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Resistance Development to Abamectin and Cross-Resistance to Multiple Insecticides in *Rhynchophorus ferrugineus* (Olivier), with Emphasis on Biochemical Analysis

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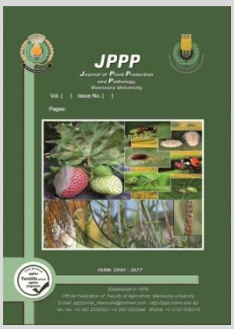

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ABSTRACT

This study investigated the development and mechanisms of resistance to abamectin in *Rhynchophorus ferrugineus* larvae across eight generations (FS0-FS8) under laboratory selection. Abamectin resistance progressively developed, with the resistance ratio (RR) increasing from 1.06 in the initial field strain (FS0) to 51.18-fold by generation FS8. This significant escalation, coupled with decreasing probit slopes, indicated increased population heterogeneity and robust resistance development. Cross-resistance was also observed against other insecticide classes: hexaflumuron (RR = 105.91-fold), dimethoate (RR = 18.20-fold), and spinetoram (RR = 14.07-fold), highlighting broad-spectrum tolerance. Enzymatic analyses revealed significant up regulation of detoxification enzymes in resistant strains. Esterase activity increased 1.39-fold in RS5 and dramatically 6.09-fold in RS8 compared to the susceptible strain. Glutathione S-transferase (GST) activity increased 3.04-fold in RS5 and 2.84-fold in RS8, while oxidase activity (P450) showed the most consistent increase, reaching 1.84-fold in RS5 and over 3.9-fold in RS8. Acetyl cholinesterase (AChE) activity also increased, suggesting broader physiological adaptations. These findings strongly indicate that metabolic detoxification by esterases, GSTs, and oxidases are a primary mechanism driving abamectin resistance and cross-resistance in *R. ferrugineus*. Therefore, effective resistance management necessitates the implementation of diverse control strategies, including insecticide rotation, use of synergists, and integrated pest management approaches, to preserve the efficacy of current chemical controls and mitigate further resistance evolution**.**

Keywords: Resistance Development, Abamectin, *Rhynchophorus ferrugineus*.

INTRODUCTION

The date palm (*Phoenix dactylifera* L.) has held significant economic and agricultural importance throughout history. In Egypt, date palms are widely distributed, covering areas from Aswan to the northern Delta, as well as oases like Siwa, Bahariya, Farafra, Kharga, and Dakhla. Egyptian date palms are vital horticultural crops, with approximately 12.5 million trees producing around 1.5 million tons annually, making Egypt the leading date producer according to FAO statistics (2013–2014). The red palm weevil (RPW), *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae), is among the most invasive pests globally, causing extensive damage to date palms. It poses a significant economic threat to date palms worldwide, with larvae being the most destructive stage as they tunnel through the soft tissues of the trunk (Henery, 1917; Butani, 1975). These tunnels penetrate deep into the lower stem, damaging internal tissues, which can lead to tree collapse and death. Infestation symptoms include trunk tunnels, oozing yellow-to-brown fluid, chewed plant tissues around openings, a fermented odor from tunnel fluids, and crown collapse (Kaakeh*et al.,* 2001). Recent estimates highlight economic losses due to RPW infestations ranging from 1% to 5%, equivalent to $5.18–25.92 million, with indirect losses amplifying these figures (El-Sabea*et al.,* 2009). RPW management is challenging due to its hidden lifestyle, as all life stages develop inside the trunk, and visible symptoms often appear only after severe infestation making control and containment difficult. Preventive and curative insecticide applications are frequently employed to control infestations (Abozuhairah*et al.,* 1996). Abamectin (ABM), a macrocyclic lactone derived from the soil-dwelling bacterium *Streptomyces avermitilis*, is extensively utilized as both an insecticide and acaricide. It acts as a γ-aminobutyric acid (GABA) agonist, affecting insect nervous systems (Burg *et al.*, 1979; Putter *et al.,* 1981). Recently in Egypt, farmers have reported the reduced effectiveness of available insecticides, possibly due to resistance development. However, mechanisms underlying insecticide resistance in *R. ferrugineus* remain poorly understood. This study investigates resistance development in *R. ferrugineus* to abamectin and cross-resistance to three insecticides, namely spinetoram, hexaflumuron, and dimethoate, which have distinct modes of action. Cross-resistance findings prompted biochemical analyses of detoxifying enzymes in resistant and susceptible colonies. Detoxification mechanisms involve three key enzymes, namely MFOs, GSTs, and ESTs that neutralize insecticides before reaching their target sites. Enzymatic activity was evaluated to understand resistance mechanisms.

MATERIALS AND METHODS

1. Chemicals Tested in the Study:

Insecticides: This study employed four different insecticides. Table (A) summarizes key details such as trade names, formulations, active ingredient percentages (%AI), application rates per feddan (4200 m²), manufacturing companies, and mechanisms of action for each insecticide examined in this research.

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Table A. Insecticides utilized in the present study.

Details about Insecticides	Mode of action
Abamectin, marketed as Agri-Mek® SC, with a 1.9% concentration, 250 ml formulation (equivalent to 4.75 g active ingredient), produced by Syngenta Crop Protection Pty Limited.	Abamectin acts by binding to glutamate-gated chloride channels in the nerve and muscle cells of invertebrates, leading to cell hyperpolarization, resulting in paralysis and ultimately death.
Spinetoram, commercial name Delegate® SC, 11.7% formulation, 100 ml containing 11.7 g active ingredient, produced by Dow AgroSciences (Australia) Ltd.	Spinosoid insecticides kill insects by disrupting their nervous system. They do this primarily by binding to nicotinic acetylcholine receptors (nAChRs), which interferes with normal acetylcholine transmission. Additionally, spinosad acts as a GABA agonist, leading to overstimulation and death in insects.
Hexaflumuron, commercial name HUMER® EC. 5% formulation. 80 ml with 4 g of active ingredient, manufactured by Syngenta Crop Protection Pty Limited.	Chitin synthesis inhibitors, a group of benzoyl phenyl urea insecticides, work by blocking the chitin synthetase enzyme, preventing the formation of chitin. This disrupts the cuticle formation, causing it to be too weak to withstand molting pressure, leading to the insect's failure to molt and eventual death. These insecticides are known as ant-molting agents.
Dimethoate, marketed as Perfekthion™ EC, with a 40% concentration, 1000 ml formulation (400 g active ingredient), produced by Dow AgroSciences (Australia) Ltd.	OPs inhibit acetylcholinesterase, leading to a buildup of acetylcholine and prolonged nerve signaling. dimethoate poisoning occurs when more than 70% of AChE is inhibited, and this mechanism also serves as the primary insecticidal action.

