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Determination of the Baseline Susceptibility of European Populations of *Cydia pomonella* (Lepidoptera: Tortricidae) to Chlorantraniliprole and the Role of Cytochrome P450 Monooxygenases

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Determination of the Baseline Susceptibility of European Populations of *Cydia pomonella* (Lepidoptera: Tortricidae) to Chlorantraniliprole and the Role of Cytochrome P450 Monooxygenases

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Abstract

The codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), is the key pest on pome fruit and walnut orchards worldwide. Its resistance to available insecticides has been widely reported. Chlorantraniliprole is an anthranilic diamide that was introduced in European countries in 2008–2009 and acts by activating the insect's ryanodine receptors. The aims of this study were to determine the baseline susceptibility of European populations of *C. pomonella* to chlorantraniliprole, to establish the discriminant concentrations (DC) to check the possible development of resistance, and to know the role of cytochrome P450 monooxygenases (P450) in the possible susceptibility decrease of field populations to the insecticide. Ten field populations from Spain along with others were used to calculate the baseline response of larvae to chlorantraniliprole incorporated into the diet. A pooled probit line was calculated, and three DC were established: 0.3 mg a.i./kg (close to the LC₅₀), 1.0 mg a.i./kg (close to the LC₉₀), and 10 mg a.i./kg diets (threefold the LC₉₉). The DC were used to test the susceptibility of 27 field populations from France, Germany, Hungary, Italy, and Spain. The corrected mortality observed in all cases ranged within the expected interval, even with Spanish populations that showed between 12.1 and 100.0% of individuals with high P450 activity. However, the mortality caused by the DC_{0.3} decreased as the mean P450 activity increased. Field populations resistant to other insecticides were susceptible to chlorantraniliprole. The determined baseline codling moth susceptibility is a valuable reference for tracking possible future alterations in the efficacy of the insecticide.

Key words: codling moth, chlorantraniliprole, baseline, resistance monitoring

The codling moth (CM), *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), is the key pest on pome fruit and walnut orchards in almost all the areas where these crops are cultivated. At present, its control is mostly achieved by a combination of pesticides and mating disruption (Witzgall et al. 2008, Ioriatti and Lucchi 2016). The existence of CM insecticide-resistant populations is a widespread problem in many pome fruit production areas in the world, and it concerns 10 out of the 11 insecticide modes of action available to control this pest, depending on the country (IRAC Codling Moth WG 2013), which are as follows: 1A—carbamates, 1B—organophosphates, 3A—pyrethroids, 4A—neonicotinoids, 5—spinosyns, 6—avermectins, 7B—phenoxy-ethyl carbamates, 15—benzoylureas, 18—diacylhydrazines, and 22A—oxadiazines (Sauphanor et al., 1998, 2000; Dunley and Welter 2000; Reuveny and Cohen 2004; Fuentes-Contreras et al.

2007; Ioriatti et al. 2007; Stará and Kocourek 2007; Knight 2010; Rodríguez et al. 2010, 2011, 2012; Voudouris et al. 2011; Grigg-McGuffin et al. 2015; Iscy and Ay 2017). CM resistance to insecticides is mainly metabolic due to three enzymatic complexes (cytochrome P450 monooxygenases (P450), glutathione transferases (GST), and esterases (EST); www.irac-online.org), but mutations in the insecticide target site protein have also been detected (the acetylcholinesterase (*AChE*) mutation, only reported in the fruit growing area of Lleida (Spain) (Cassanelli et al. 2006), and the knockdown resistance (*kdr*) mutation (Brun-Barale et al. 2005)). For example, compared with nonchemically treated orchards, an increase of the frequency of resistant CM individuals has been detected in many chemically treated orchards in the tree fruit area of Lleida, and enhanced P450 activity was the main enzymatic mechanism involved (Rodríguez et al. 2010,

2011; Bosch et al. 2016). However, enhanced GST and EST activity was also detected (Rodríguez et al. 2011) as well as the presence of *AChE* and *kdir* mutations (Bosch et al. 2014). Metabolic cross-resistance is a big concern in any management resistance strategy, which may occur either at the level of interaction with the various chemical families or at a geographical level (Dunley and Welter 2000, Reyes et al. 2007, Voudouris et al. 2011, IRAC Codling Moth WG 2013). New pesticides with new modes of action and with an environmentally safe toxicological profile are then necessary to control resistant CM populations.

Chlorantraniliprole (Rynaxypyr) and cyantraniliprole (Cyazypyr), developed by DuPont (USA), are anthranilic diamides whose mode of action has been classified in the new insecticide group 28 (ryanodine receptor modulator) within the Insecticide Resistance Action Committee (IRAC) mode of action classification scheme (Nauen 2006). By activating the insect ryanodine receptors (RyRs), they stimulate the release and depletion of intracellular calcium stores from the sarcoplasmic reticulum of muscle cells, causing impaired muscle regulation, paralysis, and, ultimately, death (Cordova et al. 2006). Chlorantraniliprole acts primarily by ingestion and by contact on the larvae of chewing pests. Newly hatched larvae from treated eggs die when eating the chorion at emergence. It has extremely broad spectrum effectiveness within the insect order Lepidoptera and some Coleoptera, Diptera, and Isoptera, acting in a broad range of crops and showing very low mammalian toxicity and high selectivity to nontarget arthropods (DuPont 2008, Lahm et al 2009). Chlorantraniliprole is active on the CM, and it has been registered in European countries since 2008. Due to the potential of *C. pomonella* to develop resistance to insecticides, it is necessary to establish a baseline susceptibility database before or early after the introduction of a new insecticide to the market (Roditakis et al. 2013). The database has to include data from a wide geographical area, to know the natural variability of the response to the chemical in field populations, and it has to consider the state of the insecticide resistance in every area.

