# Diversity of insecticide resistance mechanisms and spectrum in European populations of the Codling moth, *Cydia pomonella*

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**Abstract: Only a few of the registered insecticides against *Cydia pomonella* L. are still effective in areas where insecticide resistance has emerged in this pest. Resistance mechanisms are multiple, and their lone or cumulative effects in a single population are not completely understood. A detailed estimation of resistance spectrum is still required to define the suitable insecticides to use against a given population. The efficacy of ten insecticides was therefore investigated together with the resistance mechanisms expressed in four laboratory strains and 47 field populations of *C. pomonella* from five countries. Bioassays were performed using topical applications of diagnostic concentrations on diapausing larvae, and resistance mechanisms were analysed on adults emerging from control insects. All populations exhibited a reduced susceptibility to at least one insecticide when compared with the susceptible laboratory strain. Cross-resistances were observed between azinphos-methyl or phosalone and more recent compounds such as spinosad and thiacloprid. Resistances to azinphos-methyl, diflubenzuron, spinosad, tebufenozide and thiacloprid were significantly correlated with mixed-function oxidase activity, while increased glutathione-*S*-transferase and reduced non-specific esterase activities were correlated with resistance to azinphos-methyl and emamectin, respectively. Conversely, resistances to azinphos-methyl, tebufenozide and thiacloprid were negatively correlated with increased esterase activity. None of the observed mechanisms explained the loss of susceptibility of populations to chlorpyrifos-ethyl, and no significant correlation was detected between resistance to deltamethrin and the presence of the *kdr* mutation. The suitability of such non-target instars to monitor insecticide resistance in field populations is discussed.**


**Keywords:** *Cydia pomonella*; insecticide resistance; detoxifying enzymes; cross-resistance

## 1 INTRODUCTION

The codling moth, *Cydia pomonella* L., is a worldwide pest in apple, pear and walnut production. For many years, pesticide applications have been the dominant tools used for its control. However, increasing frequencies of chemical treatments led to the acquisition of resistance to many of the recommended pesticides belonging to various chemical groups. In this species, the first case of resistance was the resistance to arsenates reported by Hough[1] in 1928 in the USA. Since then, new cases of resistance have been and are being reported in almost all of the main apple-growing regions worldwide.[2–9]

In Europe, insecticide resistance in the codling moth was firstly detected in the 1990s with the emergence of resistance to diflubenzuron in Italy and south-eastern French populations.[3–5] Failures of control with pesticides were further observed in Switzerland and Spain.[6,7] Currently, the resistance spectrum of some of these populations has dramatically increased to include avermectins, benzoylureas, benzoylhydrazines, neonicotinoids, organophosphates, macrocyclic lactones and pyrethroids.[10–12]

Reduced insecticide efficacy in *C. pomonella* has been related to non-specific mechanisms such as increased enzymatic metabolization and/or modification of the specific molecular target of one group of insecticides. The main enzymatic systems involved in insecticide resistance of French codling moth populations are the mixed-function oxidases (MFO) and glutathione-*S*-transferases (GST).[13,14] A *kdr* mutation in the voltage-dependent sodium channel is involved in resistance to pyrethroids,[15] and an acetylcholinesterase (AchE) mutation has been identified in a laboratory strain selected for resistance to azinphos-methyl.[16]

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Although resistance mechanisms have been investigated only in a few production areas, different mechanisms are likely to induce resistance to a single compound. Resistance to diflubenzuron is associated with enhanced MFO activity in French, Swiss, Spanish and Portuguese populations of *C. pomonella*, but not in the Italian ones.[7] In the USA, a modified esterase (EST) with a lower affinity for non-specific substrates but an increased affinity for organophosphates has been suggested to explain resistance to parathion.[17] A reduced non-specific EST activity was also observed in resistant south-eastern French populations,[18] while increased EST activity was associated with organophosphate (OP) resistance in Spanish and Argentinian populations of *C. pomonella*.[19,20] However, such resistance may be due either to overexpression or to qualitative changes in the specific isozymes hydrolysing or sequestering the insecticides.[21–26]

Different resistance mechanisms can be involved in resistance to chemicals of the same class of insecticides, as demonstrated in *Bemisia tabaci* (Gennadius)[27] and *Plutella xylostella* L.[28] In *C. pomonella*, such mechanisms are usually expressed within a single population, and both their individual and coupled effects on the toxic products still remain insufficiently understood. Specific analyses indicated that resistance mechanisms can differ between geographical areas,[7,9] but current knowledge on these mechanisms does not make it possible to predict either the resistance spectrum of a given population or the range of the insecticides that would remain efficient for crop protection. Therefore, there is a need for both appropriate and convenient methods for routine resistance monitoring. One of these methods is the topical application of diagnostic concentrations on diapausing larvae.[11,29] Neonate larvae are the main target instar of pesticides, but they have to be initially reproduced in the laboratory before setting the bioassay because their collection in the field is not possible. Conversely, diapausing larvae have the advantages of being easily collectable and shippable. Hence, the use of diapausing larvae makes it possible to bypass the time-consuming process of neonate reproduction and to perform tests on large insect samples at the same developmental stage.[11] The aim of the present study was to analyse the interrelations between resistance spectrum and resistance mechanisms and their variability in southern European populations of the codling moth. For this purpose, topical applications of diagnostic insecticide concentrations on diapausing larvae were used.[9,11,30]

## 2 MATERIALS AND METHODS

### 2.1 Insects

#### *2.1.1 Laboratory strains*

Three resistant strains (Rdfb, RΔ and Raz) and one susceptible strain (Sv) of *C. pomonella* have been mass reared on artificial diet at INRA Avignon (France) since 1995. Rdfb and RΔ strains originated from the Tarascon and Les Vignères apple orchards respectively, which are located in the Avignon region and where failure of benzoylureas and pyrethroids occurred.[13,14] The Raz strain originated from a pear orchard in Torrefarrera (Lerida region, Spain), which has been mainly sprayed with organophosphates and in which control failure occurred.[7,19] The susceptible strain (Sv) was built by the maintenance of the susceptible individuals that were detected in the Les Vignères population without any insecticide exposure. The resistant Rdfb, RΔ and Raz strains were submitted to constant selection pressures for more than 50 generations by exposing larvae to diflubenzuron (500 mg $L^{-1}$), deltamethrin (2 mg $L^{-1}$) and azinphos-methyl (375 mg $L^{-1}$), respectively. Insecticides were applied on the surface of the artificial diet.[19,31]

#### *2.1.2 Field populations*

Diapausing larvae of *C. pomonella* were collected in orchards of five countries using corrugated cardboard traps.[32] Samples from France (31), Switzerland (4) and Italy (11) were collected in autumn 2003 and 2004. Samples from Armenia (1) and Spain (1) were collected in 2004 and 2005, respectively. A total of 60–1200 larvae were obtained, depending on the population. Larvae were kept at 2 °C and 12:12 light:dark for at least 3 months in order to satisfy the chill requirement, until 24 or 48 h (diflubenzuron) before their treatment with insecticide.

### 2.2 Insecticides

Tests were carried out with solutions of technical material of diagnostic concentrations of ten insecticides in organic solvents (Table 1). Diflubenzuron and spinosad were dissolved in tetrahydrofurane and dichloromethane, respectively. All other insecticides were dissolved in acetone.[11,29]

### 2.3 Bioassays

Diapausing larvae were topically treated on the middle of the dorsum using a P2 Gilson micropipette with 1 μL of the discriminating dose that was previously defined for each insecticide and that induced at least 97% mortality in the susceptible strain.[11,29] The control insects were treated with the same volume of solvent. According to sample sizes, 2–10 insecticides were assayed on two or four groups of ten larvae for each population (six groups of ten larvae for laboratory strains). In most cases, small samples allowed the testing of only a few insecticides. As far as possible, the same insecticides were tested in all populations, but the demand of growers or advisors who supplied the populations, were also responded.

