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Research Article

High detoxification enzyme and gene expression level in tomato leafminer, *Tuta absoluta*, explains resistance to fenvalerate

Ehsan Baradaran, Azam Mikani*, Saeid Moharramipour and Mohammad Mehrabadi

Department of Entomology, Faculty of Agriculture, Tarbiat Modares University, Tehran 14115-336, Iran.

Abstract: The tomato leafminer, *Tuta absoluta*, is a pest that greatly harms crops. Regrettably, the application of insecticides has resulted in the emergence of resistance in the tomato leafminer. Metabolic resistance is the most prevalent form of resistance, characterized by heightened esterase activity, cytochrome P450 monooxygenase, and glutathione S-transferase enzymes encoded by specific genes. This study investigated the metabolic resistance mechanisms associated with fenvalerate resistance in the tomato leafminer and explored the genes' status. Tomato leafminer populations in Iran showed varying levels of resistance to fenvalerate; the Tehran and Urmia populations were the most resistant and sensitive, respectively. The activity level of detoxifying enzymes, particularly cytochrome P450 monooxygenase, was found to be increased in the resistant populations (i.e., Tehran) compared to the susceptible one (i.e., Urmia). Gene expression analyses showed higher transcript levels of *P450*, *esterases*, and *GSTs* expression levels in the resistant population compared to the susceptible population. Our findings indicated that detoxification enzymes, especially cytochrome P450 monooxygenase, and differential expression of related genes contribute to fenvalerate resistance. Identifying the specific mechanisms behind resistance could assist in pest control and resistance management programs.

Keywords: fenvalerate, *Tuta absoluta*, cytochrome p450 monooxygenase, *cyp306a*

Introduction

The tomato leafminer (TLM), *Tuta absoluta* (Meyrick) (Lep.: Gelechiidae), is a devastating global pest of tomato crops and causes significant damage to yield and quality. The larvae burrow into tomato leaves, stems, and fruits, causing extensive plant damage and reducing yield. Several methods for controlling TLM include insecticides, biological control agents, and cultural practices (Ghaderi *et al*., 2017; Mirhosseini *et al*., 2019). Excessive use of insecticides leads to resistance in the pest populations and recently spread and become resisted in the United Kingdom, forming the concern that become a problem in Europe (Grant *et al*., 2019; Grant *et al*., 2023). The tomato leafminer has developed resistance to an extensive list of 17 insecticides, which includes many pesticide categories such as carbamates, organochlorines, pyrethroids and insect growth regulators as documented in reports (Guedes *et al*., 2019; Siqueira *et al*., 2000; Siqueira *et al*.,

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*Corresponding authors: a.mikani@modares.ac.ir
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2001; Lietti *et al*., 2005; Silva *et al*., 2011; Reyes *et al*., 2012; Campos *et al*., 2015; Silva *et al*., 2016; Douris *et al*., 2017; Grant *et al*., 2019; Silva *et al*., 2021).

There are several types of resistance mechanisms that pests can develop against insecticides, such as target-site resistance, metabolic resistance, penetration resistance, behavioral resistance, and cross-resistance (Hemingway and Ranson, 2000). Previous research revealed that amino acid mutations on organophosphate and carbamate target sites are responsible for insecticide resistance in the diamondback moth, *Plutella xylostella* (Liu *et al*., 1981; Yu and Nguyen, 1992). However, metabolic resistance is the most prevalent resistance mechanism in insects, which involves the enhancement of esterase, cytochrome P450 monooxygenase, and glutathione S-transferase activity (Scott and Wen, 2001; Feyereisen, 2006). Previously, scientists dedicated most of their studies to these enzymes. Nevertheless, it has become clear that exploring their encoding genes is equally important. A set of encoding genes forms detoxification enzymes belonging to the different classes. Glutathione S-transferase is mainly divided into six classes, including Delta, Epsilon, Omega, Sigma, Theta, and Zeta Cytochrome P450 monooxygenase has four clades, including CYP4, CYP3, CYP2, and mitochondrial clad; Esterases are also mainly classified into alpha, beta, acetylcholinesterase, hormone processing, and neuro/developmental classes (Feyereisen, 2011). In each class, multiple genes carry the potential for detoxifying insecticides.

