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Evaluation of Insecticide Toxicity and Enzymatic Detoxification in Neonate Larvae of European Grapevine Moth, *Lobesia botrana* Denis & Schiff. (Lepidoptera: Tortricidae)

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ABSTRACT

Lobesia botrana Denis & Schiff. (Lepidoptera: Tortricidae), also known as the European grapevine moth is a detrimental pest in grape production in Palearctic Region. Insecticides are used to control *L. botrana* infestation in grape-produced areas; however, repeated and intensive use of insecticide causes reduced efficacy in *L. botrana* management. In the present study, the efficacy of five commonly used insecticides (chlorpyrifos-ethyl, emamectin benzoate, indoxacarb, lambda-cyhalothrin, and spinosad) was evaluated in two field populations (AL and SAR) from Manisa, Turkey. In addition, detoxification enzyme activities including esterase (EST) and glutathion-S-transferase (GST) were measured via in vitro assays. LC_{50} values were found lower for chlorpyrifos-ethyl, emamectin benzoate, and spinosad and higher for indoxacarb and lambda-cyhalotrin in AL populations compared to SAR population. EST levels were slightly higher in AL population compared to SAR. GST levels were found higher in SAR population. However, no statistical difference was found in both detoxification enzyme activities and EST enzyme levels were higher than GST enzyme levels in both populations. Findings of the current study would help growers as well as the applicator to strategize their insecticide use in integrated pest management programs for *L. botrana* and possibly mitigate any insecticide resistance development.

Keywords: Detoxification enzyme, Dose-response, Grape, Insecticide toxicity, Tortricidae

1. Introduction

The European grapevine moth *Lobesia botrana* Denis & Schiff. (Lepidoptera: Tortricidae) is a major pest in grapevine production regions in Asia, Europe, North & South America (Ioriatti et al. 2011). *Lobesia botrana* can feed on approximately 40 different plant species; however, feeding on grapevine makes *L. botrana* an economically important pest in grape production. The first generations mainly feed on flower buds, which can be negligible for growers. However, the second and third generations feed on flesh and result in increased yield loss and unmarketable fruit (Pasquini et al. 2018). It is reported that in some regions 4th generation could occur due to environmental conditions (Roditakis & Karandinos 2001; Harari et al. 2007). In addition, dripping fruit juice during the feeding causes secondary damage e.g., gray mold fungal growth (*Botrytis cinerea*) (Moosavi et al. 2020). For this reason, egg and larvae stages are the primary targets to control the pest for potential crop loss.

Several control methods including mating disruption, biological control, and cultural practices are included in integrated pest management programs (IPM); however, insecticides are ubiquitously in use due to their fast and immediate effects (Ioriatti et al. 2011; Navarro-Roldan et al. 2017). Currently, commonly used insecticides include chlorantraniliprole, chlorpyrifos-ethyl, emamectin benzoate, indoxacarb, lambda-cyhalothrin, methoxyfenozide, tebufenozide, spinosad, and *B. thuringiensis*. Frequent use of insecticides causes resistance against the individual insecticide or chemical classes. In addition, factors such as canopy deposition, application rate, poor sprayer calibration, insufficient spray coverage, inappropriate timing of spray application could cause failure in insecticide application thus possibly insecticide resistance may occur (Pasquini et al. 2018).

The toxicity of insecticides has been evaluated both in the field and laboratory on *L. botrana* stages (egg, pupae, and adult) (Irigaray et al. 2005; Ioriatti et al. 2011; Civolani et al. 2014; Hatipoglu et al. 2015). Some of the insecticides were found more effective than others (e.g., emamection benzoate was highly effective compared to indoxacarb) (Civolani et al. 2014). Out of all insecticides tested, indoxacarb is the only insecticide that the resistance is observed (Civolani et al. 2014). Thus, monitoring insecticide efficacy via bioassays is necessary to strategize further insecticide used to mitigate resistance development. In addition to insecticide bioassays, enzymatic activity levels (EST; carboxylesterases and GST, glutathione S-transferases) in

neonate larvae provide detailed and consistent results on the resistance development in the field (Rodriguez et al. 2011a). Detoxification enzymes play a major role in insecticide resistance (Scott 1998; Enayati et al. 2005; Montella et al. 2012). Of the other mechanism resistance, detoxification enzymes have the ability to amplify genes through overexpression and changes in coding sequence to alter the detoxification abilities thus causing resistance (Li et al. 2007; Navarro-Roldan et al. 2017).

