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Biochemical and genetic mechanisms in *Pieris rapae* (Lepidoptera: Pieridae) resistance under emamectin benzoate stress

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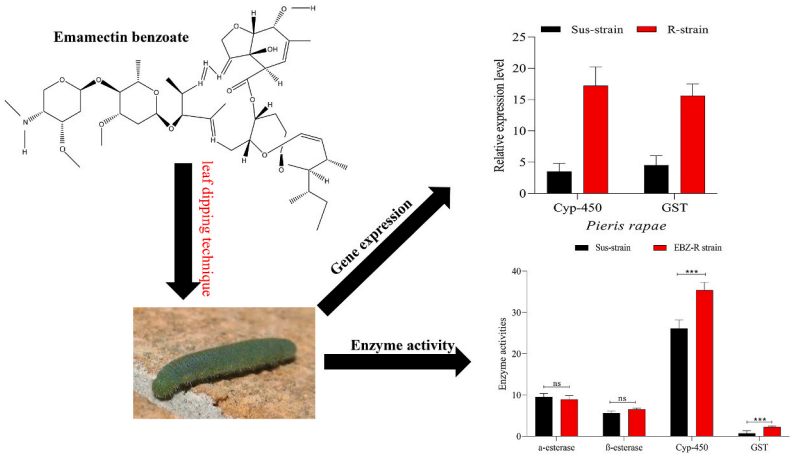
HIGHLIGHTS

- Our findings showed that *P. rapae* could promote resistance to EBZ.
- Emamectin benzoate-resistant strain exhibited a clear cross-resistance to deltamethrin.
- DEM and PBO enhanced the toxicity of EBZ in susceptibility and resistant *P. rapae* strains.
- Enzymatic and genetic analysis indicate that Cyp-450 and GST are implicated in EBZ resistance.

GRAPHICAL ABSTRACT



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ABSTRACT

Pieris rapae (Lepidoptera: Pieridae) poses a significant threat to Brassicaceae crops, leading to substantial losses annually. Repeated insecticide applications are widely used to protect crops and increase the resistance of *P. rapae*. Exploring the biochemical and molecular basis of insecticide tolerance in *P. rapae* is crucial for achieving effective insect suppuration and implementing resistance control strategies. In our research, emamectin benzoate (EBZ) resistance was developed in *P. rapae* strain through selective pressure over 15 generations. Moreover, the biochemical mechanisms underlying resistance to EBZ and its potential cross-resistance to other insecticides were studied. Additionally, the expression levels of cytochrome P450 (CYP450) and glutathione-*s*-transferase (GST) genes in *P. rapae* were quantitatively assessed upon exposure to EBZ using real-time

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PCR. Our data exhibited that the LC_{50} value of susceptible strain (Sus) and EBZ resistance strain (EBZ-R) were 0.009 and 8.09 mg/L, with a resistance ratio (RR) reaching 898.8-fold. The EBZ-R stain displayed notably low cross-resistance to lambda-cyhalothrin, spinetoram, and cypermethrin. However, it demonstrated a moderate level of cross-resistance to deltamethrin. Conversely, no cross-resistance was noted to chlorantraniliprole and indoxacarb. Notably, enzyme inhibitors of detoxification enzymes revealed that piperonyl butoxide (PBO) and diethyl maleate (DEM) enhanced the EBZ toxicity to the resistant strain, indicating the potential involvement of CYP450 and GST in avermectin resistance. A remarkable enhancement in CYP450 and GST activity was observed in the EBZ-R stain. CYP450 and GST genes are upregulated in the EBZ-R stain compared to the Sus strain, which serves as a basis for comprehending the mechanism behind *P. rapae* resistance to EBZ. The molecular docking analysis demonstrated that EBZ has a high binding affinity with CYP6AE120 and PrGSTS1 with docking energy values of −20.19 and −22.57 kcal/mol, respectively. Our findings offer valuable insights into crafting efficient strategies to monitor and manage resistance in *P. rapae* populations in Egypt.

1. Introduction

Pieris rapae L. (Lepidoptera: Pieridae) is a significant agricultural pest responsible for substantial crop yield losses in Brassicaceae crops globally (Walker et al., 2004). It has a broad host range and is capable of infesting 83 species of food plants within the Cruciferae family (Hasan, F., and Ansari, M. S., 2011). *P. rapae* has a Palearctic distribution, spanning from North Africa through Europe and extending from Asia to the Himalayan mountains (Jainulabdeen and Prasad, 2004). The caterpillars of *P. rapae* consume cabbage leaves, frequently leaving them as bare stems (Xian et al., 2017). Additionally, it pollutes plants by leaving copious amounts of excrement on the leaves (Reda et al., 2018). The larvae bore into the heads of cabbage, and a single larva of cabbage consumes 74–80 cm^2 of leaf area during development (Mohammad et al., 2004). Frequent usage of synthetic insecticides, such as pirimiphos-methyl, teflubenzuron, and pyriproxyfen, against *P. rapae* has led to the emergence of resistance (Abo-Elghar et al., 2010). Controlling *P. rapae* is becoming more difficult because of its high reproductive rate, wide temperature tolerance, larvae feeding discreetly or covertly, and resistance to many chemical pesticides (A. Aioub et al., 2021). Consequently, alternative insecticide groups featuring innovative modes of action are required to potentially postpone or impede the development of resistance (Aioub et al., 2023; Siddiqui et al., 2022).