Chemicals Applied in Enzyme Activity Assays

Chemicals such as Glucose 6-phosphate, Glucose 6-phosphate dehydrogenase, NADP, p-nitroanisol (PNA), bovine serum albumin (fraction 5), Coomassie Brilliant Blue G-250, reduced glutathione (GSH), acetylthiocholine iodide (ATCI), DTNB, and α-naphthyl acetate (α-NA) were sourced from Sigma Chemical Co. (St. Louis, MO). Phenylthiourea (PTU), trichloroacetic acid (TCA), potassium chloride (KCl), DCNB, CDNB, and Fast Blue B salt were obtained from Aldrich Chemical Co. (Milwaukee, WI).

2. Insect rearing

In this study, a laboratory colony of the fourthinstar larvae was used. Adult red palm weevils (RPWs) were collected from infested date palm trees at El-Frafra Oasis, located in the New Valley governorate of Egypt. Adults, larvae, and pupae were separated into individual plastic jars and transported to the laboratory. In the lab, larvae were provided with sugarcane (Saccharumof ficinarum L.) stems for food and pupation, while adults were given shredded sugarcane for food and oviposition in plastic containers. Pupae were placed in separate boxes to allow for adult emergence under controlled conditions (27±2°C, 60±5% RH, 12:12 light/dark cycle). Emerged adults were moved to jars for feeding and mating. Colonies were maintained in plastic boxes (15×30×30 cm) with mesh lids for aeration. The rearing was conducted at the Laboratory of Insects, Department of Plant Protection, Faculty of Agriculture, Minia University, Egypt. Adult food was replaced every three days, and the discarded sugarcane pieces were used for egg hatching. Following hatching, the neonate larvae were transferred to fresh sugarcane to feed until they molted to the fourth instar stage. Three distinct colonies were utilized in this study: a laboratory colony, a field-collected colony, and an abamectin-selected colony.

3. Insecticides

This study assessed the performance of various insecticides on freshly molted F1 fourth instar larvae of *R. ferrugineus*. The F1 generation was established from field-collected weevils and those emerging from collected larvae and pupae. Bioassays used an artificial diet prepared according to IRAC Method No. 020 guidelines, containing a mix of agar, yeast, wheat germ, corn flour, and various supplements. Insecticide concentrations were diluted in distilled water, with a distilled water control. Ten individual L4 larvae were placed in glass cups with diet pieces, covered for aeration and containment. All treatments were replicated three times and maintained under controlled conditions (27±2 °C, 65±5% RH, and a 12:12 L: D photoperiod).

4. Methodology Approach:

Resistance Development to Abamectin in *R. ferrugineus* and Cross-Resistance to Various Insecticides

The bioassay of abamectin was conducted on the fourth instar larvae of both laboratory and field-collected strains, and probit analysis was used to establish the resistance data. Field-collected RPW was selected at the fourth instar larvae with a concentration of abamectin equivalent to the 96-hour LD_{50}. Initially, the field-collected strain exhibited a 1.06-fold resistance compared to the laboratory strain. After eight generations of selection, the resistance ratio increased to 51.18-fold in comparison to the laboratory strain.

At the onset of the selection process, the fourth instar larvae from the field-collected colony were bioassayed with abamectin, as well as three other insecticides: spinetoram, hexaflumuron, and dimethoate. Once the selection was complete, the four chemicals were re-bioassayed. Probit analysis was performed on abamectin for each generation. For the remaining three chemicals, data were collected both before and after the selection process to evaluate the development of cross-resistance in the abamectin-selected colony.

Assessment of enzyme activity in the fourth instar larvae of *R. ferrugineus*, both susceptible and resistant to abamectin.

The enzyme activities of fourth instar larvae from laboratory and abamectin-selected strains were measured. For each test, 30 larvae from each strain were used (3 individuals in 10 replicates). The procedures for measuring the four enzyme activities are described below:

Preparation of test samples:

Following the methods outlined by Ibrahim and Ottea (1995), Korrat (2009), Muthusamyet al. (2011), and Reyes et al. (2012), with some adjustments, thirty fourth instar larvae from susceptible and resistant colonies were selected, grouped into ten replicates of three larvae each. After weighing, the larvae were rinsed with acetone (2 X 5ml) to eliminate surface residues. To prepare the homogenate, the larvae were ground in 1ml of ice-cold 50mM sodium phosphate buffer (pH 7.5) containing phenylthiourea to prevent oxidation. The homogenate was then placed into ten Snap-cap tubes (1 ml capacity) and centrifuged at 10,000 rpm for 20 minutes at 4ºC. The supernatant was maintained on ice and used within 30 minutes. Absorbance was determined using a Shimadzu UV-120-02 Spectrophotometer, with the wavelength selected based on the specific enzyme being analyzed.

Estimation of General Esterase Activity:

The esterase activity assay followed protocols outlined by Kranthi (2005) and Korrat (2009), with minor modifications. In this assay, 100 µl of blank buffer and supernatant (equivalent to 0.33 insects) from susceptible and

resistant larvae were mixed with 100 µl of 0.3 mM α-naphthyl acetate (substrate) and 4.8 ml of 40 mM phosphate buffer (pH 6.8). The mixtures were incubated in the dark for 20 minutes at room temperature. After incubation, 1 ml of staining solution (1% fast blue BB salt prepared in phosphate buffer [40 mM, pH 6.8] with 5% sodium dodecyl sulfate [SDS]) was added, and tubes were further incubated at 20°C for 30 minutes. Absorbance readings were taken at 450 nm. Enzyme activity per mg was calculated using an extinction coefficient based on an α-naphthol standard curve. Each sample was analyzed in ten replicates to ensure accuracy.

Estimation of *S*-transferase Activity:

Following the protocol by Habig*et al.* (1974), Glutathione S-transferase (GST) activity was assessed. The assay components included 50 µl of 50 mM CDNB, 150 µl of reduced glutathione (GSH), and 2.79 ml of 40 mM phosphate buffer (pH 6.8). To start the reaction, 10 µl of enzyme extract (equivalent to 0.033 insects) was introduced. The reaction mixture was gently mixed, then incubated at 20°C for 2–3 minutes. Absorbance readings at 340 nm were subsequently taken for 5 minutes using a UV spectrophotometer. Enzyme activity was quantified as µmol of CDNB conjugated per minute per mg protein.

