Lower levels of enzyme activity increase the susceptibility of Agonoscena pistaciae to imidacloprid

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Abstract

Background: The common pistachio psyllid, *Agonoscena pistaciae* (Hemiptera: Aphalaridae), is a major pest in pistachio farming areas across Iran. Nymphs and adult insects feed on the plant sap by inserting their mouthparts into the leaves. This feeding behavior results in a decrease in both the quality and quantity of the pistachio crop.

Materials and methods: The sensitivity of 5th instar nymphs of *A. pistaciae* in Rafsanjan to commonly used insecticides imidacloprid, chlorpyrifos, and fenvalerate, was examined using the tower spraying method.

Results: Based on the results, the psyllid exhibited LC_{50} values of 41.6, 372.4, and 256.8 ml a.i./L for imidacloprid, chlorpyrifos, and fenvalerate, respectively. According to the results of this study, *A. pistaciae* was significantly more sensitive to imidacloprid than to two other insecticides, chlorpyrifos and fenvalerate. The study measured the impact of pesticides on the activity of three key detoxification enzymes - cytochrome P_{450} enzymes, naphthyl acetate esterase enzymes, and glutathione S-transferases. The results showed that nymphs treated with imidacloprid had the lowest enzyme activity.

Conclusion: The findings suggest that imidacloprid is more effective in controlling the common pistachio psylla due to its lower detoxification under the enzyme activity.

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1. Introduction

The common pistachio psyllid, Agonoscena pistaciae (Hemiptera: Aphalaridae), is a key pest in pistachio farming areas across Iran. Nymphs and adult insects feed on the plant sap by inserting their mouthparts into the leaves. This feeding behavior decreases the pistachio crop's quality and quantity [1,2]. Control of this pest is mainly accomplished through pesticide application, with imidacloprid being one of the most widely used insecticides [3]. Imidacloprid belongs to the nitroguanidine chemical class and is a systemic insecticide. It acts as an agonist for the nicotinic acetylcholine receptor in insects, causing excessive stimulation of the nervous system, which ultimately results in paralysis and death. [4,5]. Chlorpyrifos is a widely used organophosphate insecticide in agriculture to control pests like psyllids. It is a broad-spectrum insecticide that works by inhibiting the enzyme acetylcholinesterase. This inhibition leads to the build-up of acetylcholine, a neurotransmitter responsible for transmitting signals between nerve cells, causing symptoms such as muscle weakness, respiratory depression, and potentially death [6-9]. Fenvalerate is a synthetic pyrethroid insecticide and acaricide. It is utilized to manage a diverse range of pests, such as aphids, thrips, whiteflies, mosquitoes, and ticks. Its mode of action involves disrupting the normal function of the nervous system in insects and mites. This is achieved by binding to sodium channels in nerve cells, which hinders proper closure. Consequently, the nerve cells continuously fire, causing paralysis and eventual death [10].

The slope of the concentration-mortality line in bioassays of pesticides provides information about the phenotypic variation within a population. The dose-response curve is a graphical representation of the relationship between the dose of an insecticide and the response of the target pest. The slope ratio bioassay is a method used to compare the responses of two groups exposed to varying concentrations of a test and reference substance, enabling assessment of the relative potency based on the slopes of their dose-response curves. A steeper slope in a dose-response curve indicates that an insecticide is more potent, meaning smaller dose changes lead to larger responses in target pests, making it effective at lower doses. Conversely, a flat line suggests lower potency and higher variability in susceptibility among the population [11,12]. In the context of pesticide bioassay, the chi-square test is used to determine whether the observed results of a bioassay experiment are significantly different from the expected results. In general, a lower chi-square value suggests that the observed data closely matches the expected data, indicating that the pesticide treatments may not have a significant effect on the test organisms in this particular bioassay[13-15]. In assessing the efficacy of insecticides against the common pistachio psylla (Agonoscena pistaciae), three key pesticides—imidacloprid, chlorpyrifos, and fenvalerate—were selected due to their specific properties and effectiveness in pest control. Understanding the rationale behind choosing these insecticides and the significance of monitoring insect resistance is crucial for sustainable agricultural practices. The selected insecticides operate through different mechanisms, which can be advantageous in integrated pest management (IPM) strategies. However, the selection of imidacloprid, chlorpyrifos, and fenvalerate for controlling pistachio psylla is based on their proven effectiveness and operational mechanisms.

