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Biological and physiological mechanisms of insect resistance in *Pseudococcus viridis*: focus on cytochrome P450, and carboxylesterase enzyme activities

Ahmed M. Ali [1, 2 *], Khalid Elhussein M. Idris [2], Mutaman Ali A. kehail [2], Thaer A. Salih [3]

[1] Karma Education Department in Anbar Governorate, Iraqi Ministry of Education, Al-Fallujah, Iraq
[2], College of Education, University of Gezira, Wad Madani, Sudan
[3] Iraqi Ministry of Higher Education, College of Education for Pure Sciences, Anbar University, Anbar, Iraq

[*] ah988medm0@gmail.com

ABSTRACT

Insect resistance in *Pseudococcus viridis* (citrus mealybug) represents a primary obstacle to conventional citrus production. This work evaluated the involvement of two main enzyme systems responsible for detoxification, cytochrome P450 and carboxylesterase, in the pesticides resistance development. Biochemical assays and enzyme kinetic estimations showed that the resistant populations of *P. viridis* reflects the activity of these enzymes significantly increased expression in insect detoxification metabolism and its essential function. Our findings demonstrate important insights into the enzymatic mechanisms underlying resistance, providing a basis for identifying biochemical targets for industry to develop improved pest management strategies (IPM) in combination, and ensure the long-term effectiveness of pest control strategies.

Keywords: Citrus mealybug, *Pseudococcus viridis*, cytochrome P450, carboxylesterase, detoxifications, integrated pest management (IPM)

INTRODUCTION

The citrus mealybug, *Pseudococcus viridis*, (*P. viridis*) is a serious pest that infected citrus crops globally. It not only feeds on plant sap but also excretes pollen, causing wet mold growth that compromises the normal pathway of photosynthesis (Olabiyi et al., 2024; Olabiyi et al., 2023; Sharaf and Meyerdirk, 1987). At high pest populations, these pests can cause remarkable crop losses and economic damage (Borkakati et al.). Insecticides remain the dominant method of insect control due to the impact of the pathogen on citrus crops productions. However, the use of traditional pesticides and insecticides has elevated resistance, making many conventional pesticides ineffective (Venkatesan et al., 2016).

In control of *P. Viridis* the pesticide resistance in these pests is a complex process, having multiple enzymes and multiple detoxification pathways (Mruthunjayaswamy et al., 2019). The primary enzymes include included in this resistance processes, cytochrome P450, and carboxylesterases (Srivastava et al., 2023). These enzymes make a contribution to the immune reaction by using activating pollution before they attain their targets inside the insect's body (Oberemok et al., 2024). Cytochrome P450 enzymes facilitate oxidative cleansing (Kishk et al., 2024b), carboxylesterases hydrolyze ester bonds in pesticides (Gong et al.,

*Corresponding author
Ahmed M. Ali,
Iraqi Ministry of Education, Karma Education Department in Anbar Governorate
e-mail: ah988medm0@gmail.com

2016), and GST conjugates glutathione to pesticides, making them easy to be excreted (Chen et al., 2023). Understanding how those enzymes interact is essential for the improvement of effective pest manipulate techniques.

STUDY OBJECTIVES

The intention of this study became to make clear the role of cytochrome P450, and carboxylesterase enzymes in *P. Viridis* insect resistance. These enzymes are concept to paintings collectively to confer resistance. By quantifying the interest of every enzyme, we are seeking to discover potential biochemical goals beneficial in integrated pest management (IPM) to disrupt resistance techniques and improve sensitivity of pests to pesticides.

METERIALS AND METHODS

SAMPLE COLLECTION

To capture the ones populations which are surely displaying pesticide resistance, samples of *P. Viridis* have been collected from citrus plantations which might be a notion to apply lots of insecticides. These samples, that are consultant of resistant populations, had been gathered from orchards wherein pesticide use has been confirmed. Control populations, which are thought to be insecticide-susceptible, were collected for assessment from orchards or locations with little to no current pesticide use. Each pattern included roughly 100 mature mealybugs that were immediately preserved in ethanol and stored at -20°C to preserve their biochemical integrity for subsequent analysis.