Estimation of oxidase activity:

Mixed-function oxidase activity was evaluated by measuring P-nitroanisole O-demethylation, following the modified method of Hansen and Hodgson (1971). The reaction mixture (2 mL) consisted of 0.5 mL enzyme extract (0.333 insect equivalents), 1 mL of 0.1 M sodium phosphate buffer (pH 7.8), and 0.5 mL of 0.36 mM NADPH. The reaction was initiated by adding 30 µL of ethanol containing p-nitroanisole to a final concentration of 3 mM. Samples were incubated at 25°C for 30 minutes in a water bath with constant shaking. The reaction was stopped by adding 0.5 mL of 1 M HCl. The p-nitrophenol product was extracted with chloroform (CHCl3). After centrifugation to separate the layers, the CHCl3 phase was re-extracted with 0.5 M NaOH. The aqueous phase's absorbance at 400 nm was then recorded, and product concentrations were quantified using a standard curve.

Estimation of Acetyl cholinesterase Activity:

Acetylcholinesterase (AChE) activity was determined using a modified Ellman*et al.* (1961) protocol, employing acetylthiocholine iodide as the substrate. The reaction mixture, containing 400 µL of enzyme extract (1.2 insect equivalents), 200 µL of 0.075 M acetylthiocholine iodide, and 2.4 mL of 0.1 M phosphate buffer (pH 7.4), was incubated at 27°C for 15 minutes. Enzyme activity was then quantified by measuring absorbance at 412 nm.

Protein assay:

To ensure consistency, enzyme activity data were expressed relative to protein content, facilitating comparisons between homogenates derived from distinct larval tissues of the two strains. Mean enzyme activities (± SE) for both colonies were calculated from ten replicates, each comprising three fourth instar larvae. Differences between the two colonies were analyzed using an unpaired t-test with significance thresholds of P = 0.05 and 0.01.

Protein concentrations in tissue homogenates were determined using the Bradford method (1976), with bovine serum albumin (fraction V; adjusted for impurities) serving as the standard, Fig (1).

For the preparation of the protein reagent, 100 mg of Coomassie Brilliant Blue G-250 was solubilized in 50 mL of 95% ethanol. This ethanolic solution was subsequently combined with 100 mL of 85% (w/v) phosphoric acid. The entire mixture was then quantitatively diluted to a total volume of 1 liter, yielding a reagent with final concentrations of 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid.

Protein Assay Procedure: Protein solutions (10–100 µg) in volumes up to 0.1 mL were placed in 12 × 100 mm test tubes, and their volumes were adjusted to 0.1 mL using an appropriate buffer. Five milliliters of protein reagent were then added, and the mixtures were gently inverted or vortexed to ensure proper mixing. Absorbance at 595 nm was measured between 2 minutes and 1 hour in 3 mL cuvettes. A blank containing 0.1 mL buffer and 5 mL reagent served as a reference. A standard curve, based on bovine serum albumin concentrations versus absorbance, was prepared to determine the protein content of unknown samples.

Protein concentration (µg)	Absorbance at 595nm
0	0
12.5	0.15
25	0.30
37.5	0.46
50	0.59
62.5	0.62
75	0.75
87.5	0.96
100	1.06
112.5	1.56
125	1.99

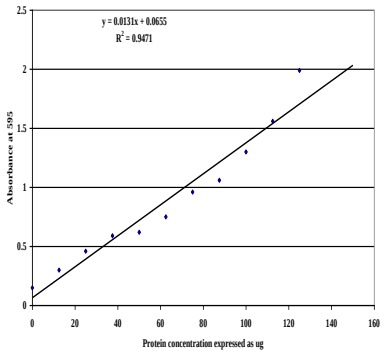

Fig. 1.Standard Curve Data for Protein Quantification Using the Bradford Assay

RESULTS AND DISCUSSION

1. Resistance Phenomenon: Insights into the Mechanisms Resistance Development in Response to Abamectin

The data presented in Table (1) illustrate the progressive development of resistance in *Rhynchophorus ferrugineus* (red palm weevil) fourth instar larvae to abamectin across successive generations (FS0 to FS8) under laboratory selection pressure using the 96-hour LD_{50} concentration. The Laboratory Strain (LS) served as the baseline for comparison, with an LD_{50} value of 0.33 µg/larva and an LD_{90} of 1.47 µg/larva. This strain is considered fully susceptible, with a resistance ratio (RR) set at 1.0.The initial field-collected strain (FS0), before any selection, showed a slight increase in LD_{50} (0.35 µg/larva) and LD_{90} (1.49 µg/larva), resulting in a negligible resistance ratio of 1.06, indicating a similar susceptibility to the laboratory strain. However, after just one generation of selection (FS1), the

LD_{50} increased markedly to 0.59 µg/larva and LD_{90} to 2.57 µg/larva, with a resistance ratio of 1.79, signaling the onset of resistance development.Resistance escalated rapidly in subsequent generations. By the second generation (FS2), the LD_{50} had more than doubled to 1.29 µg/larva, while the LD_{90} surged to 14.45 µg/larva. The resistance ratio at this stage was 3.91, indicating a significant increase in tolerance. The slope of the probit regression line also declined (from ~2.0 in FS0 and FS1 to 1.218 in FS2), suggesting greater variability in individual response to abamectin, a typical indicator of early resistance selection.The trend continued in FS3, with LD_{50} reaching 3.55 µg/larva and LD_{90} exploding to 123.03 µg/larva. The resistance ratio jumped to 10.76, demonstrating a steep rise in resistance. The slope continued to decrease (0.831), indicating even wider variability in susceptibility among larvae. By FS4, the LD_{50} doubled again to 6.46 µg/larva, and LD_{90} increased dramatically to 446.68 µg/larva, with a resistance ratio of 19.57. This reflects a substantial shift in population susceptibility, likely due to strong selection pressure.The resistance intensified sharply from FS5 onward. LD_{50} in FS5 reached 11.48 µg/larva, and LD_{90} crossed the 1000 µg/larva threshold. The resistance ratio more than doubled to 34.39. From FS6 to FS8, the LD_{50} values gradually increased to 12.88, 14.13, and 16.89 µg/larva, respectively. The corresponding LD_{90} values were extremely high, 1258.93, 1380.38, and 1862.09 µg/larva, demonstrating a plateau in resistance development but with still-growing lethality thresholds. The resistance ratios for FS6, FS7, and FS8 were 39.03, 42.82, and 51.18, respectively.The consistent decrease in the slope values across generations from 2.046 in FS0 to 0.627 in FS8 further indicates increasing heterogeneity within the selected populations. This phenomenon is common during resistance evolution, as individuals with diverse levels of tolerance emerge and survive.