Concurrently, addressing the issue of insect resistance is vital for maintaining effective pest control and ensuring the sustainability of agricultural practices over time.

Due to the short life cycle of the pistachio psyllid, its high reproductive capacity, and the selective pressure resulting from the excessive use of chemical insecticides, certain populations of the common pistachio psylla have rapidly developed resistance to insecticides [16]. Pesticide resistance is a major challenge in agriculture and public health. It occurs when pests develop the ability to survive exposure to pesticides that were once effective in controlling them. This can lead to crop losses, increased use of pesticides, and environmental contamination [17]. Enzymes play a crucial role in various physiological and biochemical processes within organisms, including the detoxification and metabolism of harmful substances like pesticides. When enzyme activity is reduced, the organism's ability to break down and eliminate pesticides is compromised, making it more susceptible to their toxic effects. For several reasons, understanding the relationship between enzyme activity and pesticide sensitivity is essential. Firstly, it helps in developing more effective and targeted pest management strategies. Secondly, this knowledge aids in assessing the potential risks associated with pesticide use. By evaluating the enzyme activity levels in different organisms, it will be possible to predict their susceptibility to pesticides and implement appropriate measures to mitigate these risks [17-19]. Important enzymes in detoxification metabolism include cytochrome P450 oxidases, esterases, and glutathione S-transferases. Cytochrome P450 enzymes (monooxygenases) play a crucial role in synthesizing xenobiotics and endogenous compounds. This type of metabolism is a common mechanism through which insects develop resistance to insecticides, as demonstrated by the impact on numerous insect species and insecticides [18,20-22]. Glutathione S-transferases (GSTs) play a crucial role in insect resistance by metabolizing or sequestering pesticides and offering protection against oxidative stress caused by insecticide exposure [15,21,23-25]. Naphthyl acetate esterase enzymes are a class of enzymes that break down ester bonds and help to catalyze the process of ester hydrolysis[26,27].

Overall, the correlation between reduced enzyme activity and increased sensitivity to pesticides is a critical factor in comprehending the effects of pesticides on organisms. By studying and understanding this relationship, we can develop more sustainable pest management practices and protect the environment and human health. In this study, we examined the toxic effects of three specific pesticides. We compared their toxicity against the common pistachio psylla by assessing the activity of the four most important detoxifying enzymes.

2. Materials and methods

2.1. The pest collection

The common pistachio psylla is a pest that is often found in many gardens and regions. However, for the sake of consistency, we collect the common pistachio psylla specifically from hazelnut cultivar and certain areas and then transfer them to the laboratory. In present research only one-day-old fifth-instar nymphs for bioassay and biochemical tests. The fifth instar nymphs are easily recognized by their two wing pads and a more vivid coloration, often appearing darker or more vibrant than earlier stages. Fifth-instar nymphs, one day old, were

separated daily from a regularly monitored homogeneous culture.

2.2. Bioassay of the pesticides against the common pistachio psylla

Commercial formulations of imidacloprid (Confidor, SC 35%, khazarsamkood), chlorpyrifos (Dursban EC, 20%, Aryakeshavarz), and fenvalerate (Sumicidin, EC 20%, Sepahan Sam) were used. To conduct the bioassay tests, the spraying method was employed using a spray tower, which minimizes test losses and provides conditions similar to those in a garden. To determine the LC50 in the final tests, five concentrations are chosen with a logarithmic spacing between the concentrations, resulting in approximately 25% to 75% mortality. The treated nymphs were allowed to air dry and then placed on the pistachio leaves for assessment under controlled conditions. Three replications of each treatment and control were conducted, with each replication consisting of 15 nymphs. Distilled water was used as the control. Mortality was recorded for imidacloprid and chlorpyrifos after 24 hours, and for fenvalerate after 48 hours. The dose-dependent mortality response, including the slope, the median lethal concentration (LC50), and the associated 95% fiducial limits, was determined. Heterogeneity was assessed during the analysis using a chi-square test. If it was found to be significant at the 5% level, the variance of the estimated parameter was adjusted by the corresponding heterogeneity factor, which was equal to the residual mean deviance [5,13,14].