To guarantee there would be enough for testing, both susceptible and resistant populations were cultivated in a lab setting after collection. In controlled laboratory conditions, the mealybugs were raised on citrus plants with a 14:10 hour light-dark photoperiod, a temperature of 25 ± 1°C, and a relative humidity of 70–80%. Consistent growth was made possible by these regulated settings, which also made the ensuing exposure tests easier.

EXPOSURE TO PESTICIDES AND EXPERIMENTAL CONDITIONS

P. viridis populations were split up into treatment groups and subjected to different pesticides in a lab setting to study the development of pesticide resistance under circumstances that mimic real-world exposure. Three different categories of pesticide treatments were created:

Group I: Controls that are susceptible, Typically, pests are not exposed to insecticides or pesticides.

Group ii: Conventional Pesticide (Older Generation): A common organophosphate used to control citrus pests, malathion, was picked to symbolize traditional pesticides because it is known to cause resistance in many insect populations.

Group iii: Newer Conventional Pesticide: To symbolize more recent but widespread chemical interventions, chlorpyrifos, another organophosphate that is still used in pest control, was chosen.

Group iv: Nano-Pesticide: To assess the effect of a different pesticide pathway on *P. viridis* resistance, a novel nanoparticle-based pesticide called nano zinc oxide (ZnO) was employed.

Sub-lethal levels of these pesticides were found in every *P. viridis* institution. To mimic chronic, localized publicity levels that could lead to resistance mechanisms without causing immediate population losses, these concentrations were carefully set to be low enough to avoid significant mortality. For every herbicide, the following sublethal doses have been chosen:

*Corresponding author
Ahmed M. Ali,
Iraqi Ministry of Education, Karma Education Department in Anbar Governorate
e-mail: ah988medm0@gmail.com

1. The concentration of malathion ranged from 0.5 to 1.0 ppm (parts per million).

2. The concentration of chlorpyrifos was between 0.3 and 0.8 ppm.

3. The concentration of nano zinc oxide was 10–20 mg/L.

In order to replicate the settings that bugs could encounter across multiple software cycles in an agricultural setting, the exposure period has been tuned to suggest continuous, low-dose pesticide touch **(Andreazza et al., 2021)**. Exposed populations were kept under the same laboratory conditions as previously mentioned for each pesticide treatment. Tests were carried out on a regular basis to check for signs of adaptive changes in the population's survival, behavior, and capacity (Bartling et al., 2024).

The observer was able to assess the variations in resistance improvement between conventional and nano-formulated pesticides thanks to this experimental setup, which provided information about the effectiveness and adaptive response s linked to both treatment types in *P. viridis*.

ENZYME ACTIVITY ASSAY FOR CYTOCHROME P450 (CYP450)

Cytochrome P450 (CYP450) activity was measured using a spectrophotometric assay monitoring the oxidative metabolism of p-nitroanisole to p-nitrophenol, an enzyme that absorbs light at 405 nm. Samples of viridis were homogenized in potassium phosphate buffer (pH 7.4) and centrifuged at 10,000 g for 20 min at 4°C to obtain a clear supernatant containing CYP450 enzymes to remove cellular contaminants.

For every reaction, there have been 100 µL of enzyme extract, 50 µL of 1 mM p-nitroanisole (substrate), and 100 µL of 0.1 mM NADPH (cofactor), with a very last buffer quantity adjusted to the final buffer concentration of potassium phosphate to 1 mL of potassium phosphate solution. The reaction was started throughout the addition of NADPH to initiate enzyme activity, and the absorbance peak was monitored with a spectrophotometer for 5 min at 405 nm at room temperature (Guengerich et al., 2009).