After treatment, batches of ten larvae were placed in PVC cages provided with corrugated cardboard pieces and transferred under suitable conditions for adult emergence (25 °C, 60% RH and 16:8 h light:dark). Mortality was recorded in terms of non-emerged adults.[11]

**Table 1.** Insecticides and discriminating concentrations used for detection of insecticide resistance in diapausing larvae of *Cydia pomonella* L.[11,29]

| Insecticide | Concentration (%)[a] | Discriminating concentration (mg $L^{-1}$) | Chemical group | Supplier |
|---|---|---|---|---|
| Azinphos-methyl | 93.0 | 400 | Organophosphate | Bayer CropScience, France |
| Chlorpyrifos-ethyl | 97.3 | 1200 | Organophosphate | Philagro, France |
| Deltamethrin | 95.0 | 100 | Pyrethoid | Aventis CropScience, France |
| Diflubenzuron | 99.0 | 25 000 | Benzoylurea | Philagro, France |
| Emamectin | 97.3 | 500 | Avermectin | Syngenta, France |
| Fenoxycarb | 99.5 | 1 | Carbamate | Syngenta, France |
| Phosalone | 98.0 | 3000 | Organophosphate | Dr Ehrenstorfer, Germany |
| Spinosad | 100 | 6000 | Macrocyclic lactone | Dow AgroScience, France |
| Tebufenozide | 97.1 | 300 | Benzhydrazide | Rohm & Haas, USA |
| Thiacloprid | 99.7 | 500 | Neonicotinoid | Bayer CropScience, France |

[a] Concentration of active ingredient in the original product.

## 2.4 Enzymatic activity

GST, MFO and EST activities were evaluated on at least 20 adults that emerged from non-treated or control larvae. Fluorescence and absorbance were measured using a microplate reader (HTS 7000, Perkin Elmer).

### *2.4.1 Enzyme extract*

Adult abdomens were individually homogenized on ice in Hepes buffer (50 mM, pH 7.0; 150 µL) and centrifuged at 15 000 × *g* for 15 min at 4 °C. The supernatant of each sample was used as enzyme source to determine GST and EST activities.[9] The protein content of each sample was measured according to Bradford,[33] using bovine serum albumin to build the standard curve.

### *2.4.2 Esterases*

Total esterase activities were measured using $\beta$-naphthyl acetate as substrate.[14] Each well was supplied with 0.1 mM substrate in 50 mM of phosphate buffer (pH 6.5), 0.5 µL of enzyme extract and 89.5 µL of Hepes buffer (50 mM, pH 7.0).[9] After 15 min of incubation at 30 °C, 20 µL of a staining reagent containing 3 g $L^{-1}$ Fast Garnet and 35 g $L^{-1}$ sodium dodecyl sulfate was added to the solution, and the absorbance was measured at 492 nm after 15 min at room temperature.[9,14] Twelve wells without enzyme were used for blanks.

### *2.4.3 Glutathione-S-transferases*

GST activities were determined using monochlorobimane (MCB) as substrate.[34] Each well was supplied with 30 µL of enzyme extract, 168 µL of 100 mM reduced glutathione (GSH) in Hepes buffer (50 mM, pH 7.0) and 2 µL of 30 mM MCB.[9] Twelve wells with Hepes buffer instead of protein extract were used as controls. Fluorescence was measured after 20 min of incubation at 22 °C with 380 nm excitation and 465 nm emission filters. Since bimane–glutathione adduct is not commercially available, the activity was expressed as fluorescence units per insect.[34]

### *2.4.4 Mixed-function oxidases*

MFO activities were determined using 7-ethoxycoumarin *O*-deethylation (ECOD)[35] adapted for *in vivo* analysis in microplate.[36] The abdomens of adults were dissected in 6 g $L^{-1}$ sodium chloride and introduced individually into wells containing 100 µL of phosphate buffer (50 mM, pH 7.2) and ethoxycoumarin (0.4 mM). After 4 h incubation at 30 °C, the reaction was stopped by adding 100 µL of 0.1 mM glycine buffer (pH 10.4)/ethanol (v/v) (buffer is prepared in water and then diluted 1:1 with ethanol). The 7-hydroxycoumarin fluorescence was quantified with 380 nm excitation and 465 nm emission filters. Twelve wells receiving glycine buffer previous to incubation were used for blanks.

Frequencies of resistant individuals within populations (RMFO, RGST and R > EST) were determined using the upper activity value of 90% of Sv individuals as a threshold. These values were established as 38 pg 7OH $insect^{-1}$ $min^{-1}$, 14.7 fluorescence units $insect^{-1}$ and 583 nmol $\beta$-naphthyl acetate $mg^{-1}$ protein $min^{-1}$ for MFO, GST and EST activities respectively. For EST activity, the rate of individuals showing an activity lower than 90% of Sv individuals (namely R < EST) was also calculated using 218 nmol $\beta$-naphtol $mg^{-1}$ protein $min^{-1}$ as threshold.

## 2.5 Detection of the *kdr* and AchE mutations

The genetic variability of fragments of 169 bp (sodium channel gene) and 206 bp (AchE1) were analysed by PCR-RFLP in 30 insects per population. The first fragment encompasses the molecular target linked with pyrethroid resistance (L1014F in transmembrane segment IIS in the amino acid sequence),[15] and the second one the molecular target linked with organophosphate resistance (mutation accounted for a F290V substitution in *cydpom-ace1*).[16] Total DNA was extracted from an adult leg with 10% Chelex 100 (Bio-rad) solution[37] and 10 mg $mL^{-1}$ proteinase K. After dilution had been carried out 4 times, this extract was used as a DNA template for PCR amplifications. PCR amplifications were carried out in a 25 µL reaction volume containing 10 mM Tris-HCl, pH 9, 50 mM potassium chloride, 1.5 mM magnesium chloride, 50 µM

of each dNTP, 0.4 μM of each primer *CpNaF* (5′-TAGAGAGCATGTGGGATTGC-3′), *CpNaR* (5′-AATTTCGTAGCCCTTGATCG-3′),[38] *AchEF* (5′-TGAATTAGTGAATAATGAATGGG-3′) and *AchER* (5′-TTCTTCTTTGGGAAATAATTCAG-3′), one unit of Taq DNA polymerase and 2 μL of DNA template. A quantity of 5 μL of the PCR product was subsequently digested with two units of the restriction enzyme Tsp509I (NEB) in 20 μL reaction volume prior to electrophoresis on 6% polyacrylamide gel. DNA fragments were visualized after silver staining coloration.[39] Tsp509I specifically cuts at the AATT site, which is specific of the *kdr* allele associated with deltamethrin resistance[15] and is characteristic of sensitive AchE in *C. pomonella*. After digestion, the *kdr* and the sensitive alleles were respectively identified by DNA fragments of 77 and 112 bp, and insensitive and sensitive AchE by DNA fragments of 190 and 152 bp, respectively.

### 2.6 Statistical analysis

Mortalities to diagnostic concentrations in the field populations and in the three resistant laboratory strains were compared with those of the Sv strain using a $\chi^2$ test after Abbott's correction,[40] and compared between countries or growing areas using analyses of variance (ANOVA). Enzymatic activities were subjected to ANOVA, and means were compared using the protected least significant difference (PLSD) Fisher test. Relationships between insecticide efficacies and frequencies of resistance mechanisms for the whole sample were investigated through linear regressions, while the resistances of 16 field populations and the laboratory strains to six common insecticides (azinphos-methyl, chlorpyrifos-ethyl, diflubenzuron, spinosad, tebufenozide and thiacloprid) were subjected to multiple correlations and a principal components analysis (PCA).

