and, any gene with $SD > 1$ was considered unstable. In addition, CV analysis calculates the overall variance of each gene, and the threshold were defined as 50%, and above considered to be highly variable (Wang *et al.* 2020). Then high variation was detected between the two group insecticide classes Py and OP. otherwise, the comprehensive gene

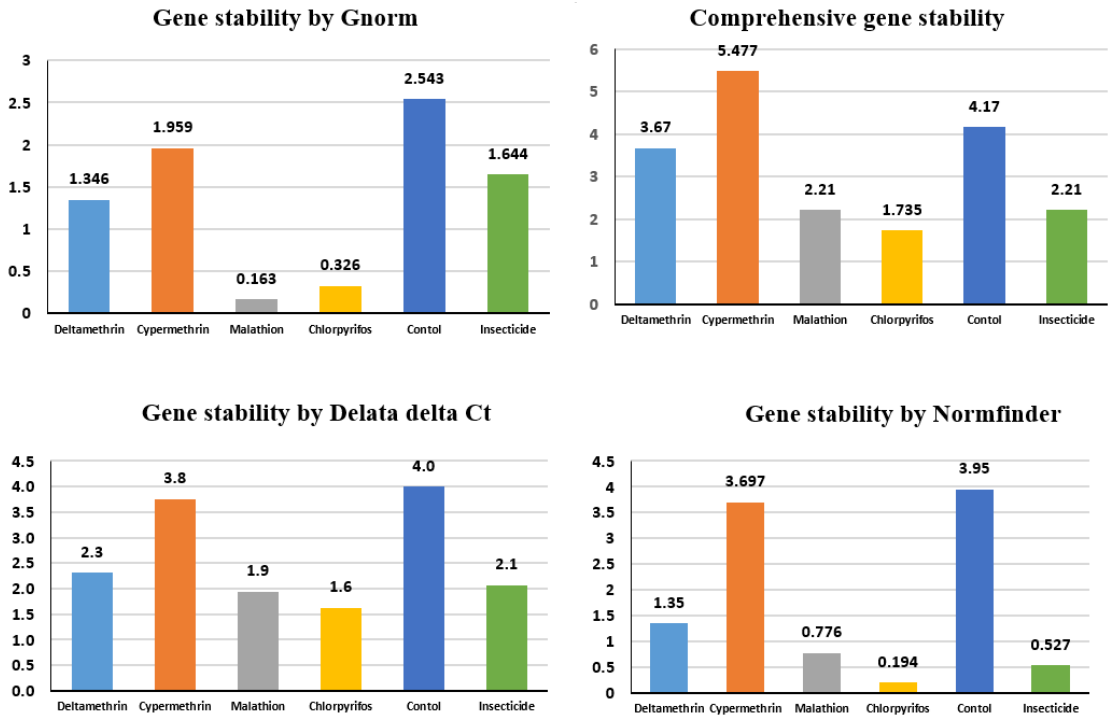

Figure 11: Selection and validation of reference genes

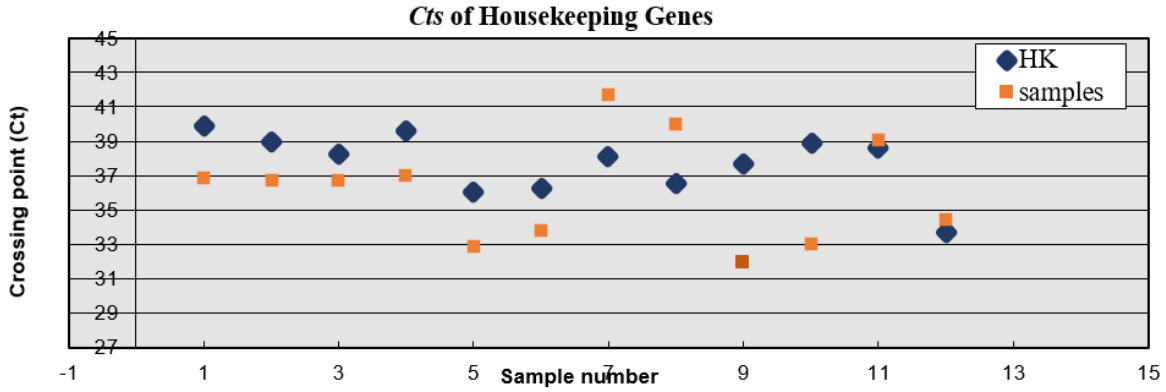

Figure 12: Expression Stability examination

expression ranking and recommended reference genes revealed average gene stability values was M ranged from 0.5 to 1 indicating high reference gene stability. From this study, data determines the optimal references of sample genes were cypermethrin followed by deltamethrin, and the most stable genes were chlorpyrifos and malathion.