Generally, the data clearly demonstrate a strong and cumulative resistance development in *R. ferrugineus* larvae to abamectin under continuous selection pressure. Each generation exposed to the LD_{50} level of abamectin exhibited progressively higher tolerance, with resistance ratios increasing more than 50-fold by the eighth generation. These results underscore the critical need for resistance management strategies, such as rotation with other insecticides, integration of biological control, and minimizing overreliance on a single active ingredient to preserve the efficacy of abamectin in field applications.

Table 1. Resistance development in field-collected *R. ferrugineus* fourth instar larvae subjected to selection pressure using abamectin at the 96-hour LD_{50} concentration.

Tested colony	Toxicity Line Equation	Slope ± SE	*df*	LD_{50} (95% FL)	LD_{90} (95% FL)	RR (Related to LS)
LS	y = 1.967x + 5.953	1.967 ± 0.088	3	0.33 (0.28 - 0.40)	1.47 (1.28 - 1.50)	1.0
FS_0	y = 2.046x + 5.924	2.046 ± 0.039	2	0.35 (0.32 - 0.39)	1.49 (1.41 - 1.53)	1.06
FS_1	y = 1.997x + 5.461	1.997 ± 0.028	2	0.59 (0.51 - 1.05)	2.57 (2.12 – 2.89)	1.79
FS_2	y = 1.218x + 4.862	1.218 ± 0.025	2	1.29 (1.11 - 1.99)	14.45 (14.04 – 15.76)	3.91
FS_3	y = 0.831x + 4.544	0.831 ± 0.060	2	3.55 (3.09 – 4.76)	123.03 (111.54 – 135.16)	10.76
FS_4	y = 0.695x + 4.441	0.695 ± 0.011	2	6.46 (6.11 – 7.12)	446.68 (434.34 – 551.46)	19.57
FS_5	y = 0.651x + 4.308	0.651± 0.062	2	11.48 (9.45 – 13.38)	1071.52 (980.29 – 1145.76)	34.39
FS_6	y = 0.648x + 4.279	0.648 ± 0.012	2	12.88 (12.07– 14.40)	1258.93 (1145.11 – 1376.06)	39.03
FS_7	y = 0.645x + 4.256	0.645 ± 0.021	2	14.13 (13.67 – 15.35)	1380.38 (310.81 – 1645.64)	42.82
FS_8	y = 0.627x + 4.230	0.627 ± 0.005	2	16.89 (14.13 – 17.88)	1862.09 (1756.34 – 2354.54)	51.18

LS: Laboratory strain; FS0: Field-collected strain prior to selection; FS1: Field-collected strain subjected to one generation of selection with abamectin; FS8: Field-collected strain subjected to eight generations of selection with abamectin. Selection pressure was based on the 96-hour LD_{50} concentration.

Cross-Resistance Induced by Abamectin Selection in Colonies Exposed to Insecticides from Different Chemical groups

The data presented in Table (2), along with the toxicity curves illustrated in Figure. (2) (Sets A, B, C, and D), provide a detailed comparison of the toxicological responses of fourth instar larvae of *R. ferrugineus* from three distinct colonies: the Laboratory Strain (LS), the Field-collected Strain (FL or FS), and the eighth generation of a Resistant Strain (RS8). These larvae were tested against four insecticides, abamectin, spinetoram, hexaflumuron, and dimethoate. The results reveal notable differences in susceptibility and resistance development among the strains, quantified by LD_{50} and LD_{90} values as well as resistance ratios (RR). Mortality assessments were conducted at time intervals tailored to each insecticide's mode of action and rate of activity, ensuring accurate evaluation of their toxic effects. The current study revealed cross-resistance to spinetoram, hexaflumuron, and dimethoate. After eight generations of selection, the resistance ratios were 51.18-fold for abamectin, 14.07-fold for spinetoram, 105.91-fold for hexaflumuron, and 18.20-fold for dimethoate. The lowest resistance was observed toward spinetoram, while hexaflumuron showed the highest resistance. These results suggest that the development of resistance to abamectin is associated with cross-resistance to spinetoram, hexaflumuron, and dimethoate. Abamectin demonstrated high toxicity to the LS colony, with an LD_{50} of 0.33 µg/larva and an LD_{90} of 1.47 µg/larva, serving as the reference for resistance comparison (RR = 1.0). The FL colony showed a similar susceptibility (LD_{50} = 0.35 µg/larva, RR = 1.06), indicating minimal natural tolerance. However, the RS8 colony, after eight generations of selection exhibited a drastic reduction in susceptibility, with the LD_{50} escalating to 16.89 µg/larva and LD_{90} exceeding 1862 µg/larva, resulting in a resistance ratio of 51.18. This extreme shift underscores the substantial resistance development due to continuous selection pressure with abamectin. Spinetoram, a spinosyn class insecticide, showed moderate toxicity in the LS strain (LD_{50} = 19.82 µg/larva). The FL colony had a modestly higher LD_{50} of 35.57 µg/larva (RR = 1.79), indicating some field-evolved tolerance. In contrast, the RS8 colony had an LD_{50} of 500.49 µg/larva, an approximate 25-fold increase over FL and 14-fold over LS (RR = 14.07). The corresponding LD_{90} value soared to 2476.98 µg/larva. This significant resistance development, although not as extreme as with abamectin or hexaflumuron, signals that spinetoram efficacy could deteriorate under sustained exposure. Hexaflumuron, an insect growth regulator (IGR), was moderately toxic to LS larvae (LD_{50} = 7.42 µg/larva), but the FS colony showed a notable increase in resistance (LD_{50} = 35.65 µg/larva; RR = 4.80). The RS8 colony, however, demonstrated a dramatic increase in tolerance, with an LD_{50} of 3775.78 µg/larva and an LD_{90} of over 31,800 µg/larva. The resistance ratio jumped to 105.91, indicating that hexaflumuron was the most affected insecticide in terms of

resistance development among those tested. The steep increase in lethal doses suggests that the active ingredient's growth-disrupting mechanism became largely ineffective against the selected population. Dimethoate, an organophosphate, showed baseline toxicity in LS (LD_{50} = 14.24 µg/larva; LD_{90} = 64.42 µg/larva). The FS colony exhibited slightly reduced susceptibility (LD_{50} = 23.62 µg/larva; RR = 1.66).

Table 2. Probit line (LD-P) data derived from toxicity assays of four insecticides on fourth instar larvae of *R. ferrugineus* from three distinct colonies. Mortality data were recorded at 48 hours for dimethoate, 96 hours for abamectin and hexaflumuron, and 144 hours for spinetoram.

