Evaluation of Azinphos-Methyl Resistance and Activity of Detoxifying Enzymes in Codling Moth (Lepidoptera: Tortricidae) from Central Chile

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ABSTRACT Regular applications of insecticides have been the main management practice against codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) in Chile. Organophosphates are the most widely used insecticides, and azinphos-methyl is an important element in spray programs. In particular, we evaluated diagnostic doses of azinphos-methyl on neonate and postdiapausing larvae from seven apple (*Malus* spp.) orchards. We also evaluated the activity of detoxifying enzymes, such as glutathione *S*-transferases (GSTs), cytochrome P450 polysubstrate monooxygenases (PSMOs), and esterases, which are likely to be involved in resistance to insecticides. Such responses were compared with an insecticide-susceptible strain that has been maintained in the laboratory for several years. Neonate larval mortality of field populations to azinphos-methyl was not significantly different from of the susceptible strain. In contrast, postdiapause larval mortality was significantly lower in the six analyzed populations than in the susceptible strain. The *C. pomonella* populations with reduced postdiapause mortality to azinphos-methyl also showed statistically higher GST activity. Finally, no significant differences were found in total esterase or PSMO activity between *C. pomonella* populations. Therefore, the observed reduction in postdiapause larval mortality to azinphos-methyl seems to be associated with an increase in GST activity.

KEY WORDS *Cydia pomonella*, azinphos-methyl, insecticide, resistance, glutathione *S*-transferases

Pest management in Chilean apple (*Malus* spp.) orchards with fruit grown for export is dependent on intensive pesticide use, mainly because of strong quarantine restrictions toward the codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), from Asian and Latin American countries. In this production scenario, even low levels of fruit damage at harvest (<0.5%) are a major concern for growers. To avoid quarantine rejection of exports, an increase in the frequency of insecticide sprays has been observed.

Regular application of insecticides has been the main management practice, with organophosphate (OP) compounds such as azinphos-methyl as important components, although more selective active ingredients also have been incorporated during the last decade (Reyes et al. 2004).

Decreased efficacy of azinphos-methyl has been reported in the main apple-producing areas worldwide, including California, Washington, and Missouri in the United States (Varela et al. 1993, Knight et al. 1994, Chapman and Barrett 1997, Dunley and Welter 2000), Australia (Thwaite et al. 1993), southeastern France (Sauphanor et al. 1998), Spain (Bosch et al. 1999), the Middle East (Reuveny and Cohen 2004a), South Africa (Giliomee and Riedl 1998), and Chile (Reyes et al. 2004). Although the mechanisms involved in OP resistance in the codling moth have not been extensively studied, the involvement of metabolic detoxification has been proposed. A reduced nonspecific esterase activity was found to be correlated with resistance to parathion (Bush et al. 1993) and enhanced glutathione *S*-transferase (GST) and cytochrome P450 polysubstrate monooxygenase (PSMO) activities were related to cross-resistances to several groups of insecticides (Sauphanor et al. 1997, 1998; Bouvier et al. 2002). More recently, reduced acetylcholinesterase (AChE) catalytic activity in azinphos-methyl-resistant codling moth populations (Reuveny and Cohen 2004b) and reduced AChE sensitivities to OP and carbaryl associated with a single amino acid substitution F399V (Cassanelli et al. 2006) have been reported.

The aim of this work was to address the presence of azinphos-methyl resistance in the codling moth from apple orchards in central Chile, by using standard laboratory bioassays on neonate and postdiapause larval stages. In addition, we wanted to evaluate the activity of detoxifying enzymes likely to be involved in resistance to this insecticide.

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Materials and Methods

Study Orchards. The apple orchards were situated in the central valley of the O'Higgins and Maule regions, which are the main apple production areas in Chile. The codling moth populations used in this study came from three managed and four abandoned orchards. The managed orchards were located in San Fernando (34° 34′ S, 70° 58′ W), Teno (34° 52′ S, 71° 12′ W) and Linares (35° 57′ S, 71° 19′ W) and were subjected to seven or eight applications of organophosphates (azinphos-methyl, diazinon, and chlorpyriphos) and a final application of pyrethroids (esfenvalerate) on a regular basis. These orchards were selected because although insecticides were heavily used there, they still experienced 1–5% codling moth damage at harvest. The abandoned orchards did not receive insecticides during the last two seasons. They were located in Gultro (34° 11′ S, 70° 46′ W), Molina (35° 5′ S, 71° 10′ W), Colín (35° 27′ S, 71° 19′ W) and Pencahue (35° 23′ S, 71° 48′ W). The infestation level of the codling moth in abandoned orchards was higher than 60% in each case.