Validation of the Selected Reference Genes for Optimization

The results of pairwise relative expression showed that expression of normalized samples according to the reference gene stability gave similar expression trends of insecticide treatment and control (Fig13). However, the variation in expression was larger between the unstable reference genes compared to the stable reference gene group. The expression levels of chlorpyrifos and malathion compared with others PSC-domain ll gene not varied significantly.

Genotyping Analysis of P. Gossypiella Samples and Gene Copy Number

Copy number variations detected by qPCR principles, carried out according to DNA amount (Jimenez *et al.* 2011), insertions, deletions in the samples of *P. gossypiella* and calculated using Copy caller software of Multiplex RT-qPCR for gene expression analysis without need a standard curve. Determining achieved by which qPCR wells have a true sample versus noise and carrying out replicate averaging, outlier detection, and statistical error

Table 6: Stability values of PSC-domain ll sample gene in *P. gossypiella* treated by insecticides, calculations performed by RefFinder site, Delta Ct, NormFinder, and geNorm and CV analysis.

Method	Insecticide	Control	Chlorpyrifos	Malathion	Cypermethrin	Deltamethrin
Delta CT	2.0	4.0	1.0	1.94	3.76	2.3
Normfinder	0.527	3.95	0.194	0.776	3.7	1.35
Genorm	1.0	2.54	0.328	0.10	1.95	1.34

Table 7: Ranking order: Control and target genes ranked in order of their expression stability

Method	1	2	3	4	5	6	7
Delta CT	Chlorpyrifos	Malathion	Insecticide	Deltamethrin	Cypermethrin	Control	
BestKeeper	Insecticide	Deltame-thrin	Chlorpyrifos	Malathion	Control	Cypermethrin	
Normfinder	Chlorpyrifos	Insecticide	Malathion	Deltamethrin	Cyperme-thrin	Control	
Genorm	Control	Malathion	Chlorpyrifos	Insecticide	Deltamethrin	Cypermethrin	Control
Comprehensive	Chlorpyrifos	Insecticide	Malathion	Deltamethrin	Control	Cypermethrin	

calculated. Data were in Fig (12) showed gene copy number was in range between 9.79 to 12 copies were detected in all insecticide samples. Similar results attained by some researchers as, Marcombe *et al.* (2019), detected high gene copy number variations between survived larvae of highly resistant larvae collected to malathion, temephos, DDT, permethrin and deltamethrin.

Sensitivity and specificity frequently used to describe the amount of hesitation of a particular test, values for these two parameters range from 0 to 1, where the higher

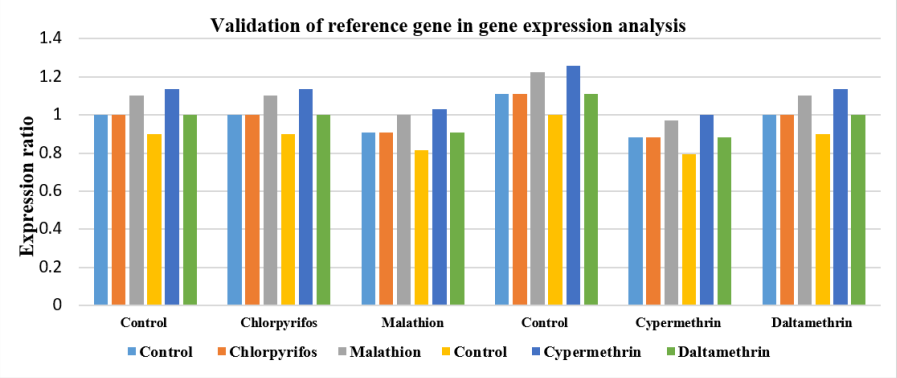

Figure 13: Pair wise variations for finding the optimal number of reference gene for precise normalization in *P. gossypiella* samples using the comparative Δ-Ct method