Insecticide detoxification in insects is undergone in two-phase metabolisms; Phase I is attaching a polar group to toxic compounds e.g., insecticides or their cleavage in the body (Navarro-Roldan et al. 2020). Phase II is adding sugar, amino acid, sulfate, or phosphate group on Phase I product to increase the polarity (hydrophilicity) to excrete from the insect body (Bernard & Philogene 1993). The main important detoxification enzyme groups are poly substrate monooxygenases (PMSO also known as cytochrome P450), EST, and GST (Despres et al. 2007). PSMO and EST belong to Phase I group enzymes while GST is in Phase II enzymes (Navarro-Roldan et al. 2020). Furthermore, detoxification enzyme levels can be measured via in vitro methods (Brown & Brogdon 1987; Navarro-Roldan et al. 2020) to monitor insecticide resistance levels. Reports about *L. botrana* enzymatic activity levels are limited (Hatipoglu et al. 2015; Navarro-Roldan et al. 2020).

The current study aims to evaluate the insecticide efficacy as well as the detoxification enzyme activity of two *L. botrana* population from Manisa, Turkey. Insecticides including chlorpyrifos, emamectin benzoate, indoxacarb, lambda-cyhalothrin, and spinosad were tested on the neonate larval stage to determine dose-response. In addition, EST and GST detoxification enzyme activities were measured via in vitro assays.

2. Material and Methods

2.1. Insect rearing

The stages of *L. botrana* (larvae, pupae, and eggs) were collected from the fields in Alaşehir (AL) and Sarıgöl (SAR), Manisa, Turkey, by visually examining the grape clusters. The area is primarily well-known with its grape (seedless and table) production. In addition, the distance between the two locations is approximately 25 km; however, types of insecticide used and frequency of the applications for *L. botrana* slightly differs due to microclimatic and geographic conditions in two locations. The insects were reared under controlled climate chamber conditions (25 °C, 60±10 RH) on a semiartificial diet (220 mL water, 4 g agar, 15 g cornmeal,15.6 g wheat germ, 15 g yeast, 1.28 g ascorbic acid, 0.4 g benzoic acid, 0.4 ml corn oil, 0.4 g nipagine, and 0.2 g benomyl) in plastic containers (Delbac et al. 2010). The photoperiod was set at 1000 lux luminosity for the first 15 h, 25 lux (twilight period) for the last hour to induce oviposition, and 8h dark period. The adults were fed with pollen: sugar: water mixture (1:1:1) to prevent starving and kept in a plastic cylinder container covered with plastic bags for egg-laying. Following laying, eggs were transferred into a container with an artificial diet for further larval feeding and adult emergence.

2.2. Chemicals

Chlorpyrifos-ethyl, lambda-cyhalothrin, indoxacarb, emamectin benzoate, and spinosad were used for mortality bioassay (Table 1). Insecticides were chosen from a different mode of action mechanism according to IRAC classification (http://www.irac-online.org/) as well as growers` preference. Insecticides were graciously donated from the corresponding companies upon request (Table 1).

Table 1- Detailed information of insecticides tested on *L. botrana* neonate larvae

Insecticides	***Active ingredient amount***	***Tested concentration range (mg AI L^{-1})***	***Formulation name***	***Chemical group****	***Manufacturer***
Chlorpyrifos-ethyl	480 g/L	60, 120, 240, 480, 960, 1920, 3840	Dursban®	Organophosphate (1B)	Dow AgroSciences, Indianapolis, IN, USA
Emamectin benzoate	5%	0.4 ,0.7, 1.5, 3.1, 6.25, 12.5, 25	Proclaim®	Avermectins (6)	Syngenta, Wilmington, DE, USA
Indoxacarb	150 g/L	2.34, 4.6, 9.3, 18.7, 37.5, 75, 150	Avaunt®	Oxadiazines (22A)	Dupont, Wilmington, DE, USA
Lambda-cyhalothrin	50 g/L	0.625, 1.25, 2.5, 5, 10, 20, 40	Karate Zeon®	Pyrethroids (3A)	Syngenta, Wilmington, DE, USA),
Spinosad	480 g/L	3, 6, 12, 24, 48, 96, 192	Laser®	Spinosyns (5)	Dow AgroSciences, Indianapolis, IN, USA