Understanding the mechanism behind resistance is crucial for effective pest management, and it requires answering how insects, especially target pests, become resistant. In this study, we investigated the detoxification enzyme activities and expression of some detoxification genes in the susceptible and fenvalerate-resistant populations of TLM collected from different regions of Iran. We showed that the resistant population imposed higher detoxification enzyme and gene expression levels.

Materials and Methods

Insect culture

Four populations of tomato leafminer were collected from the tomato greenhouse in Tehran (35°44'29.7"N 51°09'43.0"E), Urmia (37°31'41.3"N 45°07'55.2"E), Arak (34°18'03.3"N 50°15'40.3"E and Kashmar (35°15'11.9"N 58°26'59.0"E) Iran in 2020. For three generations, the populations were reared on tomato plants at 25 ± 1 °C, 65 ± 5 Relative humidity, and a photoperiod of 16L:8D h within individual cages (120 × 100 × 80 cm) situated at Tarbiat Modares University and were subsequently deployed in the research trials.

Bioassays

The toxicity of fenvalerate ($C_{25}H_{22}ClNO_3$, with 97% purity) (Sigma, Missouri) on the third-instar larvae from each population was evaluated using the leaf disc-dip method (Galdino *et al*., 2011). Distilled water (with 0.1% Triton X-100) was used to wash fresh tomato leaf discs (5 cm diameter) and air-dried at room temperature. Seven different concentrations of fenvalerate solutions (0, 100, 250, 500, 1000, 2500 and 5000 mg/l for Urmia, and Arak population, 0, 500, 1000, 2500, 5000, 7500 and 10000 mg/l for Kashmar population, and 0, 500, 2500, 5000, 12000, 25000 and 50000 mg/l for Tehran population) were used to immerse the leaf discs for 10 seconds. Acetone was utilized to dilute the insecticide. The control discs were treated solely with acetone and used for mortality correction using Abbott's formula (zero concentration of fenvalerate). All the discs were air-dried and placed in plastic containers (7 cm in diameter) along with 10 larvae. The mortality rate was observed after 24 hours. The experiment was repeated three times.

Cytochrome p450 monooxygenase activity

Cytochrome P450 monooxygenase activity was measured using a modified version of a previously established procedure (de Sousa *et al*. 1995). At 4 °C, ten third-instar larvae were homogenized in 0.1 M sodium/potassium phosphate buffer (pH 7.2) containing 1 mM EDTA, 200 mM sucrose, and 1 mM DTT. After that, the samples were centrifuged at 5,000 g for

5 minutes at 4 °C. The supernatants were centrifuged at 15,000 g for 15 minutes at 4 °C, followed by a second centrifugation at 100,000 g for 60 minutes at 4 °C. The resulting microsomal pellet was resuspended in 1000 µl of the buffer above and used as the enzyme source. Each well received a combination of 100 microliters from the mixture and 50 µl of sodium/potassium buffer (0.1 M, pH 7.2), which included 0.4 mM 7-ethoxycumarin and 1 mM NADPH. At 30 °C, the plate was on the shaker for 30 min. To eliminate the self-fluorescence of NADPH, 0.5 units of glutathione reductase and oxidized glutathione at a concentration of 1.5 mM were added and incubated for 10 minutes at room temperature. Finally, the reaction was stopped by adding 150 µl of acetonitrile (50%) in Trizma-base buffer (0.05 M, pH 10). The measurement of the released 7-ethoxycumarin was performed at 465 nm with an excitation wavelength of 390 nm. Five replicates were conducted for each population.

Esterase activity

Esterase activity was measured following the method of van Asperen (van Asperen, 1962) with a few changes to the method. To do this, third-instar larvae (n = 15) from each population were mashed in 900 µl of sodium phosphate buffer (10 mM, pH 7), containing 01% of Triton X-100. The resulting homogenate was then centrifuged for 10 min at 4 °C and 15,000 × g. Next, 50 µl of the supernatant was combined with 100 µl of 0.1 M phosphate buffer (pH 7.0) and 10 µl of α-naphthyl acetate dissolved in 10 mM acetone. Subsequently, 50 µl of fast blue RR (0.5 mg/ml in buffer) was added. Finally, using a microplate reader (BioTek, Vermont), the released naphthol was determined continuously for 25 min at 450 nm. Five replicates were performed for each population.