Emamectin benzoate (EBZ), categorized as a novel macrocyclic lactone insecticide and a subset of the avermectin family, was formulated for managing Lepidopteran pests infesting various vegetable crops (Liguori et al., 2010). The primary mechanism of action of EBZ is to induce sustained immobilization of the nervous system (Jansson and Dybas, 1998). Due to its high toxicity to Lepidoptera, EBZ is relatively less harmful to many beneficial arthropods such as parasitoids, predators, and honey bees (El-Helaly et al., 2020). Nevertheless, the indiscriminate or excessive long-term use of insecticides ultimately results in the emergence of resistance in certain populations of *P. rapae*. Consequently, the efficacy of insecticides against *P. rapae* is steadily diminishing.

Chemical insecticides exert their influence on insects through various mechanisms, including biochemical pathways (Moustafa et al., 2024). Insecticide resistance arises from an elevation in levels of detoxifying enzymes, which shield insects from the detrimental effect of pesticides, and occurs as the insecticides degrade, reducing their potency and efficacy (Serebrov et al., 2006), or due to a decline in sensitivity at the target site (Ffrench-Constant, 1999). The metabolic detoxification of insecticides entails the involvement of cytochrome P450 (CYP450) and carboxylesterases (CarE), which facilitate the oxidation or reduction of insecticides (X. Li et al., 2007). Following this process, glutathione-S-transferases (GST) transform the detoxified molecule into a more soluble form through glutathione conjugation, allowing for its prompt expulsion from the cell (Enayati et al., 2005). Another possibility is that the alteration of the target site, involving the amino acid residue mutations, may lead to insects becoming less sensitive to toxic chemicals (Rinkevich et al., 2012).

Enzyme inhibitors are compounds that interact with enzymes to hinder their proper functioning (Kuddus, 2019). Insecticide synergists are crucial in tackling resistance challenges encountered in insecticide applications (Bao et al., 2016). Three common synergists, piperonyl butoxide (PBO), triphenyl phosphate (TPP), and diethyl maleate (DEM), are regularly employed to inhibit CYP450, CarE, and GST, respectively (Qie et al., 2020). Synergists are frequently combined with various pesticide classes to improve control effectiveness while minimizing treatment rates (S.-P. Wang et al., 2013). Multiple studies on synergists demonstrated that increased enzymatic activity is a crucial process in insect resistance, potentially interfering with or breaking down the pesticide (Y. Li et al., 2016). Interestingly, resistance evolution can be shaped by biochemical and genetic analysis, as evidenced by the relationship between enzyme expression and the levels of resistance (Aioub et al., 2023), as well as cross-resistance stemming from previous exposure to various insecticides (Bolzan et al., 2019).

Accurate information on the underlying resistance mechanisms and their intensity or frequency in *P. rapae* can then inform Lepidoptera control programs and ensure timely insecticide resistance management. Our study explores the biochemical and molecular underpinnings of EBZ resistance mechanisms in *P. rapae*. Furthermore, the study evaluated cross-resistance to other insecticides.

2. Material and methods

2.1. Compounds

Emamectin benzoate (Proclaim, 5% SG), chlorantraniliprole (Coragen® 20%, SC), deltamethrin (Decis® 2.5%, EC), lambada-cyhalothrin (Axon, 5% EC), indoxacarb (Avant, 15% EC), spinetoram (Radint, 12% SC), and cypermethrin (Sparkill®, 5% EC). Formulated insecticides were acquired from the Kafr El Zayat Pesticides & Chemicals Company, Egypt. Piperonyl butoxide (PBO) (90%), diethyl maleate (DEM) (98%), and TPP (≥99%) were obtained from Sigma-Aldrich, Germany. Enzyme kits for Cyp 450, GST, and CarE were sourced from the Biotechnology (SAE) Egyptian Co., Egypt.

2.2. Strains

A field population of *P. rapae* was gathered from cabbage fields located in Zagazig, Sharqia (30.5765° N, 31.5041° E), Egypt, in March 2021 (Aioub et al., 2021). A laboratory strain of *P. rapae* was reared in the Agricultural Research Center in Dokki, Egypt, in the absence of pesticides for 10 generations to be used as a susceptibility strain (Sus). Both strains were maintained at 25 ± 3 °C and 75 ± 5% relative humidity in the laboratory on fresh cabbage leaves, which were consistently offered ad libitum, enabling the maturing larvae to autonomously switch from older to newer leaves.

2.3. Bioassay

Third-instar larvae of both Sus and field strains were subject to seven different concentrations (0.25, 0.5, 1, 2, 4, 8, and 16 μg/mL) of EBZ by

using the leaf discs technique as outlined by Aioub et al. (2021). The leaf discs (0.5 cm × 0.5 cm) were cut from fresh cabbage leaves, dipped into each concentration for 10 s, held vertically to allow the excess dilution to drip off, and placed on a rack to dry. Each concentration had prepared a total volume of 20 mL and was tested with three replicates, each containing 30 larvae. Distilled water was utilized as a control. Both strains were housed in cages (25 × 20 × 18 cm) with local cabbage leaves obtained from the collection sites as a food source. The larvae were exposed to the cabbage leaves treated with the respective concentrations in a 0.25 L glass jar for 24 h. Mortality was assessed 72 h after treatment, and larvae that survived were moved to clean containers with natural leaves. The mortality result underwent probit analysis to determine the LC_{50} values of EBZ.