*: IRAC (Insecticide Resistance Action Committee, www.irac-online.org, 2020)

2.3. Insecticide efficacy

The neonate larvae (<24 h old) were used for bioassays. Seven or eight different concentrations of insecticides were prepared in water and mixed with a semisynthetic diet accordingly (2 µl cm^{-3}) to determine the dose-response (Bosch et al. 2007; Rodriguez et al. 2011a). A minimum of 16 larvae was used and for each concentration, four replicates were performed (Table 2). The control treatments were mixed with purified water. First, wells (24-Well Plate Costar®, Corning Inc., Corning, NY, USA) were filled

with semisynthetic diet, and then larvae were transferred to the wells individually to prevent cannibalism or any interferences between the individuals. Mortality of the larvae was determined after 24 h by touching with a fine brush to examine dead, moribund, and alive larvae (Bosch et al. 2007). Missing larvae were subtracted from the total treated larvae.

2.4. Enzymatic activity

The EST and GST enzyme activities were measured according to Rodriguez et al. (2011a). The larvae extracts were prepared in 100 µl of phosphate buffer (50 mM, pH 7.2) with a 0.4 mM final concentration of phenylmethylsulfonylfluoride. Ten neonate larvae were homogenized for each enzyme (EST and GST) with a total replicate of ten were prepared for each population. All homogenates were prepared on ice and centrifuged at 4 °C for 15 min at 15000g and supernatants were used as an enzyme source to measure EST and GST levels (Bouvier et al. 2002; Rodriguez et al. 2011a). The protein concentrations for both EST and GST were calculated by using bovine serum albumin as standard (Bradford 1976).

The total EST activity was measured in 96 well microplates and β-naphthyl acetate was used as substrate. Ninety microliters of larvae extracts were added to 90 µl of sodium phosphate buffer (50 mM, pH 6.5) containing a final concentration of 0.1 mM β-naphthyl acetate. After 15 minutes of incubation, the reaction was stopped by adding 20 µl of a staining reagent containing 3 g/L Fast Garnet and 35 g/L sodium dodecyl sulfate (Bouvier et al. 2002; Rodriguez et al. 2011a). The absorbance of the Naphthol-Fast Garnet complex was measured at 492 nm, after 15 minutes of incubation at room temperature. In addition, 10 wells were supplied with sodium phosphate buffer instead of enzyme extracts and were used as controls. The units were expressed in β-naphthol mg of protein^{-1} min^{-1} (Bouvier et al. 2002; Rodriguez et al. 2011a).

GST enzyme activity was measured in 96 well microplates (COSTAR) with UV transparent bottom and 1-chloro-2,4-dinitrobenzene (CDNB) was used as substrate. Microplate wells were filled with 4 µl of enzyme extract, 184 µl of 50 mM sodium phosphate buffer (pH 7.2), 2 µl of reduced glutathione (100 mM), and 10 µl of CDNB (30 mM). Ten wells were supplied with enzyme extract were used as control. The optical density was measured at 340 nm for both at time zero (t0) and after 1 min (t1) and the absorbance was measured at 30 °C. Optical reading results were expressed as mM glutathione conjugated mg of protein^{-1} min^{-1} (Bouvier et al. 2002; Rodriguez et al. 2011a).

2.5. Data analysis

The probit analysis (Polo Plus Program, LeOra Software LLC, Petaluma, CA, USA) was used to compare LC_{50} and LC_{90} values of the two populations (Robertson et al. 2003). Abbott formula was used to correct the mortality values (Abbott, 1925). The statistical difference of enzyme assays was evaluated via ANOVA test and means were tested with Tukey HSD test at $P < 0.05$ using R studio (R Development Core Team 2004).