GST activity

With some adjustment, the Keen method (Keen *et al*., 1976) was used to assess GST activity. Fifteen third-instar larvae from each population were mashed in ice-cold 10 mM phosphate buffer (pH 7), followed by centrifugation at 10,000 ×g for ten minutes at 4 °C. In each well, 200 µl of 1 mM 1-chloro-2,4-dinitrobenzene and 5 mM reduced glutathione (GSH) in 0.1 M sodium phosphate buffer was supplemented with 10 µL of the resulting supernatants. The absorbance changes were continuously recorded for 6 min at 340 nm, using a microplate reader. Five replicates were performed for each population. The protein concentration was determined using the Bradford method (Bradford 1976).

Genes identification

Identifying and characterizing tomato leafminer-specific genes of interest (Table 1) (Baradaran *et al*., 2019b, Baradaran *et al*., 2019a, Sayani *et al*., 2019) was done by using translated mRNAs derived from whole transcriptome sequences and ESTs acquired from the NCBI server (www.ncbi.nlm.nih.gov). The genes responsible for detoxification enzymes were identified through comparative analysis in other studies conducted on different insect species, and the important genes were selected based on the most frequently reported genes in insecticide resistance. The Clustal Omega (www.ebi.ac.uk) (Sievers *et al*., 2011) was utilized to execute sequence alignments.

RNA isolation and gene expression analysis

Total RNA samples were extracted from the larvae of Tehran (resistant) and Urmia (susceptible) populations, using Trizol solution, following the guidelines provided by the Molecular Research Center. All samples underwent DNase I (Promega) treatment to eliminate the possibility of DNA contamination. The RNA concentrations were determined using a spectrophotometer, and 2 µg total RNA was reverse transcribed with MMLV-RT enzyme (Promega) at 42 °C for 1 h using an oligo (dT) primer. RT-qPCR with gene-specific primers (listed in Table 1) was used to examine transcript levels of the target genes, with RPL27 serving as a reference gene. The analysis used a Mic-qPCR cycler (BMS) with three biological replicates per experiment. The RT-qPCR experiment was conducted under the prescribed conditions, consisting of one cycle at 95 °C for 15 min, followed by a sequence of 40 cycles at 95 °C for 15 s, 57 °C

for 30 s, and 73 °C for another 30 s, with the final cycle stepping up to 95 °C for a minute.

Statistical analysis

The LC_{50} was calculated utilizing probit analysis (Finney, 1980) performed on PoloPlus software (LeOra Software 2007). For each test, the parameters were determined for all populations: LC50 with its 95% confidence interval (95% CI), regression line slope, and resistance ratio (RR). Lack of overlap in the calculated 95% CI of the LC_{50} indicated significant distinctions and comparisons among populations. Data from qPCR were analyzed with ΔΔCt method (Livak and Schmittgen, 2001). Significant differences were evaluated using t-student test. All data was normal. The Kolmogorov-Smirnov test was used for data normality. All statistical analyses were performed using SPSS 22 software, and the graphs were created using GraphPad Prism (version 7) software.

Results

TLM resistance levels

The LC_{50} values of fenvalerate for tomato leafminer populations from four different regions in Iran are shown in Table 2. The Urmia population possessed the lowest LC_{50} (892.826 mg/l), and the Tehran population possessed the highest LC_{50} (10935.823 mg/l) with 95% CI, and a good fit to the probit model was observed.

Table 1 List of primers used in this study.

Gene	Fwd./Rev.	Sequence	Amplicon (bp)	References
RPL27	Fwd.	GAAGCCAGGTAAAGTGGTGCT	106	baradaran *et al.*, 2019 a
	Rev.	GTGTCCGTAGGGCTTGTCTG		
cyp9g	Fwd.	TTCCCCGATCCTGACAAGTT	100	This study
	Rev.	TTCTAGGCCCTAATCCAAAGGG		
cyp6ab5	Fwd.	ATCCACCGCTACCGTTCTTT	110	This study
	Rev.	GACGACTCTTTCCTCGGGAT		
cyp306a	Fwd.	CACGCATGCTCACCAATTC	155	This study
	Rev.	TGGCAAAAAGTTAACGACACC		
ae3	Fwd.	AACGCAACTGCCAATCTACC	180	This study
	Rev.	GTTGCCAGGTGCAGTATCAG		
ae13	Fwd.	CGGTAACATGGGGCTAAAGGA	181	This study
	Rev.	TTGTGCGATGGCTTTGTGAA		
Ac type1	Fwd.	GTGTTCGGCGGAGGGTTTTA	115	This study
	Rev.	GAGACGCAACTCGGTACTGC		
gstd2	Fwd.	CCATAGACCTGTACGAGATGCCCA	128	Sayani (2019)
	Rev.	CTCTCCAGCCATCAGATCCACAAG		
gste2	Fwd.	GGACTACAAGGAGCCCGACC	173	Sayani (2019)
	Rev.	TTCAGCAAGAACGAAGTTACCATC		
gsts1	Fwd.	ATTCATCCTGGTCGGCATCATC	161	Sayani (2019)
	Rev.	TTCTGGACCGCAGCCTTCAC		