Insects. After harvest, codling moths were collected as diapausing larvae from corrugated cardboard strips. The larvae were removed from traps and placed in plastic boxes provided with cardboard strips under cold conditions (2–3°C) and complete darkness for 2 mo (Sauphanor et al. 1999, Boivin et al. 2001). These larvae were then transferred and maintained for 24 h in a controlled environment chamber at 25 ± 1°C and a photoperiod of 16:8 (L:D) h. Subsequently, the larvae were sexed and used in postdiapause bioassays or reared until the adult stage to obtain neonate larvae or to perform enzymatic evaluations (Sauphanor et al. 1999, Boivin et al. 2001). In addition, a laboratory strain of codling moth reared on artificial diet for >15 yr at INRA-Avignon was used as a susceptible reference (Bouvier et al. 2002).

Laboratory Bioassays. The bioassays for both larval stages were performed using diagnostic doses, which are considered to be more suitable when sample sizes are small or resistance frequencies are low (ffrench-Constant and Roush 1990, Sauphanor et al. 2000). The diagnostic dose for neonates was determined from a concentration–mortality curve performed on the susceptible reference strain. Microplates of 96 wells were filled with 150 μl of artificial diet (Stonefly Industries Ltd., Bryan, TX) and six concentrations of azinphos-methyl (Gusathion 350 g [AI]/kg WP, Bayer, Santiago, Chile), producing between 0 and 100% mortality, were tested. Six microliters of each concentration dissolved in distilled water was applied on the surface of the diet; distilled water was used as control. Newly hatched larvae were individually placed in the wells and transferred to controlled conditions (25 ± 1°C and a photoperiod of 16:8 [L:D] h). Twenty individual neonate larvae were exposed to each concentration and the entire experiment was replicated four times. Mortality was registered after 5 d. Larvae were considered dead when did not respond to a probe with dissecting forceps. Missing larvae were subtracted from the initial number and mortality-survival data were subjected to Probit analysis.

A diagnostic concentration of 26 ppm azinphos-methyl (two-fold the calculated LC_{90}) was used in the neonate bioassays. Because the fertility of adult codling moths obtained from diapause populations is rather low (ca. four neonate larvae per female) (Sauphanor et al. 2000), only ≈40 neonate larvae were obtained from each orchard for the bioassay. The diagnostic dose of azinphos-methyl was applied individually to 20 neonate larvae per orchard, and a control treatment with distilled water was applied individually to the other 20 larvae per orchard ($n = 20$). The mortality of neonates was recorded after 5 d, as described above.

For postdiapause larvae bioassays, a diagnostic dose of 400 ppm azinphos-methyl (Reyes et al. 2004) (Gusathion 350 g [AI]/kg WP, Bayer Chile) was dissolved in acetone. To improve formulated azinphos-methyl dissolution, the sample was vortexed and subjected to a short spin in a centrifuge to pellet insoluble components. A volume of 1 μl of supernatant was applied to the mid-dorsal region of the larvae of each sex. Control applications, with acetone alone, also were performed on the larvae. A sample size of nine to 65 individual larvae of each sex was subjected to the diagnostic dose, and 10–40 individual larvae of each sex were subjected to the control application. The treated larvae were left in petri dishes provided with small pieces of cardboard and transferred to a controlled environment chamber at 25 ± 1°C and a photoperiod of 16:8 (L:D) h. Postdiapause larvae were recorded as dead if they failed to produce adults after 1 mo (Sauphanor et al. 2000). Corrected mortality from both bioassays were recalculated to frequency data for each orchard and compared with the susceptible strain by using chi-square tests with Yates correction. When corrected mortality data were available for three or more orchards, differences between management (abandoned versus production) were tested with a Mann–Whitney *U* test.