the number, the more accurate the test is. The figure 14 illustrate about determination of the optimal annealing temperatures of the candidate reference genes by gradient PCR showed annealing temperatures was at 65°C were suitable for each gene. Additionally, the dissociation curves of the candidate reference genes showed a single peak, (Figures generated by Genex7). Much investigations were carried out by scientists to find Py sensitivity and PSC-domain ll -relationship. Like, Tan *et al.* (2002), found big difference in sensitivity to deltamethrin (Py) with PS-domain III. As well as Bona *et al.* (2016), about Aedes eagyptii resistant to deltamethrin have higher expression

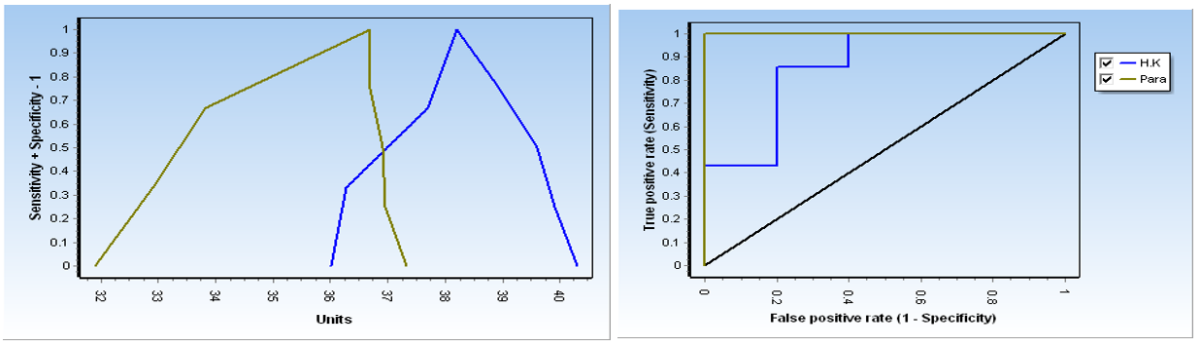

Figure 14: Amplification specificity and efficiency of the primer sets from Total RNA and DNA extraction and amplification from P.gossypiella treated samples

to PSC, and Dermauw *et al.* (2012), found the same in Tetranychus urticae resistant to abamectin. Also Zuo *et al.*, (2016), found the same, about Rhopalosiphum padi resistant to beta-cypermethrin. But Haddi *et al.* (2012), in Tuta absoluta resistant to cyhalothrin and fluvalinate, (Py) resistance was mediated by three mutations of the PSC containing confer (kdr)-type resistance. Also, Williamson *et al.* (1996), found PSC gene of the housefly, *Musca domestica*, responsible for (kdr), confers nerve insensitivity to DDT, and Py was methionine to threonine replacement located in PSC-domain ll, and leucine to phenylalanine replacement in the hydrophobic IIS6 transmembrane

segment. Those have 92% identity to the *Drosophila* PSC and 50% homology to vertebrate sodium channels. However, one amino acid substitution in PSC known to confer to profenofos, chlorpyrifos and deltamethrin pyrethroid resistance of Onion thrips, Thrips tabaci Lindeman (Nazemi *et al.* 2016).

CONCLUSIONS

For accurate quantification of insect biological samples of agriculture pests treated by insecticides the most widely usages real-time PCR expression technique as molecular biology technology procedures because more reliable noticed and provide such benefits. It becomes more rapid, cost-effective, easier to use, great sensitivity and high efficiency for detecting differences for more than much number of samples. In addition to sufficient data analysis and visualization techniques with simplest tools, such as the scatterplot, principal component analysis and clustering and algorithms classification. Furthermore, the current developed relative quantitation, mathematical mod¬els and amplification efficiency calculations, types of normalization or data correction and detection chemistries used to get and interpret results. The need to reference gene, such as a housekeeping gene to express differential gene expression, and the limited detection of twofold changes whereas less than twofold may be required especially in the field of toxicity by pesticides considered default.