The aims of this study were to determine the baseline susceptibility of European CM populations to chlorantraniliprole, to establish the discriminant concentrations (DC), to check the possible development of resistance in the field, and to understand the possible role of P450 in the lack of susceptibility of field populations to this insecticide.

Material and Methods

Experimental protocol outline

In our study, the concentration–response to chlorantraniliprole lines (mortality vs concentration) by ingestion were first determined for eight Spanish field populations collected from 2007 to 2009. These data along with others obtained from populations in Italy, France, Netherlands, and Belgium and tested since 2005 in Italy were used by DuPont to calculate the DC. Values close to different lethal concentrations (LC), the LC_{50} , LC_{90} , and to three times the LC_{99} were chosen as the DC. These concentrations were then applied to 27 European populations collected from 2008 to 2015 to test their susceptibilities. Two more concentration–response lines were later determined for two more Spanish populations collected in 2010. Their DC results were within the range of the ones calculated previously, and all the probit line data were pooled to calculate a common probit line. Additionally, the probit line of chlorantraniliprole was also calculated for a Spanish susceptible strain (S-Spain) whose response to several insecticides is well known. Finally, the activity of

P450 was measured for 12 field populations and for the laboratory susceptible strain.

Insects

The list of the CM field populations used in the bioassays is shown in Table 1. The 10 Spanish populations used to adjust probit lines were collected from 2007 to 2010 in the Ebro Valley pome fruit production area (Catalonia and Aragon, NE of Spain) when the product was not registered. The 27 populations tested with the DC were collected from 2008 to 2015 in the pome fruit growing areas of France, Germany, Hungary, Italy, and Spain. They were collected before or after the registration of chlorantraniliprole, or from orchards that had not been sprayed with it.

Except in one case (Jun 3–63 (09), that was collected from an abandoned orchard), the Spanish and most of the rest of the European field populations originated from commercial IPM orchards. The pest management strategy with chlorantraniliprole was the same in all countries, once the product was registered: only two applications per season on one single generation, preferable first generation. Most of the CM field populations were collected as diapausing larvae using corrugated cardboards installed in the field from July–August until October, but in some cases injured apples were collected or corrugated cardboards were installed in the field to obtain nondiapausing larvae or both. When it was needed, the populations were reared until their second generation (F2) to have enough progeny to carry out the bioassays (Table 1).

The susceptible CM strain, S-Spain, was collected from an abandoned apple orchard in Lleida in 1992, and it has been reared since then using a semiarificial dehydrated apple diet at the joint IRTA and UdL laboratory (Lleida, Spain). Its response to many insecticides; its P450, GST, and EST enzymatic activity; and the presence of the *AChE* mutation are well known (Rodríguez et al. 2010, 2011, Bosch et al. 2014).

The P450 activity in CM adults was determined for 12 field populations from the Ebro Valley (Spain). CM adults from the first flight were captured from seven orchards in pheromone traps in 2009 and 2010 (Table 1). As for the other five populations collected in 2010 and 2011, the P450 activity was measured on adults emerging from the collected diapausing larvae.

Insecticide and bioassays experimental procedure

Chlorantraniliprole was used as DPX-E2Y45 20SC (Coragen 20SC, DuPont de Nemours France SAS, Nambesheim, France), and a Stonefly Premix (Stonefly Industries Ltd) lyophilized diet was used as feeding substrate in the bioassays, following the IRAC susceptibility test method 017 (www.irc-online.org). To complement the diet, CD International BA-128 multiwell plastic trays (each well of 15.9 mm diameter and 15.9 mm deep) and lids were used. CM neonate larvae (<24-h old) were exposed to chlorantraniliprole in diet-incorporated assays.

To calculate the baseline probit lines, 20 g of diet was mixed with 60 g of the insecticide solution at given concentrations ranging from 0.01 to 1.0 mg a.i./kg of diet at approximately 3× series dilution. Given the results, a second series was performed to get a better fitting of the probit line. Distilled water was used as a solvent. Each well was filled with 0.5 g of treated diet, and the diet was pressed to be evenly distributed across the bottom. One neonate larvae per well was placed on the treated diet. At least three replications of 16 larvae were tested per each concentration. The trays were incubated at $22 \pm 1^\circ\text{C}$, 16:8 (L:D) h, and 45% humidity. Larval mortality was assessed after 4 d. Larvae were considered dead when they did not

Table 1. Population name, origin, year of collection, state of the insects collected (DP = diapausing), and generation tested of the field coding moth populations treated with chlorantraniliprole to obtain either the probit line (Probit) or the mortality produced by the DC