Insecticides	Colony tested	Line equation	Slope ± SE	*df*	LD_{50} (95%CL)	LD_{90} (95%CL)	RR
Abamectin	LS	y = 1.967x + 5.953	1.967 ± 0.088	3	0.33 (0.28 - 0.40)	1.47 (1.28 - 1.50)	1.0
	FL	y = 2.046x + 5.924	2.046 ± 0.039	2	0.35 (0.32 - 0.39)	1.49 (1.41 - 1.53)	1.06
	RS_8	y = 0.627x + 4.230	0.627 ± 0.005	2	16.89 (14.13 – 17.88)	1862.09 (1756.34 – 2354.54)	51.18
Spinetoram	LS	y = 1.7985x + 2.667	1.7985 ± 0.057	2	19.82 (11.33 - 25.58)	102.07 (75.25 - 210.83)	1.0
	FL	y = 2.653x + 0.885	2.653± 0.0356	2	35.57 (25.74 - 44.46)	108.03 (88.23 - 235.45)	1.79
	RS_8	y = 1.843x - 0.025	1.043 ± 0.045	3	500.49 (323.13- 756.54)	2476.98 (869.41 - 3658.64)	14.07
Hexaflumuron	LS	y = 2.023x + 3.239	2.023 ± 0.336	2	7.42 (5.45 - 11.28)	31.86 (20.74 - 47.52)	1.0
	FS	y = 1.576x + 2.554	1.576 ± 0.0135	3	35.65 (22.17 - 43.82)	231.32 (145.54 - 369.74)	4.80
	RS_8	y = 1.383x - 0.053	2.783 ± 0.339	4	3775.78 (877- 1309.18)	31807.53 (21877.52 - 51964.36)	105.91
Dimethoate	LS	y = 1.953x + 2.747	1.953 ± 0.093	3	14.24 (9.47 - 19.43)	64.42 (40.25 - 88.46)	1.0
	FS	y = 2.154x + 2.042	2.154 ± 0.141	3	23.62 (15.83 - 36.76)	92.79 (60.63 - 206.46)	1.66
	RS_8	y = 1.863x - 0.094	1.863 ± 0.068	3	429.92 (289.34- 658.36)	2091.47 (986.65- 4356.45)	18.20

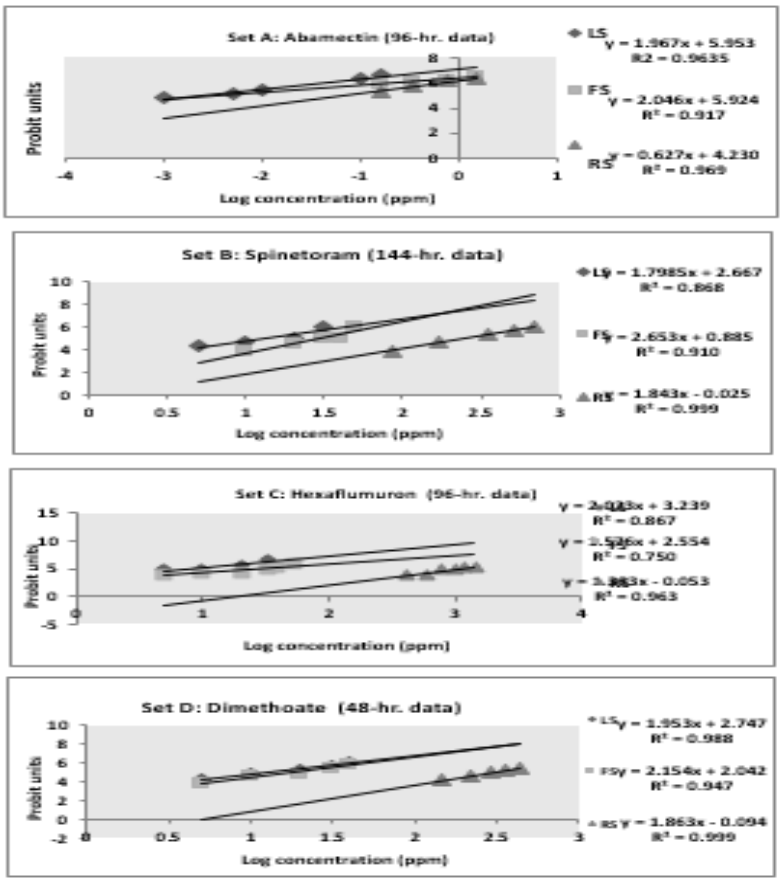

Fig. 2.Toxicity regression lines of abamectin, spinetoram, hexaflumuron, and dimethoate(Sets A, B, C, and D, respectively), based on log concentration versus probit units for laboratory, field-collected, and abamectin-resistant *R. ferrugineus* strains.

In RS8, however, resistance was significantly more pronounced (LD_{50} = 429.92 µg/larva; LD_{90} = 2091.47 µg/larva), resulting in a resistance ratio of 18.20. Though not as extreme as hexaflumuron or abamectin, these values indicate a strong resistance trend, suggesting field populations could rapidly lose sensitivity to dimethoate under repeated applications. In terms of probit line slopes, a general trend is observed where resistant colonies (particularly RS8) often exhibit lower slope values compared to LS, suggesting increased heterogeneity in larval response and broader variation in tolerance levels, typical of resistance development. For instance, the slope for abamectin dropped from 1.967 (LS) to 0.627 (RS8), indicating a wider distribution of susceptibility within the population. A similar pattern was seen with spinetoram and hexaflumuron.

Overall, this data strongly supports the notion that repeated selection pressure whether from field exposure or deliberate laboratory selection can result in profound resistance in *R. ferrugineus* larvae across different chemical classes. The most dramatic resistance was observed with hexaflumuron (RR = 105.91), followed by abamectin (RR = 51.18), dimethoate (RR = 18.20), and spinetoram (RR = 14.07). These findings highlight the urgent need for resistance management strategies such as chemical rotation, integrated

pest management, and reduced reliance on any single insecticidal mode of action to maintain control efficacy over this destructive pest.

Enzymatic Activity Evaluation in fourth Instar Larvae of Susceptible versus Resistant Colonies

This study sought to elucidate the contribution of detoxification enzymes: esterase, monooxygenase, and glutathione S-transferase in mediating resistance to abamectin in red palm weevil larvae. Their potential roles in cross-resistance to spinetoram, hexaflumuron, and dimethoate were also analyzed. In addition, target site (AChE) activity was evaluated to determine whether AChE overproduction played a role in cross-resistance to dimethoate. Enzyme activity assays were initially performed after five generations of selection (RR = 34.39-fold) and subsequently repeated after eight generations of selection (RR = 51.18-fold).

