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Research Article

# Investigating *Plutella xylostella* Resistance to Insecticides: Sensitivity Shifts to Cypermethrin, Profenofos, and Acetamiprid

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## Abstract

The Diamondback Moth, *Plutella xylostella* (L.), is a notorious pest posing a substantial threat to global agriculture due to high resistance to conventional insecticides. Some P450 related enzymes are responsible to help the Diamondback Moth to metabolize and neutralize pesticides, contributing to the resistance problem. Our study aimed to understand the expression of the cytochrome P450 genes in response to Cypermethrin, Profenofos, and Acetamiprid. The *P. xylostella* larvae were collected from three different locations, representing varying resistance histories, and subjected them to controlled laboratory conditions for insecticide susceptibility and gene expression analysis. Expression levels of three genes –CYP321E1, CYP4M22, and CYP9G2- involved in the resistance in response to insecticide exposure were investigated. The analysis revealed significant variations in the gene expression patterns among the different populations and across the insecticides tested. The CYP4M22 gene displayed significant different expression patterns depending on the specific insecticide and population. The CYP321E1 and CYP9G2 genes also showed increased expression levels with prolonged exposure to the insecticides, indicating a potential adaptive mechanism for metabolizing pesticides. These findings emphasized the complex nature of insecticide resistance and suggested that gene expression patterns can differ significantly across populations, reflecting the unique evolutionary pressures in each environment.

## Keywords

Insecticide Resistance, Diamondback Moth, Pests, Cytochrome P450, Gene Expression

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# 1. Introduction

The Diamondback moth (*Plutella xylostella*) poses a significant threat to Brassicaceae plants worldwide due to its ubiquitous nature, high adaptation, and rapid reproduction [1]. In the some parts of the world, outbreaks of the moth have led to devastating losses in crop yields [2]. Yet, efforts to control this pest infestation face challenges due to the high resistant ability to the various insecticides. Researches have indicated variations in the effectiveness of different insecticides, with Cypermethrin, Profenofos, and Acetamiprid emerging as promising options for the Diamondback moth control [3].

To encounter with the pests, farmers often resort to increase pesticide levels or using mixtures, resulting in reduced effectiveness and development of cross-resistance [4, 5]. It is now well understand that traditional insecticides have limited long-term efficacy [6]. Recent studies suggested that intermittent use of insecticides may help mitigate cross-resistance, but careful management practices are crucial due to observed cross-resistance among certain compounds.

In eukaryotic cells, one important family of enzyme e.g. cytochrome P450s (CYP450) are involved in the context of responses to the xenobiotic substances [7]. These enzymes play pivotal roles in detoxification processes, enhancing the cell's ability to cope with potentially harmful foreign compounds [8, 9]. Genetic variations within the cytochrome P450 enzyme family, specifically in response to pesticide exposure, result in the emergence of three distinct gene variants: *CYP4M22*, *CYP321E1*, and *CYP9G2* [10]. This variability stems from the intricate interplay of nucleotide sequences within the genomic region encoding cytochrome P450 enzymes, influencing the enzymatic activity and substrate specificity of these variants in response to the pesticides [10].

Within the insect species, such as *P. xylostella*, the expression of cytochrome p450 superfamilly genes is notably up-regulated in response to pesticides [10]. This up-regulation is an adaptive response aimed at countering the toxic effects of these xenobiotics and maintaining cellular homeostasis [11]. However, this heightened expression of detoxification genes can also lead to the development of pesticide resistance in *P. xylostella* populations [12].

Understanding the molecular mechanisms underlying pesticide resistance in insects like *P. xylostella* is crucial for the development of sustainable pest control strategies [11]. By elucidating the specific genes and enzymes involved in detoxification processes, researchers can identify potential targets for novel insecticides or develop alternative approaches to pest management that minimize the risk of resistance development [10]. Additionally, studying the interplay between detoxification pathways and other cellular processes can provide insights into the broader implications of pesticide exposure on insect physiology and ecology.

Based on these, our study aims to assess three specific genes to investigate populations' *P. xylostella* in Iran in terms of their history with Cypermethrin, Profenofos, and Acetamiprid, and to explore cabbage's potential for resistance to the chemical insecticides. We evaluated the susceptibility to insecticides across three cabbage larval populations in the fields located in different places associated with the outbreaks of the pest.