Population	Country/County	Collection year	Insect collection state	Tested laboratory generation	Bioassay done	P450 activity
S-Spain	Spain/Catalunya				Probit	Yes
Bal 2–371 (SP) (07)	Spain/Catalunya	2007	DP larvae	F1	Probit	
Jun 14–16 (SP) (08)	Spain/Catalunya	2008	Non-DP and DP larvae*	F2	Probit	Yes
Cal-4 (SP) (08)	Spain/Aragón	2008	Non-DP and DP larvae	F2	Probit	
Torref 15-3 (SP) (08)	Spain/Catalunya	2008	Non-DP and DP larvae*	F2	Probit	Yes
Torreg 11–160 (SP) (08)	Spain/Catalunya	2008	Non-DP and DP larvae	F2	Probit	
Bal 2–480 (SP) (08)	Spain/Catalunya	2008	Non-DP and DP larvae*	F2	Probit	Yes
SAS (SP) (09)	Spain/Catalunya	2009	DP larvae	F2	Probit	
Jun 3–63 (SP) (09)	Spain/Catalunya	2009	Non-DP and DP larvae*	F1	Probit	Yes
Tamarite (SP) (10)	Spain/Aragón	2010	Non-DP and DP larvae	F1	Probit	Yes
Riud (SP) (10)	Spain/Catalunya	2010	DP larvae	F1	Probit	
Torreg 11–166 (SP) (09)	Spain/Catalunya	2008	DP larvae*	F2	DC	Yes
La Almunia (SP) (09)	Spain/Aragón	2009	DP larvae	F1	DC	
La AlmuniaG (SP)(09)	Spain/Aragón	2009	DP larvae	F2	DC	
Tossal (SP) (09)	Spain/Catalunya	2009	Non-DP and DP larvae*	F2	DC	Yes
Malpartit (SP) (09)	Spain/Catalunya	2009	Non-DP and DP larvae*	F1	DC	Yes
Vauvert (FR) (09)	France/Languedoc-Roussillon	2009	DP larvae	F2	DC	
Isle SLS (FR) (09)	France/Vaucluse	2009	DP larvae	F2	DC	
Nedel Market (HU) (09)	Hungary/Bács-Kiskun	2009	DP larvae	F2	DC	
Aseleben (DE) (09)	Germany/Sachsen Anhalt	2009	DP larvae	F1	DC	
Isle SLS (FR) (10)	France/Vaucluse	2010	DP larvae	F1	DC	
Noves P (FR) (10)	France/Provence-A-C.A.	2010	DP larvae	F1	DC	
Albalate (SP) (11)	Spain/Aragón	2011	Non-DP and DP larvae	F2	DC	Yes
PuigvertC (SP) (11)	Spain/Catalunya	2011	DP larvae	F1	DC	Yes
PuigvertB (SP) (11)	Spain/Catalunya	2011	DP larvae	F1	DC	Yes
SAS (SP) (11)	Spain/Catalunya	2011	DP larvae	F1	DC	Yes
Noves P (FR) (11)	France/Provence-A-C.A.	2011	DP larvae	F1	DC	
Noves P (FR) (12)	France/Provence-A-C.A.	2012	DP larvae	F1	DC	
Le Thor (FR) (12)	France/Provence-A-C.A.	2012	DP larvae	F1	DC	
Isle SLS (FR) (13)	France/Vaucluse	2013	DP larvae	F2	DC	
Noves P (FR) (13)	France/Provence-A-C.A.	2013	DP larvae	F2	DC	
Noves P (FR) (14)	France/Provence-A-C.A.	2014	DP larvae	F1	DC	
Lumpiaque (SP) (15)	Spain/Aragón	2015	DP larvae	F1	DC	
Salillas (SP) (15)	Spain/Aragón	2015	DP larvae	F1	DC	
Le Thor (FR) (15)	France/Provence-A-C.A.	2015	DP larvae	F1	DC	
Meckenheim (DE) (15)	Germany	2015	DP larvae	F1	DC	
Orsingen (DE) (15)	Germany	2015	DP larvae	F1	DC	
Ravenna (IT) (15)	Italy	2015	DP larvae	F1	DC	

The P450 activity was calculated for the populations reported in column: P450 activity.

*Adults caught in pheromone traps.

move after a light touch with a brush or when they were moribund. Moribund larvae were those visibly affected and significantly different from normal ones; when they were probed and flipped on their back, the larva could not flip back right-side up, or, when it was able to do it, it did so with uncoordinated and slow movements. Only data in which control mortality was <20% were analyzed. Missing larvae were subtracted from the initial number.

Once the DC were calculated, to test the susceptibility of 27 European field populations, the same procedure was followed.

P450 enzymatic activity

The adult P450 activity was determined in the susceptible strain S-Spain and in 12 field populations (Table 1) with an *in vivo* protocol (Rodríguez et al. 2012) using 7-ethoxy-coumarin-*O*-deethylation (ECOD) in a black microplate of 96 wells. The dissected abdomens of the adults were placed individually in a well containing 100 µl

of phosphate buffer (50 mM, pH 7.2) and 7-ethoxycoumarin (0.4 mM). After 4 h of incubation at 30°C, the reaction was stopped by adding 100 µl of glycine buffer (pH 10.4, 10⁻⁴ M) with ethanol (v/v). Before the incubation, a minimum of 10% of the wells of each microplate were used as controls and received the glycine buffer to stop the reaction. The ECOD activity was measured by fluorescence with a 380 nm excitation filter and 465 nm emission filters and was expressed as picograms of 7-ethoxycoumarin (7OH)/insect min.

Data analysis

To calculate the baseline, a probit analysis using the program POLO Plus (LeOra Software 1987) was performed, and the LC₅₀, the LC₉₀, and their 95% fiducial limits were calculated. Two LC₅₀ were considered significantly different when their fiducial limits did not overlap (Robertson et al. 2007). The resistant ratio (RR) relative to the most susceptible field population (RR-F₅₀) and the resistance ratio relative

to the susceptible laboratory strain (RR-L₅₀) were calculated for the LC₅₀. Values close to the LC₅₀, to the LC₉₀, and to three times the LC₉₉ were chosen as the DC. The highest DC were expected to kill approximately 100% of larvae of the susceptible population, but a smaller percentage when applied to a resistant population (French-Constant and Roush 1990).

To evaluate the susceptibility of the European field populations, the corrected mortality produced by the DC was calculated using Abbott's formula (Abbott 1925), being the correction factor the mortality produced by the control treatment (water). The RR of each population for every DC was calculated by dividing the mortality of the most susceptible field population by the mortality produced in every field population strain (RR-F_{0.3}, RR-F_{1.0}, and RR-F_{10.0}). Mean ± SEM of the corrected mortality produced by the DCs and the coefficient of variation (CV) across all the field populations to evaluate the dispersion of the data were calculated.

