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Target-site mutations (*AChE* and *kdr*), and PSMO activity in codling moth (*Cydia pomonella* (L.) (Lepidoptera: Tortricidae)) populations from Spain

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ABSTRACT

Codling moth, *Cydia pomonella* (L.) is a key pest of global importance that affects apple fruit production and whose populations have developed resistance to insecticides in many apple production areas. In Spain, enhanced cytochrome P450 polysubstrate monooxygenase (PSMO) activity is the main mechanism involved in insecticide detoxification by codling moth, although acetylcholinesterase (AChE) target site mutations have been described in two populations. However, the extent of *AChE* and knockdown resistance (*kdr*) mutations in Spain is unknown. To assess the actual occurrence of *AChE* and *kdr* mutations concurrently with the frequency of moths with PSMO enhanced activity (R-PSMO), 32 Spanish field populations from four apple-growing areas of Spain and two susceptible laboratory strains were evaluated. R-PSMO was significantly higher in 23 chemically treated field populations from Extremadura, Catalonia and Aragon, with proportions that varied between 25% and 90%, but no significant differences among strains and the non-chemically treated orchards (organic or abandoned) were observed. The *AChE* mutation (F290 V) was detected in all field populations from Catalonia (n = 21) and in three field populations from Aragon (n = 5), with resistant phenotype proportions varying from 34.2% to 97.5% and from 7.2% to 65% in Catalonia and Aragon, respectively. In addition, the *kdr* mutation (L1014F) was detected in twelve Catalonian field populations, at rates of incidence ranging between 2.6% and 56.8%. A positive correlation between R-PSMO and *AChE* mutation was found. The origin of the mutations and their ability to persist and spread in field populations with different management systems is discussed.

1. Introduction

A key component of the integrated management of key pests such as codling moth (*Cydia pomonella* (L.), Lepidotera: Tortricidae) is establishing an insecticide-resistance management (IRM) program. Codling moth is one of the most damaging pests of pome fruit crops worldwide, although in almost all the areas where such crops are cultivated it affects mainly apple production [1]. As a result of long-term pesticide use, the codling moth has developed resistance to different insecticide modes of action and chemistries, such as neurotoxic insecticides and insect growth regulators [2–18], and even to *C. pomonella* granulovirus (CpGV) (family Baculoviridae) [19].

Worldwide, codling moth insecticide resistance is mainly associated with the increased activity of detoxifying enzymes such as non-specific esterases (ESTs), cytochrome polysubstrate P450 monooxygenases (PSMOs), and glutathione S-transferases (GSTs) [3,14,20–22]. In addition, two target-site mutations (structural changes in the insecticide target proteins that render them less sensitive to an insecticide) have been reported: a F290V replacement in acetylcholinesterase in *AChE-1* gene, *AChE* onwards, involved in resistance to organophosphates and carbamates [23], and a L1014F replacement in the voltage-gated sodium channel gene, *kdr* onwards, involved in resistance to pyrethroids [24]. The *AChE* mutation confers phenotypic resistance under both homozygote and heterozygote conditions, whereas the *kdr* mutation confers it only under the recessive homozygote condition.

In codling moth Spanish field populations, insecticide resistance has been associated with three detoxification systems, mainly PSMO in adults and larvae [14,15,21,25,26], and to a lesser extent GST and EST in larvae [14,15,25]. As for target-site mutations, Reyes et al. [21,27] found the *AChE* mutation only in a single Spanish field population from a Catalan (NE Spain) apple-growing area, in a study of the variability of resistance mechanisms worldwide that involved a total of 55 populations from Europe (1 from Spain) and 24 from other continents. This Catalan population came from the same area where the Raz population,

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selected in laboratory with azinphos-methyl by INRA in Avignon, was sampled and in which the *AChE* mutation was identified by Cassanelli et al. [23]. The *kdr* mutation was not detected in this Spanish field population [21,27,28].

The existence of multiple resistance mechanisms increases the difficulty of codling moth control and it interferes with the management programs in the orchards. Brent [29] pointed out that the existence of at least 5% of resistant individuals is required in a population to detect a resistance problem, but Denholm et al. [30] and Hoy [31] reported that resistance is very difficult to manage even when only 10% of the individuals carry resistance genes. It is therefore important to establish an IRM program for Spanish codling moth populations to avoid or delay the increase in the frequency of resistant individuals. This requires the early detection of the existing resistance mechanisms in field populations and knowledge of their extent at local scale.

Adult moths with PSMO enhanced activity (PSMO-resistant onwards) from Spanish populations did not show a greater attraction to pure kairomone-baited traps in apple orchards [32], as was reported by Sauphanor et al. [33] for French PSMO-resistant populations. However, PSMO detection in codling moth adults caught during the different flights, in orchards under different crop management systems, was found to be a good tool to assess levels of insecticide resistance in field apple crops [32]. Extensive studies of increases in the metabolic capacity of detoxifying enzymes have been carried out in the Ebro Valley pome fruit production area [14,15,25], but not on the extension in the occurrence of the target site sensitivity.

In an attempt to complete the overview of resistance mechanisms in Spanish codling moth populations, the aims of the present work were to assess the actual occurrence of *AChE* and *kdr* mutations in codling moth field populations from different pome fruit-growing areas of Spain, concurrently with the frequency of moths with PSMO enhanced activity (R-PSMO).