# 2. Materials and Methods

## 2.1. Collection and Colonization Procedures

To establish a colony, larvae of cabbage weevils sourced from cabbage fields in the central provinces of Arak (34 °04'16.6"N49 °48'47.9"E), Alborz (35 °46'59.6"N50 °55'04.2"E), and Qazvin (36 °15'12.6"N 49 °56'50.2"E) were collected and subsequently transported to the Zabol Research Institute laboratory. These larvae underwent two generations of rearing on white cabbage. The white cabbage specimens were placed within breeding containers designed for cabbage weevil rearing, and then the weevil larvae were transferred onto them. The cultivation of cabbage willow occurred under controlled laboratory conditions, with an 8-16 h dark-light period, at 25 ℃. Each of the three cabbage willow populations was bred separately within enclosed net cages (60 ×60 ×90 cm).

## 2.2. Insecticide Resistance for Gene Expression Analysis

For the examination of pesticide resistance gene expression, three insecticides were employed in the study. These compounds were included Cypermethrin (EC 40%; Green Spectrum Products Co.), Profenophos (EC 40%; Meshkfam Co.), and Acetamiprid (20% SP; Plant Co.).

## 2.3. Lethal Concentrations

To conduct the biometric test, we employed the leaf disc immersion method on three-day-old third instar larvae [13]. We investigated five compound concentrations to determine the lethal concentrations (LC50). Preliminary tests were conducted to establish a loss range of 25-75% in each water sample containing insecticides. Five final concentrations were calculated using logarithmic relationships based on the formulated substance to determine the lethal concentration of 50% [14].

Small leaves from white cabbage seedlings were isolated, and their petioles were moistened with wet cotton and wrapped in aluminum foil to preserve leaf moisture. The leaves were then immersed in prepared solutions of each insecticide for 30 sec. Control solutions consisting of water and fire tween emulsifier at a 0.02% rate were utilized. Following drying at room temperature, ten third instar larvae were placed in a petri dish with a ventilated lid. Prior to this,

the larvae underwent a 2 h fasting period. The number of larval deaths and samplings for gene expression analysis were evaluated at two time periods of 48 and 72 h post-biometry.

## 2.4. Gene Expression Analysis

Total RNA extraction from insect samples was performed using a column extraction kit (Denazist, Iran). The extracted RNA underwent qualitative evaluation using a 1% agarose gel and quantitative assessment using NanoDrop™ 1000 Spectrophotometer (Thermo scientific 2000c, USA) at wavelengths of 230, 260, and 280. Following RNA assessments, DNase (Fermentase, USA) treatment was performed to eliminate any potential contamination. The mixture was incubated in a Thermo cycler (PeQlab, 96Grad) at 37 ℃ for 30 min to activate the DNase enzyme. Finally, EDTA was used to inactivate the DNase enzyme.

For cDNA synthesis, the extracted RNA was mixed with OligodT primer and Random primer, volume by DEPC water. The mixture was then incubated at 65 ℃ for 10 minutes to facilitate the disruption of RNA secondary structures and allow the establishment of dT primers. Subsequently, a reverse transcriptase enzyme mixture (SMOBio, Taiwan) was added to the samples. The mixture was then incubated for 90 min at 42 ℃ (cDNA synthesis step). To halt the reaction, the samples were subjected to a termination stage by heating them at 70 ℃ for 5 min. The resulting cDNAs were properly stored in a -80 ℃ freezer until they were utilized in the Real-Time PCR process.

Geneious R23 and Oligoanalyzer software were utilized to design primers. The designed primers underwent primer BLAST analysis in the Gene Bank (NCBI, GenBank) to ascertain their specificity (Table 1).

***Table 1.*** *Primer specifications for the candidate genes.*

| Gene | Sequence | m | Amplicon | Accession no. |
|---|---|---|---|---|
| CYP321E1 | F: CTGGCTCACATTCTACGCAAC | 1 | 94 | KC626090 |
| | R: TGGCAGGTCTTATGATGAGGG | 1 | | |
| CYP9G2 | F: TGGTGGGAAACTCTTTGACGA | 0 | 147 | AB096739 |
| | R: TGTTTTCGCGATGCTGCATAG | 0 | | |
| CYP4M22 | F: CAGTGCAGGATAAAGTGGTGC | 0 | 111 | EU189050.1 |
| | R: CCTTGATACAGCACTCGAGGT | 0 | | |
| Actin | F: CCGTGCCCATCTACGAAGGTTA | | 128 | NM_001309126 |
| | R: AGCGGTGGTGGTGAAGGAGTATC | | | |

## 2.5. Real-time Quantitate PCR

In this study, real-time PCR reactions were conducted using the ABI StepOne Real-Time PCR machine (Applied Biosystems, USA). The reaction mix had a final volume of 10 µl, comprising 5 µl of Taq SYBR Green mix (Ampliqon 2x SYBR Green High ROX), 0.2 µl of reverse primers, and 2 µl of synthesized cDNA template.