2.4. Selection of resistance

A field-collected population of *P. rapae* was selected for resistance to EBZ through successive applications over 15 consecutive generations via the leaf dipping technique, as described above. In each generation, 500 larvae were used in the selection pressure with EBZ. The median lethal concentration (LC_{50}) of EBZ was determined to be used as a selection pressure for the following generation. The developed resistance was expressed as a resistance ratio (RR).

2.5. Screening for cross-resistance

Third-instar larvae from both *P. rapae* strains (Sus and EBZ-R) were subjected to six insecticides (chlorantraniliprole, deltamethrin, lambda-cyhalothrin, indoxacarb, spinetoram, and cypermethrin) with various mechanisms of action using the previously mentioned leaf dipping technique, and the presence of cross-resistance was evaluated. The LC_{50} value of each insecticide was calculated for both tested strains. Cross-resistance was assessed by partitioning LC_{50} for the EBZ-R strain by the LC_{50} of the Sus strain.

2.6. Synergism study

Synergism assays were performed on both Sus and EBZ-R strains of *P. rapae* to explore the metabolic resistance mechanism to EBZ in *P. rapae*. The leaf dipping technique was used to examine the potential synergistic effect of TPP, piperonyl butoxide (PBO), and diethyl maleate (DEM) at a concentration of 70 mg/L (El-Sayed et al., 2023; Qie et al., 2020). Briefly, third-instar larvae starved for 12 h and treated with enzyme inhibitors for 1 h. Afterward, the larvae were transferred to plates and subjected to EBZ at different concentrations. Three replicates for this experiment were used. Mortality rates were noted and adjusted after 72 h of treatment. The insecticidal effectiveness of EBZ was demonstrated by the LC_{50} value, along with its corresponding 95% confidence interval. The synergism ratio (SR) was calculated using the equation provided by Qie et al. (2020): LC_{50} of larvae treated with EBZ alone divided by LC_{50} of larvae treated with EBZ + synergists.

3. Detoxification enzyme assays

3.1. Enzyme preparation

The surviving larvae of both Sus and EBZ-R strains were collected from the bioassay experiment for analysis. In total, 0.5 g of collected larvae was homogenized in 1 mL phosphate buffer solution and centrifuged for 20 min (13,000 rpm, 4 °C), and the supernatants were gathered for subsequent further enzyme activity assays. The carboxylesterase (CarE; α- and β-esterase) assays were done using α-and β-naphthyl acetate, respectively, according to Van Asperen (1962). The hydrolysis of α-naphthyl acetate was measured at 600 nm, while β-naphthyl acetate was measured at 550 nm. Additionally, GST activity was determined, as described by Habig et al. (1974). The GST activity was measured at 340 nm using a Jenway-7205 UV/Vis Spectrophotometer, Staffordshire, UK. Thus, cytochrome P450 (CYP450) activity was tested according to Hansen and Hodgson (1971) using p-nitro anisole and NADPH. MFO activity was measured at 405 nm using a microplate reader (Clindiag-MR-96, ISO09001:2008, Steenberg, Belgium).

3.2. RT-qPCR analysis

The LC_{50} values of surviving larvae (Sus and EBZ) strains (0.5g) were evaluated as three biological replicates. RT-qPCR was employed to examine the expression patterns of CYP6AE120 belonging to cytochrome P450 and PrGSTS1 to Sigma GST, following the methodology outlined by Liu et al., 2017a, 2017b and Liu et al. (2018). RNA extraction from samples was conducted using the QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH). The procedure involved adding 30 mg of the sample to 600 μl RLT buffer supplemented with 10 μl β-mercaptoethanol. Tubes were inserted into adaptor sets, which were secured into the clamps of a Qiagen Tissue Lyser to homogenize the samples. Disruption occurred through a 2-min high-speed (30 Hz) shaking procedure. Following lysate clarification, an equal volume of 70% ethanol was introduced, and the subsequent steps were conducted as per the "purification of total RNA from insect tissues" protocol outlined in the QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH). DNase digestion was performed using a single-column method to eliminate any remaining DNA. Primers were employed in a 25 μl reaction mixture, consisting of 12.5 μl of the 2 × QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 0.25 μl of RevertAid Reverse Transcriptase (200 U/μL) (Thermo Fisher), 0.5 μl of each primer at a concentration of 20 pmol, 8.25 μl of water, and 3 μl of RNA template. Primers utilized for RT-qPCR are detailed in Table 1. The housekeeping gene (β-actin) served as an internal reference to standardize the expression of target genes. The reaction was carried out using a Stratagene MX3005P real-time PCR machine.

3.3. In Silico molecular docking assay

Two protein structures (CYP6AE120 and PrGSTS1) were downloaded from the National Center for Biotechnology Information (NCBI) server to construct 3D models. Swiss model tools supplied the sequences of all proteins to create a more acceptable structural template for trustworthy theoretical 3D models. The Ramachandran plot (PROCHECK analysis) was then used to analyze and validate these models. Structure models of all proteins and their active sites (pockets) were downloaded in Protein Data Bank (PDB) format and imported into the Molecular Operating Environment (MOE) 2014.13 software (Chemical Computing Group Inc., Montreal, QC, Canada) (Damayanthi Devi, 2015). The heteroatoms and crystallographic water molecules were removed from the protein after restoring the missing hydrogen chemistry (Elkanzi et al., 2022). Ligand selection and EBZ were created using the Chem Draw Professional 15 Builder module. Before starting the docking process, the ligands were reduced using the CHARMM 99 force field. Then, three-dimensional (3D) structures were constructed, duplicates were removed, and bonds were added. The ligands were made flexible and manually placed inside the catalytic site cavity of the

Table 1
Primers of the two target genes and a reference gene of *Pieris rapae* used in this study.