REFERENCES

Bala, I., Mukhtar, M. M., Saka, H. K., Abdullahi, N., & Ibrahim, S. S. (2019). Determination of insecticide susceptibility of field populations of tomato leaf miner (Tuta absoluta) in Northern Nigeria. *Agriculture, 9*(1), 7.

Bass, C., Nikou, D., Donnelly, M. J., Williamson, M. S., Ranson, H., Ball, A., & Field, L. M. (2007). Detection of knockdown resistance (kdr) mutations in Anopheles gambiae: a comparison of two new high-throughput assays with existing methods. *Malaria Journal, 6*(1), 1-14.

Bona, A. C. D., Chitolina, R. F., Fermino, M. L., de Castro Poncio, L., Weiss, A., Lima, J. B. P., & Maori, E. (2016). Larval application of sodium channel homologous dsRNA restores pyrethroid insecticide susceptibility in a resistant adult mosquito population. *Parasites & Vectors, 9*(1), 1-14.

Boom, R. C. J. A., Sol, C. J., Salimans, M. M., Jansen, C. L., Wertheim-van Dillen, P. M., & Van der Noordaa, J. P. M. E. (1990). Rapid and simple method for purification of nucleic acids. *Journal of clinical microbiology, 28*(3), 495-503.

Bustin, S. A., & Dorudi, S. (2002). The value of microarray techniques for quantitative gene profiling in molecular diagnostics. *Trends in molecular medicine, 8*(6), 269-272.

Catterall, W. A. (1988). *Structure and function of voltage-sensitive ion channels. Science, 242*(4875), 50-61.

Cetin, H., Erler, F., & Yanikoglu, A. (2006). Larvicidal activity of novaluron, a chitin synthesis inhibitor, against the housefly, *Musca domestica*. *Journal of Insect Science, 6*(1), 50

Cook, D., Hofmann, H., Lee, E. K., Yang, H., Nikolau, B., & Wurtele, E. (2007). Exploring gene expression data, using plots. *Journal of Data Science, 5*(2), 151-182

De Spiegelaere, W., Dern-Wieloch, J., Weigel, R., Schumacher, V., Schorle, H., Nettersheim, D., & Fink, C. (2015). Reference gene validation for RT-qPCR, a note on different available software packages. *PloS one, 10*(3), e0122515.

Dermauw, W., Ilias, A., Riga, M., Tsagkarakou, A., Grbić, M., Tirry, L., & Vontas, J. (2012). The cys-loop ligand-gated ion channel gene family of Tetranychus urticae: implications for acaricide toxicology and a novel mutation associated with abamectin resistance. *Insect biochemistry and molecular biology, 42*(7), 455-465

Dong, K. E. (1997). A single amino acid change in the para sodium channel protein is associated with knockdown-resistance (kdr) to pyrethroid insecticides in German cockroach. *Insect biochemistry and molecular biology, 27*(2), 93-100

Elliott, M., Janes, N. F., & Potter, C. (1978). The future of pyrethroids in insect control. *Annual review of entomology, 23*(1), 443-469.

M El-Bakry, A., AM Abdelgaleil, S., MI Kassem, S., EM Shalaby, S., & A Sammour, E. (2014). Effect of Tank-Mix Adjuvants on the Efficiency of Chlorpyrifos and Cyhalothrin Formulations against Cotton Bollworms. *Alexandria Science Exchange Journal, 35*(January-March), 1-9.

El-Saadany, G., El-Shaarawy, M. F., & El-Refaei, S. A. (1975). Determination of the loss in cotton yield as being affected by the pink bollworm *Pectinophora gossypiella* (Saund.) and the spiny bollworm Earias insulana (Boisd.). *Zeitschrift für Angewandte Entomologie, 79*(1-4), 357-360.

El-Shaarawy, M. F., El-Saadany, G., & El-Refaei, S. A. (1975). The economic threshold of infestation for the cotton bollworms on yield in Egypt. *Zeitschrift für angewandte Entomologie, 79*(1-4), 276-281.