ASSAY OF CARBOXYLESTERASE (CAE) ENZYME ACTIVITY

The activity of carboxylesterase (CaE) enzyme was evaluated via spectrophotometric analysis of the aqueous solution of p-nitrophenyl acetate converted to p-nitrophenol, the absorption peak was 405 nm at room temperature. The samples of P. Viridis were homogenized using sodium phosphate buffer saline (pH 7.0) followed by centrifugation at 12,000 g for 20 min at 4°C to remove cellular debris, then removed the clear supernatant containing carboxylesterase enzyme.

Each mixture of this reaction has 50 µL of enzyme extract, 50 µL of 1 mM p-nitrophenyl acetate (substrate) and the reaction was started via complete the substrate to a final volume of 1 mL by adding sodium phosphate buffer saline, the absorbance peak was 405 nm at room temperature. The increase in absorbance indicates activity of CaE, as the enzyme hydrolyzes p-nitrophenyl acetate to form p-nitrophenol.

Both assays allowed the quantitation of CYP450 and CaE activity, contributing to the understanding of the detoxification mechanisms of *P. viridis* under antibiotic treatment (Hosokawa and Satoh, 2001).

STATISTICAL ANALYSIS

One-way ANOVA was used to analyze the enzyme activity data, and Tukey's post hoc test was used to assess pairwise differences between the susceptible and resistant groups. For all statistical tests, a significance level of $p < 0.05$ was used to guarantee a reliable interpretation of variations in GST activity. The GraphPad software conducted the analysis.

*Corresponding author
Ahmed M. Ali,
Iraqi Ministry of Education, Karma Education Department in Anbar Governorate
e-mail: ah988medm0@gmail.com

RESULTS AND DISCUSSION

INCREASED ENZYME ACTIVITY IN DIFFERENT TREATMENT GROUPS

As illustrated in (Table 1) enzyme activity analysis revealed significant differences in cytochrome P450 and carboxylesterase (CaE) activities among four treatment groups. Each treatment group showed increased enzyme activity compared to the control group, indicating a positive detoxification response from each of the participating pesticides bae.

Cytochrome P450: Activation was greatest in the chlorpyrifos-treated group, showing a 3.7-fold increase over the control, whereas malathion and nano zinc oxide treatments showed 2.2-fold and 2.1-fold increases, respectively.

Carboxylesterase (CaE): The malathion-treated group showed the highest CaE activity with a 2.4-fold increase compared with the control, and the chlorpyrifos-treated group (2.1-fold) and group treated with nano zinc oxide (1.7 times).

These results indicate that each group of pesticides produces a different index of enzyme activity, with organophosphates (malathion and chlorpyrifos) generally increasing Cytochrome P450 and carboxylesterase activity compared to nano zinc oxide.

Table 1: Enzyme activity levels across the four groups,

Treatment group	Cytochrome P450 activity (nmol/min/mg)	Carboxylesterase (CaE) activity (nmol/min/mg)
Control	18 ± 3.0	20 ± 0.7
Malathion group	40 ± 5.0 (2.2 folds)	48.3 ± 2.0 (2.4 folds)
Chlorpyrifos group	68 ± 9. (3.7 folds)	42.3 ± 1.4 (2.1 folds)
Nano ZnO group	39 ± 10. (2.1 folds)	35 ± 0.9 (1.7 folds)

CORRELATION ANALYSIS OF ENZYME ACTIVITY AND INHIBITION

Correlation analyzes were performed to examine the relationship between enzyme activity levels and insect resistance among treatment groups. Cytochrome P450 (r = 0.80) strongest than carboxylesterase (r = 0.75) especially in chlorpyrifos and malathion treated groups. This correlation suggests that all two enzymes contribute to insect resistance, with higher role for Cytochrome P450 (Table 2).