## 3 RESULTS

### 3.1 Bioassays

Susceptibility of the field populations to most of the tested insecticides was dramatically reduced when compared with the susceptible strain (Table 2). Such a reduced susceptibility was recorded in all populations for deltamethrin and phosalone, and in more than 80% of the populations for the other insecticides. Conversely, the diagnostic concentration of emamectin was highly effective against all populations. Compared with the Sv strain, only 25% of them were significantly less susceptible to this insecticide; however, mortality was over 83% in all cases. The dose–response analysis also revealed that none of the resistant laboratory strains was resistant to this compound, and 100% mortality was already induced in the Sv strain by a concentration tenfold lower than the chosen diagnostic concentration (data not shown).

For each of the tested compounds, resistance was detected in some of the French populations. Only the Gotheron population exhibited a high susceptibility to five insecticides, whereas diapausing larvae of the populations of the Provence region were quite insensitive to the diagnostic concentrations of all the different compounds. Similar resistance patterns were recorded for insects from France, Italy and Switzerland. Populations from these countries did not differ significantly for resistance to chlorpyrifos-ethyl ($F = 2.434$, $P = 0.1082$, $\mathrm{df} = 2$), emamectin ($F = 0.952$, $P = 0.428$, $\mathrm{df} = 2$), fenoxycarb ($F = 0.243$, $P = 0.7862$, $\mathrm{df} = 2$), tebufenozide ($F = 0.935$, $P = 0.4015$, $\mathrm{df} = 2$) and thiacloprid ($F = 3.130$, $P = 0.0574$, $\mathrm{df} = 2$). Only to azinphos-methyl did French populations exhibit a higher resistance than Swiss and Italian populations (33.7, 61.4 and 74.9% mortality respectively; $F = 6.189$, $P = 0.0046$, $\mathrm{df} = 2$). Differences between regions were also recorded, i.e. 21.2 and 94.9% mortality with azinphos-methyl in Provence and Normandy, respectively. The most contrasted situation between regions was observed for thiacloprid, with mortalities ranging from 12.7% (Perigord) to 87% (Trentino). Populations from Trentino were also highly susceptible to spinosad, tebufenozide and azinphos-methyl.

One population from Armenia and one from Spain were analysed. Only diflubenzuron, spinosad and emamectin provided high mortality in the Armenian population, whereas the Spanish population was only highly susceptible to emamectin, and to a lesser extent to thiacloprid.

### 3.2 Resistance mechanisms

Most field populations expressed enhanced MFO and GST activities when compared with the susceptible strain ($F = 9.024$, $P < 0.0001$, $\mathrm{df} = 51$; $F = 10.441$, $P < 0.0001$, $\mathrm{df} = 51$, respectively), while EST activity values were mostly lower than in the Sv strain ($F = 4.836$, $P < 0.0001$, $\mathrm{df} = 51$). Based on mean values, the enzymatic activity ratios between field populations and Sv strain ranged from 0.4 to 32. The highest ratios were recorded for MFO activities (Table 2). The Spanish and some French populations exhibited higher GST and MFO activities than the resistant laboratory strains, i.e. the MFO activity in the Spanish population was twice that in the Raz strain.

Most of the French and all of the Swiss populations exhibited significantly higher frequency of RMFO than the susceptible strain. On the other hand, only a few populations showed a higher frequency of RGST than Sv strain. None of the populations studied exhibited higher frequency of R > EST, but a few populations exhibited a significantly higher frequency of R < EST than Sv strain (Table 3).

Analysis of the target site modifications in field samples indicated that AchE mutation was present only in the Spanish population, in which no individual was carrying the *kdr* mutation. Conversely, the *kdr*

**Table 2.** Susceptibility of diapausing larvae of one susceptible strain (Sv), three resistant laboratory strains (Raz, Rdfb, RΔ) and 47 field populations of *Cydia pomonella* L. to diagnostic concentrations of ten technical insecticides applied topically in 1 μL of organic solvent

| | | | Efficacy (%)[bc] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| C[a] | Region | Population | Azin | Chl-e | Delta | Diflu | Ema | Feno | Phos | Spi | Tebu | Thia |
| F | Laboratory | Sv | 97.0 | 96.9 | 99.5 | 97.3 | 100 | 97.7 | 98.54 | 100 | 97.0 | 100 |
| F | Laboratory | Raz | 0.0*** | 92.9⁻ | | 52.9*** | 100⁻ | | | 40.0*** | 64.3*** | 100⁻ |
| F | Laboratory | Rdfb | 69.0*** | 90.5* | | 27.8*** | 92.9** | | | 62.4*** | 38.1*** | 100⁻ |
| F | Laboratory | RD | 46.6*** | 90.5* | | 52.4*** | 100⁻ | | | 96.9*** | 59.4*** | 81.6*** |
| A | Armenia | Asktarak | 52.9*** | 66.7*** | 0.0*** | 84.2** | 88.2*** | 64.7*** | 11.1*** | 88.9** | 0.0*** | 64.7*** |
| F | Midi-Pyrénées | Albi | 38.9*** | 50.0*** | 5.6*** | 17.5*** | 94.4⁻ | 66.7*** | 36.5*** | 63.2*** | 38.9*** | 72.2*** |
| F | Midi-Pyrénées | Meauzac | 25.0*** | | | | | | | | 31.3*** | 56.3*** |
| F | Midi-Pyrénées | Moissac (1) | 27.8*** | 27.8*** | | 25.0*** | 100⁻ | 55.6*** | 33.3*** | 7.1*** | 44.4*** | 16.7*** |
| F | Midi-Pyrénées | Moissac (2) | 84.2** | | | 37.0*** | | | | | 68.4*** | 52.63*** |
| F | Midi-Pyrénées | Montauban (1) | 37.5*** | 87.5⁻ | 27.6*** | | 100⁻ | 43.75*** | | 66.7*** | 37.5*** | 62.5*** |
| F | Midi-Pyrénées | Montauban (2) | 45.5*** | | 0.0*** | 34.5*** | | | | | 18.2*** | |
| F | Midi-Pyrénées | Montauban (3) | 6.7*** | | | | | | | | 26.7*** | 33.3*** |
| F | Midi-Pyrénées | Montauban (4) | 53.8*** | 92.3⁻ | 15.4*** | 53.8*** | 100⁻ | 46.2*** | 53.8*** | 100⁻ | 61.5*** | |
| F | Midi-Pyrénées | Tarn | 0.0*** | 75.0*** | 0.0*** | 38.5*** | | 0.00.0 | 0.0*** | 57.1*** | 8.3*** | 0.0*** |
| F | Normandy | Boissemont | 100⁻ | 52.9*** | | | | | | | 70.0*** | 100⁻ |
| F | Normandy | Mesnil Jourdan | 85.7* | 100⁻ | | | | | | | 42.9*** | 71.4*** |
| F | Provence | Boulbon | 13.3*** | 66.7*** | 13.3*** | 42.1*** | | 66.7*** | 0.0*** | 72.2*** | 26.7*** | 26.7*** |
| F | Provence | Cabannes | 0.0*** | 64.3*** | 12.5*** | 33.3*** | 92.9** | 0.0*** | 0.0*** | 31.25*** | 0.0*** | 7.1*** |
| F | Provence | Caumont | 1.0*** | 40.7*** | 0.0*** | 48.1*** | 100⁻ | 0.0*** | 6.7*** | 61.1*** | 6.7*** | 33.3*** |
| F | Provence | Cavaillon (1) | 27.2*** | 33.8*** | 10.7*** | 17.3*** | 100⁻ | 23.9*** | 23.9*** | 47.1*** | 20.6*** | 50.4*** |
| F | Provence | Cavaillon (2) | 6.7*** | 0.0*** | 0.0*** | 38.4*** | 83.3*** | 91.2⁻ | 0.0*** | 14.3*** | 0.0*** | 25.0*** |
| F | Provence | Cavaillon (3) | 0.0*** | 38.5*** | 22.9*** | 94.3⁻ | | | | 85.9*** | 33.3*** | 73.3*** |
| F | Provence | Cavaillon (4) | 14.3*** | 40.0*** | 22.9*** | 42.4*** | 100⁻ | 51.4*** | 11.4*** | 61.5*** | 37.1*** | 42.9*** |
| F | Provence | Cavaillon (5) | 12.5*** | 31.25*** | 12.5*** | 43.8*** | 100⁻ | 45.8*** | 6.25*** | 73.3*** | 75.0*** | 29.2*** |
| F | Provence | Maillane | 50.0*** | 27.8*** | 66.7*** | 69.4*** | | 61.1*** | 36.1*** | 88.9** | 41.7*** | 75.0*** |
| F | Provence | St Andiol (1) | 72.4*** | 57.7*** | 30.8*** | 72.1*** | 100.0⁻ | 26.9*** | 65.4*** | 75.9*** | 57.7*** | 80.8*** |
| F | Provence | St Andiol (2) | 47.4*** | 47.4*** | | 38.2*** | 92.9** | 41.5*** | | 66.7*** | 47.4*** | 36.8*** |
| F | Provence | Tarascon | 16.7*** | | | | 100⁻ | | | | 50.0*** | 60.8*** |
| F | Provence | Vigneres | 14.3*** | | | | | | | | 7.1*** | 0.0*** |
| F | Pays de la Loire | Le Bailleul | 23.1*** | | | | | | | | 30.8*** | 23.1*** |
| F | Pays de la Loire | St Cristophe | 55.6*** | 78.6*** | | | | | | | 88.9⁻ | 55.6*** |



