Universitat de Lleida

Diversidad y extensión de los mecanismos de resistencia a insecticidas en poblaciones españolas de carpocapsa (*Cydia pomonella* (L.) Lepidoptera: Tortricidae) y estudio de sus posibilidades de manejo

Dolors Bosch i Serra

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Universitat de Lleida

TESI DOCTORAL

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Memòria presentada per optar al grau de Doctor per la Universitat de Lleida
Programa de Doctorat en Ciència i Tecnologia Agroalimentària

Directors
Jesús Avilla Hernández
Marcela A. Rodríguez García

Tutor
Jesús Avilla Hernández
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Als meus pares, el Pepe i la Maria, les meves arrels.
La meva mare gaudiria del moment tant o més que jo, com ho fa el meu pare.

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ÍNDICE GENERAL

Resum

La carpocapsa, *Cydia pomonella* (Linnaeus 1758) (Lepidoptera: Tortricidae), és una de les plagues més importants del cultiu dels fruiters de pinyol, principalment de la pomera. A la zona fructícola de Lleida, a l'igual que a la majoria de les zones productives del món, s'han detectat problemes de resistència a insecticides piretroïds, organofosforats, benzoilurees, fenoxycarb i diacilhidracines. El principal mecanisme de resistència detectat ha estat l'acció dels enzims detoxificadors citocrom P450 polisubstrat monooxigenases (PSMO) i, de manera menys important, glutathion-S-transferases (GST) i esterases (EST). A la present tesis doctoral, s'han determinat els nivells resistència de carpocapsa a insecticides de baix risc per a la salut humana i medi ambient i la presència i extensió de les mutacions de la acetilcolinesterasa (*AChE*) i la *knockdown* (*kdr*), en poblacions de camp procedents de tres zones productives de poma a España, amb sistemes de maneig molt diferents.

Les poblacions asturianes resultaren susceptibles a tots els insecticides provats, mentre que les poblacions de la Vall de l'Ebre (Catalunya i Aragón) presentaren resistència en alguna població i tolerància de forma freqüent a metoxifenocida, lambda-cihalotrí i tiacloprid. La resta de productes provats, etil-clorpirifos, tebufenocida, spinosad, spinetoram, indoxacarb, clorantraniliprol i emamectina, resultaren efectius fins i tot en les poblacions amb elevats nivells de resistència. La mutació *AChE* està present de forma generalitzada únicament a les parcel·les de la Val de l'Ebre, mentre que la *kdr* està present a la majoria de les parcel·les de Catalunya. A les poblacions d'Asturias i a la població d'Extremadura analitzades no es detectaren mutacions.

També s'ha determinat la línia base de susceptibilitat insecticida de la matèria activa clorantraniliprol en poblacions de camp europees de carpocapsa, i s'ha avaluat l'evolució de la seva eficàcia al llarg dels anys d'aplicació. No s'han detectat poblacions resistents a chlorantraniliprole, però, tanmateix, sí s'ha detectat una relació entre la freqüència d'individus PSMO-resistents (amb un elevat nivell d'enzims de detoxificació PSMO) i el percentatge de la mortalitat produïda per la concentració discriminant pròxima a la CL_{50}.

A més, s'estudià la freqüència d'adults PSMO-resistents de *C. pomonella* capturats en trampes de feromones amb diferents difusors, amb i sense la cairomona èster de pera ((2E, 4Z)-decadienoat d'etil), degut a la major atracció al laboratori de la cairomona pura per aquests individus. La finalitat fou la millora del monitoreig a les parcel·les amb una elevada presència d'individus resistents. No obstant, aquella major atracció no es produí al camp. Sí que es detectà una diferent freqüència d'individus resistents entre generacions i entre parcel·les amb o sense tractaments químics.

Resumen

La carpocapsa, *Cydia pomonella* (Linnaeus 1758) (Lepidoptera: Tortricidae), es una de las plagas más importantes del cultivo de los frutales de pepita, principalmente del manzano. En la zona frutícola de Lleida, al igual que en la mayoría de las zonas productoras del mundo, se han detectado problemas de resistencia a insecticidas piretroides, organofosforados, benzoilureas, fenoxicarb y diacilhidracinas. El principal mecanismo de resistencia detectado ha sido la acción de las enzimas detoxificadoras citocromo P450 polisustrato monooxigenasas (PSMO) y, en menor medida, glutathion-S-transferasas (GST) y esterasas (EST). En la presente tesis doctoral, se han determinado los niveles de resistencia de carpocapsa a insecticidas de bajo riesgo para la salud humana y el medio ambiente y la presencia y extensión de las mutaciones de la acetilcolinesterasa (*AChE*) y la *knockdown* (*kdr*), en poblaciones de campo procedentes de tres zonas productivas de manzana en España, con sistemas de manejo muy distintos.

Las poblaciones asturianas resultaron susceptibles a todos los insecticidas ensayados, mientras que las poblaciones del Valle del Ebro (Cataluña y Aragón) presentaron resistencia en alguna población y tolerancia de forma frecuente a metoxifenocida, lambda-cihalotrín y tiacloprid. El resto de productos ensayados, etil-clorpirifos, tebufenocida, spinosad, spinetoram, indoxacarb, clorantraniliprol y emamectina, resultaron efectivos incluso en las poblaciones con elevados niveles de resistencia. La mutación *AChE* está presente de forma generalizada únicamente en las fincas del Valle del Ebro, mientras que la *kdr* está presente en la mayoría de las fincas en Cataluña. En las poblaciones de Asturias y la población de Extremadura analizadas no se detectaron mutaciones.

También se ha determinado la línea base de susceptibilidad insecticida de la materia activa clorantraniliprol en poblaciones de campo europeas de carpocapsa, y se ha evaluado la evolución su eficacia a lo largo de los años de aplicación. No se han detectado poblaciones resistentes a clorantraniliprol, pero, sin embargo, sí se ha detectado una relación entre la frecuencia de individuos PSMO-resistentes (con un elevado nivel de enzimas de detoxificación PSMO) y el porcentaje de mortalidad de la concentración discriminante próxima a la CL_{50}.

Por otro lado, se ha estudiado la frecuencia de adultos PSMO-resistentes de *C. pomonella* capturados en trampas con distintos difusores, con y sin la cairomona éster de pera ((2E, 4Z)-decadienoato de etilo), debido a una mayor atracción en laboratorio de la cairomona pura por estos individuos. La finalidad era la mejora del monitoreo en las fincas con una elevada presencia de insectos resistentes. Sin embargo, esa mayor atracción no se produjo en campo. Sí se detectó una distinta frecuencia de individuos resistentes entre generaciones y entre fincas con o sin tratamientos químicos.

Summary

Codling moth, *Cydia pomonella* (Linnaeus 1758) (Lepidoptera: Tortricidae), is one of the most important pests of pome orchards, mainly of apple ones. Problems of resistance to insecticides pirethroids, organophosphates, benzoylureas, fenoxycarb, and diacylhydrazines have been detected in the fruit growing area of Lleida, as well as in the majority of the world apple-growing areas. The main resistance mechanism detected has been the action of the detoxifying enzyms cytochrome P450 polysubstrate monooxygenases (PSMO) and, in a lesser extent, glutathione-S-transferases (GST) and esterases (EST). In the present thesis, the levels of codling moth resistance to safe insecticides and the presence and the extension of the mutations acetylcholinesterase (*AChE*) and the *knockdown* (*kdr*) have been determined in field populations from three fruit growing areas of Spain, with very different production systems.

The populations from Asturias were susceptible to all the tested insecticides, while some populations from the Ebro Valley (Catalonia and Aragón) were resistant, and frequently tolerant to methoxyfenozide, lambda-cyhalothrin and thiacloprid. The rest of tested insecticides chlorpyrifos-ethyl, tebufenozide, spinosad, spinetoram, indoxacarb, chlorantraniliprole and emamectin, were effective, even against populations with high resistance levels. The *AChE* mutation is generally present only in orchards from the Ebro Valley, while the *kdr* is present in the majority of the orchards from Catalonia. No mutations were detected in the populations from Asturias and Extremadura.

The susceptibility baseline of chlorantraniliprole has been determined in field populations from Europe, and the evolution of its efficacy along time have been evaluated. Resistant populations to chlorantraniliprole have not been detected, but a relationship between the frequency of PSMO-resistant individuals (those that show a high level of PSMO detoxification enzymes) and the mortality (percentage) caused the discriminant concentration near to the LC_{50}.

Furthermore, the frequency of *C. pomonella* MFO-resistant adults caught in traps with different lures, with and without the kairomone pear ester ((2*E*, 4*Z*)-2,4-decadienoate) was studied, because a higher attraction of these individuals for the pure kairomone had been detected in the laboratory. The aim was to improve monitoring in the orchards that show a high percentage of resistant individuals. However, this higher attraction was not observed in the field. A different frequency of resistant individuals among generations and among orchards chemically or non-chemically treated was observed.

INTRODUCCIÓN GENERAL

Antecedentes

La carpocapsa, *Cydia pomonella* (Linnaeus 1758) (Lepidoptera: Tortricidae), es una de las plagas más importantes del cultivo de los frutales de pepita: manzano (*Malus domestica* Borkhausen), peral (*Pyrus spp*.) y membrillero (*Cydonia oblonga* Mill) y del nogal (*Juglans spp*.). Está presente en prácticamente todas las zonas templadas donde se encuentran estos cultivos (Barnes, 1991).

El uso de insecticidas químicos es el método más extendido para el control de esta plaga y su uso se hace necesario a pesar de utilizar de forma cada vez más extensiva el método de confusión sexual. Debido a dichos tratamientos, *C. pomonella* ha desarrollado resistencia a un elevado número de materias activas insecticidas en la mayoría de las zonas productivas del mundo (Fuentes-Contreras *et al*., 2007; Ioriatti *et al*., 2007; Knight, 2010; Reuveny & Cohen, 2004; Rodríguez *et al*., 2010, 2012; Sauphanor *et al*., 1998, 2000; Soleño *et al*., 2008, 2012; Stará & Kocourek, 2007). En la zona frutícola de Lleida también se detectaron resistencias en la mayoría de las fincas que presentaban problemas de control de carpocapsa (Rodríguez *et al*., 2010, 2011a, 2011b). Los insecticidas estudiados en ese momento fueron piretroides, organofosforados, benzoilureas, fenoxicarb y diacilhidracinas. La resistencia de los insectos a los insecticidas puede ser debida a la detoxificación del insecticida mediante tres complejos enzimáticos: citocromo P450 polisustrato monooxigenasas (PSMO), glutatión-S-transferasas (GST) y esterasas (EST) o a mutaciones en la proteína diana del insecticida que la convierten en menos susceptible al producto: mutación de la acetilcolinesterasa (*AChE*) que afecta a organofosforados y carbamatos (Cassanelli *et al*., 2006) y la mutación en el canal de sodio (*knockdown*, (*kdr*)), que afecta a piretroides (Brun-Barale *et al*., 2005). En la zona de Lleida, se demostró que los adultos y las larvas de carpocapsa de las fincas con problemas presentaban, en general, una elevada producción de enzimas PSMO (Rodríguez *et al*., 2010, 2012) y, de forma puntual, una elevada producción de enzimas GST, mientras que la elevada producción de enzimas EST fue detectada únicamente en larvas (Rodríguez *et al*., 2011a, 2011b). Por otra parte, las únicas dos poblaciones en las que se detectó la mutación *AChE* eran originarias de la zona de Lleida (Cassanelli *et al*., 2006; Reyes *et al*., 2007), zona donde las poblaciones de carpocapsa presentaban con frecuencia resistencia a metil-azinfos y donde carbaril se había utilizado para el aclareo.

Otro punto a destacar son los efectos pleiotrópicos producidos por la resistencia a insecticidas en individuos de carpocapsa, como por ejemplo presentar distintos ratios de desarrollo (Boivin *et al*., 2001, 2003) o distintos fotoperiodos críticos para inducir la diapausa (Boivin *et al*., 2005), que pueden modificar el nivel de insectos resistentes presentes en el campo durante las distintas generaciones, además de producir efectos negativos para el insecto en el momento de aparearse debido a una menor producción de feromona por parte de la hembra o una menor capacidad de los machos a la hora de detectar la fuente feromonal (Poulot *et al*., 2001; Trimble *et al*., 2004). Sauphanor et al. (2007) demostró una elevada atracción en campo de la cairomona pura ((2E, 4Z) decadienoato de etilo, éster de las peras (Light et al., 2001) en los machos carpocapsa que presentaban elevados niveles de PSMO.

Introducción

Cydia pomonella (L.)

Distribución geográfica

Cydia pomonella está establecida en prácticamente todas las zonas del mundo donde se cultivan sus huéspedes (Figura 1) y donde se acumula en verano una cantidad mínima de 600 grados-día, tomando como temperatura base 10 ºC, que son los necesarios para el desarrollo completo de una generación (UC IPM Statewide Integrated Pest Management Program, 2017).

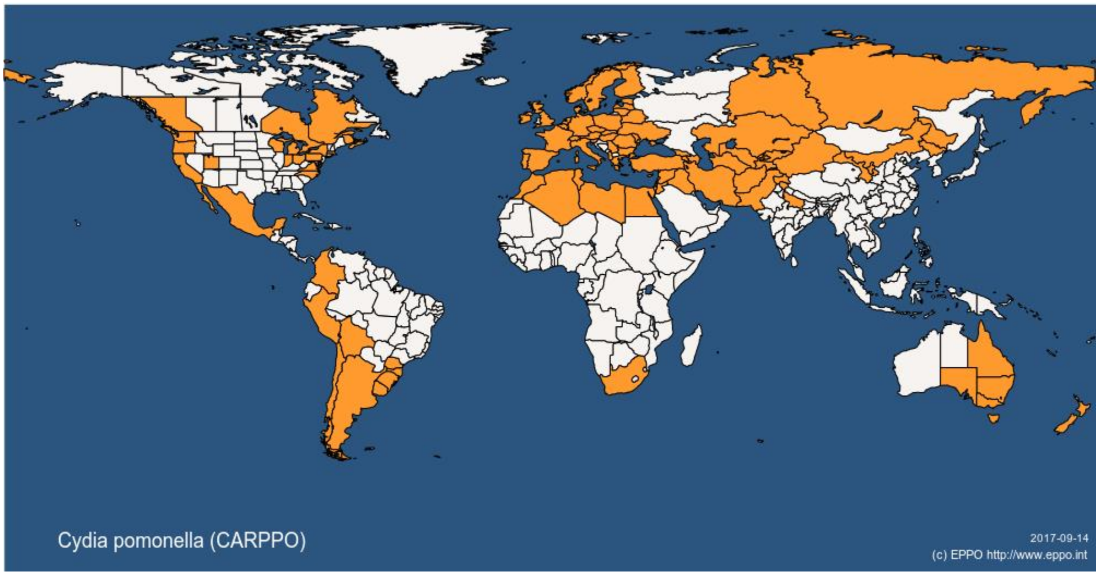

Figura 1. Distribución mundial de *Cydia pomonella*. Mapa obtenido en EPPO Global Database (2017) (https://gd.eppo.int/taxon/CARPPO/distribution.

En España, está distribuida en todas las zonas productivas de manzanos, perales y nogales (de la Cruz, 2009; Lozano, 2013; Miñarro & Dapena, 2000; Sánchez & Biurrun, 2008; Rodríguez *et al*., 2010).