2. Materials and methods

2.1. Insects

Thirty-two codling moth field populations were collected during the years 2010 to 2012 in four Spanish autonomic regions where pome fruits are cultivated (Fig. 1): Asturias (AST), n = 5; Extremadura (EXT), n = 1; Aragon (ARA), n = 5; and Catalonia (CAT) n = 21 (Table 1). Apple cultivation in Asturias (4106 ha in 2016) is dedicated to cider production, and orchards are managed mainly organically and without irrigation. In the other three regions, apple trees are grown for table apple production, and orchards are intensively managed. In 2016, Catalonia (11,066 ha, mostly in Lleida) and Aragon (4576 ha) represented 75% of the total Spanish acreage dedicated to apple production under irrigation.

Codling moth adults were either caught in monitoring delta traps baited with attractants or as they emerged from larvae caught in cardboard traps. According to the intensity of chemical insecticide treatments, the orchards were grouped as non-chemically treated orchards (UN, n = 8, abandoned or organic orchards), chemically treated orchards (CH, n = 12), and mating disruption plus chemical control orchards (MD + CH, n = 12). In several orchards, the percentage of damaged fruits at harvest was higher than 2% in spite of the control measures applied. Two codling moth-susceptible laboratory strains were used: (i) a Spanish susceptible strain, S_Spain (SSp), collected from an abandoned apple orchard in Lleida in 1992 and reared since then at the joint IRTA (Institute for Food and Agricultural Research and Technology) and UdL (University of Lleida) laboratory (Lleida, Spain) using a semi-artificial dehydrated apple diet, and (ii) a French susceptible strain, S_France (Sv), provided by INRA (Avignon, France), mass-reared on an artificial diet (Manduca Premix-Heliothis Premix, Stonefly Inc., Bryan, TX) under laboratory conditions. SSp was used as the main reference in this study.

2.2. PSMO activity

To determine PSMO activity, freshly emerged adult abdomens were dissected to be used as enzymatic source, as described by Rodríguez et al. [26], in 6 g L^{-1} sodium chloride, and were placed in black 96-well microplates. The activity was measured using 7-ethoxycoumarin *O*-deethylation activity (ECOD), adapted for *in vivo* analysis [34]. The methodology used was the same as described by Reyes et al. [21]. The reaction was initiated when an adult abdomen was individually introduced into a well containing 100 mL of phosphate buffer (50 mM, pH 7.2) and ethoxycoumarin (0.4 mM). After 4 h of incubation at 30 °C, the reaction was stopped by adding 100 mL of 0.1 mM glycine buffer (pH 10.4)/ethanol (v/v). The 7-hydroxycoumarin fluorescence was quantified using a multilabel plate counter VICTOR3 (Perkin Elmer Life and Analytical Sciences, Madrid, Spain), with 380-nm excitation and 450-nm emission filters. In each plate, twelve wells were used as controls and received the glycine buffer prior the incubation. A standard curve was obtained using 7-hydroxycoumarin, and PSMO activity was expressed as pg of 7-hydroxycoumarine $insect^{-1}$ min^{-1}.

2.3. AChE and kdr mutations

Target-site mutations related to insecticide resistance, located in *AChE-1* and *kdr* genes, were analysed using PCR–RFLP [21,24] in 1174 adults (1103 from field populations and 71 from the two susceptible laboratory strains). Total DNA was obtained from the adult thorax, using the "salting out" methodology described by Fuentes-Contreras et al. [35]. PCR amplifications of *kdr* and *AChE-1* genes were carried out separately, in a 25-μL reaction volume containing primer reaction buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 200 μM of each dNTP, 0.4 mM of each primer, 1 unit of Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) and 2 μL of DNA template. Restrictions were performed by pooling the PCR products from both genes (5 μL each) with 0.2 units of *Tsp*509I (New England Biolab, Ipswich, MA, USA) in a 20-μL reaction volume incubated at 65 °C for 16 h. DNA fragments were separated by electrophoresis on 6% polyacrylamide gel and visualized after silver staining. DNA fragments of 141 bp and 102 bp, respectively, identified the mutant and sensitive *AChE* alleles. The *kdr* mutant allele was identified by a DNA fragment of 77 bp and two different sensitive alleles were identified by fragments of 101 bp and 112 bp, respectively. The genotype frequency of *AChE* and *kdr* mutations was measured.

2.4. Data analysis

The distribution of the variable PSMO activity did not fulfil the assumption of homoscedasticity, and several standard transformations did not normalize it (Normal Q-Q plot and Shapiro-Wilkes normality test), using the *qqplot* and *shapiro.test* functions in R language [36]. Thus, a non-parametric Kruskal–Wallis test was performed, followed by a multiple comparison (*post hoc*) test [37], using the *kruskalmc* function from the *pgirmess* R package [38] to evaluate PSMO activity among populations and among orchards grouped by their management system. In both cases, only field populations with n ≥ 20 adults were used.

To calculate the relative frequency of PSMO-resistant codling moth adults (R-PSMO), an adult was classified as resistant if its PSMO activity was higher than the upper value of the 95% confidence limit of the mean PSMO activity of the susceptible strain SSp [21]. A Pearson chi-square (χ^2) test was used to compare the R-PSMO between each population and the susceptible population SSp using *chisq.test* functions [36].

To detect whether the *AChE* and *kdr* genes were under selection pressure, a Fisher's exact test was performed for each population to check the Hardy-Weinberg equilibrium (GENEPOP 4.5 [39]). Finally, Pearson correlation was used to evaluate the relationship between the R-PSMO, the percentage of resistant insects with the *kdr* mutation and