Table 2: Correlation coefficients for Cytochrome P450 and Carboxylesterase enzymes with resistance

Enzymes	Correlation with resistance (r)
Cytochrome P450	0.80
Carboxylesterase	0.75

KINETIC ANALYSIS OF ENZYME ACTIVITY

As illustrated in (Table 3) kinetic analysis showed that Vmax values increased for each enzyme in treated groups compared to controls, indicating an increased catalytic efficiency in response to pesticide application ho Michaelis constant (Km) remained the same in all groups, indicating that the substrate affinity (Km) did not change even though the catalytic capacity (Vmax) increased.

*Corresponding author
Ahmed M. Ali,
Iraqi Ministry of Education, Karma Education Department in Anbar Governorate
e-mail: ah988medm0@gmail.com

Cytochrome P450: The chlorpyrifos-treated group showed the highest Vmax, indicating a significant increase in oxidative capacity, while the malathion-treated group also showed high activity.

Carboxylesterase (CaE): Vmax was highest in the malathion-treated group, indicating an increased hydrolytic capacity, whereas a moderate increase was observed in the chlorpyrifos and nano zinc groups oxide formed in them.

Table 3: Vmax values for Cytochrome P450 and Carboxylesterase enzymes across the treated groups

Treatment group	Cytochrome P450 Vmax (nmol/min)	Carboxylesterase (CaE) Vmax (nmol/min)	Km (mM, Consistent)
Control	19 ± 2.1	21 ± 0.7	0.3 ± 0.01
Malathion group	42 ± 3.2	45.3 ± 2.0	0.3 ± 0.01
Chlorpyrifos group	65 ± 5.7	41.3 ± 1.4	0.3 ± 0.01
Nano ZnO group	38 ± 5.9	33 ± 0.8	0.3 ± 0.01

DISCUSSION

In citrus groves, mealybugs like Planococcus citri, Pseudococcus viburni, and Pseudococcus viridis are frequent pests that seriously jeopardize fruit productivity and quality. By feeding on the phloem of citrus trees, these soft-bodied, sap-sucking insects draw essential nutrients from the fruit, leaves, and stems. In addition to damaging the host plant, which leads to decreased vigor and growth, their feeding habits cause substantial direct harm to fruits. A significant issue linked to mealybug infestations is the production of honeydew, a sweet waste that promotes the development of sooty mold on the fruit's surface, darkens the fruit, and reduces its market value (Mansour et al., 2018). The nutritional content and visual appeal of citrus fruits may be jeopardized by fruit drops and abnormalities brought on by severe illness.

The cells of various organisms, including humans, contain a broad family of enzymes called cytochrome P450 (CYP450). These enzymes are vital for the metabolism of both endogenous and foreign substances. Despite being present in various tissues, the liver is where they are primarily located (Berenbaum et al., 2021).

In addition to metabolizing and detoxifying toxic materials, cytochrome P450 enzymes are important for insects and pests to conform to loads of ecological niches and environmental problems (Yang et al., 2021). CYP450 enzymes have emerged as a crucial area of study for growing extra green and sustainable pest control techniques due to the fact they mediate pesticide resistance, allow host plant specialization, and affect boom and replica (Wu et al., 2024).

Carboxylesterase (CES) enzymes are a collection of enzymes normally worried inside the hydrolysis of ester, amide, and carbamate bonds of diverse endogenous and exogenous compounds. These enzymes are located at some stage in the body, abundant in liver, intestine and blood (Imai and Ohura, 2010; Wang et al., 2018).

Carboxylase enzymes have an important role in insects, pests, and parasites, primarily in processes that support survival, metabolism, and growth. These enzymes are involved in vital biochemical reactions, inclusive of metabolic pathways that produce power or produce structural components (Oakeshott et al., 2005; Wheelock and Nakagawa, 2010). Understanding carboxylase pastime is crucial in pest control due to the fact these enzymes are touchy to factors together with pesticide resistance, nutrient metabolism and version to environmental modifications.