3. Results and Discussion

The present study evaluates the insecticides efficacy of chlorpyrifos-ethyl, emamectin benzoate, indoxacarb, lambda-cyhalothrin, spinosad in *L. botrana* management in two grapevine production locations in Turkey. In addition to bioassays, EST and GST enzyme levels were measured for AL and SAR populations. Several reports are available on insecticide efficacy of *L. botrana* developmental stages and detoxification enzymatic levels (Irigaray et al. 2005; Vassiliou 2011; Civolani et al. 2014; Pavan et al. 2014; Hatipoglu et al. 2015; Navarro-Roldan et al. 2017; Pasquini et al. 2018; Navarro-Roldan et al. 2020). Several reports are available regarding insecticide toxicity on *L. botrana* (Irigaray et al. 2005; Vassiliou 2011; Pavan et al. 2014; Hatipoglu et al. 2015; Pasquini et al. 2018) however, up until now, there is only one insecticide resistance report available for *L. botrana* (Civolani et al. 2014). For that reason, it is important to monitor the insecticide efficacy to provide optimal *L. botrana* control and mitigate the potential resistance development.

3.1. Insecticide efficacy

The mortality bioassay results indicated that LC_{50} and LC_{90} values were found different in AL and SAR populations for the insecticides tested in the experiment (Table 2). Chlorpyrifos-ethyl LC_{50} value for AL (132.4 mg AI L^{-1}) was lower than SAR (259.3 mg AI L^{-1}). However, LC_{90} values were found slightly different for AL (900.2 mg AI L^{-1}) and SAR (919.2 mg AI L^{-1}) populations. LC_{50} values for emamectin benzoate for AL was 12.8 mg AI L^{-1} while SAR is 31.4 mg AI L^{-1}. Emamectin benzoate LC_{90} values for AL and SAR populations were 123.4 mg AI L^{-1} and 338.1 mg AI L^{-1} respectively. Indoxacarb LC_{50} values of AL (14.3 mg AI L^{-1}) were almost two times higher than SAR (8.7 mg AI L^{-1}). LC_{90} values for indoxacarb for AL (39.8 mg AI L^{-1}) and SAR (37.0 mg AI L^{-1}) were found close to each other. Lambda-cyhalothrin LC_{50} value was 100.0 mg AI L^{-1} for AL while SAR was found as 42.1 mg AI L^{-1}. LC_{90} values were followed the same trend as LC_{50} values for AL 920.4 mg AI L^{-1}and SAR 361.4 mg AI L^{-1}. LC_{50} value of spinosad for AL (0.6 mg AI L^{-1}) was almost 2.5 times lower than SAR (1.6 mg AI L^{-1}) while LC_{90} values were 18.0 mg AI L^{-1} and 7.7 mg AI L^{-1} respectively.

Table 2- Toxicity of insecticides on *L. botrana* neonate larvae for two field populations (SAR and AL)

Insecticides	*Population*	*N*[a]	*Slope*	*LC_{50} (mg AI L^{-1}; 95% CI)*	*LC_{90} (mg AI L^{-1}; 95% CI)*	*HF*[b]
Chlorpyrifos-ethyl	*AL*	227	1.540±0.025	132.4 (14.43-288.58)	900.2 (396.94-20565.41)	0.7
	SAR	128	2.332±0.352	259.3 (133.69-553.59)	919.2 (458.5-6584.12)	0.42
Emamectin benzoate	*AL*	128	1.304±0.249	12.8 (2.78-32.74)	123.4 (43.72-10125.6)	0.39
	SAR	128	1.241±0.236	31.4 (19.12-53.50)	338.1 (152.89-1679.85)	0.06
Indoxacarb	*AL*	128	2.877±0.458	14.3 (6.73-19.93)	39.8 (28.22-92.61)	0.37
	SAR	128	2.047±0.329	8.7 (3.31-21.38)	37.0 (16.67-715.37)	0.51
Lambda-cyhalothrin	*AL*	253	1.330±0.177	100.0 (57.39-220.42)	920.4 (355.19-8903.78)	0.34
	SAR	128	1.373±0.302	42.1 (7.40-105.84)	361.4 (135.48-12399.89)	0.27
Spinosad	*AL*	128	0.885±0.229	0.6 (0.04-1.55)	18.0 (5.67-275.06)	0.20
	SAR	128	1.889±0.297	1.6 (1.10-2.31)	7.7 (4.90-16.42)	0.18

[a]**:** number of individuals tested; [b]**:** heterogeneity factor divided by *dF*