The frequency of P450-resistant individuals in every CM field population analyzed was compared with the susceptible S-Spain using a Pearson chi-square (χ^2) test. Moths were classified as resistant if their P450 enzyme activity exceeded the highest activity value corresponding to 90% of S-Spain individuals (Reyes et al. 2007). Regression lines between the mean P450 activity (pg 7OH/insect min) and the LC₅₀, and between the P450 activity and the mortality produced by the DC_{0.3} for each population were calculated. Only the orchards with more than 20 adults analyzed were taken into account for the regression lines.

Results

The results of the probit analysis and the RR are shown in Table 2. The LC₅₀ values for the CM field populations ranged from 0.161 to 0.446 mg a.i./kg diet. Some significant differences were found among the field populations, namely Torref 15-3 (SP) (08), Bal 2-480 (SP) (08), and SAS (SP) (09) were significantly less susceptible than five field populations, and Riud (SP) (10) was significantly more susceptible than four field populations. One population, Torref 11-160 (SP) (08), showed no significant differences with any of the other field populations. Jun 3-63 (SP) (09), from an abandoned orchard, showed no significant differences with 6 of the

10 tested field populations. The RR-F₅₀, calculated comparing each LC₅₀ value with the LC₅₀ value of the most susceptible field population (Riud (SP) 10, 0.161 mg a.i./kg diet), ranged from 1.1 to 2.8. The laboratory susceptible strain, S-Spain, was significantly more susceptible to chlorantraniliprole than any field population, and it was not included in the calculation of the pooled LC values. The RR-L₅₀, calculated comparing each LC₅₀ value with the LC₅₀ value of the laboratory strain (S-Spain, 0.086 mg a.i./kg diet), ranged from 1.9 to 5.2. SAS (SP) (09) had a high slope (4.860 ± 0.557), suggesting a more homogeneous response than the other populations and a narrow concentration range between the LC₅₀ and LC₉₀. The same happened with Riud (SP) (10), S-Spain, and Tam (SP) (10) that had parallel slopes to SAS (SP) (09) ($\chi^2 = 3.34$, $df = 3$, $P < 0.343$). Torref 11-160 (SP) (08) had the lowest slope (1.371 ± 0.168) compared with the next smallest one, Torref 15-3 (SP) (08) ($\chi^2 = 4.80$, $df = 1$, $P < 0.028$). Due to its low slope, Torref 11-160 (SP) (08) had a very wide interval of concentrations between LC₉₀ and LC₅₀ and, thus, had high intrapopulation variability, and it did not present significant differences with any other field population. The LC₉₀ values for the CM field populations ranged from 0.339 to 2.788 mg a.i./kg diet.

The LC₅₀ value of the pooled data was 0.250 mg a.i./kg of diet, the LC₉₀ was 0.888 mg a.i./kg of diet, and the LC₉₉ was 3.323 mg a.i./kg of diet. These results are according to the DC selected to test the susceptibility of CM field populations to chlorantraniliprole: 0.3 and 1.0 mg a.i./kg diets, approximately the LC₅₀ and the LC₉₀, respectively, and 10 mg a.i./kg diet that corresponded to threefold the LC₉₉ value of the pooled data probit line results.

Table 3 shows the corrected mortality and the resistance ratio relative to the most susceptible field population (RR-Fs) obtained with each DC for the European CM field populations. All the DC applied produced a 100% of mortality on the susceptible population S-Spain, as expected according to its probit line results (Table 2). Feeding the European field populations larvae with concentrations of 0.3, 1.0, and 10.0 mg a.i./kg diet produced mean corrected mortalities of 70.82, 96.44, and 99.85%, respectively. The CV was low for 0.3 mg a.i./kg diet (31.17) and very low for 1.0 (7.21) and 10.0 mg a.i./kg diets (0.52). The RR-F_{0.3} ranged between 1.1 and 4.8, and between 1.0 and 1.5 for the other two DC.

Table 2. Baseline susceptibility of *Cydia pomonella* Spanish field collected populations to chlorantraniliprole

Population (year)	n	Probit analyses parameters									
		Intercept	Slope ± SE	LC ₅₀	CI 95%	LC ₉₀	CI 95%	χ^2 (df)	P*	RR-F ₅₀	RR-L ₅₀
S-Spain	577	3.555	3.345 ± 0.252	0.087	0.079–0.095	0.209	0.182–0.249	0.36 (4)	>0.05	-	-
Bal 2-371 (SP) (07)	275	1.606	2.220 ± 0.396	0.189ab	0.124–0.254	0.714	0.488–1.502	0.71 (4)	>0.05	1.2	2.2
Jun 14-16 (SP) (08)	572	1.102	2.183 ± 0.260	0.313bc	0.220–0.426	1.209	0.786–2.861	5.09 (6)	>0.05	1.9	3.6
Cal-4 (SP) (08)	612	1.974	2.800 ± 0.269	0.197ab	0.160–0.243	0.566	0.427–0.867	8.03 (6)	>0.05	1.2	2.3
Torref 15-3 (SP) (08)	400	0.684	1.954 ± 0.260	0.446c	0.309–0.665	2.021	1.173–6.365	5.63 (5)	>0.05	2.8	5.2
Torref 11-160 (SP) (08)	262	0.671	1.371 ± 0.168	0.324abc	0.182–0.592	2.788	1.302–11.396	9.31 (3)	<0.05	2.0	3.8
Bal 2-480 (SP) (08)	436	0.809	2.079 ± 0.248	0.408c	0.312–0.536	1.687	1.114–3.531	5.70 (5)	<0.05	2.5	4.7
SAS (SP) (09)	380	2.235	4.860 ± 0.557	0.347c	0.251–0.431	0.636	0.503–1.051	37.47 (4)	<0.05	2.2	4.0
Jun 3-63 (SP) (09)	283	2.041	2.722 ± 0.317	0.178ab	0.137–0.230	0.526	0.383–0.849	4.22 (3)	>0.05	1.1	2.1
Tam (SP) (10)	684	2.624	3.748 ± 0.486	0.199ab	0.162–0.248	0.438	0.332–0.749	4.99 (4)	>0.05	1.2	2.2
Riud (SP) (10)	554	3.139	3.951 ± 0.337	0.161a	0.136–0.185	0.339	0.258–0.431	11.42 (5)	<0.05	1.0	1.9
Pooled	4458	1.401	2.324 ± 0.087	0.250	0.225–0.376	0.888	0.763–1.068	186.73 (50)	<0.05	1.6	2.9