Cultivos huésped

C. pomonella es una plaga de pomáceas y sus huéspedes principales son básicamente manzanas y peras, aunque también es considerada la plaga más importante de las nueces (Riedl & Barnes, 1979). Otros huéspedes importantes pueden ser melocotones, albaricoques, ciruelas, caquis y naranja, aunque éstos son secundarios y deben sus ataques a la proximidad de fincas de huéspedes primarios con un fuerte ataque (Barnes, 1991).

Taxonomía

La descripción original fue realizada por Linnaeus (1758) en su “Systema Naturae” (Ed. 10, Vol 1, p.538) con el nombre de *Phalaena (Tortrix) Tinea pomonella*. A lo largo del tiempo fue descrita con otros nombres considerados sinónimos: *Tortrix pomonana* Denis & Schiffermüller, 1775; *Phalaena pomonana* Denis & Schiffermüller, 1776; *Phalaena aeneana* Villers, 1778; *Pyralis pomana* Fabricius, 1793; *Carpocapsa putaminana* Staudinger, 1859; *Carpocapsa pomonella* Harris, 1862; *Cydia pomonella* Walsingham, 1897 y *Laspeyresia pomonella* Davis, 1969. Brown (1979) realizó una revisión de los nombres genéricos y tribales y estableció claramente la sinonimia de *Laspeyresia* y *Carpocapsa* con respecto a Cydia, y estableció como correcto el nombre de *Cydia pomonella* (L.).

La ubicación taxonómica de *C. pomonella* es:

Orden: Lepidoptera
Suborden: Heteroneura
División: Ditrysia
Subdivisión: Heterocera
Superfamilia: Tortricoidea
Familia: Tortricidae
Subfamilia: Olethreutinae (sensu Horak 1999)
Tribu: Grapholitini (sensu Horak 1999)
Género: *Cydia* Hübner, 1825
Especie: *Cydia pomonella* (Linnaeus 1758).

Descripción morfológica

Huevo: Los huevos son de forma lenticular planoconvexa, blanco-perlados y de 1,0 a 1,2 mm de diámetro. Están fijados a la superficie de puesta mediante una substancia adherente. La transparencia del corion permite seguir el desarrollo embrionario sin dificultad. Se reconocen distintas fases en el desarrollo del huevo, aunque generalmente se suelen distinguir tres: huevo blanco, cuando está recién puesto, anillo rojo, cuando se distingue un halo rojo que indica la formación de membranas internas, y cabeza negra, cuando se distinguen las partes esclerotizadas de la larva, especialmente la cabeza (Figura 2). Cuando la larva está completamente madura, corta con sus mandíbulas el corion y emerge.

Figura 2. Huevos de *C. pomonella* en distintas fases de desarrollo: Huevo blanco, anillo rojo y cabeza negra (Fotos: Jesús Avilla).

Larva: Las larvas recién emergidas son de color blanco cremoso y tienen la cabeza negra. A medida que se van desarrollando, su coloración varía tornándose amarillo rosáceo en los últimos dos estadios (Figura 3). El desarrollo larvario pasa por cinco estadios separados por cuatro mudas hasta transformarse en crisálida o pupa. Su tamaño varía de 1,8-2,0 mm en el primer estadio hasta 18-20 mm en el último estadio. El tamaño de la cápsula cefálica es un buen indicador del estadio larval pasando de 0,33 a 1,2 mm desde el primer al último estadio (Pons, 1992). El cuerpo de la larva es cilíndrico y se divide en 12 segmentos. Las patas se encuentran en los tres segmentos torácicos, los segmentos abdominales del 6 al 9 y el último segmento, el segmento anal. En el último estadio larval se puede ver el dimorfismo sexual a simple vista, ya que los machos tienen visibles sus órganos genitales, apreciando dos manchas oscuras en la superficie dorsal del séptimo segmento abdominal (Hansen & Harwood, 1968).

Pupa: Las pupas son fusiformes y de un color marrón que varía de claro a oscuro según la edad. Su tamaño varía entre 8 y 11 mm (Figura 4). Se encuentran 10 segmentos abdominales. Los segmentos 4-6 en las hembras y 4-7 en los machos son móviles. Los cuatro últimos segmentos en las hembras y los tres últimos en los machos están fusionados para formar la terminalia. La obertura genital está presente en el segmento 9 en los machos y en el 8 en las hembras, lo que permite sexar las pupas. Generalmente, las pupas de las hembras son de mayor tamaño que las de los machos.

Figuras 3 y 4: Larvas de *C. pomonella* en distintos estadios de desarrollo, primer (L1) y último (L5) estadio y pupa (Fotos: Jesús Avilla y Dolors Bosch).

Adulto: El adulto es una mariposa de unos 10 mm de largo y una envergadura alar que varía de 15 a 22 mm. Las alas anteriores tienen, en general, unas bandas en gris plateado y negro, y una ancha franja medio anterior más oscura. Sin embargo, existe otro prototipo cromático menos frecuente con una coloración gris uniforme y la banda terminal oscura, que fue citado por Bovey (1966) como *Laspeyresia pomonella* forma *putamina* Stgr. (Figura 5).

Figura 5. Adultos de *C. pomonella* de distinto prototipo cromático (Fotos: Jesús Avilla y Dolors Bosch).

Las alas posteriores son de color gris pálido. Las hembras tienen mayor tamaño y son más pesadas que los machos, pero existe una gran variabilidad por lo que no se puede distinguir el sexo por el tamaño. Una manera rápida de clasificar los sexos es por la forma del abdomen. La genitalia externa de la hembra es una papila anal, que se ve como una mancha circular marrón en la punta del abdomen, formada por los dos lóbulos del ovipositor. La genitalia externa del macho son un par de *claspers* (Howell, 1991).

Otra característica útil para distinguir entre sexos es una mancha gris oscuro que se puede ver en los machos en el centro de la parte interior del ala anterior (Fernández *et al*., 2007).

Biología y dinámica de población

C. pomonella es una plaga variable y adaptable, como lo demuestra estando presente en la mayor parte de las zonas donde se cultivan sus plantas huésped. La mayor parte de las investigaciones referentes a la actividad y ciclo de vida de carpocapsa, incluyendo fenología, voltinismo, tasa de desarrollo, vuelo y oviposición se han basado en los efectos de la temperatura y fotoperiodo. Por ello, las investigaciones referentes a su ciclo de vida deben realizarse de forma local.

Pasa el invierno en diapausa en forma de larva completamente desarrollada, dentro de un capullo que fabrican mayoritariamente en las grietas de la corteza de los árboles o en el suelo. En la zona de estudio, las tres generaciones de adultos se inician aproximadamente en abril, junio y agosto (Bosch *et al*., 2016). La primera generación es más extensa debido a las temperaturas y normalmente presenta dos picos de vuelo más o menos marcados. La extensión y la forma de esta curva de vuelo están muy influenciadas por la cantidad de horas frío acumuladas en invierno (Audemard, 1991).

Los machos vuelan con temperaturas mayores a 13°C y las hembras con temperaturas superiores a 15°C, pero la cópula se produce preferentemente con temperaturas crepusculares por encima de los 17°C (Batiste & Olson, 1973). Carpocapsa es un lepidóptero de hábitos crepusculares, por lo que su actividad diaria está limitada a unas pocas horas durante la tarde e inicio de la noche y, si las temperaturas son adecuadas, durante la madrugada (Howell *et al*., 1990). El componente principal de la feromona sexual, emitida por las hembras se denomina codlemona: (*E*,*E*)-8,10-dodecadien-1-ol (*E*8,*E*10-12OH) (Roelofs *et al*., 1971), pero existen dos componentes más, además de isómeros geométricos de la codlemona en distintas proporciones (Witzgal *et al*., 2008). La hembra emite feromona a partir del primer día de emergencia y la emisión alcanza su máximo en el tercer día de emergencia (Vickers & Rothschild, 1991).

Las hembras pueden poner una media de 132 a 162 huevos (Howell, 1981). La cantidad de huevos es influida por las condiciones de fotoperiodo a las que está expuesta la hembra antes de su emergencia como adulto (Howell, 1981) y al tamaño corporal (Geier, 1963). Los huevos se depositan en las hojas o en los frutos de forma aislada y eclosan entre 9 y 14 días después de la puesta, en función de las temperaturas.

Las larvas neonatas vagabundean unas horas para encontrar el fruto o determinar su punto de entrada. Este vagabundeo es más largo durante la primera generación, depende del lugar de oviposición y durante el mismo pueden alimentarse de hojas. Una vez perfora la epidermis del fruto, la larva realiza una cavidad superficial donde muda a segundo estadio y a partir de ese momento empieza una galería en la pulpa dirigiéndose a la zona carpelar para alimentarse de las semillas. La larva completa el desarrollo con cinco estadios larvarios en 25-30 días y abandona el fruto para pupar. Alimentándose en manzanas, el tiempo de desarrollo de los cinco estadios larvales incluido el periodo pupal está entre 30 y 37 días a 26 ºC y 50 % de humedad relativa (Hathaway *et al.*, 1971).

El número de generaciones larvarias al año depende en gran medida de las temperaturas (suma de temperaturas) y de la duración del día (fotofase), y por lo tanto de la latitud. Mientras que la temperatura tiene un efecto directo sobre la duración del desarrollo, la fotofase es el principal desencadenante de la diapausa (Stoeckly *et al.*, 2012). La fotofase que induce la diapausa varía según la latitud entre 13 h en el Sur de Asia (39º N) y 18 h en Leningrado (60º N) (Shel'deshova, 1967). Así pues, en función de la zona, esta plaga puede ser de univoltina a trivoltina. El nuestra zona de estudio se producen tres vuelos de adultos y, por lo tanto, tres generaciones larvarias, aunque parte de estas larvas de tercera generación no consiguen acabar su desarrollo debido a las condiciones climáticas. Ejemplos del número de generaciones en distintas zonas del mundo se encuentran en Solomon (1991). En las zonas con tres generaciones, ya sean completas o parciales, como el área del Valle del Ebro, las larvas diapausantes proceden no sólo de la progenie del tercer vuelo, sino que también proceden de parte de la progenie del segundo e incluso de una pequeña parte de la del primer vuelo (Portillo, 2000). La diapausa finaliza con una exposición durante un tiempo determinado a bajas temperaturas o por la exposición a fotofases largas (Audemard, 1991).

Daños y síntomas

Los daños son ocasionados por la alimentación de las larvas en los frutos y se producen de forma continua durante todo su desarrollo. Cualquier daño en el fruto producido por carpocapsa (Figura 6), aunque sea un ataque parado o bien en una fase inicial, produce una depreciación de la fruta y la hace inservible como fruta de mesa. Las pérdidas ocasionadas pueden llegar a ser superiores al 80% (Angeli & Rizzi, 2013).

Figura 6. Daños de *C. pomonella* en manzana

Control de *Cydia pomonella*

La base para la protección integrada de los cultivos son los controles continuos de las densidades poblacionales de las plagas, patógenos y malas hieras (Baggiolini *et al*., 1990). Para el control de carpocapsa el seguimiento del vuelo mediante trampas y los controles visuales de los frutos son fundamentales para determinar la necesidad de realizar tratamientos, así como la eficacia del método de control aplicado, sea cual sea el que se utilice.

El seguimiento del vuelo mediante trampas se ha realizado desde el descubrimiento y la posibilidad de la síntesis del componente principal de la feromona, la codlemona (Roelofs *et al*. 1971), y su uso ha sido extensivo (Charmillot, 1980; Gut & Brunner, 1996; Howell & Quist, 1980; Riedl *et al*., 1986). Sin embargo, en las fincas con confusión sexual la utilización de la misma molécula tanto para el control como para el seguimiento del vuelo hace que la eficacia atractiva de las trampas sea poco fiable. En 2001, Light *et al*. descubrieron el denominado éster de pera, 2,4-decadienoato de etilo, una cairomona que resultaba atractiva tanto para los machos como para las hembras de carpocapsa. Durante los últimos años, el uso de un difusor con la combinación de 3 mg de la cairomona y 3 mg de codlemona, CM-DA Combo®, ha ido reemplazando el uso de los difusores de 10 mg de feromona en las fincas en confusión sexual debido a que captura un mayor número de adultos en las trampas (Joshi, 2011; Torà *et al*., 2009).

El control de *C. pomonella* ha sido tradicionalmente mediante la aplicación de productos insecticidas. Sin embargo, existen distintos métodos también importantes como el control etológico, el control microbiológico, el control biológico, el control autocida y las técnicas de control cultural.

Control químico

El control químico de carpocapsa es uno de los temas mejor documentados de la literatura entomológica (Croft & Riedl, 1991). La evolución del uso de insecticidas en el control de plagas se identifica en eras o generaciones (Casadei, 2003; Croft & Riedl, 1991; Walker *et al*., 2017). La primera generación se basaba en productos naturales inorgánicos, botánicos o aceites minerales, y la segunda en organoclorados, organofosforados, carbamatos y piretroides. Sin embargo, aproximadamente en los años 60, hubo una creciente preocupación por la salud pública debido al uso extensivo de este tipo de insecticidas de amplio espectro, debido a los riesgos de toxicidad y de residuos en los alimentos, y al impacto sobre el medio ambiente y los insectos beneficiosos. A partir de ese momento aparecieron insecticidas más selectivos que actuaban en los procesos fisiológicos de los insectos pero no en los de los animales superiores. En primer lugar aparecerían los reguladores de crecimiento, y después otros insecticidas de acción específica que actuarían en distintos puntos del sistema nervioso.

Actualmente, según el Insecticide Resistance Action Committee (IRAC), existen 11 clases químicas de insecticidas disponibles para el control de carpocapsa (IRAC Codling Moth WG, 2013), de las cuales 8 están disponibles en España (Tabla 1).

Control etológico

Este control se basa en la utilización de feromonas sexuales sintéticas. El método de control etológico más utilizado es la confusión sexual, aunque las técnicas de atracción y muerte y de captura masiva también se han ensayado (Vickers & Rothschild, 1991; Witzgall *et al*., 2010). La confusión sexual está siendo ampliamente utilizada en muchos países y ha sido implementada de forma contínua durante las últimas casi tres décadas (Angeli & Rizzi, 2013; Bosch *et al*., 1998; Charmillot, 1990; Cichón & Fernández, 1999; Gut & Brunner, 1996; Witzgall *et al*., 2008).

La distribución de la feromona en campo se puede conseguir mediante una amplia gama de difusores disponibles en el mercado, mediante tratamientos con feromona microencapsulada o bien mediante la técnica de autoconfusión. Los difusores son el sistema más utilizado, y los de liberación controlada están ganando importancia debido a su fácil distribución en las fincas. Sin embargo, existen factores limitantes en el éxito de la confusión sexual: la necesidad de niveles poblacionales bajos (Vickers & Rothschild, 1991) y la necesidad de un sistema de monitoreo de la plaga fiable (Gut & Brunner, 1996).

Tabla 1. Clasificación de las materias activas insecticidas registradas contra carpocapsa en España según la clasificación de IRAC.