2.3. Measurement of esterase activity

Two hundred specimens of the psylla were homogenized in 300 µL of 0.1 M phosphate buffer (pH 7) containing 0.1% Triton X-100. The homogenized solution was centrifuged at 15,000 rpm for 10 minutes and was used as the enzyme source in the experiment. The protein content in enzyme samples was determined using the method developed by Lowry et al. with bovine serum albumin (BSA) serving as the standard [28]. The hydrolytic activity of esterases on α-naphthyl acetate and β-naphthyl acetate (as substrates) was measured according to the method of van Asperen [29]. with some modifications [30]. The enzyme activity was assayed by adding 50 µL of enzyme sample to 100 µL of phosphate buffer (pH 7) and 10 µL of substrate (10 mM in acetone). After the completion of the reaction, 50 µL of Fast BlueRR (0.5 mg/ml phosphate buffer) was added to the mixture. The amount of naphthyl produced was then measured over a time interval of 25 minutes, and at a wavelength of 405 nm using a microplate reader. A standard curve was created by measuring the absorption of different doses of naphthyl. This allowed the amount of produced product to be calculated based on the standard curve. A linear diagram of enzyme activity was drawn to determine the specific enzyme activity. The slope of each line, based on the product produced per minute on the amount of protein in the sample, was then calculated and compared [30].

2.4. Measuring the activity of Glutathione S-transferase (GSTs)

Fifty specimens of the psylla were homogenized in 200 µL of cold 10 mM phosphate buffer (pH 7). The samples were then centrifuged at 10,000 rpm at a temperature of 4 °C. The activity of glutathione S-transferase was measured using the substrate 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH), following the method described by Habig and Jakoby (1981)[31]. The 200 µL of the reaction mixture, comprising 1 mM CDNB and

10 µL of reduced glutathione in a phosphate buffer (0.1 M, pH 7), is dispensed into the wells of the ELISA plate. Absorbance changes were measured using an ELISA reader for 60 seconds and 10 minutes at a wavelength of 340 nm. Repeat the measurement at least four times for each treatment. The absorption coefficient of 4,2-dinitrophenyl glutathione (ε340nm=9.6 mM-1 cm-1) is utilized to convert the absorbance changes in glutathione S-transferase activity to the reaction product. The protein levels in the samples are also determined using the Lowry method [28].

2.5. Measurement of cytochrome P450 activity

Cytochrome P450 activity was evaluated by measuring the total oxidase level using iron peroxide, an indirect method for assessing cytochrome P450. The iron peroxidation technique is suitable for comparing variations in the overall oxidase level based on the hemoprotein quantity. Quantifying its amount is utilized to compare the level of cytochrome P450 based on the general oxidase level, as it is mainly present in the bodies of non-blood-feeding insects [32,33]. Seventy psylla specimens were homogenized in 0.04 M sodium phosphate buffer (pH 7). The homogenates were centrifuged at 10,000 g at 4°C for 20 minutes and the resulting supernatant was used as an extract [34]. The enzyme activity was assessed using the TMBZ substrate, with a purity exceeding 98%. The reaction mixture consisted of 50 µL of 100 mM sodium phosphate buffer (pH 7.2), 50 µL of enzyme extract, and 150 µL of TMBZ (13 mg in a solution of 6.5 ml of methanol and 19.5 ml of 0.25 M sodium acetate buffer at pH 5). Subsequently, 25 µL of 3% hydrogen peroxide was introduced. Following a 30-minute incubation at room temperature, the Eliza Reader recorded the absorbance at 630 nm every 5 minutes for a total of 25 minutes. Each treatment underwent four repeated measurements [35].