El-Refai, A. R. M., Abdel-Moneam, A. L. A., Mohamed, H. A., Mustafa, S. A., & Abdel-Hamid, H. F. (2009). Synergism among some insecticides and ergosterol biosynthesis inhibiting fungicides against *Pectinophora gossypiella* (Saund.). *Egyptian Journal of Agricultural Research, 87*(4), 1041-1056

Fernandez-Jimenez, N., Castellanos-Rubio, A., Plaza-Izurieta, L., Gutierrez, G., Irastorza, I., Castano, L., & Bilbao, J. R. (2011). Accuracy in copy number calling by qPCR and PRT: a matter of DNA. *PloS one, 6*(12), e28910

Finney, D. J. (1971). Probit analysis, Cambridge University Press. Cambridge, UK

ffrench–Constant, R. H., Pittendrigh, B., Vaughan, A., & Anthony, N. (1998). Why are there so few resistance–associated mutations in insecticide target genes?. Philosophical Transactions of the Royal Society of

London. *Series B: Biological Sciences, 353*(1376), 1685-1693.

Guerrero, F. D., Jamroz, R. C., Kammlah, D., & Kunz, S. E. (1997). Toxicological and molecular characterization of pyrethroid-resistant horn flies, Haematobia irritans: identification of kdr and super-kdr point mutations. *Insect biochemistry and molecular biology, 27*(8-9), 745-755

Haddi, K., Berger, M., Bielza, P., Cifuentes, D., Field, L. M., Gorman, K., & Bass, C. (2012). Identification of mutations associated with pyrethroid resistance in the voltage-gated sodium channel of the tomato leaf miner (Tuta absoluta). *Insect biochemistry and molecular biology, 42*(7), 506-513.

Hafez, A. M. (2021). First evaluation of field evolved resistance to commonly used insecticides in house fly populations from Saudi Arabian dairy farms. *Insects, 12*(12), 1120.

Horowitz, A. R., Weintraub, P. G., & Ishaaya, I. (1998). Status of pesticide resistance in arthropod pests in Israel. *Phytoparasitica, 26*, 231-240.

Huggett, J., Dheda, K., Bustin, S., & Zumla, A. (2005). Real-time RT-PCR normalisation; strategies and considerations. *Genes & Immunity, 6*(4), 279-284.

Ingham, V. A., Elg, S., Nagi, S. C., & Dondelinger, F. (2021). Capturing the transcription factor interactome in response to sub-lethal insecticide exposure. *Current Research in Insect Science, 1*, 100018.

Kozera, B., & Rapacz, M. (2013). Reference genes in real-time PCR. *Journal of applied genetics, 54*, 391-406.

LeOra Software (1989). POLO-PC: a user's guide to probit and logit analysis. LeOra Software, Berkeley, CA.

Leake, L. D., Buckley, D. S., Ford, M. G., & Salt, D. W. (1985). Comparative effects of pyrethroids on neurons of target and non-target organisms. *Neurotoxicology, 6*(2), 99-116.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *Methods, 25*(4), 402-408.

Loughney, K., Kreber, R., & Ganetzky, B. (1989). Molecular analysis of the para locus, a sodium channel gene in *Drosophila*. *Cell, 58*(6), 1143-1154..

Li, W., Freudenberg, J., Suh, Y. J., & Yang, Y. (2014). Using volcano plots and regularized-chi statistics in genetic association studies. *Computational biology and chemistry, 48*, 77-83.

Martinez-Torres, D., Foster, S. P., Field, L. M., Devonshire, A. L., & Williamson, M. S. (1999). A sodium channel point mutation is associated with resistance to DDT and pyrethroid insecticides in the peach-potato aphid, Myzus persicae (Sulzer) (Hemiptera: Aphididae). *Insect Molecular Biology, 8*(3), 339-346.

Mansee, A. H., & Montasser, M. R. (2003). Maximizing toxicity of certain insecticides against *Tribolium castaneum* (Herbst). *Journal of Agricultural and Marine Sciences [JAMS], 8*(1), 27-34.

Mao, M., Xue, Y., He, Y., Zhou, X., Hu, H., Liu, J., & Ma, J. (2021). Validation of reference genes for quantitative real-time PCR normalization in Ananas comosus var. bracteatus during chimeric leaf development and response to hormone stimuli. *Frontiers in Genetics, 12*, 716137.