Grupo principal. Punto de acción primario	Subgrupo químico	Materia activa
1. Inhibidores de la acetilcolinesterasa	1B. Organofosforados	Metil-clorpirifos, fosmet
3. Moduladores del canal de sodio	3A. Piretroides y piretrinas	Lambda-cihalotrín, betaciflutrín, deltametrín
4. Agonista del receptor nicotínico de la acetilcolina	4A. Neonicotinoides	Tiacloprid
5. Activadores del receptor alostérico nicotínico de la acetilcolina	Spinosines	Spinosad
7. Míméticos de la hormona juvenil	7B. Fenoxicarb	Fenoxicarb
18. Agonistas del receptor de ecdisona	Diacilhidracinas	Metoxifenocida, tebufenocida
22. Bloqueadores del canal de sodio dependiente del voltaje	22A. Indoxacarb	Indoxacarb
28. Moduladores del receptor de la rianodina	Diamidas	Clorantraniliprol

Control microbiológico

Distintos organismos entomopatógenos, virus, bacterias, hongos y nematodos, pueden ser medios efectivos de control de carpocapsa combinados con otras técnicas como confusión sexual o/y insecticidas de bajo riesgo. Son seguros para el medio ambiente, los enemigos naturales, los aplicadores y los consumidores, además de poder aplicarse en el momento justo antes de la recolección. Lacey & Shapiro-Ilan (2008) realizaron una revisión de los organismos de control microbiológico aplicados en frutales de pepita y hueso.

Los entomopatógenos comercializados en España son la bacteria *Bacillus thuringiensis*, los nematodos del género *Steinernema* (Família: Steinernematidae) y el virus de la granulosis de *C. pomonella* (CpGv, granulovirus o carpovirusine) (MAPAMA, 2017).

Control biológico

Los enemigos naturales de carpocapsa incluyen pájaros, arañas e insectos, además de los organismos entomopatógenos, ya citados anteriormente. Una revisión de las posibilidades de control biológico se encuentra en Falcon & Huber (1991). La elevada incidencia de la plaga en las zonas productivas más templadas, además de la presencia de otras plagas también importantes, hace muy difícil que se llegue a un control efectivo mediante enemigos naturales. La aplicación de insecticidas poco selectivos también va en detrimento de dicho control. Únicamente en las zonas donde, por condiciones climáticas, carpocapsa ve limitada su reproducción se puede conseguir un control biológico de carpocapsa aceptable.

Control autocida

Se basa en la técnica de liberación de machos estériles para que se apareen con las hembras de campo y no se produzca una descendencia. Tiene varias limitaciones para que resulte exitosa como por ejemplo la necesidad de que las áreas tratadas estén aisladas, la cría en masa de carpocapsa, la coordinación de la región tratada, y los altos costes de la producción y liberación de los insectos estériles. En el Valle de Okanagan, en British Columbia (Canadá) el año 1994 se puso en marcha un programa en 8.000 ha de huertos de manzanos y perales que ha conseguido reducir la aplicación de insecticidas contra carpocapsa un 96 %, aunque no ha conseguido la erradicación de la plaga (Tyson *et al*., 2007).

Control cultural

Se basa en medidas preventivas como el uso de trampas de cartón corrugado en los troncos para la captura de larvas diapausantes o la eliminación de los frutos atacados durante la campaña. Estas medidas culturales no sirven, por sí solas, para controlar la plaga pero sí son útiles para reducir el nivel de población (Judd *et al*., 1997).

Resistencia de *Cydia pomonella* a insecticidas

Uno de los factores más importantes en la evolución de los sistemas de control de carpocapsa a lo largo de las distintas eras ha sido el estado de la resistencia de sus poblaciones a los productos insecticidas del momento (Croft & Riedl, 1991; Walker *at al*., 2017).

IRAC define la resistencia como "la reducción en la sensibilidad de una población, que se refleja en repetidos fallos de un producto, que reduce sus expectativas de control al ser utilizado a la dosis recomendada para la plaga determinada, siempre que los problemas de almacenamiento del producto, su aplicación y los factores climáticos puedan ser eliminados" (IRAC, 2017; Silva, 2003).

Existen distintos libros que definen y describen el problema de la resistencia a insecticidas y/o pesticidas (Roush & Tabashnik, 1990; Stenersen, 2004; Yu, 2008) y una base de datos, Arthropod Pesticide Resistance Database (2017), donde se encuentran registrados los casos de resistencia en artrópodos a nivel mundial desde 1914 y que da una idea de la extensión del problema. En carpocapsa están registradas poblaciones resistentes a veintidós ingredientes activos en diecisiete países.

Mecanismos de resistencia

Existen distintos mecanismos de resistencia a los insecticidas. Yu (2008) los clasifica como resistencia por comportamiento, que define como el desarrollo de una habilidad que evita que la dosis de insecticida sea letal, y resistencia fisiológica, la cual divide a su vez en tres mecanismos más: reducida penetración del insecticida, incremento de la detoxificación del insecticida e insensibilidad en el sitio de acción del insecticida. En carpocapsa se han detectado hasta el momento dos de estos tres mecanismos de resistencia fisiológica.

Los mecanismos de la resistencia metabólica encargados de la detoxificación de los insecticidas en carpocapsa son mecanismos enzimáticos y son los más frecuentes en los insectos. Estos sistemas enzimáticos se encargan de la degradación de las sustancias activas de forma que el insecto sea capaz de metabolizarlas y eliminarlas e incluyen la participación de las citocromo P450 polisustrato monooxigenasas (PSMO), las glutatión-S-transferasas (GST) y las esterasas (EST). Existe numerosa bibliografía que explica el modo de acción de cada uno de estos mecanismos (Feyereisen, 1999; Oakeshoot *et al.*, 2005, 2010; Ranson, 2010; Ransom & Hemingway, 2005; Roush & Tabashnik, 1990; Stenersen, 2004; Silva, 2003; Yu, 2008). En las poblaciones españolas de campo de carpocapsa se han detectado altos niveles de detoxificación enzimática, debido principalmente a las PSMO, en larvas neonatas, larvas post-diapausantes y adultos, aunque también se ha detectado elevada actividad de las GST, en larvas post-diapausantes y adultos, y de las EST, en larvas post-diapausantes (Rodríguez *et al.*, 2010, 2011a, 2011b).

Se han determinado dos tipos de resistencia por insensibilidad en el sitio de acción en carpocapsa debidas a mutaciones en la proteína diana del insecticida: la mutación de la acetilcolinesterasa o acetilcolinesterasa insensitiva (*AChE*) y la insensibilidad nerviosa o *knockdown* resistance (*kdr*). La mutación *AChE*, descrita por Cassanelli *et al.* (2006) en una población seleccionada en laboratorio para resistencia a organofosforados, confiere resistencia a organofosforados y carbamatos y consiste en la sustitución de un único aminoácido en el gen *ace-1* (F399V). Esta mutación fue también detectada en una población de campo (Reyes *et al*., 2007). Ambas poblaciones eran originarias de la zona productiva de frutales de pepita de Lleida. La mutación *kdr* es también debida a una sustitución de un aminoácido en la posición 1014 de un gen en el canal de sodio dependiente del voltaje (Brun-Barale *et al*., 2005) y afecta a la eficacia de los piretroides. Esta mutación, al contrario de lo que ocurre con la mutación *AChE*, se encuentra ampliamente distribuida por Europa (Reyes *et al*., 2007, 2009).

A partir de esos mecanismos, se definen distintos tipos de resistencia:

Resistencia múltiple. Resistencia a varios insecticidas o modos de acción no relacionados debido a la presencia de múltiples mecanismos de resistencia en el mismo insecto. En general, esta resistencia es debida a la selección de individuos de la población de insectos mediante diversos insecticidas. Ejemplos de resistencia múltiple en poblaciones europeas de carpocapsa están en Reyes *et al*. (2007).

Resistencia cruzada. Se produce cuando una población resistente a un producto insecticida desarrolla automáticamente resistencia a otro insecticida al cual no ha sido nunca expuesta. Un caso documentado de este tipo de resistencia en carpocapsa se produjo con los insecticidas tebufenocida y fenoxicarb (Ioriatti *et al*., 2007, Reyes *et al*., 2007).

Resistencia cruzada negativa. Se produce cuando la resistencia a un insecticida o modo de acción conlleva al aumento de la susceptibilidad a otro insecticida o modo de acción. Existen pocos casos de este tipo de resistencia, sin embargo, en carpocapsa está documentada con los insecticidas metilazinfos y etil-clorpirifos (Dunley & Welter, 2000).

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OBJETIVOS

Objetivos

Debido al potencial que presentan al desarrollo de resistencias las poblaciones de carpocapsa de la zona del Valle del Ebro estudiadas hasta el momento es imprescindible implementar un programa de manejo de las mismas en el que se debe incluir el seguimiento continuado de los niveles y de los mecanismos de resistencia en campo, así como el desarrollo de líneas base de susceptibilidad a nuevos productos insecticidas de forma previa a su aparición en el mercado. Estas líneas base servirían como referencia a lo largo del tiempo en el seguimiento de la evolución de la resistencia a los nuevos productos insecticidas.

En la zona productiva de Cataluña y Aragón se han utilizado de forma intensiva los organofosforados, principalmente metil-azinfos, para el control de carpocapsa, mientras que carbaryl se utilizaba para el aclareo de la manzana, por lo que la primera generación de carpocapsa quedaba afectada por dicho tratamiento. Existe un problema de resistencia en nuestras fincas a nivel enzimático, pero no se conoce la posible extensión de la mutación *AChE* ni la presencia de la mutación *kdr*.

En un programa de Control Integrado de Plagas (CIP) el muestreo es un componente crucial. Para realizar un seguimiento del vuelo de los adultos de carpocapsa en campo se utilizan trampas de feromona cebadas con 1 o 10 mg de feromona, según si la finca está en confusión sexual o no, respectivamente, además de realizar muestreos visuales de frutos. Actualmente, en las fincas en confusión sexual se utiliza de forma mayoritaria trampas cebadas con el difusor Pherocon CM-DA Combo™ (Trécé Inc, USA), compuesto por la combinación de 3 mg de cairomona y 3 mg de feromona. El hecho de que los adultos de carpocapsa respondieran en mayor o menor medida a las trampas cebadas con cairomona en función de los niveles de resistencia podría implicar la posibilidad de mejorar el monitoreo en las fincas con una elevada presencia de insectos resistentes.

Objetivo general

El objetivo general de la tesis es determinar el estado general de los niveles de resistencia de *C. pomonella* y de los mecanismos de resistencia presentes en poblaciones de campo españolas para determinar su influencia en el seguimiento y control de la plaga en campo y establecer una estrategia de manejo de la resistencia.

Objetivos específicos

Los objetivos específicos son los siguientes:

a) Evaluar la resistencia a insecticidas de *C. pomonella* en 3 áreas de producción españolas con dos sistemas de manejo de plagas completamente distintos, determinando la eficacia insecticida de nuevos productos sobre larvas neonatas de poblaciones que presenten, en algún caso y dependiendo de la zona, resistencia a los insecticidas tradicionalmente utilizados, como son los organofosforados y piretroides.

b) Determinar la extensión de las mutaciones *AChE* y *kdr* en poblaciones españolas de *C. pomonella*.

c) Realizar el estudio de una línea base de resistencia insecticida de carpocapsa con chlorantraniliprole (Rynaxypyr®), materia activa de una nueva familia insecticida, en poblaciones españolas y europeas, así como el seguimiento de la evolución de la resistencia al mismo a lo largo de los años.

d) Estudiar la frecuencia de adultos resistentes (con un elevado nivel de enzimas de detoxificación PSMO) de *C. pomonella* capturados en trampas con distintos difusores, con y sin la presencia de cairomona, durante las distintas generaciones y en fincas con distintos sistemas de manejo.

CAPÍTULO 1

Monitoring resistance of *Cydia pomonella* (L.) Spanish field populations to new chemical insecticides and the mechanisms involved

Monitoring resistance of *Cydia pomonella* (L.) Spanish field populations to new chemical insecticides and the mechanisms involved

Bosch, D.[1]; Rodríguez, M.[2]; Avilla, J.[3]

[1] Institut Recerca i Tecnologia Agroalimentària (IRTA), Av. Alcalde Rovira Roure, 191. 25198 Lleida, Spain. *Corresponding autor*.

[2] Universidad de Concepción, Castilla 160-C, Concepción, Chile

[3] Department of Crop and Forest Sciences, Agrotecnio, University of Lleida, Rovira Roure, 191. 25198 Lleida, Spain

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Abstract

A widespread resistance of *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) to organophosphates was demonstrated in populations from the Spanish Ebro Valley area that showed high levels of enzymatic detoxification. To determine the efficacy of new insecticides, neonate larvae bioassays were carried out on twenty field codling moth populations collected from three different Spanish apple production areas. Synergist bioassays were performed to detect the enzymatic mechanisms involved.

The least active ingredients were methoxyfenozide, with 100% of the populations showing significantly lower mortality than the susceptible strain, and lambda-cyhalothrin, with very high resistant ratios (872.0 for the most resistant field population). Approximately 50% of the populations were resistant or tolerant to thiacloprid. By contrast, tebufenocide was very effective in all the field populations, as was chlorpyrifos-ethyl despite its widespread use during the last few years. Indoxacarb, spinosad and Rynaxypyr also provided good control, as did emamectin and spinetoram, which are not yet registered in Spain.

The resistant Spanish codling moth populations can be controlled using new reduced-risk insecticides. The use of synergists showed the importance of the concentration applied and the difficulty of interpreting the results in field populations that show multiple resistance to different active ingredients.

Introduction

Since the 1990s the integrated pest management (IPM) program in apple orchards in Spain has focused on biological control of *Panonychus ulmi* (Koch) (Acari: Tetranychidae), using naturally occurring phytoseiid populations and achieving great success in most orchards (Avilla *et al.*, 1993). Since the late 1990s, the main pest to control has been the codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae). Organophosphates (OPs), especially azinphos-methyl, have been the most important chemical insecticide group used to control codling moth in apple, pear and walnut orchards. They have been used intensively for the last thirty years in Spain and in other apple production regions of the world (Croft & Riedl, 1991; Dunley & Welter, 2000; Fuentes-Contreras *et al.*, 2007; Reuveny & Cohen, 2004; Soleño *et al.*, 2012). The present control strategy for codling moth relies mainly on mating disruption, especially in apple and walnut orchards, where the pest is more difficult to control, but chemical insecticides are widely used to reinforce the system. In Spain, the characteristics of some parts of the production area, with small orchards and mixed crops, make the application of this strategy more complex and the use of insecticides more necessary.

Codling moth resistance to pesticides is well documented and began a long time ago with arsenate and DDT (Glass & Fiori, 1955; Hough, 1928). It now affects almost every class of synthetic insecticide and is spread throughout the world's apple production regions (Dunley & Welter, 2000; Fuentes-Contreras *et al.*, 2007; Ioratti *et al.*, 2007; Iscy & Ay, 2017; Knight *et al.*, 1994; Reuveny & Cohen, 2007; Reyes *et al.*, 2007; Sauphanor *et* al., 1998, 2000; Soleño *et al.*, 2008; Stará & Kocourek, 2007; Thwaite *et al.*, 1993). The number of available insecticides against codling moth has fallen drastically in the EU since the re-registration of active substances covered by Directive 91/414/EEC, and some of the products most used to control codling moth are no longer available. When azinphos-methyl was prohibited in Spain in 2008, widespread resistance to the product and to OPs in general was demonstrated in problematic orchards of the Ebro Valley area (Catalonia and Aragon, NE Spain) (Rodríguez *et al.*, 2010, 2011a). Chlorpyrifos-ethyl then became the most widely used insecticide and was very active against neonate larvae, the main target instar of codling moth, despite also belonging to the chemical class of OPs (Rodríguez *et al.*, 2011a). Negative cross-resistance between chlorpyrifos-ethyl and two other OPs, azinphos-methyl and methyl-parathion, was observed in field populations of codling moth adults in California by Dunley & Welter (2000), and also in neonate larvae of Spanish populations by Rodríguez *et al.* (2011a). During the last few years, the use of pyrethroids has also increased due to the low prices of fruits and the attempt of growers to reduce production costs. Knight (2010) reports a gradual increase in the use of lambda-cyhalothrin since 2005. OPs and other broad spectrum insecticides, such as pyrethroids and carbamates, act by contact and/or ingestion and are effective against multiple pests and different target instars, but they have low selectivity to natural enemies and high mammalian toxicity, and cause environmental contamination.