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**Table 2.** Continued

| C[a] | Region | Population | Efficacy (%)[bc] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Azin | Chl-e | Delta | Diflu | Ema | Feno | Phos | Spi | Tebu | Thia |
| F | Pays de la Loire | St Laurent | 50.0*** | 90.9$^{-}$ | | 18.2*** | | 64.3*** | | 30.0*** | 57.1*** | 28.6*** |
| F | Perigord | St Medard | 27.3*** | 50.0*** | | | | | | | 72.7*** | 18.1*** |
| F | Perigord | Troche | 51.8*** | | | | 100$^{-}$ | | | | 42.9*** | 7.1*** |
| F | Rhône-Alpes | Gotheron | 22.2*** | 83.3** | 88.9* | 94.1$^{-}$ | 100$^{-}$ | 100$^{-}$ | | 100$^{-}$ | 22.2*** | 100$^{-}$ |
| I | Emilia-Romagna | Bologne RA-7 | 5.6*** | | | | | | | | | |
| I | Emilia-Romagna | Bologne RA-8 | 22.2*** | 11.1*** | | 0.0*** | 95.5$^{-}$ | 5.56*** | 7.86*** | 77.78*** | 0.0*** | 61.1*** |
| I | Trentino | Borgo spagolle | 100$^{-}$ | | | | | | | | 43.8*** | |
| I | Trentino | Dro | 100$^{-}$ | | | | | | | | 95.1$^{-}$ | |
| I | Trentino | Mezzocorona | | 56.9*** | 5.56*** | 16.8*** | 100$^{-}$ | 54.7*** | | | 72.9*** | 100$^{-}$ |
| I | Trentino | Revo Incolto | 100$^{-}$ | | | | | | | | 100$^{-}$ | |
| I | Trentino | Roncafort | 41.6*** | | | | | | | | 58.5*** | |
| I | Trentino | San Michele (1) | | 46.7*** | | | 100$^{-}$ | 51.5*** | | | | 74.1*** |
| I | Trentino | San Michele (2) | 84.6** | | | | | | | 81.5*** | 75.3 | |
| I | Trentino | Tuenno | 92.8$^{-}$ | | | | | 73.7*** | | 100$^{-}$ | 95.0$^{-}$ | |
| I | Trentino | Vervo | 100$^{-}$ | | | | | | | | 100$^{-}$ | |
| S | Bassin Lémanique | Luins le Vernay | 84.2** | 70.0*** | | | 100$^{-}$ | 42.1*** | | | 40.0*** | 25.0*** |
| S | Bassin Lémanique | Prangins | 53.8*** | 35.1*** | | 23.3*** | 100$^{-}$ | 12.4*** | | | 31.9*** | 38.4*** |
| S | Valais | Riddes (1) | 100$^{-}$ | | | 43.1*** | 100$^{-}$ | | | 35.5*** | 41.4*** | 60.9*** |
| S | Valais | Riddes (2) | 7.7*** | 43.1*** | | | 100$^{-}$ | 30.0*** | | | 39.1*** | 80.0*** |
| Sp | Lerida | Gimenells | 53.8*** | 7.7*** | | | 100$^{-}$ | | | | 30.8*** | 84.6*** |

[a] C (origin country): A = Armenia; F = France; I = Italy; S = Switzerland; Sp = Spain.

[b] Effectiveness of insecticides of resistant strains and the field population were compared with the sensible strain using a $\chi^2$ test: a dash ($^{-}$) denotes n.s; $^{*}P = 0.05$; $^{**}P = 0.01$; $^{***}P = 0.001$.

[c] Four groups of ten larvae were tested for efficacy of each insecticide on Cavaillon (4), Maillane and St Andiol (1), and two groups were considered for all other field populations. Final evaluation of mortalities was made on the total of individuals.

mutation was found in populations from all other countries, reaching a frequency of 100% in the only tested Armenian population (Table 3).

### 3.3 Relationships between susceptibility to insecticides and resistance mechanisms

MFO activities were positively correlated with resistance to azinphos-methyl ($r = 0.627$, $P < 0.0001$, $df = 47$), diflubenzuron ($r = 0.552$, $P = 0.0024$, $df = 26$), spinosad ($r = 0.454$, $P = 0.0126$, $df = 28$), tebufenozide ($r = 0.311$, $P = 0.0289$, $df = 48$) and thiacloprid ($r = 0.583$, $P = 0.0289$, $df = 40$) when considering the total population sample, while increased GST activity was related to resistance to azinphos-methyl ($r = 0.285$, $P = 0.0496$, $df = 47$). Moreover, for azinphos-methyl, tebufenozide and thiacloprid, the authors observed a negative correlation with enhanced esterase activity ($r = -0.421$, $P = 0.0026$, $df = 47$; $r = -0.398$, $P = 0.0043$, $df = 48$; $r = -0.341$, $P = 0.0286$, $df = 40$, respectively). The R < EST frequency was significantly correlated with insensitivity to emamectin ($r = 0.424$, $P = 0.0209$, $df = 28$). No significant correlation was observed between the frequency of *kdr* mutation and resistance to deltamethrin, even when homozygote resistant individuals were considered ($r = 0.206$, $P = 0.4031$, $df = 18$).