Target gene		Primers sequences
Pr-β-actin	F	CGGTATGGGTCAGAAGGAC
	R	AGAAGGTGTGGTGCCAGAT
Pr-CYP450	F	TGCTTGCGTGTGTGAAACTC
	R	GAACACCCGAAGGGAGAGTG
Pr-GST	F	CTTCCCTGTTAAGGCACTC
	R	TACACAGCGTCAACCACTT

enzyme model after all the default parameters were established and the minimal energy structures were obtained. A full-force field was used to investigate the binding energy, and scoring functions that generated free-binding interaction energies based on molecular force field terms were used to assess the ligand and protein's affinity. After the docking, the best ligand interaction was investigated and evaluated using scoring functions and root-mean-square deviation (RMSD) computations (N. Zhang et al., 2016).

3.4. Statistical analysis

As per Abbott (1925), the corrected mortality percentages were subjected to statistical analysis using the Probit-MSChart computer program following the method outlined by Finney (1972). Resistance levels were categorized depending on the classification by Ahmad et al. (2010) as follows: susceptible (RR $\leq$ 1-fold), very low resistance (RR = 2-10-fold), low (RR = 11–20) moderate resistance (RR = 21–50), high resistance (RR = 51–100), and very high (RR > 100). Detoxification enzyme activities underwent statistical analysis and visualization using the paired *t*-test in GraphPad Prism 8 at $P < 0.05$.

4. Results

4.1. Selection of P. rapae resistant to EBZ

The process of selecting EBZ resistance for 15 successive generations yielded a significant level of resistance. The RR enhanced from 5.55-fold in the field (parent) generation to 898.8-fold in G15. Moreover, the LC_{50} value of EBZ increased to 8.09 mg/L compared to 0.009 and 0.05 mg/L in Sus and the parent strain of *P. rapae* (Table 2).

4.2. Susceptibility and cross-resistance of Sus and EBZ-R of P. rapae to different insecticides

As shown in Table 3, the bioassays with chlorantraniliprole, malathion, lambda-cyhalothrin, indoxacarb, spinetoram, and cypermethrin performed on the EBZ-R population (G15) showed that the selection of *P. rapae* with EBZ induced very low cross-resistance to lambda-cyhalothrin (RR = 4.08-fold; LC_{50} = 7.07 mg/L), spinetoram (RR = 2.46-fold; LC_{50} = 0.032 mg/L), and cypermethrin (RR = 3.28-fold; LC_{50} = 1.94 mg/L). Concurrently, a moderate level of cross-resistance to deltamethrin (RR = 18.83-fold; LC_{50} = 1.62 mg/L). Conversely, no cross-resistance was recorded to chlorantraniliprole (RR = 1.53-fold; LC_{50} = 1.84 mg/L) and indoxacarb (RR = 1.68-fold; LC_{50} = 4.73 mg/L).

4.3. Inhibitor enzymes effect

The effects of synergists on EBZ toxicity in the Sus and EBZ-R strains (G15) are depicted in Table 4. PBO and DEM exhibited significant synergism in the Sus (SR = 2.25- and 4.5-fold) and EBZ-R (SR = 2.22- and 2.59-fold) strains, respectively. However, no TPP had any synergistic effect on Sus and EBZ-R strains, which were 0.9- and 1.02-fold, respectively.

4.3.1. Biochemical assays

The cytochrome P450 (CYP450) and GST activities significantly enhanced in EBZ-R strain (compared with those in the Sus strain of *P. rapae*. Meanwhile, no significant was recorded in α- and β-esterase activity between the two tested strains (Fig. 1).

4.4. Detoxification gene expression by qRT-PCR

As depicted in Fig. 2, the EBZ-R strain exhibited overexpression estimated as 17.21-fold for the CYP450 gene and 15.62-fold for the GST gene compared with the Sus *P. rapae* strain.

4.5. Molecular docking analysis

Basic Local Alignment Search Tool (BLAST) through the Swiss model server was used to build templates, only the A0A821U829.1.A with CYP6AE120 (Seq. Identity 91.72%) and 3vpq.1.B with PrGSTs1 (Seq. Identity 69.61%) showed a high level of sequence similarity and were selected as templates. The final stable structure of the CYP6AE120 and PrGSTs1 and their active sites so obtained are shown in Fig. S1. Moreover, the visual inspection of the Ramachandran map (Fig. S2) demonstrates good percentages equal to 96.04 % residues in favored regions of CYP6AE120 with MolProbity score 0.92 and 98.01% residues in favored regions of GST (PrGSTs1) with MolProbity score 0.95, as well as the presence of some residue in the disallowed region which does not influence the quality of the model due to its location on an extracellular loop, outside of the binding site. Furthermore, the 3D structural simulation of the best energy-ranked result of the binding mode between enzymes and ligands is shown in Fig. (3). The docking analysis demonstrated that the investigated EBZ had a higher binding affinity to

Table 2
Selection of emamectin benzoate resistance in *Pieris rapae*.