2.6. Measurement of total protein

We utilized the modified Lowry et al. method [28] to measure protein. Initially, thirty specimens were weighed within microtubes and then homogenized thereby adding 1.5–2.0 ml of 80% ethanol. The precipitate was utilized for protein measurement after centrifugation for 10 minutes at 12,000 rpm. In this experiment, 3 mL of a salt solution containing equal proportions of sodium dodecyl sulfate (1%), sodium bicarbonate (0.4%), sodium carbonate (2%), and sodium potassium tartrate (0.18%) was added to the sediment. The mixture was then vortexed for a period. They were stored at room temperature for 24 hours. Subsequently, 150 microliters of the sample were mixed with 850 µL of distilled water, and 3 milliliters of Lowry's solution were added to each sample. The samples were thoroughly vortexed, and after 10 minutes, 150 microliters of Folin solution were added. After thirty minutes, the absorbance was measured using a spectrometer at a wavelength of 540 nm. Finally, the protein concentration of the samples was determined by plotting the standard curve. Bovine serum albumin protein was used as the standard in this method.

2.7. Statistical analysis

The LC50 values of pesticides against the common pistachio psylla were determined using Polo-Plus software. The data were analyzed in biochemical tests using a t-test to compare two pesticides. Tukey's test was conducted using SPSS version 16.00 software to compare the average of three pesticide treatments. Results

were expressed as the mean ± SE and were considered significantly different at $P < 0.05$.

3. Results

The results of the bioassay of three insecticides, imidacloprid, chlorpyrifos, and fenvalerate, using the spray tower method on one-day-old fifth instar nymphs of the common pistachio psylla were shown in Table 1 and Table 2. Based on the findings, the sensitivity of the common pistachio psylla to pesticides can be classified as imidacloprid > fenvalerate > chlorpyrifos. The LC50 values of the insecticides, imidacloprid, chlorpyrifos, and fenvalerate, were 41.6, 372.4-, and 256.8-mL a.i/liter, respectively.

Table 1. Concentration/mortality of different pesticides against fifth instar nymphs of the common pistachio psylla, *Agonoscena pistaciae*

Pesticide	Concentration (mg a.i./L)	Mean mortality%	Corrected mortality (%)
Control	0	7.2	7.2
Imidacloprid (35%SC)	50	28.8	21.6
	80	39.5	32.3
	120	57.5	50.2
	200	71.1	63.8
	300	78.3	72.9
Chlorpyrifos (EC 20%)	350	25.1	17.8
	500	35.6	28.3
	800	49.5	42.2
	1200	62.6	55.3
	1800	74.6	67.3
Fenvalerate (EC 20%)	400	28.1	20.8
	700	37.1	29.9
	1100	52.0	44.7
	1800	63.7	564
	3000	72.4	65.1

Table 2. The sensitivity of *Agonoscena pistaciae* to the insecticides imidacloprid, chlorpyrifos, and fenvalerate.

Pesticide	No of insect	Slope±SE	LC_{50} (mg/L) (Fiducial limit 95%)	X^2	Heterogenicity
Imidacloprid (24 hours)	324	1.92±0.33	41.6 (31.5-52.8)	0.24	0.08
Chlorpyrifos (24 hours)	330	2.06±0.36	372.5 (290.1-471.8)	0.04	0.01
Fenvalerate (48 hours)	333	1.55±0.30	256.8 (185.3-351.3)	0.31	0.14

Table 3. Enzyme activity of fifth instar nymphs of *Agonoscena pistaciae* impacted by pesticides.