Chlorpyrifos-ethyl is one of the common insecticides used for *L. botrana* control. Although chlorpyrifos-ethyl has low residual activity (<30% efficacy) in the field (Pavan et al. 2014), *L. botrana* IPM programs still include this insecticide to rotate with other insecticides. The results indicate that approximately two-fold difference in LC_{50} values were observed when comparing AL (132.4 mg AI L^{-1}) and SAR (259.3 mg AI L^{-1}) populations. The difference in LC_{50} values suggests that AL has more susceptibility to chlorpyrifos-ethyl than SAR population. Previously, a similar trend was observed among the field populations, which suggests that *L. botrana* susceptibility varies in different grapevine production regions (Hatipoglu et al. 2015). This might indicate that production areas in SAR or surrounding areas could potentially face a resistant population against chlorpyrifos-ethyl in the near future.

Emamectin benzoate is an alternative insecticide used in grape production against *L. botrana*. Results showed that AL population is reasonably susceptible to emamectin benzoate compared to SAR population, which could be speculated that SAR population could possibly be exposed to emamection benzoate more prominently. Emamectin benzoate is pronounced as more environment safely to replace other neurotoxic insecticides e.g., organophosphate insecticides. In addition, emamectin benzoate was found highly effective in controlling *L. botrana* in the field (Civolani et al. 2014).

Indoxacarb susceptibility was higher in SAR population compared to AL population. A similar trend was also observed in field populations (Hatipoglu et al. 2015). In a previous report, indoxacarb provided moderate residual activity (~75%) in the field (Pavan et al. 2014). Also, indoxacarb is the most widely used insecticide in Emilia-Romagna region in Italy and due to frequent use of indoxacarb, resistant *L. botrana* population was previously reported (Civolani et al. 2014). Results from the study indicated that indoxacarb resistance was 72-fold higher in the field population compared to control and resistance was stable even after rearing 10 generations (Civolani et al. 2014). Low or moderate (<10-fold) resistant ratios for indoxacarb were detected for multi-resistant strains of *Choristoneura rosaceana* and *Cydia pomonella* (Ahmad et al. 2002; Dunley et al. 2006; Mota-Sanchez et al. 2008).

Lambda-cyhalothrin results indicated that SAR population is more susceptible to lambda-cyhalothrin than AL population. Up until today, limited research has been done with lambda-cyhalothrin against *L. botrana* control (Navarro-Roldan et al. 2017). It has been demonstrated that adult males are more susceptible to lambda-cyhalothrin than females (Navarro-Roldan et al. 2017). In addition, AL population showed two-fold reduced susceptibility in spinosad than SAR population. The previous report indicated that spinosad also caused similar susceptibility in the field (Hatipoglu et al. 2015). In addition, the application of insecticides in combination was also evaluated and treatment combinations of lufenuron, spinosad, and indoxacarb and a combination of chlorpyrifos-ethyl, spinosad, and indoxacarb were found effective against 1st and 2nd generations in the field (Vassiliou 2011).

Overall, different mortality values were observed for the insecticides tested. Here, the present study primarily focused on the insect species and mode of action of the insecticides which are directly related to mortality. However, other factors such as insect stage (i.e., egg, immature, adult) and development, sex, mode of application, and time of exposure could also affect the mortality (Kanga et al. 1997; Lame et al. 2001; Shearer & Usmani 2001; Irigaray et al. 2005; Rodriguez et al. 2011a).

3.2. Enzymatic activity

Enzymatic activity results showed no statistical difference in EST and GST enzyme activities for AL and SAR populations (Figure 1). EST activities were measured as 9.4 nmol β-naphthol mg protein^{-1} min^{-1} and 6.3 nmol β -naphthol mg protein^{-1} min^{-1} in AL and SAR, respectively. However, there was no statistical difference in enzymatic activities in the two populations ($F_{1,24} = 1.85$, P=0.18). GST enzymatic activity of AL was found 0.032 mM glutathione conjugate mg protein^{-1} min^{-1} and it was 0.038 mM glutathione conjugate mg protein^{-1} min^{-1} in SAR. Again, there was no significant difference in GST activities when comparing two populations ($F_{1,22} = 0.42$, P= 0.52). Although there was no statistical difference between the two locations for detoxification enzymes, the amount of EST found in the populations was found more than GST amounts.