Generations	Slope±SE	LC_{50} (μg/mL)	95% Fiducial Limits (μg/mL)	χ2	*P*-value	[a]RR	[b]RR
Sus	0.59 ± 0.17	0.009	0.007–0.012	0.41	0.93	–	–
Parent	0.98 ± 0.21	0.05	0.01–0.08	0.89	0.81	5.55	–
G1	3.21 ± 0.56	0.22	0.16–0.28	2.98	0.46	24.5	4.4
G2	3.00 ± 0.49	0.49	0.41–0.54	0.68	0.88	54.5	10
G3	2.75 ± 0.43	1.24	1.12–1.29	3.17	0.32	138	25
G4	2.38 ± 0.36	1.85	1.72–1.90	0.73	0.82	205.5	37
G5	2.17 ± 0.19	2.01	1.96–2.08	0.86	0.84	233	40
G6	1.89 ± 0.12	3.53	3.44–3.74	1.67	0.69	392	70.6
G7	2.68 ± 0.47	3.98	3.24–4.13	2.51	0.54	422	79.6
G8	1.71 ± 0.08	4.76	4.47–4.91	0.48	0.91	528.8	95.2
G9	2.26 ± 0.31	4.49	4.31–4.79	0.26	0.98	498.8	89.8
G10	1.77 ± 0.10	5.52	5.37–5.63	1.16	0.76	613	110.5
G11	2.08 ± 0.16	5.89	5.72–5.99	1.29	0.72	654.5	117.8
G12	1.93 ± 0.14	6.78	6.54–6.96	2.42	0.59	753.3	135.6
G13	1.71 ± 0.03	7.17	7.08–7.28	0.25	0.98	796.6	143.4
G14	2.44 ± 0.41	7.51	7.39–7.59	1.18	0.73	834.4	150.2
G15	2.14 ± 0.18	8.09	7.96–8.19	0.37	0.97	898.8	161.8

LC_{50} is the concentration of pesticide that is lethal to 50% of a population of test insects.
Chi-square value (χ2) was calculated using Probit analysis (Polo Plus 2.0).
The degrees of freedom (df) in each test was 4.
[a] Resistance ratio (RR) = LC50 (Gn)/LC50 (G0).
[b] RR = LC50 of resistant strain/LC50 of parent generation.

Table 3
Cross-resistance of emamectin benzoate-resistant population (EBZ-R) of *Pieris rapae* to 6 insecticides.

Population	Insecticide	Slope ± SE	LC_{50} (μg/mL)	95% Fiducial Limits (μg/mL)	*P*-value	χ2	[a]RR
Susceptible	Chlorantraniliprole	2.41 ± 0.27	0.83	0.66–0.97	0.78	1.04	–
	Deltamethrin	2.07 ± 0.17	0.086	0.076–0.092	0.17	4.12	–
	Lambda-cyhalothrin	1.51 ± 0.12	1.73	1.17–2.09	0.99	0.04	–
	Indoxacarb	0.67 ± 0.07	2.81	2.39–2.99	0.97	0.30	–
	Spinotram	0.39 ± 0.03	0.013	0.04–0.026	0.79	0.84	–
	Cypermethrin	2.15 ± 0.09	0.59	0.33–0.087	0.54	2.61	–
EBZ R-Strain	Chlorantraniliprole	2.89 ± 0.87	1.53	1.29–1.82	0.61	2.14	1.84
	Deltamethrin	1.96 ± 0.34	1.62	1.37–1.84	0.98	0.07	18.83
	Lambda-cyhalothrin	0.76 ± 0.26	7.07	6.89–7.21	0.93	0.41	4.08
	Indoxacarb	0.98 ± 0.37	4.73	4.44–4.93	0.74	1.67	1.68
	Spinotram	1.84 ± 0.74	0.032	0.014–0.047	0.96	0.22	2.46
	Cypermethrin	1.89 ± 0.41	1.94	1.68–2.14	0.79	1.56	3.28

LC_{50} is the concentration of pesticide that is lethal to 50% of a population of test insects.
Chi-square value (χ2) was calculated using Probit analysis (Polo Plus 2.0).
The degrees of freedom (df) in each test was 4.
[a] Resistance ratio (RR) = LC_{50} of resistant strain/LC_{50} of susceptible strain.

Table 4
Effects of four enzyme inhibitors on the activity of emamectin benzoate to both emamectin benzoate resistant (EBZ-R) and susceptible (Sus) populations of *Pieris rapae*.

Treatment	Slope ± SE	LC_{50} (μg/mL)	95% Fiducial Limits (μg/mL)	χ2	[a]SR
Sus-strain					
Emamectin benzoate	0.59 ± 0.17	0.009	0.007–0.012	0.41	–
Emamectin benzoate + TPP	2.23 ± 0.55	0.010	0.09–0.13	0.85	0.9
Emamectin benzoate + DEM	1.83 ± 0.28	0.002	0.001–0.003	0.59	4.5
Emamectin benzoate + PBO	2.51 ± 0.39	0.004	0.002–0.006	0.19	2.25
EBZ R-strain					
Emamectin benzoate	2.14 ± 0.18	8.09	7.96–8.19	0.37	–
Emamectin benzoate + TPP	2.54 ± 0.34	7.91	7.68–7.99	0.54	1.02
Emamectin benzoate + DEM	1.52 ± 0.19	3.12	3.02–3.22	0.31	2.59
Emamectin benzoate + PBO	1.98 ± 0.41	3.64	3.94–4.10	0.91	2.22

LC_{50} is the concentration of pesticide that is lethal to 50% of a population of test insects.
Chi-square value (χ2) was calculated using Probit analysis (Polo Plus 2.0).
The degrees of freedom (df) in each test was 4.
[a] Synergistic ratio (SR) = LC_{50} of emamectin benzoate alone/LC_{50} of emamectin benzoate with the synergist.