A number of codling moth insecticides classified as reduced-risk or OP alternatives have been registered in Spain since the 1980s and are recommended by IPM programs: the insect growth regulator fenoxycarb, the neonicotinoid thiacloprid, the ecdysone receptor agonists methoxyfenozide and tebufenozide, the voltage-dependent sodium channel blocker indoxacarb, the nicotinic acetylcholine receptor allosteric modulator spinosad and, more recently, the ryanodine receptor modulator chlorantraniliprole. Codling moth cross-resistance among some of these reduced-risk insecticides groups and OPs and pyrethroids has been detected in some European countries (Reyes *et al.*, 2007; Stará & Kocourek, 2007; Voudouris *et al.* 2011), the USA (Magalhaes *et al.* 2012; Mota-Sanchez *et al.,* 2008) and Canada (Grigg-McGuffin *et al.*, 2015), even with pesticides that have been registered recently (Knight *et al.,* 2001; Reyes *et al.,* 2007) or have not yet been registered (Sauphanor & Bouvier, 1995). No codling moth susceptibility study has been reported with these new chemical insecticides, except thiacloprid, which has been tested in Spanish field populations, showing general high levels of enzymatic detoxification, mainly due to cytochrome P450 polysubstrate monooxygenases (PSMO) (in neonate larvae, adults and post-diapausing larvae), but also due to glutatione S-transferases (GST) (in adults and post-diapausing larvae) and esterases (EST) (in post-diapausing larvae) (Rodríguez *et al.,* 2010, 2011a).

The objectives of this work were to evaluate the insecticide resistance of *C. pomonella* in three areas of apple production in Spain with two completely different management systems and to determine the efficacy of some new insecticides in controlling codling moth neonate larvae, paying special attention to populations that showed insecticide resistance with the most commonly used products. French field populations from the neighboring area were also tested. Tests with synergists were performed on some field populations to explain the enzymatic mechanisms that may be involved in resistance.

Material and Methods

Insects

Twenty field populations of codling moth were collected as diapausing larvae in 2010, 2011 and 2012 (Table 1). The populations came from three different Spanish apple production areas: eleven from Catalonia, three from Aragon (both located in the Ebro Valley, northeast Spain, with a maximum distance of about 190 km between orchards) and three from Asturias (northern Spain). Three field populations from the southeast of France (Provence-Alpes-Côte d'Azur region) were also studied. The populations were mostly from IPM orchards, but the population Coll (from Catalonia) was an organic orchard with codling moth control problems. The three Asturian orchards produced cider-apples and the rest produced table apples, so we had two, well-distinguished pest management systems. The susceptible strain S_Spain was collected from an abandoned apple orchard in Lleida in 1992 and has been reared since then using a semi-artificial dehydrated apple diet at the joint IRTA (Institute for Food and Agricultural Research and Technology) and UdL (University of Lleida) laboratory (Lleida, Spain).

Insecticides and synergists

Ten insecticides (expressed below with mode of action followed by chemical class) were tested using commercial formulations (Table 2). Two of them are or were commonly used in the IPM orchards to control codling moth: the acetylcholinesterase inhibitor organophosphate chlorpyrifos-ethyl (prohibited at present), and the sodium channel modulator pyrethroid lambda-cyhalothrin. Four of them are hardly used: the nicotinic acetylcholine receptor competitive modulator neonicotinoid thiacloprid, the ecdysone receptor agonist diacylhydrazines methoxyfenozide and tebufenozide, and the voltage-dependent sodium channel blocker oxadiazine indoxacarb. Other products tested were recently or not yet registered in Spain at the moment of field population collection: the nicotinic acetylcholine receptor allosteric modulator spinosyns spinosad (registered in 2013) and spinetoram (not yet registered), the ryanodin receptor modulator diamide chlorantraniliprole, or Rynaxypyr, (registered in October 2011), and the glutamate-gated chloride channel allosteric modulator avermectin emamectin benzoate (not yet registered) (Insecticide Resistance Action Committee, 2017). The synergists used were piperonyl butoxide (PBO, 90% purity, distributed by Fluka) as a microsomal monooxygenase inhibitor (PSMO), diethyl maleat (DEM, 97% purity, distributed by Sigma Aldrich) as a

Table 1. *Cydia pomonella* field population name and origin, year of collection, orchard management system and assay performed with them. The neonate larva generation treated is also indicated.

Name	*Country* / Region	Collection year	Generation treated	Assay	Management
PuigverdB	*Spain* / Catalunya	2011	F1	Synergists / bioassays	IPM
PuigverdC	*Spain* / Catalunya	2011	F1	Synergists / bioassays	IPM
Tossal	*Spain* / Catalunya	2010	F2	Synergists	IPM
Tossal	*Spain* / Catalunya	2012	F2	Bioassays	IPM
Torregrossa	*Spain* / Catalunya	2010	F1	Synergists	IPM
Coll	*Spain* / Catalunya	2010	F1	Synergists	Ecological
Linyola	*Spain* / Catalunya	2010	F2	Bioassays	IPM
Paradet	*Spain* / Catalunya	2010	F2	Bioassays	IPM
SAS	*Spain* / Catalunya	2011	F2	Bioassays	IPM
Mir7/84	*Spain* / Catalunya	2011	F1	Bioassays	IPM
Poalbud	*Spain* / Catalunya	2011	F2	Bioassays	IPM
La Almunia	*Spain* / Aragón	2010	F2	Bioassays	IPM
Tamarite	*Spain* / Aragón	2010	F1 / F2	Synergists / bioassays	IPM
ADC	*Spain* / Aragón	2011	F1	Bioassays	IPM
AstAb	*Spain* / Asturias	2012	F1	Bioassays	Cider production
AstN	*Spain* / Asturias	2012	F1 /F2	Bioassays	Cider production
AstC	*Spain* / Asturias	2012	F1 /F2	Bioassays	Cider production
Le Thor	*France* / Provence-A.C.A.	2012	F2	Bioassays	IPM
Pomiers	*France* / Provence-A.C.A.	2012	F2	Bioassays	IPM
Noves	*France* / Provence-A.C.A.	2010	F1	Synergists	IPM

GST inhibitor, and S,S,S-tributyl phosphorotrithioate (DEF, 98% purity, distributed by Sigma Aldrich) as an EST inhibitor. The insecticides were diluted in distilled water and the three synergists in 96% acetone.

Insecticide efficacy bioassays

The bioassay to test the insecticide efficacy of each product was performed using the diagnostic concentrations that produced approximately 90% mortality in the susceptible population, S-Spain, hereinafter LC_{90}. These concentrations (Table 2) were previously determined from a concentration-mortality curve and were corroborated every year in the susceptible population during the field population treatments (from 2012 to 2014). Microplate wells were filled with 150 µL of artificial diet (Stonefly Industries Ltd) and six µL of each insecticide's LC_{90} was applied to the surface of the diet. Distilled water replaced the insecticide in the controls. Thirty minutes after the treatment, newly hatched larvae (0-24 h old) were individually placed in each well and transferred to controlled conditions (25 ± 1ºC and 16:8 [L:D] h photoperiod). Mortality was recorded after four days. Larvae were considered dead when they did not respond to a probe with dissecting forceps. Missing larvae were subtracted from the initial number. Fourteen Spanish (eight from Catalonia, three from Aragon and three from Asturias) and two French field codling moth populations were treated. Depending on the neonate larvae obtained in the progenies, 3–10 insecticides per population were tested.

Synergist bioassays

The synergists were dissolved in acetone and the concentrations used were 2.5 mg a.i./L for PBO, 10 mg a.i./L for DEM and 5 mg a.i./L for DEF. The concentration of each synergist to be applied was previously calculated with the laboratory population, S-Spain, and was the one that produced approximately 10% mortality. The neonate larvae were exposed by contact to the synergist for one h before feeding on the treated diet following the same methodology as in the insecticide efficacy bioassays. The insecticide concentrations used are shown in Table 2 and were those that produced approximately 50% mortality in S-Spain, hereinafter LC_{50}. Six Spanish (five from Catalonia and one from Aragon) and one French field codling moth populations were treated. Only three field populations had a sufficient number of progeny for insecticide and synergist bioassays to be performed. The insecticides chlorpyrifos-ethyl and lambda-cyhalothrin and the synergist PBO were prioritized in the assays.

Table 2. Active ingredients and commercial products. The concentration (mg a.i./L) applied corresponds approximately to the LC_{90} and LC_{50} of the *C. pomonella* susceptible laboratory population for the insecticide efficacy and the synergist tests, respectively.

Active ingredient	**Commercial product - Supplier**	**Assay**	**Concentration (mg a.i./L)**
Chlorpyrifos-ethyl	Cuspide - 25 % - Comercial Química Massó, Spain	Insecticide bioassay Synergists bioassay	90.0 31.3
Lambda-cyhalothrin	Karate Zeon CS - 10 % - Singenta España S.A., Spain	Insecticide bioassay Synergists bioassay	0.5 0.1
Rynaxypyr	Coragen 20 SC - DuPont Ibérica S.L. , Spain	Insecticide bioassay Synergists bioassay	5.0 4.0
Emamectin	Affirm - 0,855 % - SG- Syngenta, Italy	Insecticide bioassay Synergists bioassay	0.6 0.3
Methoxyfenozide	Runner CS - 24 % - Dow AgroSciences Ibérica S.A., Spain	Insecticide bioassay	4.0
Tebufenozide	Mimic 2F CS - 24 % - Nisso Chemical Europe GMBH, Germany	Insecticide bioassay	20.0
Thiacloprid	Calypso SC - 48 % - Bayer CropScience S.L., Spain	Insecticide bioassay	15.0
Indoxacarb	Steward - 30 % - WP - DuPont Ibérica, S.L., Spain	Insecticide bioassay	40.0
Spinosad	Spintor 480 SC - 48 % - Dow AgroScience, Spain	Insecticide bioassay	16.0
Spinetoram	Delegate - 25 % - Dow AgroSciences, France	Insecticide bioassay	1.0

Data analysis

The mortalities were corrected using Abbot's formula (Abbot, 1925). To calculate the insecticide efficacy, the correction factor was the mortality of the solvent-treated control (water), and for the synergistic effect, the correction factor was the mortality produced by the synergist. In the insecticide efficacy studies, the difference between the efficacies of each insecticide in the field populations was compared with that in S-Spain, tested in the same year of the field population bioassay using a Pearson χ^2 test. Resistance ratios (RR) were determined by dividing the mortality of S-Spain, obtained with the LC_{90}, by that of the field population. Populations were classified as resistant ($RR \geq 10$), tolerant ($1 < RR < 10$) and susceptible ($RR \leq 1$) (Grigg-McGuffin *et al.*, 2015). To assess the degree of synergism, synergistic ratios (SR) were calculated by dividing the mortality obtained by the LC_{50} of the insecticide plus synergist application by that of the insecticide alone. The differences between the corrected mortality obtained by each insecticide was compared with that obtained by the synergist plus the insecticide using a Pearson χ^2 test.

Results

Insecticide efficacy bioassays

During the study period, when the efficacy bioassays were done, S-Spain showed a similar mortality to the LC_{90} of the insecticides tested (Table 3). The only exception was during the 2013 bioassays, when methoxyfenozide produced 84.2% mortality, which was significantly lower than that obtained in the 2012 and 2014 bioassays, 92.4% and 97.1%, respectively.

The level of susceptibility of the codling moth field populations to the tested insecticides differed greatly depending on the apple production area. The field populations from Asturias were in general as susceptible as, or even significantly more susceptible than, the S-Spain population in 86% of the bioassays (AstAb to lambda-cyhalotrin and Rynaxypyr: dF = 1, $\chi^2 = 4.43$, p = 0.0354 and dF = 1, $\chi^2 = 4.43$, p = 0.0012, respectively). The products that showed significantly lower mortality than S-Spain in some field populations were chlorpyrifos-ethyl (AstN: dF = 1, $\chi^2 = 4.39$, p = 0.0362) and methoxyfenozide (AstN and AstC: dF = 1, $\chi^2 = 8.11$, p = 0.0044 and dF = 1, $\chi^2 = 4.33$, p

= 0.0373, respectively). The Asturian population with the lowest susceptibility was AstN, which was significantly different from S-Spain in two of the seven bioassays.

In the efficacy bioassays, the three field populations from Aragon showed significantly lower mortality than S-Spain in 28% of the bioassays tested. The Tamarite and La Almunia populations were as susceptible as S-Spain in all the bioassays, but the Albalate de Cinca (ADC) population was significantly less susceptible than S-Spain in five of the ten bioassays: lambda-cyhalothrin (dF = 1, $\chi^2 = 57.47$, p = 3.43 x 10^{-14}), methoxyfenozide (dF = 1, $\chi^2 = 34.68$, p = 3.90 x 10^{-9}) thiacloprid, (dF = 1, $\chi^2 = 34.68$, p = 27.85 x 10^{-7}), indoxacarb (dF = 1, $\chi^2 = 11.36$, p = 0.0008) and Rynaxypyr (dF = 1, $\chi^2 = 10.45$, p = 0.0012). However, the ADC population was significantly more susceptible than S-Spain to spinosad (dF = 1, $\chi^2 = 7.71$, p = 0.0301).