Enzyme Activity Assays. All enzymatic activity evaluations were performed on adults (Bush et al. 1993) emerged in the laboratory after collected as diapausing larvae from the studied orchards (Reyes et al. 2004). One- or 2-d-old adults were dissected, and their abdomens were homogenized in 150 μl of 50 mM HEPES buffer, pH 7, and stored at −70°C. GST activity was evaluated with the fluorimetry methodology described by Nauen and Stumpf (2002) by using monochlorobimane. In brief, black microplate wells were filled with 30 μl of abdomen extract, 168 μl of 100 mM reduced glutathione in 50 mM HEPES buffer, pH 7, and 2 μl of 30 mM monochlorobimane. The microplates were incubated at 22°C for 20 min, and fluorescence was measured at 390 nm for excitation and 460 nm for emission with a Victor 3 Wallac microplate reader (PerkinElmer Life and Analytical Sciences, Boston, MA). Because fluorescent bimane-glutathione adduct (GS-mCB) is not commercially available, the GST activity was expressed as fluorescence units of GS-mCB per insect equivalent (Nauen and Stumpf

2002). Esterase activity was measured with the photometry methodology described by Bouvier et al. (2002) by using β-naphthyl-acetate. Microplate wells were filled with 0.5 μl of abdomen extract, 89.5 μl of HEPES buffer (50 mmol; pH 7), and 90 μl of 0.1 mM β-naphthyl-acetate in 50 mM sodium phosphate buffer, pH 6.5. After incubation at 30°C for 15 min, the reaction was stopped by adding 20 μl of a staining reagent solution of Fast Garnet (3 g/liter) and 35 g/liter sodium dodecyl sulfate. After 15 min at room temperature, absorbance was measured at 490 nm with a Victor 3 Wallac microplate reader (PerkinElmer Life and Analytical Sciences). Protein concentration was evaluated with the Bradford assay (Bouvier et al. 2002). Esterase activity was expressed as nanomoles of colored β-naphthol per milligram of protein per minute. PSMO activity was evaluated with the in vivo protocol for ethoxycoumarin-*O*-deethylase activity (De Sousa et al. 1995). Freshly dissected abdomens in saline solution (6 g/liter NaCl) were placed in black microplate wells provided with 100 μl of 0.4 mM ethoxycoumarin in 50 mM phosphate buffer, pH 7.2. After incubation of the microplates at 30°C for 4 h, 100 μl of glycine buffer (10^{-4} M; pH 10.4)/ethanol (vol: vol) was added to stop the reaction. Finally, fluorescence was measured at 390 nm for excitation and 460 nm for emission with a Victor three Wallac microplate reader (PerkinElmer Life and Analytical Sciences) (Bouvier et al. 1998). PSMO activity was expressed as picograms of fluorescent 7-hydroxycoumarin (7OH) per insect equivalent per minute. A sample size of nine to 27 individual adults of each sex was used to evaluate activity of GST, PSMO, and esterases.

Differences in enzymatic activity between orchards were evaluated with the Kruskal–Wallis test. When enzymatic activity data were available for three or more orchards, differences between management (abandoned versus production) were tested with the Mann–Whitney *U* test. Finally, association between bioassay mortality and enzymatic activity was calculated using Spearman rank order correlations.

Results

Concentration–mortality of neonate larvae from the susceptible laboratory strain to azinphos-methyl allowed the estimation of LC_{50} (95% CI) = 5.8 (5.2–6.4), LC_{90} (95% CI) = 13.2 (11.4–16.3) ($\chi^2 = 2.17$, df = 3, $P = 0.54$), and a diagnostic concentration (twice the LC_{90}) to perform the bioassays. Neonate larvae from the studied orchards showed high levels of mortality with no significant difference in relation with the susceptible strain (Table 1). There was no significant difference in mortality between abandoned and production orchards for neonate larvae (Mann–Whitney Z adjusted = 0.76; df = 4, 3; $P > 0.05$, N.S.). However, postdiapausing larvae of both sexes showed significantly lower mortality than did the susceptible strain in six of the seven studied orchards (Table 1). There were no significant differences in mortality between abandoned and production orchards for either male (Mann–Whitney Z adjusted = 0.35; df = 4, 3; $P > 0.05$, N.S.) or female postdiapausing larvae (Mann–Whitney Z adjusted = 0.71; df = 4, 3; $P > 0.05$, N.S.).

Table 1. Corrected mortality of neonate and postdiapausing larvae of codling moth from different orchards and an insecticide-susceptible laboratory strain when exposed to a diagnostic dose of azinphos-methyl under laboratory conditions

Pop	Total mortality neonate larvae (%)[a]	Mortality postdiapausing larvae (%)[a]	
		Male	Female
Susceptible strain	100 (20)	97.9 (65)	90.9 (35)
Abandoned orchard			
Gultro	90 (20)	66.7* (12)	78.6* (40)
Molina	100 (20)	60.0* (40)	77.5* (40)
Colín	100 (20)	27.3* (20)	45.5* (20)
Pencahue	94.7 (20)	100 (32)	100 (29)
Managed orchard			
San Fernando	90 (20)	61.8* (40)	63.0* (12)
Teno	90 (20)	14.3* (35)	11.1* (9)
Linares	100 (20)	75.0* (40)	79.0* (50)

*Significant differences in relation to the susceptible strain after a chi-square test at $P < 0.05$.