CYP6AE120 and PrGSTs1 with docking energy scores of −20.19 and −22.57 kcal/mol, respectively. Moreover, EBZ as a ligand deeply enters PrGSTs1's hydrophobic pocket (5.29 Å) through two H-pi bonds with Lys 132 and Glu 135, surrounded by the residues Lys 139, Thr 45, Gln 90, Pro 47, PGO3, and PGO9. Conversely, EBZ surrounded the CYP6AE120 with a hydrophobic pocket (2.83 Å) by the following residues: Asn 49, Asn 50, Asn 57, His 54, His 409, His 410, His 412, Asp 422, Asp 61, Gln 486, Lys 484, and Phe 487.

5. Discussion

Undeniably, the urgent need to address the serious issue of insecticide resistance stemming from the excessive use of insecticides is paramount (Philippou et al., 2009). Understanding the mechanisms behind insecticide resistance is vital for developing more efficient strategies to manage resistance (Silva et al., 2016). In our findings, we selected and characterized *P. rapae* to EBZ for 15 generations. The RR to EBZ was significantly increased after selection from 24.5-fold in G1 to 898.8-fold

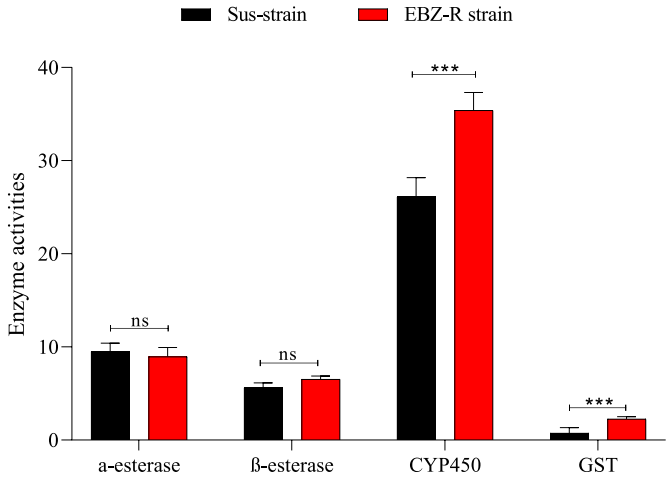

Fig. 1. α and β-esterase (μmole/min/mg protein), cytochrome P450 (CYP450, μmole/min/mg protein), and glutathione-*s*-transferase activities (GST, μmole/min/mg protein) in emamectin benzoate resistant (EBZ-R) and susceptible (Sus) populations of *Pieris rapae*. Each column represents the mean ± SEM of three independent experiments ($P < 0.05$).

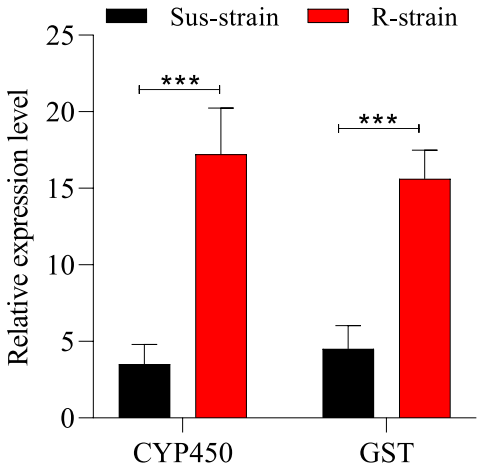

Fig. 2. Relative quantity of cytochrome P450 (CYP450) and glutathione-*s*-transferase activities (GST) gene for emamectin benzoate resistant (EBZ-R) and susceptible (Sus) populations of *P. rapae*. Each column represents the mean ± SEM of three independent experiments ($P < 0.05$).