Values of the LC are mg a.i./kg diet. LC₅₀ followed by the same letter are not significantly different (LC₅₀ are considered significantly different when their CI do not overlap).

n, number of individuals tested; RR-F₅₀, resistance ratio calculated by dividing the LC₅₀ of the strain tested by the LC₅₀ of the most susceptible field population (Riud (SP) (10)); RR-L₅₀, resistance ratio calculated by dividing the LC₅₀ of the strain tested by the LC₅₀ of the susceptible laboratory population (S-Spain).

*The P value >0.05 indicates that the observed bioassay data are not significantly different from the expected data for the probit model.

Table 3. Susceptibility, expressed as corrected mortality (%), of *Cydia pomonella* European field collected populations to DC of chlorantraniliprole (0.3, 1.0, and 10.0 mg a.i./kg diet)

Population	Control mortality (%)	Discriminant concentration (mg a.i./kg diet)					
		0.3	RR-F _{0.3}	1.0	RR-F _{1.0}	10.0	RR-F _{10.0}
S-Spain		100.0 (44)		100.0 (47)		100.0 (48)	
Torreg 11–166 (SP) (08)	4.2 (48)	44.3 (45)	2.3	97.8 (48)	1.0	100.0 (69)	1.0
La Almunia (SP) (09)	0.0 (50)	78.6 (47)	1.3	95.7 (47)	1.0	100.0 (46)	1.0
La Almunia G (SP) (09)	2.5 (40)	36.9 (53)	2.7	97.1 (52)	1.0	100.0 (68)	1.0
Tossal (SP) (09)	6.7 (45)	35.4 (48)	2.8	97.7 (44)	1.0	100.0 (48)	1.0
Malpartit (SP) (09)	2.4 (42)	51.0 (46)	2.0	93.6 (16)	1.1	97.8 (46)	1.0
Vauvert (FR) (09)	0.0 (48)	54.2 (48)	1.8	85.3 (44)	1.2	100.0 (49)	1.0
Isle SLS (FR) (09)	0.0 (48)	89.1 (46)	1.1	100.0 (47)	1.0	100.0 (47)	1.0
Nedel Market (HU) (09)	6.1 (50)	93.3 (48)	1.1	96.5 (51)	1.0	-	-
Aseleben (DE) (09)	1.6 (64)	90.9 (59)	1.1	100.0 (60)	1.0	100.0 (64)	1.0
Isle SLS (FR) (10)	2.1 (48)	48.9 (40)	2.0	98.0 (50)	1.0	100.0 (48)	1.0
Noves P (FR) (10)	2.0 (49)	69.6 (47)	1.4	100.0 (48)	1.0	100.0 (45)	1.0
Albalate (SP) (11)	0.0 (48)	69.4 (48)	1.4	100.0 (48)	1.0	100.0 (48)	1.0
PuigvertC (SP) (11)	8.3 (48)	81.4 (47)	1.2	93.0 (47)	1.1	100.0 (48)	1.0
PuigvertB (SP) (11)	14.6 (48)	95.0 (47)	1.1	100.0 (47)	1.0	100.0 (48)	1.0
SAS (SP) (11)	0.0 (48)	82.5 (48)	1.2	100.0 (48)	1.0	100.0 (48)	1.0
Noves P (FR) (11)	4.7 (64)	79.9 (47)	1.3	100.0 (63)	1.0	100.0 (46)	1.0
Noves P (FR) (12)	0.0 (62)	20.8 (48)	4.8	100.0 (48)	1.0	100.0 (48)	1.0
Le Thor (FR) (12)	4.7 (64)	75.4 (47)	1.3	100.0 (63)	1.0	100.0 (46)	1.0
Isle SLS (FR) (13)	2.1 (48)	38.1 (61)	2.6	67.7 (79)	1.5	98.9 (95)	1.0
Noves P (FR) (13)	0.0 (48)	82.2 (45)	1.2	100.0 (47)	1.0	100.0 (48)	1.0
Noves P (FR) (14)	0.0 (48)	93.3 (45)	1.1	100.0 (33)	1.0		
Lumpiaque (SP) (15)	4.3 (82)	77.8 (63)	1.3	87.2 (122)	1.1	100.0 (45)	1.0
Salillas (SP) (15)	0.0 (27)	72.3 (47)	1.4	100.0 (55)	1.0	100.0 (55)	1.0
Le Thor (FR) (15)	0.0 (64)	64.8 (88)	1.5	94.3 (138)	1.1		
Meckenheim (DE)(15)	8.3 (48)	100.0 (53)	1.0	100.0 (45)	1.0	100.0 (48)	1.0
Orsingen (DE) (15)	0.0 (16)	93.2 (44)	1.1	100.0 (45)	1.0		
Ravenna (IT) (15)	4.2 (96)	93.7 (95)	1.1	100.0 (94)	1.0		
Mean ± SEM		70.82 ± 4.25		96.44 ± 1.34		99.85 ± 0.11	
CV		31.17		7.21		0.52	

Mean ± SEM of the corrected mortality produced by the DC and CV across all the field populations were calculated. Numbers in brackets are the number of individuals tested.