The data presented in Table (3) provide a comprehensive evaluation of the enzymatic activity in *R. ferrugineus* fourth instar larvae from both abamectin-susceptible (SS) and resistant colonies (RS5 and RS8). Enzyme activity was assessed across two resistance selection trials (RS5 and RS8) to monitor the biochemical mechanisms potentially contributing to abamectin resistance. The enzymes investigated general esterases (ESTs), glutathione S-transferases (GSTs), oxidases, and acetylcholinesterase (AChE) are commonly associated with metabolic detoxification processes and resistance development in insects.In the first trial (RS5), general esterase activity increased from 0.5115 ± 0.0436 µmol/min/mg protein in the SS colony to 0.7106 ± 0.0344 µmol/min/mg in RS5, indicating a 1.39-fold elevation in enzymatic activity. This increase was statistically significant at both P = 0.05 and P = 0.01 levels (P-values: 6.57E-07, 1.08E-07), suggesting the role of esterases in hydrolyzing abamectin or related ester bonds, thereby contributing to detoxification and resistance. In the second trial (RS8), esterase activity increased dramatically to 0.3576 ± 0.0114 compared to only 0.0586 ± 0.0118 in the SS colony, representing a 6.09-fold increase. The sharp elevation in RS8 compared to RS5 suggests that esterase activity accumulates progressively with continued selection, reinforcing their central role in abamectin resistance. Glutathione S-transferase (GST) activity also showed a significant rise with resistance development. In RS5, the GST activity reached 5.5131 ± 0.3454 µmol/min/mg protein, which was approximately 3.04 times higher than the SS value of 1.8124 ± 0.3689 µmol/min/mg protein (P = 1.59E-06, 2.87E-06). Similarly, in RS8, GST activity was nearly tripled (2.84-fold increase), reaching 0.3983 ± 0.0110 µmol/min/mg versus 0.1399 ± 0.0126 in SS. GSTs are known to catalyze the conjugation of reduced glutathione to toxic electrophilic compounds, such as those found in many insecticides. Their upregulation implies enhanced detoxification of abamectin metabolites, thus contributing significantly to resistance. Oxidase activity, measured using p-nitroanisole as a substrate, demonstrated the most consistent and significant increases across both trials. In RS5, oxidase activity rose from 0.1289 ± 0.00073 nmol/min/mg protein in SS to 0.2372 ± 0.00076 nmol/min/mg in RS5, representing a 1.84-fold increase (P = 2.44E-11, 4.67E-11). In RS8, the oxidase activity was more than 3.9-fold higher than in SS, increasing from 0.0035 ± 0.0005 to 0.01365 ± 0.0009 nmol/min/mg protein.

Table 3. Measurement of enzymatic activity in fourth instar larvae derived from abamectin-susceptible and resistant colonies.

Measured enzymes	Substrates	Extinction coefficient	Trial 1 with RS_5 Mean enzyme activity (metabolized substrate/min/mg protein) ± SE				Trial 2 with RS_8 Mean enzyme activity (metabolized substrate/min/mg protein) ± SE			
			SS	RS_5	ER (RS_5/SS)	*t*-test (*P0.05, P0.01*	SS	RS_8	ER (RS_8/SS)	*t*-test (*P0.05, P0.01*
General esterase	ά naphthylacetate	14.28 mM^{-1} cm^{-1}	0.5115 ± 0.04358	0.7106 ± 0.03436	1.39	6.57E-07, 1.08E-07	0.0586 ± 0.0118	0.3576 ± 0.0114	6.09	2.05E-10, 4.09E-10
G-S-T	Chlorodinitrobenzene	10.63 mM^{-1} cm^{-1}	1.8124 ± 0.3689	5.5131 ± 0.3454	3.04	1.59E-06, 2.87E-06	0.1399 ± 0.0126	0.3983 ± 0.0110	2.84	1.03E-09, 2.08E-09
Oxidase	P- nitroanisol	14.28 mM^{-1} cm^{-1}	0.1289 ± 0.000728	0.2372 ± 0.000759	1.84	2.44E-11, 4.67E-11	0.0035 ± 0.0005	0.01365 ± 0.0009	3.90	1.09E-08, 2.19E-08
ACh esterase	AcetylthiocholineIodide	13.6 mM^{-1} cm^{-1}	0.2339 ± 0.00826	0.3989 ± 0.00656	1.71	1.06E-06, 2.1E-07	0.0081 ± 0.0005	0.01875 ± 0.0019	2.31	3.96E-05, 7.9E-05

Enzyme activities were measured in µmol for ESTs, GSTs, and AChEs, but in nmol for oxidases.

The elevation of oxidase activity implies enhanced phase I metabolism, likely through cytochrome P450 monooxygenases, which play a crucial role in oxidative detoxification of abamectin and structurally related compounds.Acetylcholinesterase (AChE), although not directly involved in abamectin detoxification (since abamectin primarily targets glutamate-gated chloride channels), also showed increased activity in both resistant strains. AChE activity in RS5 was 0.3989 ± 0.00656 µmol/min/mg, compared to 0.2339 ± 0.00826 in SS, a 1.71-fold increase (P = 1.06E-06, 2.1E-07). In RS8, the increase was 2.31-fold (from 0.0081 ± 0.0005 to 0.01875 ± 0.0019 µmol/min/mg protein), suggesting a broader physiological adaptation or compensatory up regulation in resistant individuals. Although AChE is not the direct target of abamectin, its increased activity might be indicative of a general up regulation of detoxification or neural protection systems under insecticidal stress.

Generally**,** all examined enzymes exhibited statistically significant elevations in activity in both RS5 and RS8 compared to the SS strain. The consistent and progressive increase across selection generations strongly supports the hypothesis that metabolic resistance, especially via esterases, GSTs, and oxidases, is a key mechanism underlying abamectin resistance in *R. ferrugineus*. These findings underline the importance of biochemical surveillance in resistance management and the potential benefit of using synergists or enzyme inhibitors in integrated pest control strategies.