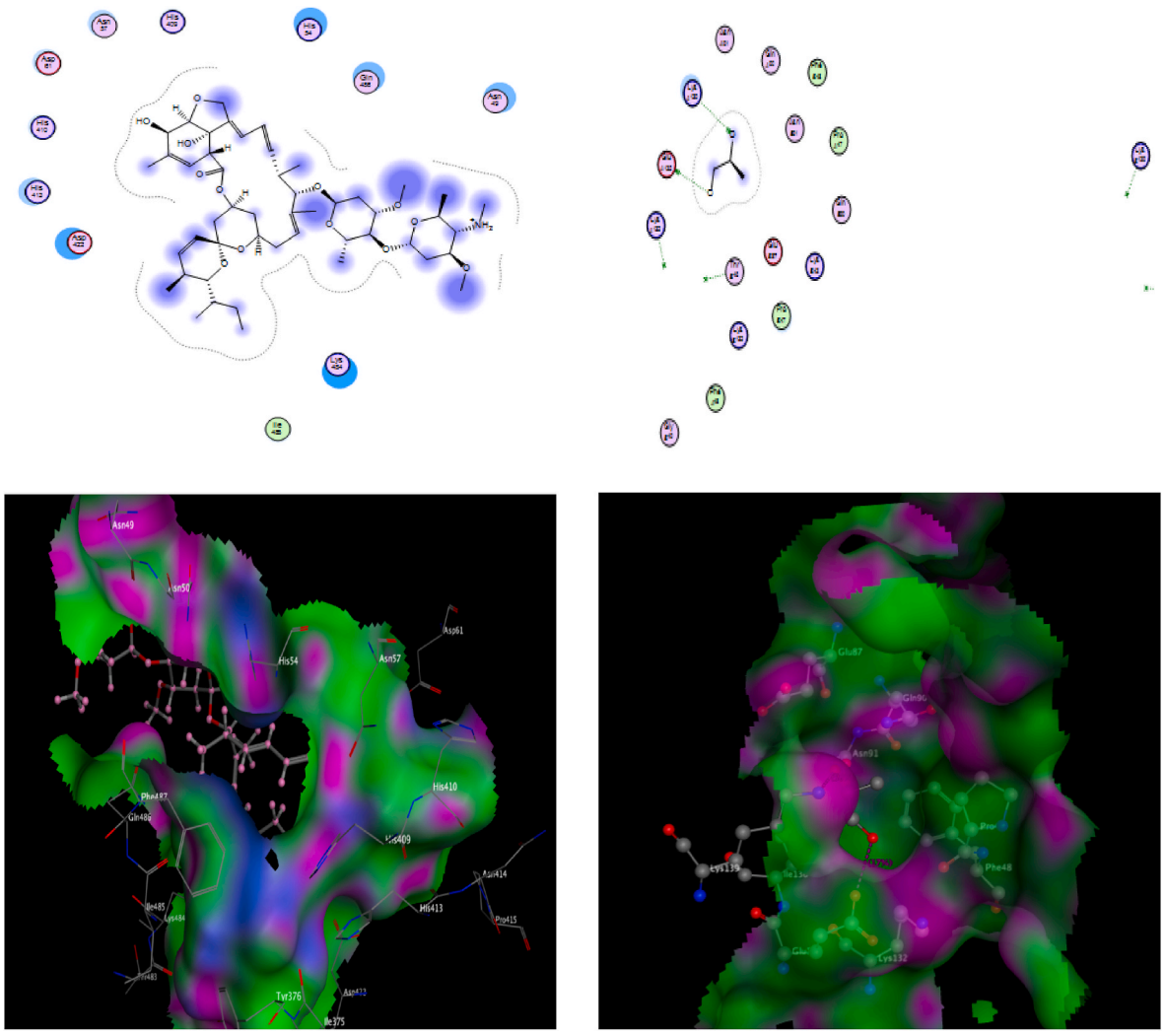

Fig. 3. Molecular docking of Emamectin benzoate with homology modeled for Cytochrome P-450 (CYP6AE120, **left**) and Glutathione-S-transferase (PrGSTs1, **right**) of Cabbage butterflies (*Pieris rapae*) created by Molecular Operating Environment (MOE) program. **Above**: two-dimensional interaction diagram of insecticide–receptor complexes. **Down**: the 3D complex structure and ligand bonds are depicted.

in G15 for *P. rapae*. This indicates that *P. rapae* can swiftly attain a significant resistance level to EBZ throughout 15 generations. Resistance selection to EBZ has been studied in *Spodoptera litura* (911.25-fold (Zaka et al., 2014); 730-fold (Shad et al., 2010):), *Spodoptera exigua* (526-fold) (M. Ishtiaq et al., 2014), *Chrysoperla carnea* (318-fold) (Mansoor et al., 2017), *Phenacoccus solenopsis* (159-fold) (Afzal et al., 2015), and *Plutella xylostella* (610-fold) (Oplopoiou et al., 2024).

Identifying cross-resistance to insecticides is crucial for devising approaches to delay resistance evolution in field settings (Wang et al., 2012). Our findings revealed that the EBZ-R strain exhibited a low cross-resistance to lambda-cyhalothrin, spinetoram, and cypermethrin but a moderate level of cross-resistance to deltamethrin. Conversely, no cross-resistance was recorded to chlorantraniliprole and indoxacarb compared to the Sus *P. rapae* strain. This may be due to insecticide resistance being raised mainly by insensitive targets or enhanced detoxification (Hilliou et al., 2021). Thus, cross-resistance among different insecticides could be due to target site mutation only when the cross couples share the same target (Brengues et al., 2003). For example, cross-resistance between abamectin and EBZ in *P. xylostella* is expected because of their common mode of action that works by stimulating the gamma-aminobutyric acid (GABA) system (Pu et al., 2010b). For those insecticides with different action targets, cross-resistance might result from enhanced detoxification, as well as reduced penetration rate or even increased excretion, which acts on both insecticides of the cross couples (Elzaki et al., 2018). Therefore, elevated metabolic detoxification might be responsible for this cross-resistance between EBZ and tested insecticides in this study because they do not share a common mode of action and belong to different chemical groups (M. Ishtiaq et al., 2014). Cross-resistance among various insecticide groups may arise from metabolic detoxification processes (Von Stein et al., 2013). Several studies clarified that the CYP450 enzyme plays a vital role in the resistance to pyrethroid insecticides and EBZ (Song et al., 2017; Che et al., 2015). Z. Wang et al. (2009) reported that an abamectin-selected strain of B-type *Bemisia tabaci* (NJAbm) showed some cross-resistance to imidacloprid, and this cross-resistance was associated with enhanced metabolism mediated by CYP450 monooxygenase and GST. Another study showed that the resistance to deltamethrin is stable in *S. exigua* and is related to metabolic mechanisms (CYP450 monooxygenase and esterase), which can confer cross-resistance to other pyrethroids (Ishtiaq et al., 2012b). Che et al. (2015) observed a slight correlation between EBZ and cypermethrin resistance. Concurrently, indoxacarb showed a lack of cross-resistance to EBZ in *S. exigua*. Moreover, there is a negative resistance between EBZ and indoxacarb in *Musca domestica* (Khan et al., 2016) and *S. litura* (Shad et al., 2010). Additionally, the chlorantraniliprole-resistant population of *Spodoptera littoralis* exhibited a negative correlation to EBZ (Moustafa et al., 2024). Furthermore, the

EBZ-R of *Dysdercus koenigii* exhibited a significant level of resistance to deltamethrin (Saeed et al., 2020). Additionally, there is a lack of cross-resistance in different field populations of *S. exigua* among representatives of EBZ, indoxacarb, spinosad (Ishtiaq et al., 2012a). Hence, successful management approaches in the field are needed to postpone continued resistance development and prevent its failure.