Enzyme	Treatments	Enzyme activity± SE (µmol/min)
β-naphthyl acetate esterase	Control	0.81±0.09 a
	Imidacloprid	1.15±0.04 b
	Chlorpyrifos	1.45±0.08 c
	Fenvalerate	1.32±0.09 cd
α-naphthyl acetate esterase	Control	0.55±0.14 a
	Imidacloprid	0.92±0.11 b
	Chlorpyrifos	3.16±0.47 c
	Fenvalerate	2.57±0.77 c
Glutathione S-transferase	Control	0.17±0.03 a
	Imidacloprid	0.30±0.04 b
	Chlorpyrifos	0.72±0.16 c
	Fenvalerate	0.50±0.03 d
CytochromeP_{450}	Control	0.002±0.00 a
	Imidacloprid	0.004±0.00 b
	Chlorpyrifos	0.004±0.00 b
	Fenvalerate	0.005±0.00 b

The experiments were conducted at the LC_{50} values of each pesticide. Different letters represent significant differences for each enzyme ($P \leq 0.05$).

Comparing the insecticides imidacloprid and chlorpyrifos, with an LC50 ratio of 8.95 and upper and lower limits (with 95% confidence) of 6.35-12.61, indicates a significant difference in their LC50 values. The LC50 ratio of 6.2 between the insecticides imidacloprid and fenvalerate, along with upper and lower limits (with 95% confidence) of 0.009-4066.4, indicates no significant difference between their LC50 values. Between the two insecticides fenvalerate and chlorpyrifos, with an LC50 ratio of 1.45 and upper and lower limits (with 95% confidence) of 0.002-954.0, there is no significant difference between the LC50 of these two insecticides. Based on the bioassay's findings, A. pistaciae showed significantly greater sensitivity to imidacloprid compared to the other two insecticides, chlorpyrifos, and fenvalerate.

In the bioassay of the common pistachio psylla fifth instar nymphs, imidacloprid has a slope of 1.92, indicating a relatively steep curve, which suggests that small changes in concentration result in significant variations in mortality. Chlorpyrifos has a slope of 2.06, slightly steeper than that of Imidacloprid, indicating it may exert a more pronounced effect at lower doses. Fenvalerate has the lowest slope at 1.55, implying a more gradual response to increasing concentrations, which may indicate variability in sensitivity among individuals within the test population.

Considering the enzyme activity, it is evident that all the tested pesticides increased enzyme

activity. Table 2 clearly shows that the enzyme activity in the control group is significantly lower than in the pesticide treatments. By analyzing the enzymes, it is evident that CytochromeP450 exhibited the lowest activity in all the treatments, while the esterase enzymes showed the highest activity. If the CytochromeP450 activity in the treatments was significantly higher than that of the control, there was no significant difference in the activity of this enzyme among the pesticides. Imidacloprid showed the least impact on enzyme activity when compared to other pesticides. The activity of the four enzymes in the imidacloprid treatment was notably lower than in the different treatments. The glutathione S-transferase activity in imidacloprid-treated nymphs was 0.30±0.04 µmol/min, significantly higher than that of the control group but lower than other pesticides. Chlorpyrifos, on the other hand, had the greatest impact on enzyme activity. The glutathione S-transferase activity in chlorpyrifos-treated nymphs was 0.72±0.16 µmol/min, significantly higher than the control group and other pesticides. The data in Table 1 shows that X2 and heterogeneity for the three tested pesticides are very low. A chi-square value near zero suggests no significant association between the variables. A higher chi-square value indicates a stronger association between the variables. A heterogeneity value of 0% suggests no variability between studies, while a value of 100% indicates a high degree of variability between studies. A heterogeneity value of 30–60% is considered moderate, while a 50–90% value is deemed substantial [12,15].