Discussion

The present study provides compelling evidence for the rapid and progressive development of resistance to abamectin in *Rhynchophorusferrugineus* larvae under continuous laboratory selection pressure, as demonstrated by

a marked increase in LD_{50} and LD_{90} values from FS0 to FS8 generations. This resistance escalation, reaching over 51-fold by the eighth generation, aligns closely with previous reports on the capacity of *R. ferrugineus* to develop significant resistance within a few generations when subjected to persistent insecticidal exposure Abdel Rahman *et al.* (2020). The initial susceptibility baseline in our laboratory strain (LD_{50} = 0.33 µg/larva) matches the thresholds reported by Al-Ayedh*et al.* (2015), confirming the validity of our reference population. The progressive decline in probit slope values from 2.046 in FS0 to 0.627 in FS8 reflects increasing heterogeneity in larval response, consistent with the emergence of multiple resistance-conferring alleles or physiological states within the population (Bass *et al.,* 2014). This phenomenon is well-documented in insect resistance literature and indicates diversifying selection pressures favoring individuals with varying mechanisms of tolerance (Feyereisen, 2015). The data from this study confirmed abamectin's superior larvicidal efficacy compared to the other insecticides, aligning with previous studies. For instance, Farag*et al.* (2021) tested various non-conventional insecticides on *R. ferrugineus*, with the LD_{50} values for abamectin, hexaflumuron, and spinetoram being 0.04, 0.15, and 0.9 µg per larva, respectively. In a study by Huang and Wenjun (2003) on *Plutellaxylostella*, abamectin was the most toxic (72h LC_{50} = 1.59 ppm), followed by spinosad (7.77 ppm). More recently, El-Sheik (2015) confirmed that emamectin benzoate exhibited faster and more potent effects than spinosad and lufenuron. In the present study, the field-collected strain exhibited slightly higher tolerance to abamectin and the other three insecticides compared to the laboratory strain. The LD_{50} values for the four insecticides showed a modest increase in the field-collected strain (0.35, 35.57, 35.65, and 23.62 ppm) compared to the laboratory strain (0.33, 19.82, 7.42, and 14.24 ppm). Consequently, the resistance ratios were 1.06-, 1.79-, 4.80-, and 1.66-fold, respectively. This finding is consistent with that of Al-Ayedh*et al.* (2016), who observed no resistance in field-collected *R. ferrugineus* when LC_{95} concentrations of abamectin and spinetoram (0.80 and 10 ppm, respectively) were applied. Furthermore, Azizi and Khajehali (2022) confirmed similar results when studying *Tuta absoluta* (Lepidoptera: Gelechiidae), where no resistance was detected in field-collected populations from three different locations when exposed to the LC_{95} of a laboratory-susceptible strain. The lack of significant difference in susceptibility between field-collected and laboratory strains toward abamectin and other insecticides were also reported by Qiang (2005) for *Helicoverpaarmigera*. These findings highlight the importance of rotating insecticides like abamectin and hexaflumuron for effective *R. ferrugineus* management. The explanation for the insignificant difference between the susceptibility of larvae from the laboratory and field strains of *R. ferrugineus* may be related to one or more of the following reasons. Cross-resistance patterns observed with spinetoram, hexaflumuron, and dimethoate further emphasize the complexity of resistance in *R. ferrugineus*. The highest resistance ratio against hexaflumuron (RR = 105.91) suggests that insect growth regulators (IGRs) may be particularly vulnerable to cross-resistance due to shared detoxification pathways (Moustafa*et al.,* 2018). Similar cross-resistance trends were reported by El-Sayed *et al.* (2017), who demonstrated that IGR resistance often accompanies metabolic detoxification upregulation triggered by unrelated insecticides. The moderate yet significant resistance to spinetoram and dimethoate supports findings by Aly *et al.* (2021), which indicated that *R. ferrugineus*can develop cross-resistance via enhanced metabolic processes that confer broad-spectrum tolerance.

The current study revealed cross-resistance to spinetoram, hexaflumuron, and dimethoate. After eight generations of selection, the resistance ratios were 51.18-fold for abamectin, 14.07-fold for spinetoram, 105.91-fold for hexaflumuron, and 18.20-fold for dimethoate. The lowest resistance was observed toward spinetoram, while hexaflumuron showed the highest resistance. These results suggest that the development of resistance to abamectin is associated with cross-resistance to spinetoram, hexaflumuron, and dimethoate. Previous studies, such as Safiye*et al.* (2022) with *Tetranychusurticae*, also found cross-resistance between abamectin and newer insecticides like spinetoram and hexaflumuron. Moreover, Hu *et al.*(2008a) observed a 1078-fold resistance in *Plutellaxylostella* to avermectin, alongside significant cross-resistance to ivermectin and emamectin benzoate. This high degree of cross-resistance among compounds in the same chemical group is likely due to target site alterations or overproduction. Similarly, Sayyed *et al.* (2008a) found resistance ratios of 15-, 23-, 37-, and 16-fold for indoxacarb, spinosad, abamectin, and emamectin, respectively, in a field population of *Spodopteralitura* compared to a laboratory susceptible population. The resistance observed in this study suggests that the cross-resistance mechanism may be related to either target site mutations or metabolic pathways, as the insecticides tested target different biological pathways. The recent study observed significant cross-resistance from abamectin-resistant *R. ferrugineus* to spinetoram, though it was less pronounced compared to the resistance observed to hexaflumuron and dimethoate. The resistance ratios were found to be 14.07-fold for spinetoram, 105.91-fold for hexaflumuron, and 18.20-fold for dimethoate. Although cross-resistance to spinetoram was confirmed in the current study, Panel *et al.* (2012) did not find significant cross-resistance between spinetoram-resistant tobacco budworms and abamectin. The low cross-resistance to spinosad observed in our study is consistent with findings from Xin *et al.* (2010), who found field populations of *P. xylostella* selected with abamectin showed a high degree of cross-resistance to emamectin benzoate, while resistance to spinosad remained relatively low. Pei *et al.* (2003) also found that the resistance ratios to abamectin, dimethoate, and indoxacarb in *P. xylostella* were all below 20-fold. The resistance to spinosyns showed a moderate increase from 1.98–5.31-fold in 2008 to 14.31–64.20-fold in 2010, while resistance to chlorfluazuron increased sharply from 31.49–88.19-fold in 2008 to 1184.39–2789.67-fold in 2010. Rehan and Freed (2014) recently reported cross-resistance in *Spodopteralitura* after thirteen generations of selection with methoxyfenozide (an IGR). The resistance ratios observed was 28.82-fold for deltamethrin, 12.87-fold for abamectin, and a low 2.36-fold for emamectin benzoate. In another study, Vojoudi (2011) investigated cross-resistance in dimethoate-resistant *Helicoverpaarmigera*. A field-collected strain was exposed to dimethoate in the laboratory (Profen-SEL). After 14 generations, the strain developed 52-fold resistance to dimethoate and showed high cross-resistance to dimethoate (62-fold). However, it exhibited very low or no cross-resistance to lambda-cyhalothrin (2.34-fold) and spinosad (0.80-fold).