Similar relationships were found when considering six insecticides for which 16 populations and the laboratory strains were assayed (Table 4). On this reduced sample (mostly French populations and the Armenian one), resistance to thiacloprid was also related to the frequency of R > EST ($r = -0.491$, $P = 0.027$, $df = 19$), and resistance to tebufenozide was no longer related to the frequency of RMFO ($r = 0.087$, $P = 0.72$, $df = 19$). The PCA built with the same reduced population sample provided a synthetic representation of these relationships, associating high

**Table 3.** Resistance ratio of enzymatic systems, frequencies of resistant individuals and percentage of presence of AchE and *kdr* mutations in one susceptible strain (Sv), three resistant laboratory strains (Raz, Rdfb, RΔ) and 47 field populations of *Cydia pomonella* L

| | | | | | | Frequency (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Resistance ratio[b] | | | Enzymatic activity[c] | | | | Mutation[d] | |
| C[a] | Region | Population | GST | EST | MFO | GST | EST > | EST < | MFO | AChE | *kdr* |
| F | Laboratory | Sv | 1 | 1 | 1 | 10 (0.0) | 10 (0.0) | 10 (0.0) | 10 (0.0) | 0 | 0 |
| F | Laboratory | Raz | 2.6 | 1 | 12.6 | 61 (4.5)*** | 14 (3.2)$^-$ | 16 (3.4)$^-$ | 92.2 (2.4)*** | 100 | 0 |
| F | Laboratory | Rdfb | 0.6 | 1 | 6.7 | 3.3 (1.4)$^-$ | 13 (2.7)$^-$ | 10 (2.4)$^-$ | 60 (3.9)*** | 0 | 0 |
| F | Laboratory e | RΔ | 1.1 | 0.9 | 8.1 | 13 (2.6)$^-$ | 8.1 (2.1)$^-$ | 9.7 (2.3)$^-$ | 67.2 (3.6)*** | 0 | 100 |
| A | Armenia | Asktarak | 0.5 | 0.7 | 0.8 | 0 (0.0)$^-$ | 0 (0.0)$^-$ | 27 (3.5)* | 6.3 (1.9)$^-$ | 0 | 100 |
| F | Midi-Pyrénées | Albi | 1.3 | 0.5 | 8.5 | 5 (1.0)$^-$ | 0 (0.0)$^-$ | 63.6 (2.3)*** | 65.4 (2.7)*** | 0 | 48.3 |
| F | Midi-Pyrénées | Meauzac | 2.3 | 1 | 8.6 | 66.7 (2.2)*** | 0 (0.0)$^-$ | 0 (0.0)$^-$ | 66.7 (2.4)*** | 0 | 73.8 |
| F | Midi-Pyrénées | Moissac (1) | 1.9 | 0.9 | 5.8 | 38.1 (1.2)$^-$ | 4.8 (0.5)$^-$ | 19 (1.0)$^-$ | 60 (2.2)*** | 0 | 16.7 |
| F | Midi-Pyrénées | Moissac (2) | 1.5 | 0.7 | 11.1 | 20 (1.8)$^-$ | 5 (1.0)$^-$ | 40 (2.2)*** | 90.9 (1.3)*** | 0 | 0 |
| F | Midi-Pyrénées | Montauban (1) | 1.3 | 0.8 | 15.4 | 0 (0.0)$^-$ | 20 (1.8)$^-$ | 40 (2.2)$^-$ | 81 (1.7)*** | 0 | 23.3 |
| F | Midi-Pyrénées | Montauban (2) | 1 | 0.5 | 9.1 | 0 (0.0)$^-$ | 0 (0.0)$^-$ | 75 (1.0)*** | 83.3 (1.7)*** | 0 | 100 |
| F | Midi-Pyrénées | Montauban (3) | 0.8 | 0.8 | 10.5 | 0 (0.0)$^-$ | 0 (0.0)$^-$ | 22 (1.7)$^-$ | 62.5 (1.2)*** | 0 | 47.9 |
| F | Midi-Pyrénées | Montauban (4) | 1.1 | 0.4 | 11.9 | 5.9 (1.0)$^-$ | 0 (0.0)$^-$ | 59 (2.1)*** | 83.3 (1.8)*** | 0 | 100 |
| F | Midi-Pyrénées | Tarn | 2.7 | 0.7 | 7.4 | 73.3 (1.8)*** | 0 (0.0)$^-$ | 33 (1.9)** | 73.3 (1.1)*** | 0 | 20 |
| F | Normandy | Boissemont | 1.2 | 0.6 | 1.9 | 33.3 (1.8)$^-$ | 0 (0.0)$^-$ | 33 (1.8)$^-$ | 21.1 (2.2)$^-$ | 0 | 0 |
| F | Normandy | Mesnil Jourdan | 2 | 0.4 | 4.8 | 50 (0.9)$^-$ | 0 (0.0)$^-$ | 50 (0.9)$^-$ | 47.6 (2.2)*** | 0 | 2.5 |
| F | Provence | Boulbon | 1 | 0.8 | 14.5 | 13 (0.5)$^-$ | 6.3 (0.3)$^-$ | 38 (0.7)*** | 80.6 (1.8)*** | 0 | 13 |
| F | Provence | Cabannes | 1.4 | 0.9 | 12.3 | 25 (3.0)$^-$ | 10 (2.1)$^-$ | 10 (2.1)$^-$ | 78.3 (4.3)*** | 0 | 14.6 |
| F | Provence | Caumont | 1.7 | 0.7 | 8.6 | 33 (2.1)** | 0 (0.0)$^-$ | 29 (2.0)* | 63.6 (2.3)*** | 0 | 57.1 |
| F | Provence | Cavaillon (1) | 1.3 | 0.9 | 8.7 | 24.4 (1.9)** | 8.9 (1.3)$^-$ | 6.7 (1.1)$^-$ | 82.8 (2.2)*** | 0 | 92.5 |
| F | Provence | Cavaillon (2) | 1.1 | 0.9 | 5.1 | 10 (2.0)$^-$ | 5 (1.5)$^-$ | 15 (2.4)$^-$ | 66.7 (3.8)*** | 0 | 70.4 |
| F | Provence | Cavaillon (3) | 1.8 | 0.9 | 4.4 | 26.3 (1.9)* | 5.3 (1.0)$^-$ | 5.3 (1.0)$^-$ | 51.9 (2.7)*** | 0 | 59.3 |
| F | Provence | Cavaillon (4) | 1.2 | 0.9 | 8.4 | 13 (1.5)$^-$ | 0 (0.0)$^-$ | 0 (0.0)$^-$ | 54.5 (2.6)*** | 0 | 81.4 |
| F | Provence | Cavaillon (5) | 1.7 | 0.7 | 6 | 25 (2.1)$^-$ | 5 (1.0)$^-$ | 25 (2.1)$^-$ | 70.6 (2.6)*** | 0 | 85.2 |
| F | Provence | Maillane | 1 | 0.7 | 1.8 | 6.8 (1.1)$^-$ | 3.4 (0.8)$^-$ | 22 (1.8)** | 20.5 (2.4)*** | 0 | 14 |
| F | Provence | St Andiol (1) | 1 | 0.6 | 3.7 | 4.8 (1.6)$^-$ | 0 (0.0)$^-$ | 38 (3.7)*** | 29.7 (4.0)*** | 0 | 19.6 |
| F | Provence | St Andiol (2) | 1.8 | 0.7 | 11.9 | 30.8 (2.1)* | 0 (0.0)$^-$ | 15 (1.6)$^-$ | 66.7 (2.9)*** | 0 | 46.7 |
| F | Provence | Tarascon | 2.7 | 0.8 | 6.3 | 60 (1.8)** | 0 (0.0)$^-$ | 20 (1.4)$^-$ | 75 (2.1)*** | 0 | 67.5 |
| F | Provence | Vignères | 3.4 | 0.8 | 8.1 | 83.3 (0.8)*** | 0 (0.0)$^-$ | 17 (0.8)$^-$ | 77.8 (1.9)*** | 0 | 0 |
| F | Pays de la Loire | Le Bailleul | 3.2 | 0.7 | 9.7 | 73.3 (1.1)*** | 0 (0.0)$^-$ | 27 (1.1)$^-$ | 63.6 (2.0)*** | 0 | 6.7 |
| F | Pays de la Loire | St Cristophe | 2.1 | 0.7 | 3.7 | 60 (1.9)** | 0 (0.0)$^-$ | 20 (1.5)$^-$ | 38.1 (2.3)*** | 0 | 0 |
| F | Pays de la Loire | St Laurent | 3 | 0.6 | 9.2 | 62.5 (1.1)*** | 0 (0.0)$^-$ | 50 (1.1)** | 85 (1.0)*** | 0 | 0 |
| F | Perigord | St Medard | 2.4 | 0.8 | 8.3 | 48.1 (1.4)*** | 3.7 (0.5)$^-$ | 19 (1.1)$^-$ | 85.7 (1.6)*** | 0 | 7.1 |
| F | Perigord | Troche | 1.9 | 0.6 | 9.9 | 23.8 (2.2)$^-$ | 0 (0.0)$^-$ | 52 (2.6)*** | 68 (2.5)*** | 0 | 6 |
| F | Rhône-Alpes | Gotheron | 1.8 | 0.9 | 1 | 28.6 (2.1)$^-$ | 14 1.6$^-$ | 29 (2.1)$^-$ | 17.4 (1.9)$^-$ | 0 | 1.7 |