In the efficacy bioassays the eight field populations of Catalonia showed significantly lower mortality than S-Spain in 43% of the bioassays tested. The populations SAS, Mir7/84 and Tossal showed lower susceptibility than S-Spain in 50% of the products tested and PuigverdC in 80%. Linyola was the only field population as susceptible as S-Spain to all the five products tested. Methoxyfenozide showed significantly lower mortality than S-Spain in 100% of the Catalan field populations: SAS (dF = 1, $\chi^2 = 40.09$, p = 8.71 x 10^{-11}), PuigverdB (dF = 1, $\chi^2 = 13.93$, p = 0.0002), Poalbud (dF = 1, $\chi^2 = 11.78$, p = 0.0006), PuigverdC (dF = 1, $\chi^2 = 59.55$, p = 1.19 x 10^{-14}), Mir7/84 (dF = 1, $\chi^2 = 60.55$, p = 7.19 x 10^{-15}) and Tossal (dF = 1, $\chi^2 = 50.92$, p = 9.60 x 10^{-13}). Lambda-cyhalothrin and thiacloprid showed significantly lower mortality than S-Spain in 75% of the populations: SAS (dF = 1, $\chi^2 = 30.30$, p = 3.70 x 10^{-8}), PuigverdB (dF = 1, $\chi^2 = 67.61$, p = 1.99 x 10^{-16}), PuigverdC (dF = 1, $\chi^2 = 73.63$, p = 9.42 x 10^{-18}), Mir7/84 (dF = 1, $\chi^2 = 73.63$, p = 9.42 x 10^{-18}), Tossal (dF = 1, $\chi^2 = 52.17$, p = 5.10 x 10^{-13}) and Paradet (dF = 1, $\chi^2 = 5.39$, p = 0.020). Thiacloprid showed significantly lower mortality than S-Spain in 66% of the populations: SAS (dF = 1, $\chi^2 = 9.87$, p = 0.0017), PuigverdB (dF = 1, $\chi^2 = 6.99$, p = 0.0082), PuigverdC (dF = 1, $\chi^2 = 46.10$, p = 1.12 x 10^{-11}) and Mir7/84 (dF = 1, $\chi^2 = 23.40$, p = 1.32 x 10^{-6}). Fifty percent of the populations tested with indoxacarb obtained significantly lower susceptibility than S-Spain (PuigverdC: dF = 1, $\chi^2 = 1.17$, p = 0.0074, and Mir7/84: dF = 1, $\chi^2 = 5.95$, p = 0.0147). All the populations tested with spinosad were as susceptible as S-Spain, but two populations of the five tested with spinetoram were significantly less susceptible than S-Spain (PuigverdB: dF = 1, $\chi^2 = 4.38$, p = 0.0363 and PuigverdC: dF = 1, $\chi^2 = 17.02$, p = 3.70 x 10^{-5}). Despite being hardly used, chlorpyrifos-ethyl showed significantly lower mortality than S-Spain in just two of the seven populations tested: PuigverdC (dF = 1, $\chi^2 = 21.17$, p = 4.20 x 10^{-6}) and Mir7/84 (dF = 1, $\chi^2 = 6.21$, p = 0.0127). This was the same proportion as for the new active ingredient Rynaxypyr in SAS (dF = 1, $\chi^2 = 26.39$, p = 2.79 x 10^{-7}) and PuigverdC (dF = 1, $\chi^2 = 11.72$, p = 0.0006). Emamectin obtained significantly lower mortality than S-Spain in only one of the eight field populations treated, SAS (dF = 1, $\chi^2 = 12.91$, p = 0.0003). In contrast with the methoxyfenozide results, all five field populations tested with tebufenozide were as susceptible as S-Spain.

The two field populations from France were significantly less susceptible than S-Spain in 56% of the bioassays. Neither of them was treated with chlorpyrifos-ethyl due to the number of available neonate larvae. Both the Pompiers and Le Thor populations were significantly less susceptible than S-Spain to lambda-cyhalothrin (dF = 1, $\chi^2 = 12.79$ and p = 0.0003 and dF = 1, $\chi^2 = 19.52$ and p = 9.93 x 10^{-6}, respectively), methoxyfenozide (dF = 1, $\chi^2 = 38.87$, p = 4.54 x 10^{-10} and dF = 1, $\chi^2 = 13.64$ and p = 0.0002, respectively) and thiacloprid (dF = 1, $\chi^2 =19.82$, p = 8.53 x 10^{-6} and dF = 1, $\chi^2 =9.27$ and p = 0.0023, respectively), and both were as susceptible as S-Spain to indoxacarb, spinetoram and emamectin. Only Le Thor was treated with tebufenozide and spinosad, and tebufenozide produced a significantly lower mortality in the field population than in S-Spain (dF = 1, $\chi^2 = 13.63$, p = 0.0002).

According to these results, methoxyfenozide was the least effective insecticide, obtaining significantly lower mortality than in S-Spain in 100% of the field populations tested, regardless of their origin, followed by lambda-cyhalothrin and thiacloprid, which obtained significantly lower mortality in 60% and 54% of the populations, respectively. The most effective insecticides were spinosad and emamectin, which were as effective as in S-Spain in 100% and 94% of the field populations, respectively, while tebufenozide, spinetoram and chlorpyrifos-ethyl were significantly less effective in 13%, 20% and 23% of the treated populations, respectively. Indoxacarb and Rynaxypyr obtained significantly lower mortality than in S-Spain in 33% and 25% of the populations, respectively.

Considering RRs, lambda-cyhalothrin was the insecticide with most resistant field populations (PuigverdC, Mir7/84 and PuigverdB, with RRs of 872.0, 148.1 and 15.4, respectively). PuigverdC and Mir7/84 were also resistant to methoxyfenozide (RRs of 14.6 and 15.9, respectively) and PuigverdC was also resistant to thiacloprid (RR of 11.2). All the populations treated with methoxyfenozide were resistant or tolerant to the product.

Of the field populations 81% and 87% were susceptible to emamectin and spinosad, respectively (RR ≤ 1). For the rest of the products, the percentage of field populations as susceptible as the S-Spain ranged from 42% to 60%.

Synergist bioassays

Only three field populations in which insecticide efficacy bioassays were performed had enough larvae to also allow bioassays to be carried out with synergists (Tamarite, PuigverdC and PuigverdB). The field populations named Tossal were collected in two different years and were considered distinct populations. Table 4 shows the corrected mortality produced by the LC_{50} used in the synergist bioassays. The mortality obtained in the control treatment of the eight field populations ranged between 0.00% and 11.11%. Lambda-cyhalothrin was the product with a highest number of populations with significant lower mortality than S-Spain in PuigverdB (dF = 1, $\chi^2 = 43.75$, p = 3.73×10^{-11}), PuigverdC (dF = 1, $\chi^2 = 20.04$, p = 7.59×10^{-6}), Torregrossa (dF = 1, $\chi^2 = 5.03$, p = 0.0249) and Tossal (dF = 1, $\chi^2 = 41.86$, p = 9.80×10^{-11}), and chlorpyrifos-ethyl showed significantly lower mortality than in S-Spain only in PuigverdC (dF = 1, $\chi^2 = 5.63$, p = 0.0177). By contrast, some emamectin treatments showed significant higher mortality than in S-Spain: PuigverdC (dF = 1, $\chi^2 = 9.47$, p = 0.0021), Torregrossa (dF = 1, $\chi^2 = 14.29$, p = 0.0002) and Tossal (dF = 1, $\chi^2 = 18.01$, p = 2.20×10^{-5}).

The corrected mortality obtained with the application of the different synergists before the treatment with chlorpyrifos-ethyl, lambda-cyhalothrin, emamectin and Rynaxypyr to some field populations is shown in Table 5. In no cases did the application of a synergist modify the mortality obtained in the susceptible population, S_Spain. A significant synergistic effect was observed with PBO only in a few field populations when it was applied before any tested insecticides. The corrected mortality significantly increased with the synergist in one of the eight field populations treated with chlorpyrifos-ethyl, Torregrossa (dF = 1, $\chi^2 = 13.79$, p = 0.0002), and in one of the eight field populations treated with lambda-cyhalothrin, Tossal (dF = 1, $\chi^2 = 21.86$, p = 2.90×10^{-6}). A significant increase in mortality was also observed when PBO was applied before emamectin in the field population PuigverdB (dF = 1, $\chi^2 = 3.94$, p = 0.0470). No significant synergistic effect was observed with DEM in lambda-cyhalothrin treatments. In two field populations treated with chlorpyrifos-ethyl, treatment with DEM resulted in a significant increase of mortality: Noves (dF = 1, $\chi^2 = 4.12$, p = 0.0424) and Torregrossa (dF = 1, $\chi^2 = 8.42$, p = 0.0037). The same occurred in two other treatments of emamectin: Tamarite (dF = 1, χ^2

= 15.86, p = 6.80 x 10^{-5}) and PuigverdB (dF = 1, χ^2 = 10.77, p = 0.0010). The synergist DEF significantly increased mortality to chlorpyrifos-ethyl and emamectin in the same field populations as DEM did. It increased mortality to chlorpyrifos-ethyl in Noves (dF = 1; χ^2 = 4.01, p = 0.0453) and Torregrossa (dF = 1; χ^2 = 10.01 and p = 0.0016), and it increased mortality to emamectin in Tamarite (dF = 1, χ^2 = 15.86, p = 6.80 x 10^{-5}) and PuigverdB (dF = 1, χ^2 = 6.31, p = 0.0120). The mortality also increased significantly when DEF was applied to the Torregrossa field population before the treatment with lambda-cyhalothrin (dF = 1, χ^2 = 4.68, p = 0.0306), which showed significantly less susceptibility than S-Spain.

The highest SR obtained was 17.1, when PBO was applied before lambda-cyhalothrin in the field population Tossal. For the rest of the field populations and treatments in which the application of synergists significantly increased the mortality, the SR ranged between 2.0 (PBO + chlorpyrifos-ethyl in Torregrossa) and 1.1 (DEM or DEF + chlorpyrifos-ethyl in Noves). The SRs obtained when Rynaxypyr was applied after a synergist ranged between 0.6 and 1.0. Significantly lower mortality was obtained in all the field populations previously treated with PBO and in two of the three field populations treated with DEM.

Table 3. Corrected mortality (%) of insecticides at the diagnostic concentration, LC$_{90}$ (mg a.i./L), on *C. pomonella* neonate larvae of the susceptible strain and of field populations. Numbers in parentheses show the number of insects treated. RR = corrected mortality of S-Spain / corrected mortality of the field population. The mortality obtained was compared using χ^2 (df = 1; *p = 0.05; **p = 0.01; ***p = 0.001).

Population	**Active ingredient (mg a.i./L)**									
	Chlorpyrifos-ethyl (90.0)		**Lambda-cyhalothrin (0.5)**		**Methoxyfenozide (5.0)**		**Tebufenozide (20.0)**		**Thiacloprid (15.0)**	
	C. Mort. (%)	**RR**	**C. Mort. (%)**	**RR**	**C. Mort. (%)**	**RR**	**C. Mort. (%)**	**RR**	**C. Mort. (%)**	**RR**
S-Spain-12	93.8 (33) a	-	87.2 (71) a	-	97.1 (36) a	-	86.4 (37) a	-	91.0 (35) a	-
S-Spain-13	95.5 (90) a	-	93.7 (48) a	-	84.2 (96) b	-	97.9 (47) a	-	93.7 (48) a	-
S-Spain-14	91.5 (87) a	-	93.3 (45) a	-	92.4 (46) a	-	89.2 (81) a	-	-	-
SAS	100.0 (35) ns	0.9	30.4 (26) ***	2.9	22.2 (36) ***	4.4	75.0 (36) ns	1.2	59.7 (34) **	1.5
PuigverdB	85.7 (36) ns	1.1	5.7 (37) ***	15.4	64.5 (70) ***	1.5	97.1 (36) ns	0.9	68.6 (72) **	1.3
Poalbud	85.6 (35) ns	1.1	71.5 (32) ns	1.2	65.9 (35) ***	1.5	97.2 (36) ns	0.9	94.4 (36) ns	1.0
PuigverdC	42.4 (36) ***	2.2	0.0 (35) ***	872.0	6.6 (35) ***	14.6	68.0 (27) ns	1.3	8.1 (33) ***	11.2
Mir7/84	72.0 (34) *	1.3	0.6 (35) ***	148.1	6.1 (36) ***	15.9	93.9 (34) ns	0.9	36.4 (36) ***	2.5
Linyola	100.0 (48) ns	0.9	82.7 (35) ns	1.1	-	-	-	-	77.2 (32) ns	1.2
Tossal	-	-	18.8 (48) ***	5.0	17.6 (43) ***	5.3	-	-	-	-
Paradet	100.0 (36) ns	0.9	69.2 (35) *	1.3	-	-	-	-	-	-
ADC	100.0 (35) ns	0.9	11.4 (35) ***	7.7	30.6 (36) ***	3.2	97.2 (36) ns	0.9	29.6 (34) ***	3.1
Tamarite	88.6 (36) ns	1.1	96.9 (33) ns	0.9	-	-	-	-	100.0 (16) ns	0.9
La Almunia	88.6 (31) ns	1.1	-	-	-	-	-	-	87.0 (37) ns	1.0
AstAb	92.6 (72) ns	1.0	100.0 (69) *	0.9	-	-	-	-	100.0 (36) ns	0.9
AstN	84.7 (48) *	1.1	98.0 (48) ns	1.0	63.7 (46) **	1.3	-	-	-	-
AstC	95.0 (91) ns	1.0	100.0 (49) ns	0.9	79.8 (89) *	1.2	95.3 (47) ns	0.9	91.6 (25) ns	1.0
Pompiers	-	-	35.9 (47) ***	2.6	28.1 (38) ***	3.0	-	-	53.5 (45) ***	1.8
Le Thor	-	-	53.7 (48) ***	1.7	56.4 (45) ***	1.5	69.2 (39) ***	1.4	70.1 (46) **	1.3

Table 3 cont. Corrected mortality (%) of insecticides at the diagnostic concentration, LC_{90} (mg a.i./L), on *C. pomonella* neonate larvae of the susceptible strain and of field populations. Numbers in parentheses show the number of insects treated. RR = corrected mortality of S-Spain / corrected mortality of the field population. The mortality obtained was compared using χ^2 (df = 1; *p = 0.05; **p = 0.01; ***p = 0.001).

Population	**Active ingredient (mg a.i./L)**									
	Indoxacarb (40.0)		**Spinosad (16.0)**		**Spinetoram (1.0)**		**Rynaxypyr (5.0)**		**Emamectin (0.6)**	
	C. Mort. (%)	**RR**	**C. Mort. (%)**	**RR**	**C. Mort. (%)**	**RR**	**C. Mort. (%)**	**RR**	**C. Mort. (%)**	**RR**
S-Spain-12	97.1 (36) a	-	81.9 (44) a	-	88.1 (35) a	-	88.5 (71) a	-	95.7 (71) a	-
S-Spain-13	95.8 (48) a	-	86.1 (95) a	-	88.2 (50) a	-	89.2 (48) a	-	86.6 (99) a	-
S-Spain-14	97.8 (47) a	-	86.6 (47a	-	95.1 (45) a	-	-	-	88.9 (48) a	-
SAS	87.8 (34) ns	1.1	94.4 (36) ns	0.9	91.7 (36) ns	1.0	40.6 (32) ***	2.2	70.6 (35) ***	1.4
PuigverdB	94.3 (71) ns	1.0	97.1 (36) *	0.8	69.8 (74) *	1.3	82.9 (36) ns	1.1	94.3 (36) ns	1.0
Poalbud	-	-	100.0 (29) *	0.8	100.0 (36) *	0.9	85.1 (34) ns	1.0	97.2 (36) ns	1.0
PuigverdC	75.8 (37) **	1.3	86.1 (32) ns	1,0	40.4 (30) ***	2.2	60.1 (32) ***	1.5	93.9 (33) ns	1.0
Mir7/84	79.2 (32) *	1.2	68.1 (37) ns	1.2	96.0 (26) ns	0.9	93.9 (35) ns	0.9	93.9 (36) ns	1.0
Linyola	-	-	-	-	-	-	91.3 (36) ns	1.0	91.3 (39) ns	1.0
Tossal	-	-	-	-	-	-	97.9 (48) n.s.	1.0	77.1 (48) ns	1.2
Paradet	-	-	-	-	-	-	-	-	100.0 (16) ns	1.0
ADC	66.7 (36) ***	1.5	97.2 (36) *	0.8	86.1 (36) ns	1.0	62.1 (34) **	1.4	85.4 (34) ns	1.1
Tamarite	-	-	-	-	-	-	88.6 (36) ns	1.0	91.4 (36) ns	1.0
La Almunia	-	-	-	-	-	-	-	-	94.2 (38) ns	1.0
AstAb	-	-	-	-	-	-	100.0 (97) **	0.9	87.5 (39) ns	1.0
AstN	97.8 (41) ns	1.0	-	-	80.3 (46) ns	1.1	85.5 (48) ns	1.0	82.5 (48) ns	1.0
AstC	99.0 (96) ns	1.0	93.5 (45) ns	0.9	92.2 (140) ns	1.0	93.1 (45) ns	1,0	96.8 (96) ns	0.9
Pompiers	85.6 (49) ns	1.1	-	-	85.7 (86) ns	1.0	-	-	95.6 (47) ns	0.9
Le Thor	98.2 (50) ns	1.0	82.2 (44) ns	1.0	76.0 (92) ns	1.2	-	-	96.0 (48) ns	0.9

Table 4. Corrected mortality (%) of insecticides at the diagnostic concentration, LC$_{50}$ (mg a.i./L), on *C. pomonella* neonate larvae of the susceptible strain and of field populations. Numbers in parentheses show the number of insects treated. RR = corrected mortality of S-Spain / corrected mortality of the field population. The mortality obtained was compared using χ^2 (df = 1; *p = 0.05; **p = 0.01; ***p = 0.001).