The real-time PCR protocol involved a series of precise thermal cycling steps. Initially, the reaction mixture was subjected to a denaturation step at 95 ℃ for 15 min. This step effectively denatures the DNA, separating the double-stranded DNA into single strands, ready for amplification. The standard thermal cycling protocol was initial 15 min denaturation step at 94 ℃, 40 cycles of 95 ℃ for 15 s, 40 s annealing step at 59 ℃, and 20 s extension step at 72 ℃. The 2-ΔΔCt method devised by [15] was employed to quantify the alterations in the expression levels of target genes. This involved the subtraction of the mean cycle threshold (Ct) value of the primary gene from that of the internal control gene, yielding the CtΔ value. The relative expression levels of the target genes were then expressed as the mean ± standard deviation across various treatment conditions.

## 2.6. Statistical Analysis

Data were tested for normality using Kolmogorov-Smirnov test. We used multivariate ANOVA analysis with the type of pesticide, insect populations, and time points of biometry as the fixed factors and expression of the genes as dependent variables followed by Duncan's multiple range tests. Also, Paired-samples T-tests were used to investigate differences in expression levels of the genes between the time points of biometry. Data are reported as mean ±SE and all the statistical analyses were conducted using SPSS (version 22.0; IBM Statistics) at the significant level of $p$ values < 0.05, unless otherwise stated.

# 3. Results

## 3.1. Assessing Insecticide Toxicity Across Willow Cabbage Populations

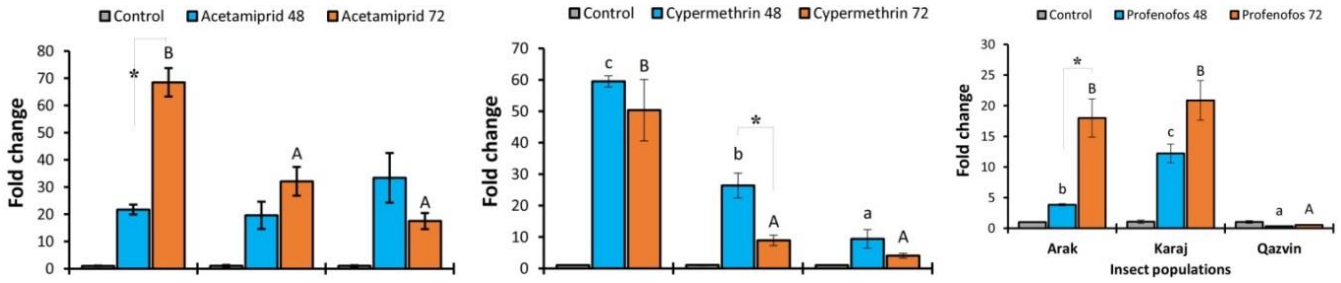
The initial examination of three willow cabbage populations originating from Qazvin, Karaj, and Arak, subjected to the insecticides Acetamiprid, Cypermethrin, and Profenofos, revealed notable variations in lethal concentrations (LC50). The Karaj population exhibited the highest susceptibility to Acetamiprid, while the Qazvin and Arak populations demonstrated elevated LC50 values for Cypermethrin and Profenofos, respectively. Notably, Acetamiprid displayed the highest toxicity across all populations, with Profenofos and Cypermethrin exhibiting comparatively lower lethality. The LC50 values for Acetamiprid after 48 h of larval exposure highlighted Qazvin as the least sensitive (19.586 mg a.i./L), while Arak and Karaj populations displayed greater susceptibility, with 529.13 mg a.i./L and 491.61 mg a.i./L, respectively. Over 72 h, Arak exhibited the highest sensitivity (405.35 mg a.i./L), contrasting with Karaj as the most resistant (524 mg a.i./L) to Acetamiprid.