Table 2 Susceptibility of four populations of *Tuta absoluta* to fenvalerate using leaf disc-dip method.

Population	N[1]	Slope ± SE	LC_{50} (LCL–UCL)[2]	$\chi 2$ (df)	RR[3]
Tehran (Tehran)	210	1.280 ± 0.186	10935.8 (7780.1-16244.9)	3.783(19)	12.25
Kashmar (Khorasan Razavi)	210	2.194 ± 0.327	4598.6 (3780.8-5797.4)	2.253(19)	5.15
Arak (Markazi)	210	1.474 ± 0.209	1202.5 (893.8-1676.2)	4.117(19)	1.34
Urmia (Azarbaijan Gharbi)	210	1.287 ± 0.194	892.8 (638.0-1273.4)	2.017(19)	-

[1] Number of tested insects.
[2] The LC50 value is expressed as mg/ml; LCL, lower confidence limits at 95%; UCL, upper confidence limits at 95%.
[3] Resistance ratio: LC_{50} of resistant population / LC50 of Urmia population.

Tehran population possessed a resistance level that was 12.25 times more than their Urmia counterparts. Comparing Kashmar and Arak populations to the Urmia population also exhibited 5.15 and 1.34 times greater resistance levels, respectively (Table 2). Therefore, we considered the Tehran population the most resistant and the Urmia population the susceptible population throughout the study for further analyses.

Detoxifying enzyme activities in the resistant and susceptible populations

Cytochrome P450 monooxygenase activity

The resistant and susceptible populations of TLM exhibited considerable distinctions in their cytochrome P450 monooxygenase activity. P450 monooxygenase activity was up to 2.3 times higher in the Tehran population compared to the susceptible population (Fig. 1A).

Esterase Activity

The level of esterase activity in the Tehran population was almost 1.9 higher than the Urmia population using alpha-naphthyl acetate as a substrate (Fig 1B).

GST activity

Analysis of the GST activity of the two populations revealed that the level of GST activity in the Tehran population was 1.5-fold higher than Urmia population (Fig. 1C).

Gene Expression of the Detoxification Enzyme Using RT-qPCR the expression levels of detoxification genes on third-instar larvae of the susceptible (Urmia) and resistant (Tehran) populations of *T. absoluta* were examined, including those encoding for Cytochrome P450 Monooxygenases (*cyp9g*, *cypAB5*, and *cyp306a*), esterases (*ae13*, *ae3*, and *ace type1*), and GSTs (*gstd2*, *gstd4* and *gsts1*). The results showed that all cytochrome P450 monooxygenase genes have significantly higher expression levels in the resistant population than the susceptible one. The expression level of *cyp306a* was 13 times greater than in the susceptible insects. Also, *cypB5* and *cyp9g* showed higher expression levels relative to the susceptible population (Fig. 2A). The resistant population also displayed a significant increase in expression levels of the esterase genes, particularly *ae13* that showed a 14.64 folds higher than the susceptible population (Fig. 2B). Also, the expression level of all the GST genes was significantly higher in the resistant population compared to the susceptible population (Fig. 2C).