Elevated levels of detoxification enzyme activities may significantly impact the initial phases of resistance development (Lira et al., 2020). The toxicity of EBZ against *P. rapae* was enhanced by PBO and DEM, indicating that CYP450 and GST enzymes may have crucial roles in reducing susceptibility to EBZ in *P. rapae*. This hypothesis was corroborated by the heightened activities of CYP450 and GST in the Sus and EBZ-R *P. rapae* population. CYP450 and GST genes were also overexpressed (17.21- and 15.62-fold) as detected with qRT-PCR, respectively. This result may be attributed to the insecticide's decreased binding affinity for the target site, which could provide detoxification enzymes with additional time to metabolize the insecticide (Samantsidis et al., 2020). Our result agrees with the same trend observed by Oplopoiou et al. (2024), who reported that the coadministration of EBZ with PBO and DEM exhibited significant synergistic effects on the resistant strain of *P. xylostella*, respectively. Specifically, the PBO application enhanced the sensitivity of resistant strains *P. xylostella* (Pu et al., 2010a) and *S. exigua* (Ahmad et al., 2018) to avermectin. Moreover, PBO and DEM significantly enhanced the toxicity of EBZ-R *S. littoralis* compared to the Sus strain (Ismail, 2023). Furthermore, enzymatic activities of CYP450 and GST in *S. frugiperda* were remarkably increased in EBZ-R strain in comparison to the susceptible strain (A. Wang et al., 2024). Therefore, the upregulated CYP450 and GST enzymes are included in the detoxification process of avermectin in insects. Boaventura et al. (2020) exhibited that CYP450 genes were significantly upregulated in field-resistant *S. frugiperda* under EBZ stress. Likewise, CYP450 genes may be associated with EBZ detoxification in *S. frugiperda* (A. Wang et al., 2024). Similarly, abamectin significantly increased the expressions of CYP450 genes (Liu et al., 2018) and GST genes (Liu et al., 2017a,b) in *P. rapae*. Notably, 16 GST genes showed increased expression in *Chilo suppressalis* larvae under abamectin stress (Meng et al., 2022). Additionally, EBZ significantly upregulated GST genes in *S. frugiperda* (A. A. A. Aioub et al., 2023) and *Grapholita molesta* (S. Zhang et al., 2023). Conversely, a weak and nonsignificant correlation was observed between esterase activity and susceptibility to EBZ. This could imply that esterase activity does not notably impact EBZ resistance in *P. rapae*. Furthermore, α- and β-EST activities were reduced as EBZ-R *S. littoralis* strain (Khalifa et al., 2023) and *S. frugiperda* (Ismail, 2023). Another study showed that carboxyesterase activity of *Sogatella furcifera* reduced after 48 and 72 h of abamectin treatment compared with the susceptible strain (Zhou et al., 2018).

Molecular docking was applied to predict the relationship between EBZ and the active site of CYP6AE120 and PrGSTs1. The aim was to understand how the EBZ interacts with the vital amino acids of the two tested enzymes and their binding modes. Our findings demonstrated that the binding affinity between EBZ was high with CYP6AE120 and PrGSTs1, indicating the potential of two tested enzymes to metabolize EBZ. This result may be because the amino acids in the receptor are linked by double or triple bonds and the docking binding affinity is high with lower binding energy (Yang et al., 2022). Furthermore, the type and position of amino acids in the active site of GSTs and CYP-450 play essential roles in insecticide binding affinity and catalytic functions (Harris et al., 2014; Feng et al., 2019). Our result was confirmed by Liu et al. (2017, 2018), who proved that CYP6AE120 and PrGSTs1 are considered one of the most upregulated genes in CYP450 and GST groups of *P. rapae* under lambda-cyhalothrin, chlorantraniliprole, and abamectin stress, respectively.

6. Conclusion

Gaining insight into the biochemical and molecular mechanisms underlying insect resistance to EBZ can be instrumental in devising successful resistance management strategies. Moreover, tracking the expression of CYP450 and GST can offer valuable insights into resistance development and aid in promptly identifying resistant populations. Furthermore, the outcomes of cross-resistance investigations can inform suitable rotations of pesticide application and pinpoint alternative insecticide groups capable of potentially postponing or impeding resistance resurgence. In conclusion, ongoing research is essential for deepening our understanding of resistance mechanisms and devising successful approaches to manage resistance in *P. rapae*.

CRediT authorship contribution statement

Ahmed A.A. Aioub: Writing – review & editing, Writing – original draft, Resources, Methodology, Data curation, Conceptualization. **Moataz A.M. Moustafa:** Formal analysis, Writing – review & editing. **Ahmed S. Hashem:** Writing – review & editing, Software, Methodology. **Samy Sayed:** Conceptualization, Writing – review & editing. **Hanan M. Hamada:** Validation, Writing – review & editing. **Qichun Zhang:** Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Sarah I.Z. Abdel-Wahab:** Writing – review & editing, Methodology, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

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