In terms of Cypermethrin toxicity after 48 h, Qazvin displayed resistance (LC50 = 5036.4 mg a.i./L), while Karaj was the most sensitive (LC50 = 3049.12 mg a.i./L). Further assessment at 72 h confirmed Qazvin's resistance and Arak's sensitivity, albeit with statistically significant differences. Regarding Profenofos, at 48 h evaluation, Karaj (LC50 = 2566.92 mg a.i./L) and Arak (LC50 = 5494 mg a.i./L) populations emerged as the most sensitive and resistant, respectively. Confirmatory analysis at 72 h reinforced these findings, though statistical significance was not observed due to overlapping confidence limits of LC50 values.

## 3.2. Gene Expression

### 3.2.1. CYP321E1 Transcription Assay

Expressions of CYP321E1 gene in the insects of three different populations in response to three common insecticides are illustrated in Figure 1. The statistics showed the significant effects of pesticides ($F_{3,72} = 93.31$, $p < 0.001$) and populations ($F_{2,72} = 58.33$, $p < 0.001$) on the CYP321E1 expression; however, time of the samplings had no effects ($F_{1,72} = 3.65$, $p > 0.05$). Arak population showed higher levels of CYP321E1 expression in response to Acetamiprid and Cypermethrin than the two other insect populations. On the other hand, Qazvin population had the lowest expression when exposed to Cypermethrin and Profenofos (Figure 1). Higher expressions of the gene at 72 h sampling time in the insects from the Arak population were also observed in response to Acetamiprid ($t_2 = -7.50$, $p < 0.05$) and Profenofos ($t_2 = -4.29$, $p < 0.05$). In an exception, the 72 h sampling time in the Karaj population showed lower levels of expression than the 48 h ($t_2 = 4.53$, $p < 0.05$) (Figure 1). Also, significant interaction effects of pesticides ×populations ($F_{6,72} = 21.50$, $p < 0.001$), pesticides ×sampling time ($F_{3,72} = 12.85$, $p < 0.001$), populations ×sampling time ($F_{2,72} = 12.69$, $p < 0.001$), and pesticides ×populations ×sampling time ($F_{6,72} = 8.98$, $p < 0.001$) were measured.




* shows significant differences ($p < 0.05$) between the sampling times in each population.

***Figure 1.*** *The expression levels (±SE) of the CYP321E1 gene in the three populations of Diamondback Moth in response to three insecticides at 48 h and 72 h challenge times. Control groups are included for each population to establish a baseline gene expression. Small and capital letters indicate significant differences at 48 h and 72 h sampling times between the populations, respectively.*

### 3.2.2. *CYP4M22* Transcription Assay

The expression patterns of the *CYP4M22* of the three insect populations in response to the pesticides at two sampling time are shown in Figure 2. The results showed significant effects of the main variables including pesticides ($F_{3,72} = 31.62$, $p < 0.001$), populations ($F_{2,72} = 19.53$, $p < 0.001$), and sampling times ($F_{1,72} = 24.18$, $p < 0.001$) on the *CYP4M22* expression levels. Also, all of the interactions were significant; pesticides ×populations ($F_{6,72} = 25.98$, $p < 0.001$), pesticides ×sampling time ($F_{3,72} = 10.14$, $p < 0.001$), populations ×sampling time ($F_{2,72} = 7.40$, $p < 0.01$), and pesticides ×populations ×sampling time ($F_{6,72} = 6.90$, $p < 0.001$). Interestingly, the Karaj population exhibited lower gene expression in response to the both Acetamiprid and Cypermethrin than the other populations. In contrast, the Qazvin pop-

ulation demonstrated significantly lower expression, with readings at 48 h and 72 h dropping below the control level, indicating almost no response to Profenofos. In response to the later pesticide, Profenofos, the Karaj population displayed the highest level of expression at 72 h sampling time. When the times of sampling were considered, the Arak ($t_2$ = -3.38, $p < 0.05$) and Karaj ($t_2$ = -7.64, $p < 0.05$) populations at 72 h showed significant higher levels of the *CYP4M22* expression than the 48 h in response to Profenofos.