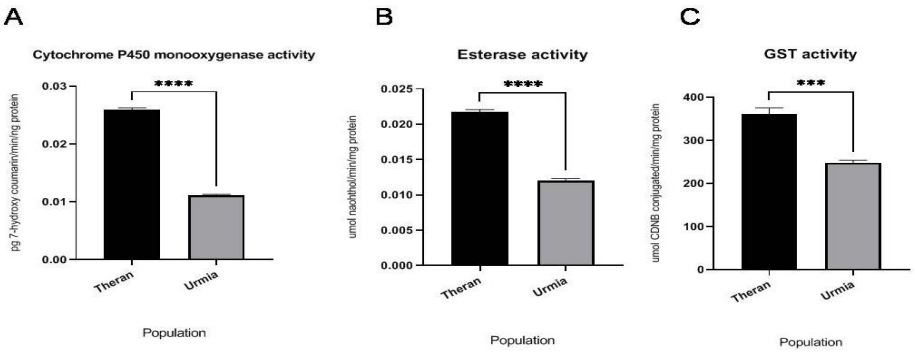

Figure 1 The detoxification enzyme activity in third larvae of tomato leafminer. The cytochrome P450 monooxygenase activity (A), esterase activity (B), and GST activities (C) in the population of Tehran (resistant) and Urmia (susceptible) showed. Data are reported as means ± SE (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; *t*-test).

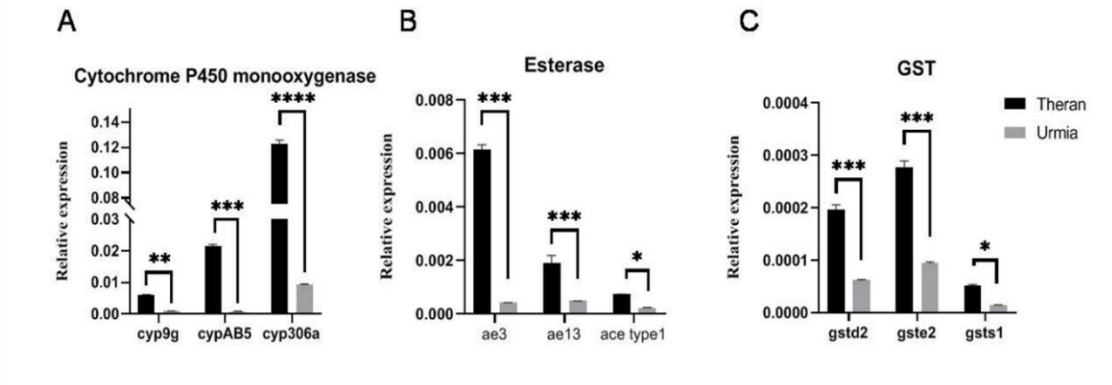

Figure 2 Expression of the detoxification genes in third larvae of tomato leafminer. The Expression level of cytochrome P450 monooxygenase genes including *cyp306a*, *cypAB5*, *cyp9g* (A), esterase genes including *ae3*, *ae13*, *ace type1* (B), GST genes including *gstd2*, *gste2*, *gsts1* (C) in the population of Tehran (resistant) and Urmia (susceptible). RPL-27 was used as the reference gene. Data are reported as means ± SE (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; *t*-test).

Discussion

Despite the existence of alternative insecticides, pyrethroids such as fenvalerate remain a popular choice thanks to their high potency and limited impact on mammalian life (Palmquist *et al*., 2012). One commonly used insecticide for TLM control is fenvalerate, a pyrethroid insecticide. This insecticide is also widely used to control the vegetable leaf miner (VLM), *Liriomyza sativae*, which usually inflicts damage on crops alongside the TLM (Gitonga *et al*., 2010). Although fenvalerate is used to control TLM, other effective alternative insecticides are also used. However, fenvalerate is one of the primary insecticides for VLM chemical control. The similar damage symptoms caused by VLM and TLM led to missed diagnosis and frequent application of fenvalerate on TLM, followed by the development of resistance in TLM populations.

In this study, we evaluated the resistance of TLM to fenvalerate. Our result showed that the Tehran population exhibited the highest level of fenvalerate resistance among the four populations investigated, while the Urmia population was the most sensitive (Table 2). TLM-resistant populations to insecticides have been frequently reported, especially resistant to pyrethroids (www.pesticideresistance.org).

Tehran population showed higher detoxification enzyme activities than the sensitive Urmia population, with cytochrome p450 monooxygenase having the highest activity, followed by esterases and GSTs. According to these, it can be concluded that the mixed function oxidases (monooxygenase P450, esterase, and GST), which are crucial in detoxifying various pesticides (Fan *et al*., 2023; Guo *et al*., 2014), besides others, are involved in metabolic resistance of TLM to fenvalerate. For example, Kasai (Kasai *et al*., 1998) demonstrated that resistance to permethrin in *Culex quinquefasciatus* is caused by increased activity of cytochrome P450 monooxygenase enzyme.