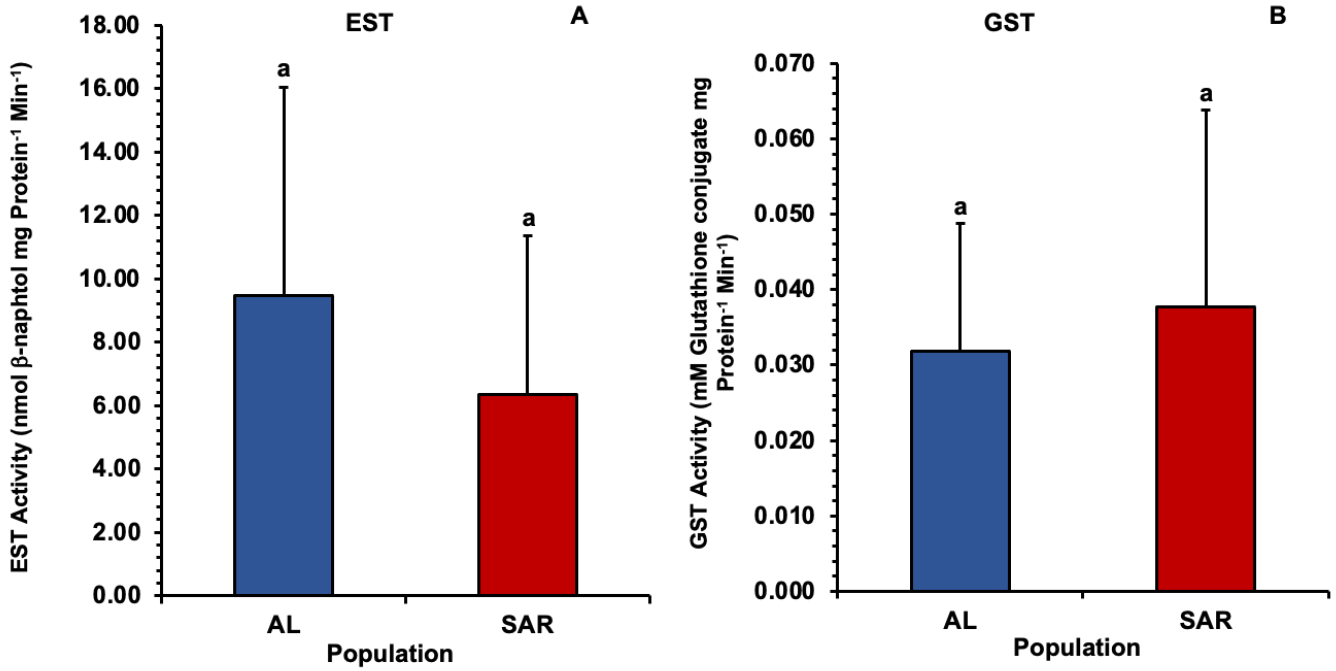

Figure 1- EST (A) and GST (B) enzymatic activities of *L. botrana* neonate larvae (n=200) from AL and SAR populations. Different letters indicate there is a statistical difference (P<0.05)

In the present study, two detoxification enzymes did not show any statistical difference between AL and SAR populations. However, EST enzyme levels were found drastically higher than GST levels for both populations. Enzymatic activity is directly related to insecticide pressure and thus it can vary in locations due to the insecticide application regime (Navarro-Roldan et al. 2020). A study reviewed 92 cases of lepidopteran detoxification mechanism and found that EST was affected only at 63% of the cases while GST was observed at 36% of the cases (Navarro-Roldan et al. 2020). Enzymatic activity of EST increased in *C. pomonella* treated with chlorpyrifos (Parra Morales et al. 2017). Several insecticides are detoxified via EST in species such as *Myzus persicae*, which could potentially induce resistance against broad-spectrum insecticides and cause cross-resistance (Devonshire & Moores 1982; Navarro-Roldan et al. 2020). On the other hand, the activity of GST was found higher when *Spodoptera littoralis* larvae treated with spinetoram (Ismail 2020). In addition, GST plays significant roles in environmental adaptation and detoxification for *Drosophila melanogaster* and *Anopheles gambiae* species (Ranson et al. 1998; Enayati et al. 2005). Previous findings suggest that EST and GST play primary roles in insecticide detoxification in lepidopteran species. Phase I enzyme families i.e., EST were actively responsible for detoxification in *C. pomonella*, *G. molesta*, *L. botrana* while Phase II enzyme i.e., GST was less changed for detoxification except for *G. molesta* (Navarro-Roldan et al. 2020). *Lobesia botrana* is the only species that the EST enzymatic activity was higher than *C. pomonella* and *G. molesta* (Navarro-Roldan et al. 2020), and different activity levels were found between sexes i.e., higher GST activity in females (Navarro-Roldan et al. 2020). As indicated in the literature, EST has the ability to detoxify a broad spectrum of insecticides in *L. botrana* compared to GST, which supports the finding from AL and SAR populations in the present study.