Marcombe, S., Fustec, B., Cattel, J., Chonephetsarath, S., Thammavong, P., Phommavanh, N., & Brey, P. T. (2019). Distribution of insecticide resistance and mechanisms involved in the arbovirus vector Aedes aegypti in Laos and implication for vector control. *PLoS neglected tropical diseases, 13*(12), e0007852.

Miyazaki, M., Ohyama, K., Dunlap, D. Y., & Matsumura, F. (1996). Cloning and sequencing of the para-type sodium channel gene from susceptible and kdr-resistant German cockroaches (Blattella germanica) and house fly (*Musca domestica*). *Molecular and General Genetics MGG, 252*, 61-68.

Moustafa, H. Z., Rizk, A. M., & El-Lebody, K. A. (2015). Biological studies of Earias insulana (Boisd.) field strains at different constant temperature degrees. *Egyptian Academic Journal of Biological Sciences. A, Entomology, 8*(3), 127-135.

Moustafa, H.Z., Kandil, M.A. & El-Bassouiny, H. M (2021). Genetic profile characterization of Earias insulana (Lepidoptera: Noctuidae) in Egypt. *Egypt. J. Plant Prot. Res. Inst. 4*(2): 341–351.

Nazemi, A., Khajehali, J., & Van Leeuwen, T. (2016). Incidence and characterization of resistance to pyrethroid and organophosphorus insecticides in Thrips tabaci (Thysanoptera: Thripidae) in onion fields in Isfahan, Iran. *Pesticide biochemistry and physiology, 129*, 28-35.

Paramasivam, M., & Selvi, C. (2017). Laboratory bioassay methods to assess the insecticide toxicity against insect pests-A review. *Journal of Entomology and Zoology Studies, 5*(3), 1441-1445.

Peredo, E. L., & Cardon, Z. G. (2020). Shared up-regulation and contrasting down-regulation of gene expression distinguish desiccation-tolerant from intolerant green algae. *Proceedings of the National Academy of Sciences, 117*(29), 17438-17445.

Pfaffl, M. W., Horgan, G. W., & Dempfle, L. (2002). Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic acids research, 30*(9), e36-e36.

Prusty, A. K., Meena, D. K., Mohapatra, S., Panikkar, P., Das, P., Gupta, S. K., & Behera, B. K. (2015). Synthetic pyrethroids (Type II) and freshwater fish culture: Perils and mitigations. *International aquatic research, 7*, 163-191.

Qin, H., Dong, K., Huang, J., He, S., & Wu, J. (2021). Identification of reference genes for gene expression analysis at different developmental stages of the bumblebee Bombus terrestris (Hymenoptera: Apidae). *Apidologie, 52*(4), 825-836.

Reigart, J. R., & Roberts, J. R. (1999). Organophosphate insecticides. Recognition and management of pesticide poisonings,, 34-40

RM, S., & El-Ghobary, A. M. (2019). Molecular diversity in Earias insulana populations from different Egyptian governorates. *Mol Divers, 4*(3)

Saad, A., Tayeb, E., Ibrahim, M., & Elhabal, N. (2015). Biological and Chemical Techniques for Integrated Control of the Spiny Bollworm, Earias insulana (Boisd.)(Lep., Noctuidae). *Journal of the Advances in Agricultural Researches, 20*(4), 586-601

Salama, M. A., Abd El-Baki, M. A., El-Naggar, J. B., & El-Naggar, E. Y. (2013). Efficiency of some insecticides sequence on cotton bollworms and histopathological effects of some biocides on pink bollworm larvae. *Egypt. J. Agric. Res, 91*(2), 429-448

Silver, N., Best, S., Jiang, J., & Thein, S. L. (2006). Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC molecular biology, 7*(1), 1-9.

Sonoda, S., Shi, X., Song, D., Zhang, Y., Li, J., Wu, G., & Gao, X. (2012). Frequencies of the M918I mutation in the sodium channel of the diamondback moth in China, Thailand and Japan and its association with pyrethroid resistance. *Pesticide biochemistry and physiology, 102*(2), 142-145.