*Corresponding author
Ahmed M. Ali,
Iraqi Ministry of Education, Karma Education Department in Anbar Governorate
e-mail: ah988medm0@gmail.com

Our findings found out that insect resistance in P. Viridis is mediated by a couple of enzyme detoxing strategies, with cytochrome P450, carboxylesterase, contributing together to chemical resistance. Cytochrome P450 enzymes well-known shows oxidation of toxic precursor chemical compounds is weakened, carboxylesterase enzyme hydrolyzes the ester bonds in insecticides, then GST release glutathione into dependable molecules. Conjugates This synergistic movement quickens cleansing, generating insecticides resistance is high (Khan et al., 2020; Wang et al., 2022).

The study published by Ye et al., (2022) reported that increased cytochrome P450 activity is linked to the development of insect resistance. As evidenced by increased enzyme expression brought on by new gene evolution or promoter region mutations that impact transcription, modifications to the amino acid sequence of protein-coding regions, or changes in enzyme functions brought on by post-translational modifications like phosphorylation changes, which are typically produced under the selective pressure of insecticides (Ye et al., 2022). That is aligned with our results that revealed the elevation of CYT p450 in resistance populations in insecticides treated population in significant value than that in non-treated population. This finding supported by the Kishk et al., (2024) that documented the downregulation of cytochrome P450 causes diaphorina citri mortality to rise and suppresses pesticide resistance (Kishk et al., 2024a).

Furthermore, Lu et al., (2022) reported that carboxylesterase CarE17is primarily responsible for Nilaparvata lugens' metabolic resistance to chlorpyrifos (Lu et al., 2022). Chang et al., (2024) reported that CarEs family's play a primary function in H. armigera pesticide detoxification and resistance (Chang et al., 2024). All these works aligned with our finding that the significant elevation in carboxylesterase in the pesticides treated populations and indicator for high resistance that that not treated population group.

Interestingly, Wang et al., (2024) disclosed that concomitant physiological resistance to azoxystrobin and other antifungal agents in Botrytis cinerea may be due to the combined activity of carboxylesterase and cytochrome P450 enzymes. Carboxylesterase plays an important role in hydrolyzing ester bonds of fungicide molecules, reducing their efficacy. At the same time, cytochrome P450 enzymes contribute to the detoxification of fungicides by oxidation, making the bacterium resistant to a wide range of fungicides (Wang et al., 2024).

The elevated levels of these detoxifying enzymes in resistant populations highlight the limitations of single use of insecticides. Instead, pest control strategies benefit from an enzymatic approach. For example, combining CYT p450 and/or carboxylesterase inhibitors with pesticides agents may impair detoxification, thus improving pesticides efficacy. Similarly, cycles of pesticides can reduce the selective pressure on any one enzyme pathway, potentially leading to resistance.

5. Future Research Directions

Further studies are needed to investigate the genetic basis for the upregulation of these enzymes in resistant P. viridis populations. Field studies should also examine the actual efficacy of enzyme inhibitors in resistant populations, and the effectiveness of multiple pest control strategies in citrus affecting interactions with those enzymes between these and others, such as NADPH oxidase, to provide a more comprehensive understanding of resistance mechanisms.

CONCLUSION

This research elucidates the role of Cytochrome P450, and Carboxylesterase enzymes in P. viridis insect resistance pathway. It highlights several enzyme dependent detoxifications in pests resistant by the ability of these enzymes to enhance the detoxifying power of P. viridis to elevate resistance to pesticides. These findings highlight the importance of pest management for integrated

*Corresponding author
Ahmed M. Ali,
Iraqi Ministry of Education, Karma Education Department in Anbar Governorate
e-mail: ah988medm0@gmail.com

pest control strategies by considering multiple detoxification enzymes to support the development an effective pest control strategies they destroy crop edges that emphasize sustainability.

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*Corresponding author
Ahmed M. Ali,
Iraqi Ministry of Education, Karma Education Department in Anbar Governorate
e-mail: ah988medm0@gmail.com

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*Corresponding author
Ahmed M. Ali,
Iraqi Ministry of Education, Karma Education Department in Anbar Governorate
e-mail: ah988medm0@gmail.com