**Table 3.** Continued

| | | | Resistance ratio[b] | | | Frequency (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Enzymatic activity[c] | | | | Mutation[d] | |
| C[a] | Region | Population | GST | EST | MFO | GST | EST > | EST < | MFO | AChE | *kdr* |
| I | Emilia-Romagna | Bologne RA-7 | 0.9 | 0.4 | 6.9 | 0 (0.0)$^-$ | 0 (0.0)$^-$ | 100 (0.0)*** | 65.9 (2.3)*** | 0 | 15 |
| I | Emilia-Romagna | Bologne RA-8 | 1.3 | 0.5 | 7.6 | 11 (0.8)$^-$ | 0 (0.0)$^-$ | 44 (1.3)*** | 68.1 (2.9)*** | 0 | 5 |
| I | Trentino | Borgo spagolle | 1.3 | 1 | 1.5 | 0 (0.0)$^-$ | 20 (2.1)$^-$ | 0 (0.0)$^-$ | 13.6 (2.4)$^-$ | 0 | 0 |
| I | Trentino | Dro | 0.5 | 1 | 0.8 | 0 (0.0)$^-$ | 20 (0.9)$^-$ | 20 (0.9)$^-$ | 4.2 (0.9)$^-$ | 0 | 0 |
| I | Trentino | Mezzocorona | 0.4 | 0.6 | 1.6 | 0 (0.0)$^-$ | 0 (0.0)$^-$ | 43 (1.1)* | 10.5 (1.5)$^-$ | 0 | 0 |
| I | Trentino | Revo Incolto | 0.6 | 1.3 | 1.8 | 0 (0.0)$^-$ | 23.1 (1.1)$^-$ | 0 (0.0)$^-$ | 16.7 (1.6)$^-$ | 0 | 0 |
| I | Trentino | Roncafort | 0.6 | 0.9 | 6.2 | 0 (0.0)$^-$ | 0 (0.0)$^-$ | 0 (0.0)$^-$ | 25 (1.1)* | 0 | 0 |
| I | Trentino | San Michele (1) | 0.5 | 0.9 | 2.1 | 0 (0.0)$^-$ | 0 (0.0)$^-$ | 0 (0.0)$^-$ | 17.8 (0.8)$^-$ | 0 | 0 |
| I | Trentino | San Michele (2) | 1.5 | 1.3 | 4.3 | 16.7 (0.9)$^-$ | 33.3 (1.2)$^-$ | 0 (0.0)$^-$ | 29.4 (3.1)* | 0 | 0 |
| I | Trentino | Tuenno | 1.6 | 1.1 | 1 | 20 (1.4)$^-$ | 20 (1.4)$^-$ | 0 (0.0)$^-$ | 13.8 (1.4)$^-$ | 0 | 0 |
| I | Trentino | Vervo | 1.2 | 1.2 | 6.3 | 0 (0.0)$^-$ | 40 (1.5)$^-$ | 0 (0.0)$^-$ | 19 (2.1)$^-$ | 0 | 0 |
| S | Bassin Lémanique | Luins le Vernay | 1.4 | 0.7 | 3.6 | 10.5 (0.7)$^-$ | 5.3 (0.5)$^-$ | 37 (1.1)*** | 41.4 (2.3)*** | 0 | 10 |
| S | Bassin Lémanique | Prangins | 1 | 0.5 | 4.3 | 7.69 (1.2)$^-$ | 0 (0.0)$^-$ | 50 (2.2)*** | 34.4 (2.6)*** | 0 | 2.5 |
| S | Valais | Riddes (1) | 1.8 | 1 | 3.9 | 28.6 (2.3)* | 14 (1.8)$^-$ | 14 (1.8)$^-$ | 54.5 (4.0)*** | 0 | 0 |
| S | Valais | Riddes (2) | 1.2 | 1.2 | 2.9 | 13.3 (1.6)$^-$ | 20 (1.8)$^-$ | 0 (0.0)$^-$ | 36.7 (2.3)*** | 0 | 0 |
| Sp | Lerida | Gimenells | 1.6 | 0.9 | 32 | 69.2 (2.5)*** | 10 (1.6)$^-$ | 5 (1.2)$^-$ | 25 (2.4)* | 66.7 | 0 |

[a] C (origin country): A = Armenia; F = France; I = Italy; S = Switzerland; Sp = Spain.
[b] Resistance ratio = enzymatic activity of field population or resistant strain divided by enzymatic activity in the sensible strain.
[c] Frequency of resistant individual was compared with the sensible strain using a $\chi^2$ test: a dash ($^-$) denotes n.s; * $P = 0.05$; ** $P = 0.01$; *** $P = 0.001$. Standard deviations for frequencies are indicated in parentheses.
[d] Frequency of resistant alleles.

**Table 4.** Correlation matrix for resistance to six insecticides and frequency of enzymatic activity on four laboratory reference strains and 16 field populations of *Cydia pomonella*[a]

| | MFO | EST < | EST > | GST | Azin | Chl-e | Diflu | Spin | Tebu | Thia |
|---|---|---|---|---|---|---|---|---|---|---|
| MFO | 1 | 0.044 | 0.052 | 0.530* | 0.555* | 0.049 | 0.811** | 0.710** | 0.087 | 0.507* |
| EST < | | 1 | −0.434 | 0.137 | −0.09 | −0.114 | 0.221 | 0.051 | 0.015 | 0.129 |
| EST > | | | 1 | 0.019 | −0.403 | −0.226 | −0.244 | −0.066 | −0.165 | −0.491* |
| GST | | | | 1 | 0.461* | −0.285 | 0.253 | 0.392 | 0.005 | 0.359 |
| Azinphos-methyl | | | | | 1 | 0.377 | 0.264 | 0.436 | 0.535* | 0.538* |
| Chlorpyrifos-ethyl | | | | | | 1 | 0.151 | 0.259 | 0.369 | 0.405 |
| Diflubenzuron | | | | | | | 1 | 0.650* | 0.143 | 0.553* |
| Spinosad | | | | | | | | 1 | 0.347 | 0.556* |
| Tebufenozide | | | | | | | | | 1 | 0.48 |
| Thiaclopride | | | | | | | | | | 1 |

[a] Correlation coefficients followed by asterisks were statistically significant according to the Fisher test. df = 19; * $P = 0.05$; ** $P = 0.01$.

frequencies of RMFO and RGST to resistance to most insecticides, in opposition to high frequencies of R > EST. This representation also suggests that other mechanisms than these three metabolization systems may be involved in resistance to chlorpyrifos-ethyl, and to a lesser extent to tebufenozide and thiacloprid (Fig. 1).