Population	**Control**	**Insecticide (mg a.i./L)**							
		Chlorpyriphos- ethyl (31.3)		**Lambda-cyhalothrin (0.1)**		**Emamectine (0.3)**		**Rynaxypyr (4.0)**	
	Mort. (%)	**C. Mort. (%)**	**RR**	**C. Mort. (%)**	**RR**	**C. Mort. (%)**	**RR**	**C. Mort. (%)**	**RR**
S-Spain-11	3.79 (107)	56.56 (70)	-	69.07 (72)	-	55.57 (72)	-	69.69 (72)	-
PuigverdB	2.78 (35)	54.29 (36) n.s.	1.0	0.00 (33) ***	62.8	66.75 (34) n.s.	0.8	80.04 (65) n.s.	0.9
Puigvert	6.25 (48)	33.74 (34) *	1.7	23.72 (37) ***	2.9	85.19 (35) **	0.7	80.00 (46) n.s.	0.9
Torregross	11.11 (36)	43.75 (36) n.s.	1.2	46.88 (36) *	1.5	90.63 (36) ***	0.6	-	-
Coll	5.56 (36)	67.65 (36) n.s.	0.8	85.29 (36) n.s.	0.8	-	-	-	-
Tossal	0.00 (36)	75.00 (36) n.s.	0.7	3.03 (35) ***	22.8	97.22 (33) ***	0.6	-	-
Noves	5.56 (36)	88.24 (36) ***	0.6	53.21 (36) n.s.	1.3	-	-	-	-
Tamarite	2.78 (36)	54.29 (36) n.s.	1.0	58.10 (36) n.s.	1.2	62.86 (36) n.s.	0.9	62.86 (80) n.s.	1.1

Table 5. Effect of metabolic synergists on *C. pomonella* neonate larvae expressed as corrected mortality (%) of 4 insecticides at the diagnostic concentration, LC$_{50}$ (mg a.i./L) of the susceptible strain. Numbers in parentheses show the number of insects treated. SR = synergistic ratio = corrected mortality with synergist / corrected mortality without synergist. The mortality obtained was compared using χ^2 (df = 1; *p = 0.05; **p = 0.01; ***p = 0.001).

Population	**Chl-e**	**PBO synergist**		**DEM synergist**		**DEF synergist**	
		Chl-e + PBO	**SR**	**Chl-e + DEM**	**SR**	**Chl-e + DEF**	**SR**
S-Spain	56.6 (70)	45.5 (36) n.s.	0.8	48.3 (36) n.s.	0.9	46.1 (36) n.s.	0.8
PuigverdB	54.3 (36)	60.8 (35) n.s.	1.1	43.5 (58) n.s.	0.8	75.0 (37) n.s.	1.4
PuigvertC	33.7 (34)	27.0 (29) n.s.	0.8	26.3 (31) n.s.	0.8	52.1 (34) n.s.	1.5
Torregrossa	43.8 (36)	87.1 (36) ***	2.0	76.5 (36) **	1.7	79.4 (36) **	1.8
Coll	67.7 (36)	78.0 (36) n.s.	1.2	56.3 (36) n.s.	0.8	77.8 (36) n.s.	1.1
Tossal	75.0 (36)	60.0 (36) *	0.8	-	-	-	-
Tamarite	54.3 (36)	62.9 (36) n.s.	1.2	69.7 (36) n.s.	1.3	56.3 (36) n.s.	1.0
Noves	88.2 (36)	97.2 (36) n.s.	1.1	100.0 (35) *	1.1	100.0 (34) *	1.1

Population	**λ-cyhal**	**λ-cyhal + PBO**	**SR**	**λ-cyhal + DEM**	**SR**	**λ-cyhal + DEF**	**SR**
S-Spain	69.1 (72)	62.5 (36) n.s.	0.9	65.5 (36) n.s.	0.9	62.9 (36) n.s.	0.9
PuigverdB	0.00 (33)	0.0 (34) n.s.	1.0	6.2 (35) n.s.	-	-	-
PuigvertC	23.7 (37)	27.0 (29) n.s.	1.1	-	-	-	-
Torregrossa	46.9 (36)	64.5 (36) n.s.	1.4	67.7 (36) n.s.	1.4	73.5 (36) *	1.6
Coll	85.3 (36)	74.9 (36) n.s.	0.9	93.8 (36) n.s.	1.1	-	-
Tossal	3.0 (35)	51.4 (36) ***	17.1	-	-	-	-
Tamarite	57.1 (34)	51.4 (34) n.s.	0.9	48.5 (36) n.s.	0.8	53.1 (36) n.s.	0.9
Noves	53.2 (34)	74.2 (34) n.s.	1.4	63.1 (34) n.s.	1.2	-	-

Table 5 cont. Effect of metabolic synergists on *C. pomonella* neonate larvae expressed as corrected mortality (%) of 4 insecticides at the diagnostic concentration, LC_{50} (mg a.i./L) of the susceptible strain. Numbers in parentheses show the number of insects treated. SR = synergistic ratio = corrected mortality with synergist / corrected mortality without synergist. The mortality obtained was compared using χ^2 (df = 1; *p = 0.05; **p = 0.01; ***p = 0.001).

Population	**Emam**	**PBO synergist**		**DEM synergist**		**DEF synergist**	
		Emam + PBO	**SR**	**Emam + DEM**	**SR**	**Emam + DEF**	**SR**
S-Spain	55.6 (72)	48.9 (36) n.s.	0.9	48.3 (36) n.s.	0.9	49.4 (36) n.s.	0.9
PuigverdB	66.8 (34)	87.5 (33) *	1.3	97.1 (36) **	1.5	91.4 (36) *	1.4
PuigvertC	85.2 (35)	100.0 (12) n.s.	1.2	90.0 (34) n.s.	1.1	-	-
Torregrossa	90.6 (36)	83.9 (36) n.s.	0.9	-	-	-	-
Tossal	97.2 (33)	97.1 (36) n.s.	1.0	-	-	-	-
Tamarite	62.9 (36)	65.7 (36)n.s.	1.0	100.0 (36) ***	1.6	100.0 (36) ***	1.6
Population	**Ryn**	**Ryn + PBO**	**SR**	**Ryn + DEM**	**SR**	**Ryn + DEF**	**SR**
S-Spain	69.7 (72)	59.1 (36) n.s.	0.8	62.1 (36) n.s.	0.9	59.6 (36) n.s.	0.9
PuigverdB	80.0 (65)	59.0 (56) *	0.7	50.1 (58) ***	0.6	83.8 (63) n.s.	1.0
PuigvertC	80.0 (46)	53.7 (51) **	0.7	71.4 (34) n.s.	0.9	71.5 (34) n.s.	0.9
Tamarite	62.9 (80)	42.9 (36) *	0.7	39.4 (36) *	0.6	53.1 (36) n.s.	0.8

Discussion

Insecticide efficacy bioassays

As it was expected, the field populations from Asturias were susceptible to all the insecticides tested, unlike the field populations from the other three areas of production. The Asturian apple orchards are for cider production and the most important pests to control are the rosy apple aphid, *Dysaphis plantaginea* Passerini (Homoptera: Aphididae), and the codling moth (Miñarro & Dapena, 2001). In the production area of Asturias, the codling moth has 1.5 generations per year, while in the other areas it has 2.5 generations. Since the early 1990s, in the new semi-intensive Asturian orchards, using mating disruption and selective insecticides, such as granulovirus and insect growth regulators (IGR), the level of codling moth damage has been maintained below 2% and biological control of the European Red Mite (*Panonychus ulmi* (Koch) (Acari: Tetranychidae)) has been achieved with phytoseids (Miñarro & Jaques, 2011). To control rosy apple aphid, neem derivates are currently used, though some less selective treatments are applied occasionally. The efficacy bioassays showed a significantly lower susceptibility of the Asturian field populations to methoxyfenozide, although the RR in these populations ranged between 1.3 and 1.2. These values are very low, and it should be noted that with S-Spain it was also possible to calculate a ratio of 1.2 when the mortalities obtained with methoxyfenozide were compared during the years studied (Table 3). In addition, an RR of 1.1 or 1.2 was found in many of the efficacy bioassays where no significant differences from the susceptible strain were found. These results imply that RR values ranging between 1.1 and 1.2 cannot be attributed to the population's tolerance to the insecticide but may be due to assay variability coupled with population response variability.

The field populations from Catalonia and Aragon showed a similar low susceptibility to the tested insecticides. In both areas it was possible to find field populations as susceptible as S-Spain to all or almost all the products (Tamarite and La Almunia in Aragon, and Linyola and Poalbud in Catalonia) and populations that had low susceptibility to half or more than half the insecticides used in the bioassay (ADC in Aragon and PuigverdC, Mir7/84 and some others in Catalonia). No French field population was susceptible to all the products but few orchards were tested, and a similar situation to that of Catalonia and Aragon can be expected (Reyes *et al.*, 2007).

In the apple production area of Lleida (Catalonia), since azinphos-methyl use was prohibited, 24% and 16% of the chemical applications against codling moth have been with chlorpyrifos-ethyl and lambda-cyhalothrin, respectively (data obtained from the record of treatments of 2,875 ha of apples and pears during the year 2008 in Lleida). Chlorpyrifos-ethyl was surprisingly effective and the susceptibility of the field populations had not diminished in the last five years in spite of its high frequency of use. This high efficacy was also found by Rodríguez *et al*. (2011a) in field populations collected in the same area in 2006 and 2007, in which a great loss of susceptibility to azinphos-methyl was detected. Reyes *et al*. (2001), in laboratory selected populations, found that the azinphos-methyl–resistant laboratory strain was significantly more susceptible to chlorpyrifos-ethyl than the sensitive strain. Dunley & Welter (2000) found a negatively correlated cross-resistance between chlorpyrifos-ethyl and azinphos-methyl in codling moth and suggested the possibility of developing a resistance management strategy based on it, which was in fact done by advisors and growers some years ago in Spain when azinphos-methyl was banned. On the other hand, a high frequency of populations tolerant or resistant to lambda-cyhalothrin, the insecticide with the highest RRs, was found particularly in the production area of Catalonia, where 148- and 872-fold resistance was found in two orchards. The use of pyrethroids, specifically lambda-cyhalothrin, has gradually increased in the last few years in Spanish apple production areas. It is applied not only to control codling moth, particularly near harvest, but also to control *Ceratitis capitata* (Wied.) (Diptera: Tephritidae) and other pests. This practice is currently threatening the mite control strategy with indigenous phytoseids, widely established in the area since the 1990s, and is being favored by the stringent market requirements in terms of number of active substances detected and their level in fruit at harvest, obviating the need for active ingredient rotation for resistance management.

According to the results, methoxyfenozide was the least effective insecticide, obtaining significantly lower mortality than in S-Spain in all the field populations tested, regardless of their origin. The product is not registered in France against codling moth (Ministère de l'Agriculture et de l'Agroalimentaire, France, 2016), but the two French populations tested were tolerant to the insecticide. The highest methoxyfenozide RRs were found in field populations from Catalonia, but this active ingredient was hardly used in the area. This finding may suggest a cross-resistance with organophosphates, particularly azinphos-methyl (heavily used before 2008) and phosmet, which was suitably demonstrated in previous works (Reyes *et al.*, 2007; Rodríguez *et al.*, 2010). Cross-resistance of methoxyfenozide with organophosphates has been proven by several authors in codling moth (in North Carolina (USA) (Magalhaes *et al.,* 2012), in Michigan (USA) (Mota-Sanchez *et al.*, 2008) and in Canada (Grigg-McGuffin *et al.*, 2015)) and other tortricid pests (obliquebanded leafroller, *Choristoneura rosaceana* (Harris) in New York (USA) (Waldstein *et al.,* 1999) and Michigan (USA) (Ahmad *et al.*, 2002), and *Planotortrix octo* Dugdale in New Zealand (Wearing, 1998). The Spanish field populations resistant to methoxyfenozide were susceptible to tebufenozide, but the French population tolerant to methoxyfenozide was also tolerant to tebufenozide. Reyes *et al*. (2007) suggested cross-resistance between azinphos-methyl and tebufenozide in codling moth field populations from Switzerland, and this cross-resistance has been proven in other tortricids (Ahmad *et al.,* 2002; Waldstein *et al.,* 1999), but it was not the case for Spanish field populations. A high selection pressure with IGRs (mainly diflubenzuron) has occurred in southern France since the 1980s and has produced a cross-resistance with tebufenozide, even when it was a new mode of action (Sauphanor & Bouvier, 1995). Therefore, the tolerance in the tested populations may be due to an intensive use of the product or to a cross-resistance previously described in the area.

Tolerance or resistance to thiacloprid was found in 50% of the field populations. The product is rarely used in apple orchards of the Ebro Valley but it is often applied to control aphids or psylla in pears. The residual activity of the insecticides and the flux of populations between neighboring orchards may also contribute to these results. Brunner *et al*. (2005) obtained high levels of mortality of *C. pomonella* neonates even 28 days after the treatment in the field with different neonicotinoids. The possibility of a negative cross-resistance between thiacloprid and chlorpyrifos was also pointed out by Isci & Ay (2017) in some field populations from Turkey. The field population PuigverdC showed an 11.2-fold resistance to thiacloprid and an 872.0-fold resistance to lambda-cyhalothrin, and the rest of the populations tolerant to thiacloprid coincided with those tolerant or resistant to lambda-cyhalothrin. Reyes & Sauphanor (2008) found a significant decrease in susceptibility to thiacloprid in neonate larvae of laboratory strains resistant to azinphos-methyl, diflubenzuron and the pyrethroid deltamethrin. In Canada and Greece, Grigg-McGuffin *et al*. (2015) and Vodouris *et al*. (2011), respectively, obtained low susceptibility to thiacloprid in neonate larvae and fifth-instar diapausing and non-diapausing larvae when the product was recently registered. These field populations, as in the present study, showed tolerance to multiple active ingredients.