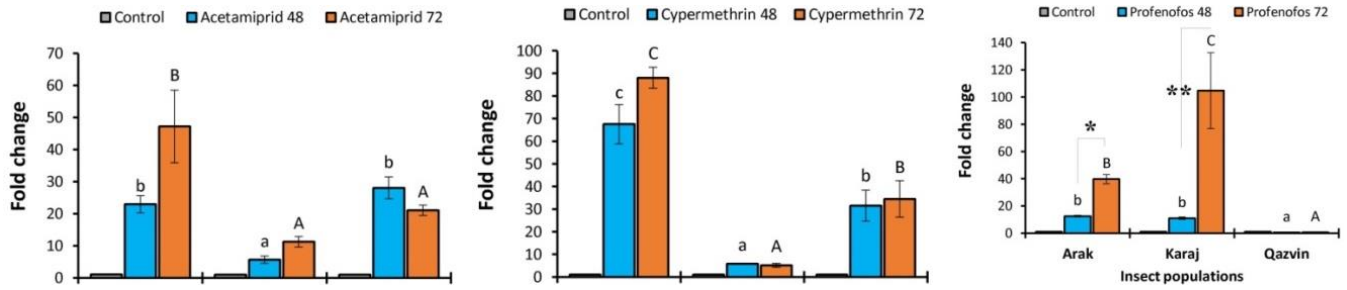



***Figure 2.*** *The expression levels (±SE) of the CYP4M22 gene in the three populations of Diamondback Moth in response to three insecticides at 48 h and 72 h challenge times. Control groups are included for each population to establish a baseline gene expression. Small and capital letters indicate significant differences at 48 h and 72 h sampling times between the populations, respectively.*

* shows significant differences ($p < 0.05$) between the sampling times in each population.
** shows significant differences ($p < 0.01$) between the sampling times in each population.

### 3.2.3. *CYP9G2* Transcription Assay

The expression of the *CYP9G2* gene appears to be closely linked to the duration of exposure to the insecticides ($F_{1,72}$ = 47.84, $p < 0.001$) (Figure 3). After a 72 h exposure period, we found significant higher levels of the gene expression in the Karaj ($t_2$ = -7.32, $p < 0.05$) and Qazvin ($t_2$ = 6.93, $p < 0.05$) populations in response to the Cypermethrin, and Qazvin population alone when exposed to the Profenofos ($t_2$ = -20.34, $p < 0.01$) than the 48 h sampling time.

Pesticides caused significantly different pattern in the *CYP9G2* gene expression ($F_{3,72}$ = 44.82, $p < 0.001$). Also, the expression levels affected by the different three insects' populations ($F_{2,72}$ = 74.90, $p < 0.001$). All of the interaction effects were also significant including pesticides ×populations ($F_{6,72}$ = 23.12, $p < 0.001$), pesticides ×sampling time ($F_{3,72}$ = 13.34, $p < 0.001$), populations ×sampling time ($F_{2,72}$ = 14.23, $p < 0.001$), and pesticides ×populations ×sampling time ($F_{6,72}$ = 3.53, $p < 0.01$).

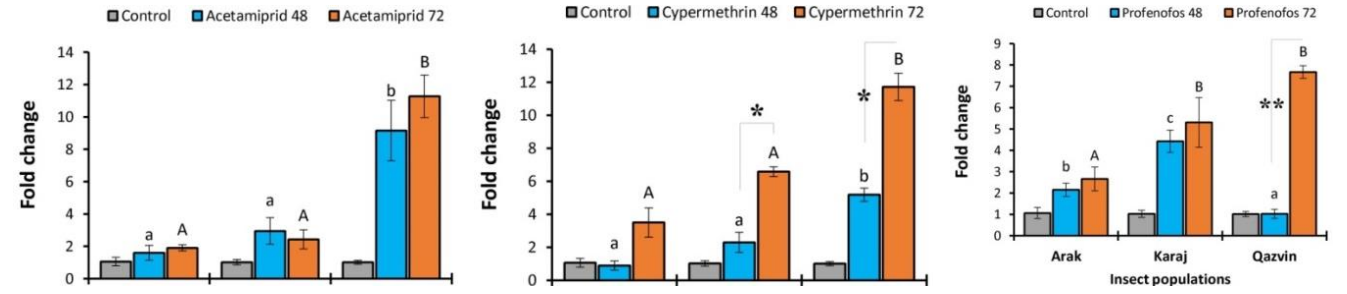



***Figure 3.*** *The expression levels (±SE) of the CYP4M22 gene in the three populations of Diamondback Moth in response to three insecticides at 48 h and 72 h challenge times. Control groups are included for each population to establish a baseline gene expression. Small and capital letters indicate significant differences at 48 h and 72 h sampling times between the populations, respectively.*

* shows significant differences ($p < 0.05$) between the sampling times in each population.
** shows significant differences ($p < 0.01$) between the sampling times in each population.