## 4 DISCUSSION

### 4.1 Methodological constraints

The diagnostic concentrations for bioassays on diapausing larvae were defined using two different laboratory susceptible strains, one from Avignon (France) and the other from Changins (Switzerland). Diagnostic concentrations to test resistance to diflubenzuron, deltamethrin, azinphos-methyl and tebufenozide were established with the Avignon strain.[11,13] Resistance to the other compounds was established with the Changins strain.[29] High discrepancy occurred in the susceptibility of the two reference strains for emamectin. The Changins susceptible strain exhibited a 12-fold resistance to emamectin when compared with the Avignon susceptible strain, while no significant resistance to emamectin occurred in the laboratory strains selected for resistance to deltamethrin, diflubenzuron or azinphos-methyl. The absence of resistance to emamectin recorded in field populations may therefore be the result of an overestimated diagnostic dose owing to the initial variability of response to this compound among 'susceptible' strains.

On account of the limited size of the samples available for monitoring studies in *C. pomonella*, the analysis of resistance mechanisms was performed on

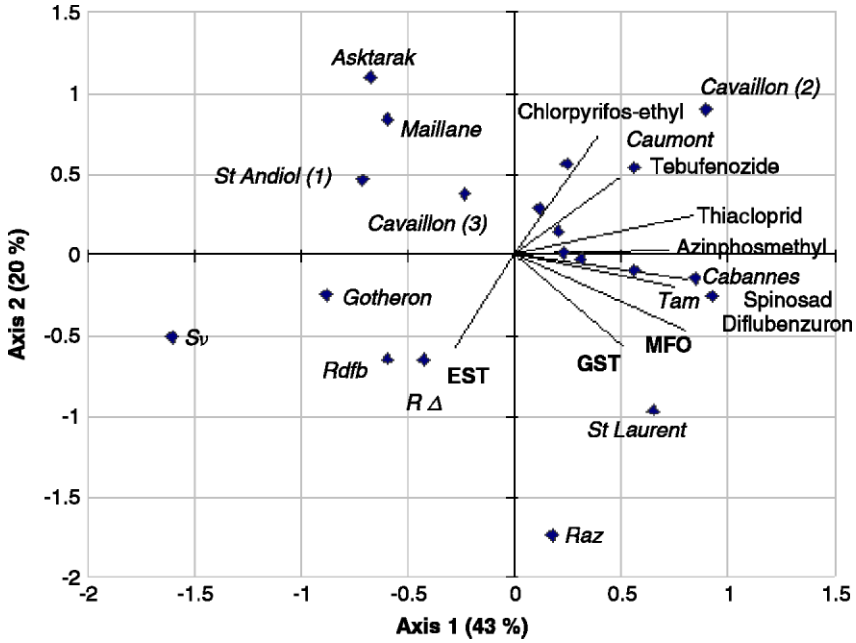


**Figure 1.** Principal components analysis for the frequency of individuals with enhanced enzymatic activity from 16 field populations and four laboratory strains of *Cydia pomonella* L. exposed to six insecticides. Each point represents a population, while lines represent enzymatic systems and insecticides. Only populations significantly contributing to the analysis are named in the figure.

the surviving adults of the control insects. However, the expression of resistance mechanisms and resistance ratios also differ strongly between developmental instars,[14] which was expected to restrict the conclusions of the present analysis relating resistance patterns to resistance mechanisms. Another pitfall of sampling is that the available populations were mostly obtained from orchards where pest control had failed, and consequently where resistance was observed. Only a few populations were collected in untreated orchards, which constrained the understanding of relationships existing between resistance mechanisms and susceptibility to insecticides.

Currently, insecticide resistance in *C. pomonella* is widely expanding in Europe.[10–12] Owing to the low genetic structuration, indicating a high gene flow (degree of migration among populations), that is observed in this species,[38,41,42] getting true susceptible populations from the field might not be an easy task. Therefore a single susceptible laboratory strain (Sv) was used for the whole study.[29] This was generated from isofemale lines for which all the treated fraction of the progeny died. Further biochemical and molecular analyses of this strain revealed homozygosity for susceptible alleles at the MFO, GST, Ache1 and *kdr* loci.[13,14,16]

### 4.2 Spread of resistance

Highly resistant populations showing reduced susceptibility to various insecticide groups were found in orchards of the five analysed countries. Moreover, it seems that susceptible populations have been replaced by resistant ones in most of the production areas. A few susceptible populations were still found in Trentino (Italy), but significant reductions in susceptibility to several compounds associated with increased activity of detoxication systems were also recorded in populations originating from untreated orchards. This was the case for the French untreated orchards at Gotheron, St Christophe, Mesnil Jourdan and Maillane. The last one had not received any insecticide treatments for the last 6 years. Such similarity of response to insecticides in *C. pomonella* populations of treated and untreated orchards was previously reported in the USA and attributed to the high migration rates of this species at the regional level.[43] Population genetic analyses also pointed to the very low genetic structure among populations owing to high gene flow in *C. pomonella*.[41] Otherwise, it was demonstrated that resistance alleles may persist in pest populations for several years after relaxing selection pressure, on account of their low fitness cost, as previously shown in *C. pomonella* for resistance associated with enhanced GST, and to a lesser extent on account of enhanced MFO.[44]

### 4.3 Resistance patterns and cross-resistances

The broad spectrum of insecticide resistance observed in southern European populations of *C. pomonella* is congruent with the resistance patterns already reported from French and Swiss codling moth populations.[12,45] However, new cases of cross-resistance were also observed. As a first example it would be possible to cite the low efficacy of azinphos-methyl against diapausing larvae of some *C. pomonella* populations from Switzerland, where this compound is not registered. This supports cross-resistance between azinphos-methyl and tebufenozide, which has been used since 1997 to control codling moth in Switzerland. Such cross-resistance between azinphos-methyl and tebufenozide has also been recorded

in other tortricid species such as *Planotortrix octo* (Walker)[46] and *Choristoneura rosaceana* (Harris).[47,48] A second example concerns the reduced susceptibility of diapausing larvae from each of the five studied countries to recent insecticides with novel modes of action (thiacloprid and spinosad) that are not or were only recently registered against this species. Resistance to these insecticides was correlated with resistance to diflubenzuron (Table 4). Both thiacloprid and spinosad are activators of nicotinic acetylcholine receptors in insects.[49] However, the low to moderate level of cross-resistance between these two insecticide groups in *L. decemlineata* suggests that resistance to both compounds can occur independently.[50] Because of the absence of previous applications of neonicotinoids and spinosad against French codling moth populations, resistance to these compounds in *C. pomonella* is more likely to result from non-specific metabolic detoxication.

### 4.4 Mechanisms involved in resistance

The PCA representation indicates that most of the cases of resistance observed in this study are associated with the expression of detoxication systems, and particularly with enhanced MFO activity. These enzymatic systems are potentially detrimental to the efficacy of different insecticide groups and are consequently implicated in cross-resistance.