[a] Data are corrected mortality. Number in parentheses is the sample size (i.e., number of treated larvae with diagnostic dose).

Enzymatic activity of GST was significantly higher for adult males and females in two orchards in relation with the susceptible strain (Table 2). One of the orchards was abandoned (Colín) and the other was in production (Teno). Also, a significant correlation between postdiapause larval mortality and adult GST activity was detected (Spearman R = −0.89, $t = -4.4$, df = 5, $P \leq 0.05$), which was accounted for by both male (Spearman R = −0.86, t = −3.7, df = 5, $P \leq 0.05$) and female data (Spearman R = −0.69, $t = -2.3$, df = 6, $P \leq 0.05$). The opposite effect was found for adult esterase activity, with a significantly lower activity compared with the susceptible strain in two abandoned orchards (Table 2). This trend is clearer with female data, where four orchards (two of each management group) showed a significantly lower adult esterase activity relative to the susceptible strain (Table 2). However, no significant correlation was found between postdiapause larval mortality and adult esterase activity. No significant differences were found for adult PSMO activity between the studied orchards and the susceptible strain (Table 2). Similarly, no significant correlation between postdiapause larval mortality and adult PSMO activity was detected. Finally, no significant differences were found in adult enzymatic activity between abandoned and production orchards for GST (females: Mann–Whitney Z adjusted = −0.35; df = 4, 3; $P > 0.05$, N.S.), PSMO (males: Mann–Whitney Z adjusted = −0.18, females: Mann-Whitney Z adjusted = −1.41) or esterases (females: Mann–Whitney Z adjusted = 0.00; df = 4, 3; $P > 0.05$, N.S.).

Discussion

Because neonate larvae represent the main target of insecticide sprays against codling moth, bioassays performed on this instar could provide information for management recommendations. In the current study,

Table 2. Mean enzymatic activity in adults of the codling moth from different orchards and in an insecticide susceptible laboratory strain

Pop	GST activity[a,b] (GS-mCB fluorescence units insect^{-1})[c]		Esterase activity[a,b] (nmol β-naphthol mg protein^{-1} min^{-1})[d]		Cytochrome P450 polysubstrate monooxygenase activity[a,b] (pg 7OH insect^{-1} min^{-1})[e]	
	Male	Female	Male	Female	Male	Female
Susceptible strain	7,528 ± 1,354a (10)	10,190 ± 1,149ab (12)	603 ± 33c (10)	505 ± 42c (12)	6.9 ± 2.4 (9)	8.0 ± 2.6 (12)
Abandoned orchard						
Gultro	9,849 ± 1,389ab (14)	12,799 ± 1,934ab (25)	430 ± 64bc (12)	400 ± 40bc (25)	8.4 ± 2.1 (9)	8.5 ± 2.1 (19)
Molina	8,592 ± 1,078a (34)	10,398 ± 1,874ab (20)	208 ± 28a (24)	141 ± 33a (10)	8.1 ± 1.5 (20)	8.1 ± 1.1 (18)
Colín	12,662 ± 2,333b (11)	17,524 ± 1,679b (27)	784 ± 124c (10)	505 ± 88bc (14)	5.0 ± 1.2 (20)	9.5 ± 2.2 (18)
Pencahue	7,792 ± 1,121a (17)	7,580 ± 892a (26)	232 ± 39ab (17)	212 ± 28a (26)	5.8 ± 1.4 (19)	10.8 ± 2.0 (19)
Production orchard						
San Fernando	12,338 ± 1,330ab (12)	9,822 ± 1,611ab (12)	443 ± 47bc (12)	320 ± 29abc (11)	6.4 ± 1.1 (9)	18.3 ± 4.2 (11)
Teno	13,754 ± 1,903b (18)	18,845 ± 3,298b (17)		292 ± 27ab (18)	8.1 ± 4.7 (12)	12.4 ± 4.2 (18)
Linares		11,108 ± 1,760ab (17)		322 ± 66ab (17)	5.9 ± 1.2 (10)	9.4 ± 2.6 (10)

[a] Letters in the same column indicate significant differences after Kruskal–Wallis test at $P < 0.05$.
[b] Data are means ± SE. Number in parentheses is sample size.
[c] Fluorescence units of enzyme product ([GS-mCB]) per insect equivalent.
[d] Nanomoles of colored enzyme product (β-naphthol) per milligram of protein per minute of reaction.
[e] Picograms of fluorescent enzyme product (7OH) per insect equivalent per minute of reaction.