RR-F, resistance ratio for every DC was calculated by dividing the mortality of the most susceptible field population (Meckenheim (DE) (15)) by the mortality produced in every field population strain.

The P450 enzymatic activity of CM adults and the relative frequency of resistant individuals (those whose P450 activity was higher than 23.92 pg 7OH/adult min) are shown in Table 4. The mean P450 activity ranged from 10.52 to 74.61 pg 7OH/insect min, and the frequency of resistant individuals in the field populations ranged from 12.1 to 100.0% (populations Jun 3–63 (SP) (09) and Balaguer 2–480 (SP) (08) for both variables, respectively). The variance of the frequency of resistant insects is strongly explained by the P450 mean activity of the field populations ($R^2 = 95.32$), which implies that there are few individuals with a very high resistance level that strongly influence the mean P450 value (Fig. 1). Although data were restricted to four field populations, the regression line between the frequency of resistant insects and the LC_{50} values showed a high coefficient of determination ($R^2 = 89.77$; Fig. 2). When the regression line was adjusted between the P450 mean activity and the mortality produced by the DC_{0.3}, the coefficient of determination was lower than in the other cases, but it explained the 69.82% of the variance (Fig. 3). The regression lines obtained with the higher DC (1.0 mg a.i./kg of diet and 10.0 mg a.i./kg of diet) did not have good adjustments due to the high mortalities obtained in all the field populations treated.

Discussion

To determine the DC, populations collected before the registration of chlorantraniliprole were used. The first European countries to get the product registered on pome fruits were Romania in 2008 and Italy in 2009, and in Spain, the product was registered in 2011. The CM Spanish field populations showed a high and relatively uniform susceptibility to chlorantraniliprole. This is likely because of the RR relative to the most susceptible population (RR-F₅₀) was lower than 2.8, even though significant differences on the LC_{50} s were observed among populations (Mota-Sanchez et al. 2002). All the field populations, even the abandoned orchard Jun 3–63 (SP) (09), were significantly less susceptible to chlorantraniliprole than the laboratory strain, S-Spain. French-Constant and Roush (1990) stated that susceptible strains held for long periods in the laboratory may bear little resemblance to susceptible strains currently found in the field. Thus, it would be more useful to determine the appropriate DC on the basis of field strains before wide commercial introduction of the specific pesticide. Therefore, to establish a useful baseline to know the natural response variability to the insecticide in the field populations, the laboratory susceptible strain was not included in the pooled data to determine the DCs. Nevertheless, once the product is applied in

Table 4. Enzymatic P450 activity, expressed as frequency of resistant insects (%) and mean activity \pm SEM (pg 7OH/insect min), of *Cydia pomonella* field adults

Population (year)	n	Resistance frequency (%)	Mean P450 activity \pm SEM	LC ₅₀	DC _{0.3} mortality (%)
S-Spain	223	10.0	9.64 \pm 0.74	0.086	100.0
Jun 14–16 (08)	47	66.0***	34.20 \pm 3.18	0.313	51.8
Torref 15-3 (08)	125	76.0***	54.47 \pm 2.95	0.446	34.2
Bal 2–480 (08)	8	100.0***	74.61 \pm 9.57	0.408	49.3
Jun 3–63 (09)	107	12.1n.s.	10.52 \pm 0.98	0.178	83.0
Torreg 11–166 (09)	48	45.8***	31.52 \pm 4.50	-	44.3
Tossal (09)	41	85.4***	62.05 \pm 6.24	-	35.4
Malpartit (09)	82	91.0***	62.34 \pm 3.87	-	51.0
Tam (10)	29	31.0**	20.60 \pm 2.73	0.192	75.6
SAS (11)	29	20.7n.s.	16.60 \pm 2.68	-	82.5
Albalate (11)	23	27.6***	20.20 \pm 3.23	-	69.4
PuigverdC (11)	39	53.9***	30.00 \pm 3.34	-	81.4
PuigverdB (11)	29	48.3***	28.31 \pm 3.24	-	81.4

The value of the P450 threshold obtained using the S-Spain population was 23.92 pg 7OH/insect-min. The frequency of resistant individuals was compared using a Pearson χ^2 test (df = 1).

n.s., not significant. ** P = 0.01. *** P = 0.001.

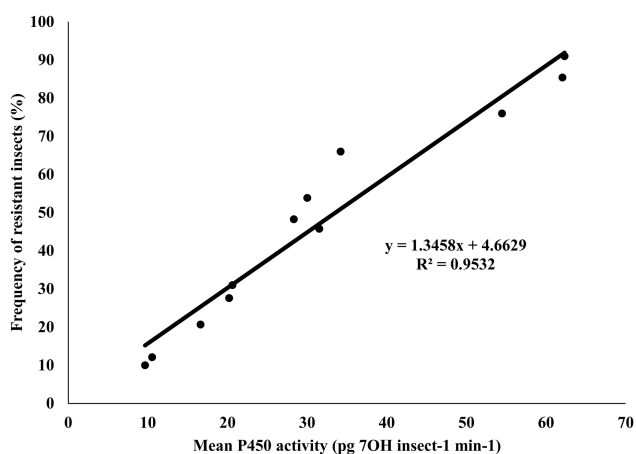


Fig. 1. The frequency of resistant codling moth adults as explained by the mean enzymatic P450 activity in 11 Spanish field populations and the laboratory strain S-Spain.

the field, susceptibility of insect populations might reduce over time due to potential resistance evolution (French-Constant and Roush 1990). In this case, susceptible strains can be helpful as an invariable mortality reference point, which are commonly used (Zheng et al. 2011, Lai et al. 2011, da Silva 2012, Caballero et al. 2013).