EST activity was found to be involved in resistance to thiacloprid, while MFO activity was related to resistance to both thiacloprid and spinosad. Mechanisms of resistance to neonicotinoids and spinosad in insects have been investigated previously.[28,51–57] Differential effects of the esterase inhibitor DEF in neonicotinoid-resistant and susceptible strains of *P. xylostella*[28] and *B. tabaci*[51] strongly support the involvement of this enzyme system in resistance to this insecticide group. However, MFOs have also been suggested to be partially involved in resistance to neonicotinoids, corroborating the present results. In *Drosophila melanogaster* (Meigen), enhanced cytochrome P450 activity (specifically *Cyp6g1* gene) confers moderate resistance to imidacloprid, resulting in cross-resistance with other neonicotinoids and DTT.[52,53] In *Leptinotarsa decemlineata* (Say), the MFO inhibitor PBO does not completely suppress resistance to imidacloprid, suggesting that complementary mechanisms may be involved in resistance to neonicotinoids.[50] In *Nilaparvata lugens* (Stål), a target site mutation corresponding to the replacement of a tyrosine with a serine in the post-membrane acetylcholine receptor was also proved to induce imidacloprid resistance.[54]

Resistance to spinosad may be associated with an altered target site in *Musca domestica* (L.), as demonstrated by the absence of synergism observed using specific inhibitors of detoxification systems.[55] The present results are corroborated by the involvement of enhanced MFO activity in spinosad resistance in *B. tabaci*.[27]

In spite of the high discriminating concentration that was chosen for emamectin, a few significant reductions in efficacy were detected among European populations of *C. pomonella*. Such reductions were found to be associated with EST activity. Several mechanisms may be involved in resistance to avermectins in insect species. Synergism studies have demonstrated the involvement of both EST and MFO in resistance to abamectin in *P. xylostella*[56] and *L. decemlineata*,[24] while resistance to this compound is related to MFO and GST in *B. tabaci*.[27] An adequate discriminating concentration would probably allow a better understanding of resistance mechanisms to emamectin in *C. pomonella*.

MFOs were overexpressed in populations of four of the studied countries, with up to 15.4, 7.6, 4.3 and 32-fold ratios for France, Italy, Switzerland and Spain, respectively, when compared with the susceptible laboratory strain. Surprisingly, in the Armenian population, which is one of the most resistant to the different pesticides, the activity of the three enzymatic systems is lower than that of the susceptible laboratory strain. This population exhibited a significant increase only in the frequency of individuals with reduced EST activity, which could be the expression of a modified EST with a reduced affinity for the non-specific substrate $\beta$-naphthyl acetate and a higher activity on insecticide substrates.[17] It has been suggested that resistance may result from a mutation in a carboxylesterase that simultaneously reduces its carboxylesterase activity and confers an OP hydrolase activity (the 'mutant aliesterase hypothesis'). The event has been described at the biochemical level for *Lucilia cuprina* (Wiedemann),[21] *Chrysomya putoria* (Wiedemann),[22] *Plodia interpunctella* (Hübner)[24] and *Musca domestica*.[21,25] In *L. cuprina*, the association is due to a single amino acid substitution that converts a carboxylesterase to an organophosphorus hydrolase and confers insecticide resistance.[26]

Evidence for such a mechanism is also suggested by the negative correlations recorded here between enhanced EST activity and resistance to azinphos-methyl, tebufenozide and thiacloprid. The similarity observed between resistance mechanisms in the four countries of southern Europe and their discrepancy with mechanisms involved in insecticide resistance in Armenia may be the result of isolation due to distance between these countries. Different resistance mechanisms to azinphos-methyl were recorded from Latin America, i.e. enhanced EST activity in Argentina[20] and enhanced GST in Chile,[19] both without any involvement of enhanced MFO activity. This clearly demonstrates that different detoxication mechanisms were selected in different parts of the world in association with eventual resistance to the same insecticides. Such independent adaptations probably result from specific genetic backgrounds in Europe and in South America.[57] Resistance to most insecticides was found to be related to the activity of at least one detoxification system, with the exception of chlorpyrifos-ethyl,

in spite of its total inefficiency on diapausing larvae of some populations of Provence, Emilie-Romagne and Lerida. The AChE mutation identified in Spanish populations from the Lerida region[16] does not induce reduced susceptibility of AChE to chlorpyrifos-ethyl oxon nor reduced susceptibility of neonates to this compound,[19] as also observed in Cavaillon (2) and Bologne RA8 populations (data not shown). Moreover, the presence of the AChE mutation was restricted here to the Gimenells orchard and to the Raz laboratory strain, both originating from the Lerida region, in spite of the intensive use of organophosphorus insecticides in Provence orchards. Taking into account the specificity of AChE mutation to restricted organophosphorus or carbamate insecticides,[58,59] the French guidelines for insecticide resistance management recommend the alternation of OPs between generations (SPV, http://www.srpv-centre.com, accessed 29 November 2006). Actually, no specific resistance to OPs occurred in French populations of *C. pomonella* in spite of their reduced efficacy owing to generalized metabolic resistance. None of the known resistance mechanisms in this species can thus be related to the resistance to chlorpyrifos-ethyl.

While the *kdr* mutation was detected in several populations of France, Italy, Switzerland and Armenia, and while all the populations owning high frequencies of this mutation were strongly resistant to deltamethrin, these two parameters were found to be unrelated in the whole sample. This result may be explained by the high resistance ratio conferred by enhanced MFO activity in late instars of *C. pomonella*.[14,60] Diapausing larvae of populations displaying such metabolic resistance were thus found to be resistant to deltamethrin whatever the occurrence of *kdr* mutation, which was also proved to be differentially expressed in different developmental stages of *D. melanogaster*.[61] As this metabolic resistance does not confer a high level of resistance to pyrethroids in first-instar nymphs of *C. pomonella*,[14] bioassays on diapausing larvae are not appropriate for a diagnostic field resistance test with pyrethroids in this species.

### 4.5 Resistance monitoring

When considering the whole sample, resistance to most compounds was found to be related to modified activity of the three metabolic systems that were investigated. It is clear that populations with enhanced GST or MFO or with reduced EST activities are likely to be less susceptible to a large range of pesticides. Such enzymatic diagnostic may be performed on a rather small sample, i.e. 30–50 individuals, whereas a similar number would be needed for topical testing of a single insecticide (including the control insects) on diapausing larvae. However, the characterization of these mechanisms in a single population failed satisfactorily to predict its resistance pattern, especially regarding chlorpyrifos-ethyl, for which complementary and unknown mechanisms may be involved. It was also demonstrated that both enzymatic analysis and bioassays on diapausing larvae failed to detect resistance to deltamethrin. Thus, either bioassays on neonate larvae or molecular detection of the *kdr* mutation may be required with this compound.

Interestingly, the present data indicated that most of the southern European populations of the codling moth are likely to be resistant to most of the registered insecticides. However, this was partially congruent with knowledge of the field efficacy of some of these compounds. Whereas azinphos-methyl and thiacloprid induced respectively only 13.3 and 26.7% mortality on diapausing larvae collected in 2003 in the Boulbon orchard, the field efficacy of these two compounds in 2000 on the same orchard reached 89.5 and 84.5%, and neonates of a resistant laboratory strain exhibited only 2–3-fold resistance to these compounds.[62] Similar field efficacy of azinphos-methyl, thiacloprid, chlorpyrifos-ethyl and spinosad was also found in Cavaillon (5) (data not shown), from which diapausing larvae appeared to be strongly resistant to these insecticides when applied topically.

The identification of both resistance mechanisms and cross-resistance is essential for the rational development of resistance management strategies. In other Lepidoptera, it is generally assumed that metabolic resistance is fully expressed in metabolically active stages such as in mature larvae, a stage at which the resistance should be monitored.[63] If resistance is due to a target site modification, its expression is expected to occur during both larval and adult stages of the pest, allowing resistance monitoring whatever the life stage.[64]

It is therefore concluded that none of these diagnostic tests may provide an accurate overview of insecticide resistance patterns in *C. pomonella*. It is postulated that topical applications on diapausing larvae may preferentially explain the field susceptibility to most insect growth regulators while they may overestimate resistance to most of the neurotoxic compounds. Thus, there may be a need for comparisons of resistance levels expressed in diapausing larvae and neonates of the same population for each of the tested compounds. So far, topical applications or enzymatic and molecular analyses have provided a first indication of the potential for insecticide resistance, which still has to be further and individually confirmed for each compound, for example through field trials or bioassays on neonates.

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