when neonate larvae were exposed to a diagnostic concentration of azinphos-methyl their mortality was satisfactory, which provides evidence that the level of azinphos-methyl resistance in the studied orchards has not yet reached the threshold for field control failure. In contrast, the main part of the studied orchards also showed a reduced mortality of postdiapausing larvae subjected to a diagnostic dose of azinphos-methyl. Although the diagnostic dose used for postdiapausing larvae only caused ≈99% mortality in the susceptible strain, this dose has been reported previously and used in orchards of the Maule Region (Reyes et al. 2004) and other regions in Europe (Reyes et al. 2007). Although this instar is not the target for insecticide sprays, the observed reduction in mortality could represent an early stage of resistance development toward this insecticide. The lower mortality of neonate larvae in relation to later instars of the codling moth exposed to teflubenzuron also was found in laboratory strains selected for resistance to diflubenzuron and deltamethrin (Bouvier et al. 2002).

For the evaluation of an association between resistance and possible biochemical mechanisms, LC_{50} and confidence intervals from each studied orchard are more statistically robust than the diagnostic concentration approach (ffrench-Constant and Roush 1990, Sauphanor et al. 2000). However, diagnostic concentration is more efficient for small sample sizes and low frequencies of resistant individuals; this is because a major proportion of the sampled individuals are exposed to an informative concentration of the insecticide (ffrench-Constant and Roush 1990, Sauphanor et al. 2000). This is the situation of codling moth in Chile, which has rather low population densities (1–5% fruit damage at harvest) and resistance frequency, probably below the insecticide efficacy threshold for managed orchards.

Age-dependent response to insecticides in codling moth larvae is known to be based on differences in GST and PSMO enzymatic activity (Bouvier et al. 2002). A significantly higher activity of GST in adults was found in our study, and this enzymatic activity was also significantly correlated with azinphos-methyl mortality of the postdiapausing larvae. No differences in PSMO activity were found, but codling moths from a few orchards showed lower esterase activity than did the susceptible strain. Nonetheless, the activity of esterases or PSMOs was not significantly correlated with azinphos-methyl mortality. Bush et al. (1993) reported lower esterase activity in a parathion-resistant population of codling moth, which could be related to a modified aliesterase in resistant populations. Such aliesterases would present enhanced hydrolysis of OP and little activity on noninsecticidal substrates generally used in bioassays (e.g., naphthyl-acetate) (Hemingway 2000, Wheelock et al. 2005). However, the lack of negative correlation between esterase activity and azinphos-methyl mortality did not support this hypothesis in our study.

The results of enzymatic activity also were supported by previous analyses performed in the codling moth from the Maule Region (Reyes et al. 2004). Enhanced GST activity seems to be the main mechanism for azinphos-methyl resistance in the studied populations, representing a nonspecific detoxification metabolic pathway that also could be associated with cross-resistance toward active ingredients with other modes of action. Although GST activity has not been previously associated with azinphos-methyl or organophosphate resistance in the codling moth, it has been reported to be involved in diflubenzuron and tebufenozide resistance and partially with deltamethrin resistance (Sauphanor et al. 1997; Bouvier et al. 1998, 2002). Furthermore, GST was associated with azinphos-methyl resistance in other species of Lepidoptera that are also apple pests in North America, for example, the tufted apple bud moth (Carlini et al. 1995, Karoly et al. 1996) and the obliquebanded leafroller (Smirle et al. 1998).

Our azinphos-methyl bioassays were performed on two larval stages, whereas the activities of detoxifying enzymes were evaluated on adults. Previous experi-

ments have correlated enzyme activity of larval and adult stages for the codling moth, providing a meaningful background for using the adult stage for enzymatic evaluations (Reyes et al. 2004).

A significant absence of higher levels of enzymatic activity or lower mortality was found in managed orchards compared with abandoned orchards. This lack of major difference in relation to management also has been reported for the United States (Knight et al. 1994) and suggests that there were high rates of codling moth migration between abandoned and managed orchards. Alternatively, azinphos-methyl resistance based on enhanced GST activity could have rather low fitness costs, which might allow its maintenance in the codling moth populations of abandoned orchards for rather long periods. Moreover, codling moth populations resistant to azinphos-methyl have shown a slow decline (≈50% in 17 generations) of their resistance level when reared under laboratory conditions in the absence of pesticides (Reuveny and Cohen 2004a).

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