The LC₅₀ values obtained varied between 0.086 mg a.i./kg of diet (S-Spain) and 0.446 mg a.i./kg of diet (Torref 15-3 (SP) (08)), and are equivalent to 0.110 and 0.59 mg a.i./liter, respectively. The same insecticide diet incorporation bioassay obtained an LC₅₀ value range in different laboratory susceptible strains of different species of Lepidoptera: 0.014 mg a.i./liter in *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) (Lai et al. 2011) and 0.28 mg a.i./liter in *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae) (Sial and Brunner 2012). Different species respond in a different way to the same insecticide, independently of the body mass differences. For example, CM is heavier than *Lobesia botrana* (Denis & Schiffmüller) (Lepidoptera: Tortricidae), but females of CM were 115 times more susceptible to thiacloprid than females of *L. botrana* were (Navarro et al. 2017). In the laboratory, where the

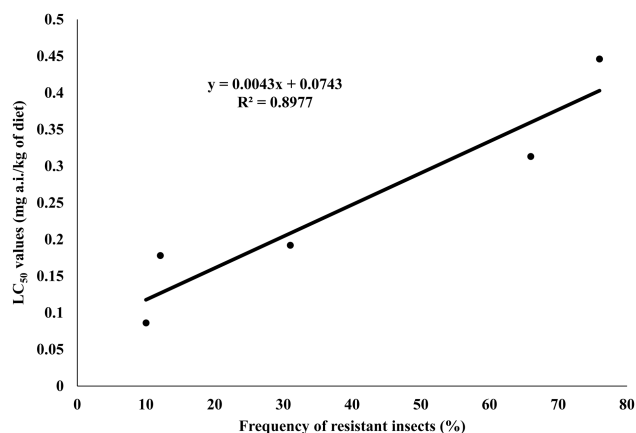


Fig. 2. The LC₅₀ of chlorantraniliprole for four Spanish field populations and the laboratory strain S-Spain as explained by the frequency of resistant codling moth adults.

larval feeding behavior is conditioned, these different responses are mainly due to that different species may have different metabolic procedures or detoxification methods (Rodríguez et al. 2012). The highest resistance ratio relative to the laboratory strain of S-Spain (RR-L₅₀) was 5.2 (Table 2), which is a low value compared with the highest value observed for Chinese *S. exigua* field populations (17.1; Lai et al. 2011) in which chlorantraniliprole was either briefly introduced or, in some cases, never used against. As found in our study, narrow variations in the LC₅₀ values were also found in some other Lepidoptera species: *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) (Campos et al. 2015), *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae) (Wang et al. 2010), or *Cnaphalocrocis medinalis* (Güenée) (Lepidoptera: Pyralidae) (Zheng et al. 2011).

The mortality caused by the DC_{0.3} (concentration close to the LC₅₀ of the pooled field population) on European field populations also showed a low variability among populations, with a mean mortality of 70.82% and a CV equal to 31.17 (Table 3). The RR-F_{0.3} obtained was similar to the RR-F₅₀, demonstrating its reliability in comparing populations. The DC_{1.0} produced a mean mortality of 96.44%, and

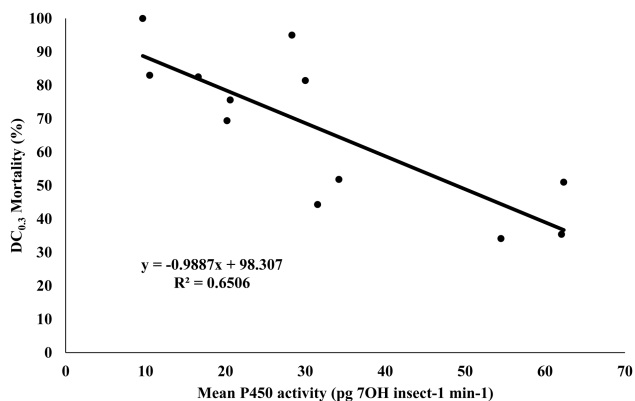


Fig. 3. The mortality caused by the chlorantraniliprole DC_{0.3} on larvae of 11 Spanish field populations and the laboratory strain S-Spain as explained by the mean enzymatic P450 activity in adults.

all the mortalities were higher than 85.3% except in the French population, Isle SLS (FR) (13), where it was 67.7%. The highest DC tested, 10 mg a.i./kg of diet, caused 100.0% mortality in all the field populations except in Isle SLS (FR) (13) and Malpartit (SP) (09), suggesting the presence of a small proportion of resistant individuals in these two populations, but generally supporting the lack of resistance to chlorantraniliprole in the field. Malpartit (SP) (09) was collected before the introduction of the product in the market, but the populations had a very high mean P450 activity (Table 4), what can play a role in the detoxification of the active ingredient, as is discussed later. Eleven European field populations were collected after registration of the product, from 2012 onwards, and these populations proved as susceptible as the previously tested Spanish populations. Some of them were collected over more than a year, as in Le Thor (2 yr), Isle SLS (3 yr), and Noves P (5 consecutive years). The mortality obtained with Isle SLS with the DC of 0.3 mg a.i./kg of diet decreased over the years from 89.1% in 2009 to 38.1% in 2013. This population was from a location with very high pest pressure and where trials with Rynaxypyr were done over several years, so, the loss of efficacy of the product may be due to the presence of resistant individuals in the population. Nevertheless, the field population, Noves, showed a similar level of mortality for all the years, ranging between 69.6 and 93.3%, except in 2012, when the efficacy of the DC_{0.3} decrease to 20.8% (obtaining an RR_{0.3} of 4.8). In these field populations, the DC_{1.0} and DC_{10.0} reached 100.0% of mortality suggesting an error in the assay or an unexplained variation in the mortality obtained, something that can occur in unexposed field populations (Sawicki 1987) and demonstrating the utility of using more than one DC.