The Spanish populations of this study were not exposed to indoxacarb (not used to control codling moth) and Rynaxypyr (registered in Spain at the end of 2011). However, 33% and 36% of the tested populations were tolerant to these products, respectively, although with RR of only 1.2 to 2.2. Some codling moth field populations tolerant to indoxacarb were also found in Michigan (USA) (Mota-Sanchez *et al.,* 2008) when it was a new compound. No resistance to Rynaxypyr has been reported in codling moth (Grigg-McGuffin *et al.,* 2015; Knight, 2010), but a slight reduction in susceptibility was found in some of our field populations when the LC_{90} was applied. The three field populations tested with the LC_{50} of Rynaxypyr (Table 4) were as susceptible as the laboratory population, but when they were treated with the LC_{90} (Table 3), one of them (PuigvertC) showed an RR of 1.5. Previous studies found an increase in RR at higher concentrations for some populations of *C. pomonella* and in other Lepidoptera, which can lead a population to be susceptible or tolerant, or tolerant or resistant, according to the discriminant concentration applied (Kodandaram & Dhingra, 2006; Mota-Sanchez *et al.*, 2008; Thwaite *et al.,* 1993). Sial *et al.* (2010) also found some Rynaxypyr-tolerant field populations of *C. rosaceana* in Washington (USA), and high levels of resistance were reported in some field populations of *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) in China after only 2 years of intensive use or misuse of the product (Wang & Wu, 2012), showing the importance of a management resistance strategy in pests with a strong ability to develop resistance.

Neither of the insecticides from the spinosyn group, spinetoram (not yet registered in Spain) or spinosad (registered in Spain in 2013), had ever been used against the tested field populations. Two of them were significantly less susceptible to spinetoram than S-Spain, with RR of 1.3 and 2.2, but spinosad was very effective with all the field populations and, in some cases, even more than with S-Spain. Mota-Sanchez *et al.* (2008) also found no resistance to spinosad in some field populations from Michigan (USA) that were resistant to the organophosphates azinphos-methyl and phosmet, the pyrethroid lambda-cyhalothrin and the diacylhydrazine methoxyfenozide. In Washington (USA), codling moth field populations that had high tolerance to azinphos-methyl and acetamiprid showed low susceptibility to spinosad and methoxyfenozide when they were not widely use (Knight, 2010). Sial *et al.* (2010) found a high correlation between spinetoram and spinosad resistance at the level of LC_{50} in *C. rosaceana* and also suggested the possibility of cross-resistance. In our case, the most tolerant field population to spinetoram (PuigverdC), in which the efficacy of the product was 40% mortality, was resistant to lambda-cyhalothrin (872.0-fold), methoxyfenozide (14.6-fold) and thiacloprid (11.2-fold), but a second population (Mir7/84), also with high resistance levels to lambda-cyhalothrin (148.1-fold) and methoxyfenozide (15.9-fold), was susceptible to spinetoram, which rules out cross-resistance with these products. In addition, all the populations were susceptible to spinosad, even more than S-Spain. Emamectin (not yet registered in Spain), together with spinosad, was the most effective product, being as effective as in S-Spain in 94% of the field populations treated. In the only tolerant population, SAS, the mortality produced by emamectin was over 70.0%. These results were similar to the ones obtained by Reyes *et al*. (2007) with different field populations from European countries, mainly France.

Synergist bioassays

To evaluate the involvement of the enzymatic systems in insecticide resistance, the maximum concentration of the synergists that produces a minimum larval mortality should be used. We used the concentration of the synergist that produced a maximum mortality of 10% in S-Spain. These values were not perhaps the optimum ones for the field populations suspected, in general, of being less susceptible than S-Spain. Reyes *et al*. (2001) found high differences in the concentrations of synergists to apply in laboratory resistant populations compared with a susceptible population (2.7-fold with PBO and DEF, and 80-fold with DEM in the field population) when they were seeking the maximum concentration to apply that did not produce larval mortality. However, obtaining enough progeny to do all the tests is usually a limiting factor for the field populations.

The sensitivity of the S-Spain strain to all the tested insecticides was not significantly modified by the synergists, as happened in other susceptible populations previously tested (Reyes *et al.,* 2001). The same response was obtained with the ecological field population Coll when it was treated with any of the synergists tested plus chlorpyrifos-ethyl and lambda-cyhalothrin.

Generalized enhanced levels of the metabolic detoxifying system PSMO were found in several studies of codling moth resistance in field populations from the same Spanish area in adults, post-diapausing larvae and neonate larvae (Rodríguez *et al.,* 2010, 2011a).

Therefore, a general increase in the mortality produced by the LC_{50} of some insecticides was to be expected when PBO was applied, particularly in the pyrethroid and organophosphate treatments, which are proven to be detoxified with PSMO (Rodríguez *et al.,* 2011a; Sauphanor & Bouvier, 1995; Voudouris *et al.,* 2011). Lambda-cyhalothrin obtained the lowest mortalities in both bioassays (LC_{90} and LC_{50} treatments). However, with the application of the synergist, the mortality produced by lambda-cyhalothrin increased numerically in Noves and Torregrossa but significantly only in Tossal (SR = 17.1), where the resistance to the product was heavily overcome with the previous application of PBO, reaching 51.4% mortality with the LC_{50}, so PSMO seems to be the main mechanism involved in this case of resistance. PuigvertC and PuigverdB, which were very resistant to lambda-cyhalothrin, with RRs of 872.0 and 15.4, respectively, did not increase their mortality with the previous application of PBO. This may be because the insects did not receive a sufficient amount of synergist to block their PSMO enzymatic system activity, and they were able to detoxify the insecticide. Increasing concentrations of PBO applied before the application of a diagnostic dose of temephos in the mosquito *Aedes aegipty* (L.) (Diptera: Culicidae) produced a significant mortality increase in a naturally resistant strain (Pereira *et al.,* 2014), but the lower concentration did not increase the mortality, the same happened with *Rhizopertha dominica* (F.) (Coleoptera: Bostrychidae) and deltamethrin (Lorini & Galley, 2000). Two major enzymatic systems are involved in the metabolism of pyrethroid insecticides: cytochrome P450 monooxygenases (P450s) and EST (Yu, 2008). Only one lambda-cyhalothrin_tolerant field population was tested with the product after being previously treated with DEF, Torregrossa, and a significant mortality increase was obtained. Previous results also reported a possible involvement of EST in the pyrethroid resistance of codling moth larvae (Sauphanor *et al.,* 1997; Voudouris *et al.,* 2011).

The only chlorpyrifos-ethyl–tolerant field population, PuigverdC, showed no significant increase with the application of the synergists. DEM and DEF significantly increased the mortality obtained in the field populations Torregrossa (SR = 1.7 and 1.8, respectively) and Noves (SR = 1.1 for both synergists), although in Noves the increases were very low, and DEF also produced mortality increases with SRs of between 1.4 and 1.5 in PuigverdC and PuigverdB. PBO produced a significant increase only in Torregrossa, but the results in the rest of the field populations were very variable, as happened with the application of DEM. Reyes and Sauphanor (2008) found a positive correlation between PSMO activity and chlorpyrifos-ethyl tolerance in neonate larvae from resistant laboratory populations, but no correlation with EST and GST in these life stages. These two mechanisms were not involved as a generalized insecticide-resistant mechanism in neonate larvae from some field populations collected in 2006 and 2007 in the production area of Lleida (Rodríguez *et al.,* 2011a), though both were detected, but were generalized in the post-diapausing larvae (Rodríguez *et al.,* 2010, 2011b). By contrast, a significant correlative association between lower EST activity in adults and fifth-instar larvae and resistance to organophosphates was found in European codling moth field populations (Reyes *et al.*, 2007, 2015; Rodríguez *et al.*, 2010; Voudouris *et al.*, 2011). These variable results were attributed in some cases to the different affinity for the substrates used in the studies but confirm the results of Reyes & Sauphanor (2008), who found no correlation for EST activity between the developmental stages neonate larvae, diapausing larvae and adults, and no correlation for GST with the larval stages.

None of the Spanish field populations tested with the synergists were resistant to the new active ingredient emamectin, which was the expected result. By contrast, three of the five field populations treated with the LC_{50} were significantly more susceptible than S-Spain (Table 4). The use of DEM and DEF caused a significantly increased mortality in PuigvertB and Tamarite, which were as susceptible to the product as S-Lleida, so, GST and EST seem to influence the efficacy of the product in these field populations. If the susceptible field populations are exposed to a mixture of synergist+insecticide, the specific detoxification pathway will be blocked and the small proportion of resistant insects to the insecticide will die as if they were susceptible. For this reason, when treating our laboratory susceptible population, we obtained SR lower than one in all cases. Nevertheless, DEM had no significant influence in PuigverdC, which was very susceptible to the product (85.2%), even more than the reference susceptible population (55.6%). Reyes *et al.* (2007) linked the efficacy reduction of emamectin in some European field populations to EST, despite the fact that it was the most effective product, with a mortality over 83% in all cases. Unfortunately, we had insufficient larvae to test these synergists with the other field populations. PSMO had no effect on the response to the insecticide in four of the five field populations tested, except in PuigvertB, which had a low SR (1.3). Civolani *et al.* (2014) suggested that monooxygenases were not responsible for emamectin benzoate detoxification in *Lobesia botrana* (Denis &

Schiffermüller) (Lepidoptera: Tortricidae), but opposite results were found with other Lepidoptera (Liang *et al.,* 2003; Tabashnik, 1991).

The LC_{50} concentration of Rynaxypyr, the other new insecticide for the Spanish field populations, was very effective against the three field populations tested, even in PuigverdC, which was tolerant to the LC_{90} concentration. However, the application of PBO and DEM led to a surprising significant decrease in mortality in three and two field populations treated, respectively. In a selected chlorantraniliprole-resistant laboratory population of *C. rosaceana*, EST was responsible for detoxifying the product (Sial & Brunner, 2012; Sial *et al.,* 2011) and the application of DEF increased the mortality even in the susceptible laboratory population, although not significantly.

Conclusions and further directions

Control of *C. pomonella* in Spain is possible with the use of mating disruption combined with reduced-risk insecticides already registered such as tebufenozide, spinosad, indoxacarb and Rynaxypyr, which have been shown to be effective in controlling resistant populations in the area. As the not yet registered insecticides emamectin and spinetoram are also effective, their registration would be valuable. If a rational strategy of resistance management is to be achieved in IPM production, growers need to have several insecticides at their disposal, and the excessively strict residue requirements of commercial market, far higher than the legal ones, need to be reduced.

The methodology to be applied when trying to determine the enzymatic systems involved in insecticide resistance with synergists need further refinement. One difficulty is the need to adjust the concentrations to be applied for each population and synergist in order to obtain credible information, and another is the interpretation of the results, particularly in field populations, in which co-occurrence of different resistance mechanisms may be present and interactions between them may occur.

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CAPÍTULO 2

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

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

Bosch, D.[1]; Avilla, J.[2]; Musleh, S.[3]; Rodríguez, M.A.[4]

[1] Institut Recerca i Tecnologia Agroalimentària (IRTA), Av. Alcalde Rovira Roure, 191.
25198 Lleida, Spain.

[2] University of Lleida, Av. Alcalde Rovira Roure, 191. 25198 Lleida, Spain.

[3] Universidad de Concepción, Castilla 160-C, Concepción, Chile.

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Abstract

Codling moth, *Cydia pomonella* (L.) is a key pest of global importance that affects apple fruit production and whose populations have developed resistance to insecticides in many apple production areas. In Spain, cytochrome P450 polysubstrate monooxygenase (PSMO) activity is the main mechanism involved in insecticide detoxification by codling moth, although acetylcholinesterase (*AChE*) target site mutations have been described in two populations. However, the extent of *AChE* and knockdown resistance (*kdr*) mutations in Spain is unknown.

To assess the actual occurrence of *AChE* and *kdr* mutations concurrently with the frequency of PSMO-resistant moths (R-PSMO), 32 Spanish field populations from four apple-growing areas of Spain and two susceptible strains (SSp, from Spain, and Sv, from France) were evaluated.

R-PSMO was significantly higher than the SSp one in 23 chemically-treated field populations from Extremadura, Catalonia and Aragón, with proportions that varied between 25% and 90%, but no significant differences among the non-chemically treated orchards (organic or abandoned) and the susceptible strains were observed. The *AChE* mutation was detected in all field populations from Catalonia (n=21) and in three field populations from Aragon (n=5), with resistant phenotype proportions varying from 34.2% to 97.5% and from 7.2% to 65.0% in Catalonia and Aragón, respectively. In addition, the *kdr* mutation was detected in 12 Catalonian field populations, at rates of incidence ranging between 2.6% and 56.8%. A positive correlation between R-PSMO and *AChE* was found. The origin of the mutations and their ability to persist and to spread in field populations with different management systems is discussed.

Introduction

A key component of the integrated management of key pests such as codling moth (*Cydia pomonella* (L.), Lepidotera: Tortricidae) is establishing an insecticide-resistance management (IRM) program. Codling moth is one of the most damaging pests of pome fruit crops worldwide, although in almost all the areas where such crops are cultivated it affects mainly apple production (Barnes, 1991). As a result of long-term pesticide use, the codling moth has developed resistance to different insecticide modes of action and chemistries, such as neurotoxic insecticides and insect growth regulators (IGRs) (Bouvier *et al*., 2001; Bush *et al*., 1993; Chapman & Barrett, 1997; Cichón *et al*., 2013; Dunley & Welter 2000; Ioriatti *et al*., 2007; Knight, 2010; Moffitt *et al*., 1988; Mota-Sanchez *et al*., 2008; Reuveny & Cohen, 2004; Reyes *et al*., 2015; Rodríguez *et al*., 2010, 2011a; Sauphanor & Bouvier, 1995; Sauphanor *et al*., 1998; Varela *et al*., 1993; Whalon *et al*., 2013), and even to codling moth granulosis virus (CpGV) (Asser-Kaiser *et al*., 2007).

Worldwide, codling moth insecticide resistance is mainly associated with the increased activity of detoxifying enzymes, such as non-specific esterases (ESTs), cytochrome P450 polysubstrate monooxygenases (PSMOs), and glutathione S-transferases (GSTs) (Bush *et al*., 1993; Sauphanor *et al*., 1997; Reyes *et al*., 2007; Rodríguez *et al*., 2010; Soleño *et al*., 2008). In addition, two target-site mutations (structural changes in the insecticide target proteins that render them less sensitive to an insecticide) have been reported: *AChE*, a F290V replacement in acetylcholinesterase, involved in resistance to organophosphates and carbamates (Cassanelli *et al*., 2006) and *kdr*, a L1014F replacement in the voltage-gated sodium channel involved in resistance to pyrethroids (Brun-Barale *et al*., 2005). The *AChE* mutation confers phenotypic resistance under both homozygote and heterozygote conditions, whereas the *kdr* mutation confers it only under the recessive homozygote condition.

In codling moth Spanish field populations, insecticide resistance has been associated with three detoxification systems, mainly PSMO in adults and larvae (Reyes *et al*. 2007; Rodríguez *et al*. 2010, 2011a, 2011b, 2012), and to a lesser extent GST and EST in larvae (Rodríguez *et al*., 2010, 2011a, 2011b). As for target-site mutations, Reyes *et al*. (2007, 2009) found the *AChE* mutation only in a single Spanish field population from a Catalan (NE Spain) apple-growing area, in a study of the variability of resistance mechanisms worldwide that involved a total of 55 populations from Europe (1 from Spain) and 24 from other continents. This Catalan population came from the same area in which the Raz population (the origin of the azinphos-methyl resistant strain selected in the INRA Avignon laboratory in which the *AChE* mutation was identified by Cassanelli *et al*. (2006)) was collected. The *kdr* mutation was not detected in this Spanish field population (Franck *et al*., 2012; Reyes *et al*., 2007, 2009).