A positive correlation was reported between PMSO activation and chlorpyrifos-ethyl in neonate larvae from resistant *C. pomonella* population; however, there was no correlation in neonate larvae between EST and GST enzymes and chlorpyrifos-ethyl resistant population (Reyes & Sauphanor 2008). EST and GST were not involved in resistance mechanisms in neonate larvae from the field population in Spain (Rodriguez et al. 2011b). However, a significant correlation was found between EST and adult and fifth instar larvae to organophosphate resistance in *C. pomonella* (Reuveny & Cohen 2007; Reyes et al. 2007; Voudouris et al. 2011; Reyes et al. 2015). The chlorpyrifos-ethyl activity was reduced via PMSO detoxification (Feyereisen 1999) and high-level PMSO activity was detected in the field population (Dunley et al. 2006; Bosch et al. 2018). Increased mortality by chlorpyrifos at 48 h could be due to the oxidative desulfurization of the P=S group to its metabolites chlorpyrifos-

oxon (P=O) by PMSO, which can result in increased toxicity of chlorpyrifos over time (Yu 2008). Also, it is reported that the higher tolerance of *L. botrana* males to chlorpyrifos and difference in susceptibility between sexes is associated with the differences in enzymatic activities and quantities (Navarro-Roldan et al. 2017). The previous reports suggest that PMSO could be the major detoxification enzyme for chlorpyrifos-ethyl; however, whether EST and GST enzymes play an active role is still unclear for *L. botrana* larvae.

On the contrary of other lepidopterans, no relationship was found between PMSO and emamectin benzoate detoxification in *L. botrana* (Tabashnik 1991; Liang et al. 2003; Bosch et al. 2018). Reduction of emamectin benzoate efficacy detected in *C. pomonella* in Europe and found to be related to increased EST activity while *B. tabaci* resistance to emamectin benzoate is related to PMSO and GST activity (Kang et al. 2006; Reyes et al. 2007; Yu 2008). Reduced efficacy of emamectin benzoate is associated with increased EST activity in *C. pomonella*, which could possibly be similar in *L. botrana* as well. It is suggested that all three major detoxification enzymes (PMSO, EST, and GST) play important roles in indoxacarb detoxification for *C. rosaceana* larvae (Hafez et al. 2020). However, in other insects (e.g., *Spodoptera litura*, *Plutella xylostella*, *Phenacoccus solenopsis*) the primary enzymes that detoxify indoxacarb were PMSO and EST (Sayyed & Wright 2006; Sayyed et al. 2008; Afzal & Shad 2015; Hafez et al. 2020). All three major enzymes could likely be associated with indoxacarb detoxification in *L. botrana*. Reports indicated that oxidative metabolism might also be involved in the detoxification of indoxacarb for other insects including *C. rosaceana* and *C. pomonella* (Ahmad & Hollingworth 2004; Rodriguez et al. 2011a). It is highly possible that a similar detoxification pathway for indoxacarb could play role in *L. botrana*.