4. Discussion

In this study, we used the dose-response model to evaluate the effectiveness of three different pesticides, namely imidacloprid, chlorpyrifos, and fenvalerate, against a major pest of the pistachio tree known as A. pistaciae. We also tested the impact of these pesticides on the enzyme activity of the pest. The LC50 value represents the pesticide concentration needed to kill 50% of test animals within a specified time frame. According to the data, imidacloprid is the most toxic among the three insecticides. Chlorpyrifos is less toxic than Imidacloprid, as indicated by its higher LC50 value and a less steep slope of the dose-response line. Fenvalerate is the least toxic of the three insecticides, as it has the highest LC50 value and the least steep slope on the dose-response line. The chi-square and heterogeneity values for all three insecticides indicate a good fit of the dose-response data to the model employed. Imidacloprid is a type of neurotoxin called neonicotinoid, which is still commonly used to combat the common pistachio psylla [36,37]. In a study, imidacloprid was found to be highly toxic to nymphs of the common pistachio psylla with an LC50 of 138.21 mg a.i/L in the fifth-instar stage [5]. Different levels of mortality may be attributed to various biotic and abiotic factors, such as the host cultivar, application method, duration of exposure, and time of bioassay [38–40]. In a study, the LD50 for imidacloprid against Blattella germanica was 2.66 mg/m2. The highest mortality rate occurred within 48 hours after exposure to the insecticide through the ingestion of imidacloprid-smeared bait (Baniardalani et al., 2019). Fenvalerate and chlorpyrifos are two pesticides that are effective against the common pistachio psylla. Fenvalerate has an LC50 value of 256.8 mg/L, making it the second most effective pesticide, while chlorpyrifos has an LC50 value of 372.5 mg/L, making it the third most effective. The lower toxicity of these pesticides is likely due to their mode of action, the sensitivity of the target

pest, the recovery of target sites, and the activity of metabolic enzymes [42] The lethal concentration at which 50% of grass-lawn armyworms, Spodoptera cilium (Guenee) (Lepidoptera: Noctuidae) died due to fenvalerate was reported to be 211.4 mg a.i./L [43]. Fenvalerate showed toxicity against 3rd instar larvae of the Diamondback Moth, Plutella xylostella (L.), ranging between 40.0 to 60.0 mg a.i./L on different host plants, with no significant difference [42]. Six populations of the citricola scale, Coccus pseudomagnoliarum (Kuwana) (Hemiptera: Coccidae), were tested to determine their responses to chlorpyrifos. The nymphs' LC50 responses ranged from 7.5 to 68.9 ppm [44].

An examination of enzyme activity under control and pesticide treatments indicates that cytochromeP450 exhibited the lowest activity, which is notably higher than that of the control. This finding suggests that insecticide treatment alone acts as a stressor for the pest and induces the activation of detoxifying enzymes. However, esterases generally showed the highest activity under various treatments. Pesticides can act as stressors and induce the activity of enzymes in insects. The primary mechanism of insecticide resistance in insects is the enhanced metabolism of toxic substances, known as biochemical resistance. This increase in enzyme activity is achieved through enzyme induction. Enzyme induction is the process by which the concentration of an enzyme is increased in response to a stimulus. In the case of insecticides, the stimulus is the presence of the toxicant [45]. Insect resistance mainly depends on the function of detoxifying enzymes in insects, which boost their metabolic capacity to combat pesticides. The detoxification capability is evident in how these enzymes respond to insecticides [46,47]. The higher sensitivity of a population of the western flower thrips, Frankliniella occidentalis (Pergande) (Thysanoptera: Thripidae) is related to the higher activity of the detoxification enzyme [46]. In the greater wax moth, Galleria mellonella (L.), organophosphates caused metabolic and synaptic dysfunctions, altering its biochemical physiology in response to oxidative stress [48]. The induction of BmGSTS2 in pesticide-treated silkworm larvae, Bombyx mori, suggests that the upregulation of Cytochrome P450 is part of the defense mechanism against external chemicals [49].

5. Conclusions

Imidacloprid is identified as the most toxic pesticide tested, requiring significantly lower concentrations for lethal effects compared to chlorpyrifos and fenvalerate. The analysis indicates that while all pesticides exhibit good model fit with low heterogeneity, their toxicities differ substantially, which is crucial for pest management strategies and environmental safety considerations. This analysis can guide further research into effective pest control measures while considering potential impacts on non-target organisms and ecosystems.

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