Our enzymatic assays highlight the critical biochemical mechanisms underpinning this resistance

development. The significant elevation in general esterase activity, reaching a 6.09-fold increase in the RS8 colony, underscores the pivotal role of esterases in hydrolyzing esterbonds common in abamectin and structurally related compounds, consistent with observations by Li *et al.* (2007) and Bass *et al.* (2014). Esterase activity against α-naphthyl acetate (α-NA) was assessed in susceptible and abamectin-resistant larvae. Enzyme activity (µmol/min/mg protein) showed significant elevation in resistant strains. In RS5 (RR = 34.39-fold), esterase activity was 1.39-fold higher, while in RS9 (RR = 51.18-fold), it increased to 6.09-fold. α–NA was chosen based on Mohamed *et al.*, (2021), who observed equivalent esterase activity in *R. ferrugineus* when tested with α- and β–naphthyl acetate. These findings corroborate Salaheldin*et al.*(2022) and Al-Rajhy (2005). The concurrent upregulation of GSTs, with a 2.84–3.04-fold increase, aligns with their known role in conjugating glutathione to electrophilic metabolites Feyereisen (2015). GST activity was assessed via CDNB conjugation in larval homogenates. In RS5, larvae showed 3.04-fold higher activity than susceptible strains; in RS8, the increase was 2.84-fold. Despite significant resistance increases (RR = 34.39 to 51.18), GST activity plateaued, indicating a supportive but not exclusive role in resistance. These results match findings from Wei *et al.* (2014) and Che-Mendoza *et al.*(2009).Elevated P450 monooxygenase activity further supports oxidative detoxification involvement, as described by Scully *et al.* (2019). Liying*et al.* (2015) observed tissue-specific induction of these enzymes, and we measured activity in whole-body homogenates. Ahmed *et al.* (2021) also found MFO activity correlated with diflubenzuron and spinosad resistance in *R. ferrugineus*. Other studies such as Sayyed*et al.* (2008b) and Achaleke*et al.* (2009) linked oxidase activity with resistance in *Helicoverpaarmigera*. While our study did not assess penetration resistance, prior works in mosquitoes (Che-Mendoza *et al.*, 2009; Korrat, 2009) suggest this is another important mechanism. Interestingly, increased AChE activity was observed, despite abamectin targeting glutamate-gated chloride channels, suggesting broader physiological adaptations. This mirrors findings by Hirata *et al.* (2021) and Siegfried *et al.* (2012), who noted neural enzyme modulation in response to organophosphates and carbamates. Environmental stressors also affect enzymatic responses. Roe *et al.*(2010) reported reduced insecticide penetration in resistant *Heliothisvirescens*. Panel *et al.* (2008) found *P. xylostella* selected with abamectin had high cross-resistance to tebufenozide and low to spinosad, linked to elevated P450s. Reyes *et al.* (2012) also reported spinosad tolerance and elevated MFO and esterase in field-collected *Tuta absoluta*. In *Frankliniellaoccidentalis*, Chen *et al.* (2011) found 159-fold abamectin resistance after four selection cycles, with synergist tests confirming involvement of esterases and P450s. Resistance was polygenic and autosomal, highlighting the central role of metabolic detoxification.

CONCLUSION

This study highlights the rapid development of abamectin resistance in *Rhynchophorusferrugineus*, driven by elevated esterase, GST, and oxidase activity, and accompanied by significant cross-resistance to multiple insecticides. These findings underscore the urgent need for integrated resistance management strategies, including insecticide rotation, IPM approaches, resistance monitoring, use of synergists, and the development of new chemistries. Without proactive measures, continued reliance on current insecticides may lead to control failure and severe economic impacts on palm agriculture.

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تطور المقاومة للأبامكتين والمقاومة العبورية لمبيدات حشرية متعددة في حشرة سوسة النخيل الحمراء, مع التركيز على التحليل البيوكيميائي

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الملخص

تبحث هذه الدراسة في تطور وآليات المقاومة ضد مبيد الأبامكتين في يرقات سوسة النخيل الحمراء (*Rhynchophorusferrugineus*) عبر ثمانية أجيال متتالية (من FS0 إلى FS8) تحت ظروف الإنتخاب المعملي المستمر. أظهرت النتائج تطورًا تدريجيًا في مقاومة الأبامكتين، حيث ارتفعت نسبة المقاومة (RR) من ١,٠٦ في السلالة الحقلية الأولية (FS0) إلى ٥١,١٨ ضعفًا في الجيل FS8. يشير هذا الارتفاع الكبير، جنبًا إلى جنب مع الميل المتناقص لمنحنيات البروبيت، إلى زيادة في تجانس السكان وتطور مقاومة قوية. كما لوحظت مقاومة عبورية تجاه مبيدات من مجاميع أخرى، مثل الهكسافلومورون (RR = 105.91 ضعفًا)، والدايميثويت (RR = 18.20 ضعفًا)، والسبينيتورام (RR = 14.07 ضعفًا)، مما يدل على تطور المقاومة الكبير. كشفت التحاليل الكيموحيوية عن زيادة ملحوظة في نشاط إنزيمات إزالة السمية في السلالات المقاومة. فقد ارتفع نشاط الإستيراز بمقدار ١,٣٩ ضعفًا في RS5، وبشكل كبير إلى ٦,٠٩ ضعفًا في RS8 مقارنة بالسلالة الحساسة. كما زاد نشاط إنزيم الجلوتاثيون إس-ترانسفيراز بمقدار ٣,٠٤ ضعفًا في RS5 و٢,٨٤ ضعفًا في RS8، بينما أظهر نشاط الأوكسيداز (P450) زيادات ثابتة، حيث وصل إلى ١,٨٤ ضعفًا في RS5 وتجاوز ٣,٩ ضعفًا في RS8. كذلك، زاد نشاط إنزيم الأستيل كولين إستيراز، مما يشير إلى حدوث تأقلم فسيولوجي كبير. تشير هذه النتائج بقوة إلى أن إزالة السمية الأيضية بواسطة الإستيرازات، والـ GST، وإنزيمات الأوكسيداز تمثل الآلية الرئيسية وراء تطور المقاومة للأبامكتين والمقاومة العبورية في *R. ferrugineus*. وبناءً عليه، فإن إدارة المقاومة بشكل فعال تتطلب تطبيق استراتيجيات مكافحة متنوعة، تشمل تدوير المبيدات، واستخدام المواد المنشطة (synergists)، وتبني برامج المكافحة المتكاملة (IPM) للحفاظ على فعالية المبيدات الحالية وتقليل فرص تطور المقاومة مستقبلًا.