Skern, R., Frost, P., & Nilsen, F. (2005). Relative transcript quantification by quantitative PCR: roughly right or precisely wrong?. *BMC molecular biology, 6*(1), 1-3.

Tan, J., Liu, Z., Nomura, Y., Goldin, A. L., & Dong, K. (2002). Alternative splicing of an insect sodium channel gene generates pharmacologically distinct sodium channels. *Journal of Neuroscience, 22*(13), 5300-5309

Tan, J., Liu, Z., Tsai, T. D., Valles, S. M., Goldin, A. L., & Dong, K. (2002). Novel sodium channel gene mutations in Blattella germanica reduce the sensitivity of expressed channels to deltamethrin. *Insect biochemistry and molecular biology, 32*(4), 445-454

Teng, X., Zhang, Z., He, G., Yang, L., & Li, F. (2012). Validation of reference genes for quantitative expression analysis by real-time RT-PCR in four lepidopteran insects. *Journal of Insect Science, 12*(1), 60.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., & Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology, 3*(7), 1-12

VanGuilder, H. D., Vrana, K. E., & Freeman, W. M. (2008). Twenty-five years of quantitative PCR for gene expression analysis. *Biotechniques, 44*(5), 619-626

Wang, W., Chen, K., & Xu, C. (2006). DNA quantification using EvaGreen and a real-time PCR instrument. *Analytical Biochemistry, 356*(2), 303-305

Wang, Y., Shan, Q., Meng, Y., Pan, J., & Yi, S. (2017). Mrpl10 and Tbp are suitable reference genes for peripheral nerve crush injury. *International journal of molecular sciences, 18*(2), 263.

Wang, H., Foquet, B., Dewell, R. B., Song, H., Dierick, H. A., & Gabbiani, F. (2020). Molecular characterization and distribution of the voltage-gated sodium channel, Para, in the brain of the grasshopper and vinegar fly. *Journal of Comparative Physiology A, 206*, 289-307

Warmke, J. W., Reenan, R. A., Wang, P., Qian, S., Arena, J. P., Wang, J., & Cohen, C. J. (1997). Functional expression of *Drosophila* para sodium channels: modulation by the membrane protein tipE and toxin pharmacology. *The Journal of general physiology, 110*(2), 119-133

Williamson, M. S., Martinez-Torres, D., Hick, C. A., & Devonshire, A. L. (1996). Identification of mutations in the housefly para-type sodium channel gene associated with knockdown resistance (kdr) to pyrethroid insecticides. *Molecular and General Genetics MGG, 252*, 51-60

Wong, M. L., & Medrano, J. F. (2005). Real-time PCR for mRNA quantitation. *Biotechniques, 39*(1), 75-85.

Wu, C. F., & Ganetzky, B. (1980). Genetic alteration of nerve membrane excitability in temperature-sensitive paralytic mutants of *Drosophila* melanogaster. *Nature, 286*(5775), 814-816.

Zhang, Q., Gao, B., Qu, C., Luo, C., Wang, J., & Wang, R. (2022). Selection and Evaluation of Reference Genes for miRNA Expression Analysis in Bemisia tabaci Under Insecticide Tolerance. *Frontiers in Genetics, 13*, 899756

Zhao, S., & Fernald, R. D. (2005). Comprehensive algorithm for quantitative real-time polymerase chain reaction. *Journal of computational biology, 12*(8), 1047-1064

Zhao, N., Xu, J., Jiao, L., Qiu, M., Zhang, J., Wei, X., & Fan, M. (2021). Transcriptome-based selection and validation of reference genes for gene expression analysis of Alicyclobacillus acidoterrestris under acid stress. *Frontiers in Microbiology, 12*, 731205

Zhou, C., Yang, H., Wang, Z., Long, G. Y., & Jin, D. C. (2019). Protective and detoxifying enzyme activity and ABCG subfamily gene expression in *Sogatella furcifera* under insecticide stress. *Frontiers in physiology, 9*, 1890

Zuo, Y., Peng, X., Wang, K., Lin, F., Li, Y., & Chen, M. (2016). Expression patterns, mutation detection and RNA interference of Rhopalosiphum padi voltage-gated sodium channel genes. *Scientific reports, 6*(1), 30166