Metabolic resistance is caused by an increase in the activity of detoxifying enzymes, and one of the reasons for this increase is the upregulation of genes associated with these enzymes. Our results showed that the level of detoxification gene expression in the resistant population was higher than in the susceptible population, according to detoxification enzyme

activities. There might be many copies of a detoxification gene. However, a few can be responsible for developing resistance against pesticides. For example, in *Drosophila*, there are 89 encoding genes for cytochrome P450 monooxygenase; some of these genes reported for involving resistance, such as CYP6A2 and CYP6G1 (Ranson *et al*., 2002). Our results demonstrated that among the assessed detoxification genes encoding cytochrome P450 monooxygenase enzymes, *cyp306a* had the highest level of expression, which is incongruent with other studies that reported the role of *cyp306a* as a resistance factor (Arouri *et al*., 2015, Vlogiannitis *et al*., 2021; Nauen *et al*., 2022). For example, CRISPR-cas9 mediated knocking down the *cyp306* gene in *Helicoverpa armigera* resulted in increased sensitivity to various insecticides, including fenvalerate (Wang *et al*., 2018). Besides these reports, our results highlight the importance of metabolic resistance imposed by detoxification genes/enzymes in developing resistant insect populations.

We demonstrated that metabolic resistance in tomato leafminers correlates with increased activity of detoxifying enzymes, particularly cytochrome P450 monooxygenase, which contributes to genes associated with these enzymes. Identifying the precise mechanisms involved in resistance needs further investigation, which could assist in controlling this pest.

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Conflict of interest

The authors declare no conflict of interest.

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افزایش فعالیت آنزیم‌های سم‌زدا و افزایش بیان ژن‌های آن‌ها در شب‌پره مینوز گوجه‌فرنگی *Tuta absoluta* مقاومت به فنوالریت را توجیه می‌کند

احسان برادران، اعظم میکانی*، سعید محرمی‌پور و محمد مهرآبادی

دانشگاه تربیت مدرس، دانشکده کشاورزی، گروه حشره‌شناسی کشاورزی، تهران، ایران.
پست الکترونیکي نویسنده مسئول مکاتبه: a.mikani@modares.ac.ir
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چکیده: شب‌پره مینوز گوجه‌فرنگی *Tuta absoluta*، آفتی است که به‌شدت به محصولات گوجه‌فرنگی آسیب می‌زند. متأسفانه، استفاده از حشره‌کش‌ها منجر به بروز مقاومت در این آفت شده است. مقاومت متابولیکی شایع‌ترین نوع مقاومت است که با افزایش فعالیت آنزیم‌های استراز، سیتوکروم P450 مونوکسیژناز و گلوتاتیون اس ترنسفراز مشخص می‌شود که توسط ژن‌های خاصی کدگذاری شده‌اند. در این مقاله، ما مکانیزم‌های مقاومت متابولیکی مرتبط با مقاومت به فن والریت در شب‌پره مینوز گوجه‌فرنگی و نیز و وضعیت ژن‌ها را بررسی کردیم. جمعیت‌های شب‌پره مینوز گوجه‌فرنگی در ایران سطوح مختلفی از مقاومت به فن والریت را نشان دادند؛ جمعیت‌های تهران و ارومیه به‌ترتیب مقاوم‌ترین و حساس‌ترین بودند. میزان فعالیت آنزیم‌های سم‌زدا به‌ویژه سیتوکروم P450 مونوکسیژناز، در جمعیت مقاوم (تهران) نسبت به جمعیت حساس (ارومیه) افزایش نشان داد. بیان ژن‌ها نشان داد که سطح ترانسکریپت‌های P450، استرازها وGSTها در جمعیت مقاوم نسبت به جمعیت حساس بالاتر است. یافته‌های ما نشان داد که آنزیم‌های سم‌زدا به‌ویژه سیتوکروم P450 مونوکسیژناز، و بیان متفاوت ژن‌های مرتبط با آن‌ها به ایجاد مقاومت به فن والریت مرتبط هستند. شناسایی مکانیزم‌های مقاومت می‌تواند به کنترل آفات و برنامه‌های مدیریت مقاومت کمک کند.

واژگان کلیدی: شب‌پره مینوز گوجه‌فرنگی، سیتوکروم P450 مونوکسیژناز، cyp306A1