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Combined detoxification mechanisms and target mutation fail to confer a high level of resistance to organophosphates in *Cydia pomonella* (L.) (Lepidoptera: Tortricidae)

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ABSTRACT

Despite the frequent and widespread applications of organophosphates against *Cydia pomonella* this species has developed low levels of resistance to this chemical group. Investigations concerning the mechanisms involved in resistance are scarce, and usually consider only one of the potential mechanisms. With the aim of a better understanding the resistance mechanisms and their possible interaction, four of these mechanisms were investigated simultaneously in one sensitive (Sv) and two resistant strains (Raz and Rdfb) of this insect. Resistant strains displayed an increased mixed function oxidase activity, whereas carboxylesterase activity varied upon the substrate used. The three strains had similar β-naphtyl acetate activity, and the hydrolysis of α-naphthyl acetate and *p*-nitrophenyl valerate was higher in the Sv strain. The *p*-nitrophenyl acetate activity was highest in the resistant strains and was strongly inhibited by azinphos and DEF. The Raz strain has a modified acetylcholinesterase (AChE), which resulted in a 0.7-, 3.2- and 21.2-fold decrease in the susceptibility to chlorpyriphos-ethyl-oxon, azinphos-methyl-oxon, and paraoxon-methyl, respectively. These combined resistance mechanisms only conferred to Raz a 0.6-, 7.9- and 3.1-fold resistance to the related insecticides. Organophosphates resistance in *C. pomonella* results from a combination of mechanisms including modified affinities to carboxylesterase substrates, and increased metabolisation of the insecticide. The apparent antagonism between increased functionalisation and reduced sensitivity of the AChE target is discussed.

1. Introduction

Different detoxifying enzymatic systems may provide to phytophagous arthropods, wide protection against the toxins of their host plants [1]. The biotransformation of xenobiotics occurs in two stages. An initial phase of functionalisation results in the addition of a functional group to the exogenous molecule, making it hydrophilic and therefore more easily excretable. In the second phase named conjugation, a polar group is added to the functionalized or original molecule. The first phase, primarily involves the mixed-function oxidases (MFO) and the carboxylesterases (CbE), whereas in the second phase, the glutathione *S*-transferases (GST) are the most important enzyme group involved [1,2]. Mutations or overexpression of these three enzymatic systems are responsible for insecticide resistance in different species [3,4]. Resistance can also be due to target specific mutations. Modifications of the voltage-dependent sodium channel (the target of pyrethroids, DDT and its analogues), and acetylcholinesterase (the target of carbamates and organophosphates) have been extensively described [5–7]. Organophosphates (OPs) have been the chemical insecticide group most extensively used in crop protection over the past four decades [8,9]. This was particularly the case for the codling moth *Cydia pomonella* (L.), one of the main pests in apple, pear and walnut orchards worldwide. Despite the propensity of this species for developing a quick resistance to insecticides, as it has occurred with arsenate [10] and DDT [11], the phenomena was only observed after 20 years of intensive OPs use [12]. The acquisition of resistance to new chemical groups such as pyrethroids [13] and insect growth regulators as diflubenzuron [14,15] has also occurred relatively promptly. Resistance to microbiological insecticides such as an entomopathogenic virus has also been recorded in orchards [16,17]. Even though the loss of effectiveness of this insecticide group against the codling moth has been detected in the United States, Europe, Australia, South Africa and South America [18–22], the OPs are still registered and

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continue to be the main insecticide group used. Most of the potential resistance mechanisms to OPs have been reported for different populations of *C. pomonella*. They can be metabolic, as a result of modified CbE or increased MFO and GST [21–25], as well as through acetylcholinesterase (AChE) modification [21,26,27]. A laboratory strain selected for resistance to azinphos-methyl cultured from a Spanish codling moth population combines all these mechanisms [28]. However, it does not present a high level of resistance to this insecticide [21,22,28]. It appears thus that it is not possible to fully understand the resistance mechanisms and how they interact without evaluating all the mechanisms within a single study using consistent methodologies. We therefore investigated the expression and interactions between the different detoxification mechanisms (MFO, GST, and CbE) and the AChE target site mutation on a sensitive and two resistant strains of codling moth.

2. Materials and methods

2.1. Insects

One sensitive (Sv) and one resistant laboratory strains (Rdfb) were mass reared on artificial diet at the INRA of Avignon (France) since 1995 [29]. A second resistant strain originating from northern Spain (Raz), was introduced at the INRA of Avignon in 1998 and mass reared [27]. The Sv strain has never been exposed to insecticides. Rdfb and Raz have been submitted to selection pressure for more than 50 generations by exposing the larvae to diflubenzuron (500 mg L^{-1}) and azinphos-methyl (375 mg L^{-1}), respectively. Insecticides are sprayed on to the surface of the artificial diet prior to penetration by newly hatched larvae [21,27,30].