Previous reports indicated that two major enzymes, PMSO and EST play key roles in pyrethroid detoxification in *C. pomonella* larvae (Yu 2008). Reduced susceptibility to deltamethrin in *C. pomonella* larvae is associated with EST detoxification (Voudouris et al. 2011). Forty-one genes were demonstrated to show gene amplification by EST in *Aedes aegypti* against deltamethrin resistance (Faucon et al. 2015). This could be an indication that PMSO and EST might be responsible for lambda-cyhalothrin detoxification in *L. botrana* neonate larvae (Bosch et al. 2018). It was stated that enzymatic detoxification of spinosad was related to insect species (Wang et al. 2006) and several detoxification mechanisms were presented in the previous reports (Scott 1998; Shono & Scott 2003; Wang et al. 2006; Reyes et al. 2007). One study indicated that PMSO is associated (GST has no role) with spinosad resistance in *Musca domestica* (Scott 1998; Reyes et al. 2007). However, resistance to spinosad was reported to be related to an altered target site and not to PMSO or GST in *M. domestica* (Shono & Scott 2003) in another study. The previous report suggested that the increased activity of PMSO is related to spinosad resistance in *Bemisia tabaci* (Kang et al. 2006; Reyes et al. 2007) and *Spodoptera exigua* (Wang et al. 2006). According to the previous literature, one assumes that PMSO could be the main detoxification enzyme for spinosad in *L. botrana* neonate larval stage.

Different detoxification mechanisms were detected for *C. pomonella*. For example, increased EST activity was observed against azinphos-methyl in Argentina while increased GST (Reyes et al. 2007) activity in Chile (Fuentes-Contreras et al. 2007) and Europe (Reyes & Sauphanor 2008). Such differences in detoxification mechanisms could be related to the genetic background of insects in different locations and countries (Pashley 1983). It is obvious that genetic studies should also be incorporated in such studies to better understand the detoxification mechanisms of the target insects. Variations in previous reports may be related to different substrate affinity-binding capacities. Reduced EST activity could also be related to a reduced affinity for the non-specific naphthyl acetate (β) substrate and increased affinity for the insecticide substrate (Bush et al. 1993; Reyes et al. 2007). Binding affinity could also be different based on the substrate used during the experiment (Sole et al. 2018). Such issues can be an important factor in determining EST detoxification and potentially be minimized using another substrate e.g., p-nitrophenyl acetate. This could be a possible reason why our detoxification enzyme results are different than previous reports (Hatipoglu et al. 2015; Navarro-Roldan et al. 2020). Limited research has been conducted on *L. botrana* detoxification enzyme levels and only neonate larvae and adult stages were used in the previous reports (Hatipoglu et al. 2015; Navarro-Roldan et al. 2020). No correlation was found in EST and GST activity levels among developmental stages (neonate larvae, diapausing larvae, and adult) in *C. pomonella* (Reyes & Sauphanor 2008). However, one study indicated that using neonate larvae provided more consistent results when the field population was considered (Rodriguez et al. 2011a). This implies that mortality bioassay and enzyme activity measurements need optimization and standardization in future research. A high number of individuals (neonate larvae) were used during the bioassay and enzymatic activity (EST and GST) experiments; thus, we were unable to rear enough individuals to further evaluate PMSO activity levels in our study. Previous reports indicated that such issues could potentially happen and could be minimized by reducing the number of neonate larvae used for the experiment to obtain more detailed data on detoxification enzyme levels (Rodriguez et al. 2011a; Bosch et al. 2018).

4. Conclusions

Monitoring the efficacy of insecticide from the field population has a great impact on future insecticide use for achieving efficient pest control. Metabolic resistance is an irreversible process that could potentially limit insecticide use once it occurs. Applicators and growers should train properly to create better insecticide application programs that focus on prioritization and understanding of the potential risks of insecticide resistance. Using insecticide in proper order and efficiently could help growers to provide insecticide use for a longer-term. This could also give researchers the necessary time to discover and register new insecticides for efficient pest control. In addition, there is a need for standardization in bioassays and enzymatic analyses for *L. botrana* in order to provide comparable results between different experiments from various locations. Lastly, more research is needed to

monitor insecticide toxicity and measuring detoxification enzyme levels for effective *L. botrana* control in grape production areas.

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Abbreviations

AL	Alasehir population
CDNB	1-chloro-2,4-dinitrobenzene
EST	Carboxylesterase
GST	Glutathione S-transferases
IPM	Integrated pest management
IRAC	Insecticide resistance action committee
LC_{50}	Lethal concentration that kills 50% of the population
PMSO	Poly substrate monooxygenases
SAR	Sarigol population

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