## 4. Discussion

It is now well understood that the Cytochrome P450 monooxygenases (P450s) in Diamondback Moth are involved in the insecticide resistance [10]. Here, in the present study we showed the expression patterns of P450-related genes, including *CYP9G2* and *CYP4M22* in the three different populations of Diamondback moth in response to three effective pesticides. This distinct variation in gene expression across different populations underscores the critical role that genetic background and strain diversity play in determining the response to insecticidal challenges [16]. P450s, a superfamily of heme-containing proteins, are ubiquitous across the spectrum of life, spanning from bacteria to humans [17]. This extensive group serves as a vital metabolic system, essential in the oxidative metabolism of both foreign and internal compounds [8]. In a previous study [18] a comprehensive transcriptomic analysis was conducted on three strains of the diamondback moth characterized by varying degrees of resistance to pesticides: low, moderate, and highly resistant strains. The study revealed that 19 individual genes belonging to the cytochrome P450 superfamily were significantly overexpressed in the pesticide-resistant strains. This finding underscores the pivotal role of these genes in mediating resistance to pesticides within the insect population [18].

Variable in the genes expression levels across different populations and insecticides have been observed in the present study. This variability could be attributed to distinct regulatory mechanisms, potentially influenced by epigenetic factors [19]. The overexpression of P450s genes in response to pesticide exposure represents a fundamental aspect of the cellular defense mechanisms against xenobiotics in eukaryotic organisms [16]. However, this adaptive response can also contribute to the development of pesticide resistance [16], underscoring the importance of comprehensive studies aimed at deciphering the molecular basis of resistance mechanisms and informing the design of more effective and sustainable pest management strategies. These findings suggest that the regulatory mechanisms governing P450s gene expression are complex and likely involve both genetic and epigenetic factors.

The *CYP9G2* gene in *Plutella xylostella* was discovered and thoroughly examined in previous researches [20]. In accordance to our results, it has been reported a noteworthy up-regulation of this gene in the herbicide-resistant strains. Also *CYP4M22* gene showed a significant increase in expression, more than doubling, when exposed to certain herbicides [21]. In this study, we observed different levels of expressions of these two genes in the insects of three populations suggesting that distinct regulatory mechanisms govern *CYP9G2* and *CYP4M22* in response to different insecticides. These findings demonstrate the complexity of gene regulation in *P. xylostella* and highlight the necessity of understanding genetic diversity and its impact on pesticide resistance.

In the present study, some similarities have been observed in the expression patterns of the genes in response to Cypermethrin and Acetamiprid. This consistency in the expression behavior between theses pesticieds, and their distinct variance from Profenofos, suggests a common regulatory mechanism in response to these two insecticides. These results imply that the regulatory elements governing P450s gene expression -including gene promoters, enhancers, transcription factors, and their binding sites- may share significant similarities between Cypermethrin and Acetamiprid [22]. This observation reinforces the idea that regulatory pathways are insecticide-specific, and understanding these variations can be critical for addressing pesticide resistance in these populations of Diamondback moths.

The phenomenon of pesticide resistance arises due to genetic variations that confer a selective advantage to individuals carrying these resistance-conferring alleles [10]. In the case of *P. xylostella*, repeated exposure to pesticides exerts evolutionary pressure on the insect population, favoring the survival and proliferation of individuals with genetic mutations that enhance their ability to metabolize or otherwise neutralize the toxic effects of the pesticides [23]. As a result, over time, the frequency of these resistance alleles within the population increases, leading to reduced efficacy of pesticide treatments and posing significant challenges for pest management strategies.

In conclusion, our study sheds light on the intricate dynamics of insecticide resistance in Diamondback Moth populations, revealing population-specific variations in gene expression and susceptibility to common insecticides. The differential responses observed underscore the need for targeted pest management strategies that consider the genetic diversity and adaptive mechanisms of the pest. By elucidating the molecular basis of resistance, we provide valuable insights for the development of more effective and sustainable pest control measures. Future research should continue to explore the interplay between genetic and environmental factors to enhance our understanding of pest resistance mechanisms and help the design of innovative control strategies.

## Abbreviations

| | |
|---|---|
| P450 | Cytochrome P450 |
| CYP321E1 | Novel Cytochrome P450 Gene Number 321 |
| CYP4M22 | Novel Cytochrome P450 Gene Number 4 |
| CYP9G2 | Novel Cytochrome P450 Gene Number 9 |
| LC50 | Lethal Concentration 50 |
| EC | Emulsifiable Concentrate |

## Conflicts of Interest

The authors declare no conflicts of interest.

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