2.2. Insecticides and synergists

The commercial formulations of azinphos-methyl (Gusathion XL WP 25%) and parathion-methyl (Oleoblaban) were obtained from Bayer CropScience (France), and chlorpyriphos-ethyl (Pyrinex ME CS 250 g L^{-1}) from Philagro (France). The synergists piperonyl butoxide (PBO, 94% purity), *S,S,S*-tributyl phosphorotrithioate (DEF, 97.5% purity) and diethyl maleate (DEM, 97% purity) were obtained from CIL-Cluzeau (France). The insecticides were diluted in distilled water and the three synergists in 96% ethanol. For AChE inhibition analysis, the oxon forms of the organophosphates azinphos-methyl (98.7% purity), chlorpyrifos-ethyl (99.7% purity) and methyl parathion (98.7% purity) were obtained from CIL Cluzeau (France). Insecticides were dissolved in an ethanol/methanol (3:1) mixture.

2.3. Toxicological tests

The sensitivity of the three strains (Sv, Raz and Rdfb) to azinphos-methyl, chlorpyrifos-ethyl and parathion-methyl was evaluated. The products were applied to the artificial diet using a spray tower [31], which allowed a uniform spraying of 1.7 ± 0.1 mg of each concentration per cm^2. At least six concentrations, including distilled water instead of insecticide as control, were used. For each concentration, a minimum of 20 newly hatched larvae (0–4 h old), were individually placed with a brush on the plastic box (2 × 2 × 2 cm), and mortality was recorded after 4 days at 25 ± 1 °C, RH 40 ± 5% and 16:8 h light:dark [30]. Larvae were considered dead if did not respond to mechanical stimuli. Missing larvae were subtracted from the initial number.

2.4. Synergism tests

In order to evaluate the involvement of the enzyme systems on azinphos-methyl resistance, maximum concentration of the synergists to be used without inducing larval mortality were assessed (Table 1). Microplate tests, more convenient for synergist applications, were used [32]. PBO, DEM and DEF which inhibit MFO, GST and CbE, respectively were used. Wells were filled with 150 µL of artificial diet (Stonefly Industries Ltd.), and 6 µL of synergist prepared in ethanol at different concentrations (or ethanol alone as control), were applied to the surface of the diet, with a repetition micropipette. After 1 h, newly hatched larvae (0–4 h old) were individually placed in the wells. Mortality was determined as described above after 4 days [17,23]. Sixteen larvae were used per concentration and tests were replicated four times.

Table 1
Concentration of the synergists PBO, DEM and DEF used on a susceptible (Sv) and two resistant strains (Raz, Rdfb) of *C. pomonella* L.

Strain	Synergist concentration (mg L^{-1})		
	PBO	DEF	DEM
Sv	450	150	12.5
Raz	1200	400	1000
Rdfb	1200	200	500

2.5. Enzymatic activities

Newly hatched larvae (0–4 h old) of all strains were maintained on artificial diet for the period of four days at 25 °C, and then used for determination of GST, MFO and CbE activities. This allowed the evaluation at the same age that toxicological and synergism tests. Fluorescence and absorbance were measured using a microplate reader (HTS 7000, Perkin Elmer). The total protein contents of the crude extracts were determined according to the Bradford method [33].

2.5.1. Mixed-function oxidases

The MFO activity was determined measuring 7-ethoxycoumarin *O*-deethylation activity (ECOD) [34] adapted for in vivo analysis in a microplate [35]. Twenty whole 4-day-old larvae from each strain were dissected in 6 g L^{-1} sodium chloride and they were individually placed in the wells of black microplates. The reaction was started with the addition of 100 µL of phosphate buffer (50 mM, pH 7.2) and 7-ethoxycoumarin (0.4 mM). After 4 h incubation at 30 °C, it was stopped by adding 100 µL of 0.1 mM glycine buffer (pH 10.4)/ethanol (v/v). The 7-hydroxycoumarin fluorescence was quantified with 380 nm excitation and 450 nm emission filters. Twelve wells receiving glycine buffer prior to incubation were used as controls. A standard curve was established using hydroxycoumarine and MFO activity was expressed as pg of 7-OH $insect^{-1}$ min^{-1}.

2.5.2. Glutathione S-transferases

Four groups of 10 larvae from each strain were homogenized on ice in 100 µL of Hepes buffer (50 mM, pH 7.0), then centrifuged at 15,000*g* for 15 min at 4 °C. The supernatants were used as enzyme source. GST activity was determined in black microplates using monochlorobimane (MCB) as substrate [36]. The reaction mixture consisted of 30 µL of enzymatic extract, 168 µL of 100 mM GSH in Hepes buffer (50 mM, pH 7.0) and 2 µL of MCB 30 mM [27]. Wells with the buffer alone were used as controls instead of the enzyme extract. Fluorescence was measured after 20 min of incubation at 22 °C with 380 nm excitation and 450 nm emission filters. Since the bimane-glutathione adduct is not commercially available [36], the activity was expressed as fluorescence units µg $protein^{-1}$.