Widespread resistance of CM field populations from the Ebro Valley area (NE of Spain) has been demonstrated, mainly to azynphos-methyl and other OP, lambda-cyhalothrin and methoxyfenozide, among other active ingredients (Rodríguez et al. 2010, 2011). Insecticide bioassays (Bosch et al. 2017) were done with some of the Spanish field populations tested with chlorantraniliprole. Compared with the susceptible population S-Spain, PuigverdB (11) was resistant to the pyrethroid lambda-cyhalothrin (RR = 15.4), and PuigverdC (11) was resistant to lambda-cyhalothrin (RR = 872.0), methoxyfenozide (RR = 14.6), and thiacloprid (RR = 11.2), besides being tolerant (2 < RR < 10) to other active ingredients such as chlorpyrifos-ethyl and spinetoram. Tam (10) was susceptible to all the tested active ingredients, and SAS (11) and Le Thor (12) were tolerant to different P450 active ingredients but with RRs always lower than 4.4. None of these populations were resistant

to chlorantraniliprole, with SAS (11) the only one that had a RR slightly higher than 2 (RR = 2.2). Despite being multiresistant populations, PuigverdB (11) and PuigverdC (11) responded to the DCs of chlorantraniliprole with high mortalities, at the same or a higher level than the rest of the tested populations (Table 3). These results showed the absence of cross-resistance among chlorantraniliprole and lambda-cyhalothrin, methoxyfenozide, and thiacloprid in these field populations, suggesting that the resistant mechanisms involved do not affect the proper activity of the product. This lack of cross-resistance was also found in *P. xylostella* field and laboratory-selected populations (Wang et al. 2010), *Spodoptera litura* (Fabricius) (Lepidoptera: Tortricidae) (Sang et al. 2016) and with cyantraniliprole, another anthranilic diamide, in selected resistant populations of *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) (Grávalos et al. 2015). Therefore, a high correlation between the two anthranilic diamides was found in *T. absoluta* (Campos et al. 2015) and in *S. litura* (Sang et al. 2016); and with flubendiamide, a phthalic diamide, in *P. xylostella* although this active ingredient had never been used (Wang et al. 2013). Chlorantraniliprole is the only diamide currently registered for pest control in pome fruits, but formulated products of cyantraniliprole are under development to control a cross-spectrum of chewing and sucking pests from the insect orders Hemiptera, Lepidoptera, and Coleoptera (Selby et al. 2013). They have obtained promising results in the control of aphid pests with no evidence of cross-resistance with other aphid insecticides (Foster et al. 2012). In the case of using both active ingredients in the same crop to control different pests, an intensive check to detect an increase in the resistance levels would be necessary due to the possible cross-resistance between IRAC Group 28 products, together with an accurate resistance management strategy combining its use.

With reference to enzymatic detoxification mechanisms, synergism assays in *S. litura* (Su et al. 2012), *S. exigua* (Lai et al. 2011), and *P. xylostella* (Wang et al. 2010) demonstrated that P450, EST, and GST were not the main mechanisms involved in chlorantraniliprole resistance and neither were they involved in the cyantraniliprole resistance of *S. litura* in China (Sang et al. 2016). However, in *T. absoluta*, Campos et al. (2015) found a moderate correlation between the cytochrome-P450-monooxygenases activity (P450) and a susceptibility to chlorantraniliprole and cyantraniliprole. Sial et al. (2011) found that EST could be involved in the detoxification of chlorantraniliprole in a resistant selected laboratory population of *C. rosaceana*, and Cao et al. (2010) found that there was an increase of EST and GST activity in chlorantraniliprole-treated insects of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). The main enzymatic mechanism for insecticide detoxification of the Spanish CM field populations is P450 (Rodríguez et al. 2010, 2011), and in the studied populations, the frequency of P450-resistant insects present in the field explained the 69.72% of the mortality obtained with the lower chlorantraniliprole DC (0.3 mg a.i./kg of diet). The coefficient of determination was higher when the LC₅₀ was used instead of the mortality of the DC_{0.3} (R² = 89.77); however, we considered that five points (the laboratory and four field populations) to adjust a regression line are too few to draw any conclusion. Despite these results, due to the high efficacy of the product in the tested field populations, P450 seems not to be the main mechanism implied in the detoxification of the product although it may have a certain role in it.

C. pomonella is a key fruit pest that has extensively demonstrated its ability to develop resistance to most of the registered insecticides. Chlorantraniliprole, a new reduced risk insecticide that can control a wide range of lepidopteran pests, has proved its high efficacy in

European field populations by obtaining low RR and variability when the LC were calculated and DC tested. The efficacy of the product in this assay not only has shown the natural variability in the response concentration-mortality in a broad geographical area, but also the lack of cross-resistance of the product with other commonly used insecticides in Spanish field populations, such as lambda-cyhalothrin, methoxyfenozide, or thiacloprid. Nevertheless, it seems there is a relationship between the frequency of resistant individuals due to high P450 enzymatic activity and the mortality produced by the approximate LC₅₀ used as DC. As many insecticides can induce a P450 enzymatic activity increase, the use of a strict resistance management strategy would be necessary to maintain the efficacy of the product for a long time (<http://www.irac-online.org>). In fact, this strategy has been considered from the beginning by DuPont, which recommend a restricted number of applications per season on the same generation, within spray programs that include other effective insecticides with different modes of action. The CM baseline susceptibility data established provide a valuable reference for tracking possible future alterations in the efficacy of the product.

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