The existence of multiple resistance mechanisms increases the difficulty of codling moth control and it interferes with the management programs in the orchards. Brent (1986) pointed out that the existence of at least 5 % of resistant individuals is required in a population to detect a resistance problem, but Denholm *et al*. (1984) and Hoy (1995) reported that resistance is very difficult to manage even when only 10% of the individuals carry resistance genes. It is therefore important to establish an Insecticide Resistance Management (IRM) program for Spanish codling moth populations to avoid or delay the increase in the frequency of resistant individuals. This requires the early detection of resistant mechanisms in field populations and a knowledge at a local scale of the extent of the resistance mechanisms involved.

PSMO-resistant male adults from Spanish populations did not show a greater attraction to pure kairomone-baited traps in apple orchards (Bosch *et al.,* 2016), as reported by Sauphanor *et al.* (2007) for French PSMO-resistant populations. However, PSMO detection in codling moth adults caught during flight, in orchards under different crop management systems, was found to be a good tool to assess levels of insecticide resistance in field apple crops (Bosch *et al*., 2016). Extensive studies on increase in the metabolic capacity of detoxifying enzymes have been carried out in the Ebro Valley pome fruit production area (Rodríguez *et al*., 2010, 2011a, 2011b), but not on the geographical distribution of resistance.

In an attempt to evaluate resistance mechanisms in Spanish codling moth populations, the aims of the present work were to assess the actual occurrence of *AChE* and *kdr* mutations in codling moth field populations from pome fruit-growing areas of Spain, and to assess PSMO activity expressed as the frequency of PSMO-resistant codling moth individuals (R-PSMO).

Material and Methods

Insects

Thirty-two codling moth field populations were collected in four Spanish autonomic regions where pome fruits are cultivated (Figure 1): Asturias (AST), n=5; Extremadura (EXT), n=1; Aragon (ARA), n=5; and Catalonia (CAT), n=21 (Table 1). Apple cultivation in Asturias (4,106 ha in 2016) is dedicated to cider production, and orchards are managed organically without irrigation. In the other three regions, apple trees are grown for table apple production, and orchards are intensively managed. In 2016, Catalonia (11,066 ha, mostly in Lleida) and Aragón (4,576 ha) represented 75% of the total Spanish acreage dedicated to apple production under irrigation (MAPA, 2017).

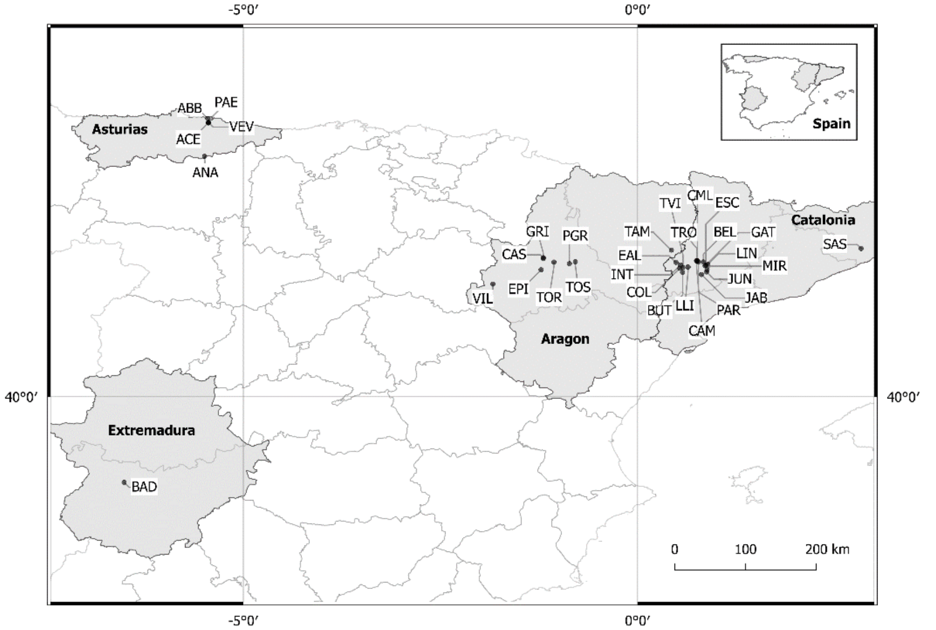

Figure 1. Sampled site locations. Shaded areas represent four Spanish autonomic regions (Asturias: Gijón-Oviedo province; Extremadura: Badajoz province; Aragón: Huesca and Zaragoza provinces; Catalonia: Lleida and Girona provinces).

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

PSMO activity

To determine PSMO activity, freshly emerged adult abdomens were dissected and individually homogenised, as described by Rodríguez *et al.* (2012), in 6 g·L^{-1} sodium chloride, and they were placed in black 96-well microplates. The activity was measured using 7-ethoxycoumarin O-deethylation activity (ECOD), adapted for *in vivo* analysis (De Sousa *et al*., 1995). The methodology used was the same as described by Reyes *et al.* (2007). The reaction was initiated when an adult abdomen was individually introduced into a well containing 100 mL of phosphate buffer (50 mM, pH 7.2) and ethoxycoumarin (0.4 mM). After 4 h of incubation at 30 ºC, the reaction was stopped by adding 100 mL of 0.1 mM glycine buffer (pH 10.4)/ethanol (v/v). The 7-hydroxycoumarin fluorescence was quantified using a multilabel plate counter VICTOR3 (Perkin Elmer Life and Analytical Sciences, Madrid, Spain), with 380 nm excitation and 450 nm emission filters. In each plate, twelve wells filled only with glycine buffer prior to incubation were used as controls. A standard curve was obtained using 7-hydroxycoumarine, and PSMO activity was expressed as pg of 7-ethoxycoumarin (7OH)·adult^{-1}·min^{-1}.

AChE *and* kdr *mutations*

Target-site mutations related to insecticide resistance, *AChE* and *kdr* genes, were analysed using PCR–RFLP (Brun-Barale *et al*., 2005; Cassanelli *et al*. 2006, Reyes *et al.* 2007) in 1.174 adults (1.103 from field populations and 71 from the two susceptible laboratory strains). Total DNA was obtained from the adult thorax, using the “salting out” methodology described by Fuentes-Contreras *et al*. (2014). PCR amplifications of *kdr* and *AChE* genes were carried out separately, in a 25 µL reaction volume containing primer reaction buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 200 µM of each dNTP, 0.4 mM of each primer, 1 unit of Taq DNA polymerase and 2 µL of DNA template. Restrictions were performed by pooling the PCR products from both genes (5 µL each) with 0.2 units of Tsp509I (NEB) in a 20 µL reaction volume incubated at 65 ºC for 16 h.

DNA fragments were separated by electrophoresis on 6% polyacrylamide gel and visualized after silver staining. DNA fragments of 141 bp and 102 bp, respectively, identified the mutant and sensitive *AChE* alleles,. The *kdr* mutant allele was identified by a DNA fragment of 77 bp and two different sensitive alleles were identified by fragmenst of 101 bp and 112 bp, respectively.

Data analysis

The distribution of the variable PSMO activity did not fulfil the assumption of homoscedasticity and several standard transformations did not normalize it (Normal Q-Q plot and Shapiro-Wilkes normality test), using the *qqplot* and the *shapiro.test* functions in R language (R Core Team, 2017). Thus a non-parametric Kruskal-Wallis test was performed, followed by a multiple comparison test (Siegel & Castellan, 1988), using the *kruskalmc* function from the *pgirmess* R package (Giraudoux, 2017), to evaluate PSMO activity among populations and among orchards grouped by their management system. In both cases, only field populations with $n \geq 20$ adults were used.

To calculate the relative frequency of codling moth resistant adults (R-PMSO), an adult was classified as resistant if its PSMO activity was higher than the upper value of the 95% confidence limit of the mean PSMO activity of the susceptible strain_SSp (Reyes *et al*., 2007). A Pearson chi-square (χ^2) test was used to compare the R-PSMO between each population and the Spanish susceptible strain (SSp), using *chisq.test* functions (R Core Team, 2017).

To detect whether the *AChE* and the *kdr* genes were under selection pressure, a Fisher's exact test was performed for each population to check the Hardy-Weinberg equilibrium (GENEPOP 4.5., Rousset, 2008). Finally, Pearson correlation was used to evaluate the the relationship between the R-PSMO, the percentage of resistant insects with the *kdr* mutation and the percentage of resistant insects with the *AChE* mutation. The same relationships were subjected to principal component analysis, using the *rda* function implemented in the *vegan* package (Oksanen *et al.*, 2017).

Table 1. List and characteristics of *C. pomonella* field populations and laboratory strains.

Spanish region	Province and municipality	Population code	Management practices	Orchard management	Latitude - Longitude
Catalonia	IRTA-Lleida, Spain	SSp	Laboratory	UN	- -
PACA (France)	INRA-Avignon, France	Sv	Laboratory	UN	- -
Asturias	Villaviciosa, Gijón	ABB	Organic	UN	43º 31' 48.03'' N - 5º 27' 05.18'' W
Asturias	Villaviciosa, Gijón	ACE	Organic	UN	43º 28' 33.24'' N - 5º 26' 29.49'' W
Asturias	Nava, Gijón	ANA	Organic	UN	43º 02' 56.88'' N - 5º 29' 32.69'' W
Asturias	Villaviciosa, Gijón	PAE	Organic	UN	43º 28' 28.51'' N - 5º 26' 37.74'' W
Asturias	Villaviciosa, Gijón	VEV	Organic	UN	43º 28' 46.23'' N - 5º 26' 31.25'' W
Extremadura	Badajoz, La Garrobilla	BAD	Chemically treated	CH	38º 54' 34.47'' N - 6º 30' 41.35'' W
Aragon	Zaragoza, Figueruelas	CAS	Chemically treated	CH	41º 45' 46.85'' N - 1º 11' 46.52'' W
Aragon	Zaragoza, Epila	EPI	Experimental treated	CH	41º 36' 34.96'' N - 1º 13' 27.16'' W
Aragon	Zaragoza, Figueruelas	GRI	Chemically treated	CH	41º 45' 06.34'' N - 1º 11' 48.99'' W
Aragon	Huesca, Tamarite	TAM	Chemically treated	CH	41º 51' 35.40'' N - 0º 25' 41.85'' W
Aragon	Zaragoza, Villalengua	VIL	Chemically treated	CH	41º 25' 42.95'' N - 1º 50' 05.13'' W
Catalonia	Lleida, Coll	COL	Organic	UN	41º 37' 41.43'' N - 0º 32' 07.85'' E
Catalonia	Lleida, Almacelles	EAL	Organic	UN	41º 42' 11.86'' N - 0º 29' 10.88'' E
Catalonia	Lleida, Juneda	JAB	Abandoned	UN	41º 32' 59.49'' N - 0º 48' 38.59'' E
Catalonia	Lleida, Menàrguens	CAM	Chemically treated	CH	41º 43' 30.56'' N - 0º 44' 48.19'' E
Catalonia	Lleida, Torre Villalba	TVI	Chemically treated	CH	41º 38' 19.75'' N - 0º 34' 26.14'' E
Catalonia	Lleida, Termens	PAR	Chemically treated	CH	41º 42' 25.72'' N - 0º 46' 16.04'' E

Table 1 (cont.) List and characteristics of *C. pomonella* field populations and laboratory strains.

Spanish region	Province and municipality	Population code	Management practices	Orchard management	Latitude - Longitude
Catalonia	Lleida, Butsenit	BUT	Chemically treated	CH	41º 34' 37.11" N - 0º 34' 11.68" E
Catalonia	Llivia, Lleida	LLI	Chemically treated	CH	41º 38' 41.46" N - 0º 38' 24.96 E
Catalonia	Lleida, Menàrguens	CML	Chemically treated	CH	41º 43' 20.65" N - 0º 45' 31.14" E
Catalonia	Lleida, Menàrguens	TRO	Mating Disruption + CH	MD+CH	41º 43' 20.61" N - 0º 44' 51.95" E
Catalonia	Lleida, Termens	TOS	Mating Disruption + CH	MD+CH	41º 42' 38.83" N - 0º 47' 22.07" W
Catalonia	Lleida, Linyola	PGR	Mating Disruption + CH	MD+CH	41º 41' 14.05" N - 0º 51' 52.10" W
Catalonia	Lleida, Palau d'Anglesola	GAT	Mating Disruption + CH	MD+CH	41º 39' 37.23" N - 0º 51' 25.92" E
Catalonia	Llaida, Alpicat	INT	Mating Disruption + CH	MD+CH	41º 40' 14.75" N - 0º 32' 37.36" E
Catalonia	Lleida, Tornabous	TOR	Mating Disruption + CH	MD+CH	41º 42' 24.49" N - 1º 03' 42.36" W
Catalonia	Lleida, Palau d'Anglesola	ESC	Mating Disruption + CH	MD+CH	41º 40' 6.30" N - 0º 51' 52.80" E
Catalonia	Lleida, Bellvis	BEL	Mating Disruption + CH	MD+CH	41º 42' 30.66" N - 0º 50' 02.24" E
Catalonia	Lleida, Linyola	LIN	Mating Disruption + CH	MD+CH	41º 40' 43.00" N - 0º 53' 19.76" E
Catalonia	Lleida, Juneda	JUN	Mating Disruption + CH	MD+CH	41º 34' 56.86" N - 0º 52' 22.60 " E
Catalonia	Lleida, Miralcamp	MIR	Mating Disruption + CH	MD+CH	41º 36' 48.21" N - 0º 52' 53.48" E
Catalonia	Girona, St Andreu Salou	SAS	Mating Disruption + CH	MD+CH	41º 52' 42.95" N - 2º 50' 5.13" W

Results

PSMO activity and R-PSMO

Table 2 shows the PSMO activity in adults of 32 *C. pomonella* Spanish field populations and two laboratory susceptible strains. The mean PSMO activity was significantly different among codling moth populations (p-value < 0.0001, $\chi^2 = 461.71$, d.f. = 33). In general, the chemically sprayed orchards (CH or MD+CH) showed a PSMO activity significantly higher than the susceptible strains, except in five cases: VIL (MD+CH) from Aragon, and CAM, BUT, LLI, (MD+CH) and SAS (CH) from Catalonia (Figure 2). No significant differences were found among non-chemically treated orchards and the susceptible strains (Figure 2). The mean PSMO activity was also significantly different among orchard management system populations (p-value < 0.0001, $\chi^2 = 369.2$, d.f. = 3). No significant differences were found among orchards with or without mating disruption, but a significantly higher PSMO activity was observed in orchards subject to insecticide treatment (CH and MD+CH) compared to non-chemically treated orchards and laboratory susceptible strains (Figure 3). Taking into account the origin of the populations, the resistance ratio (R/S), based on the mean PSMO activity, ranged from 0.7 to 1.6 for Asturias; from 3.9 to 15.6 for Aragón; was 10.1 for Extremadura; and ranged from 2.3 to 18.9 for Catalonia (Table 2). Consistently with PSMO activity, the lowest R/S values corresponded to non-chemically treated orchards, while higher R/S values were found in orchards receiving chemical insecticide treatments.

The frequency of resistant *C. pomonella* adults, R-PSMO, was calculated using 37.04 pg of 7-hydroxycoumarin·insect^{-1}·min^{-1} as the threshold. The R-PSMO ranged from 0.0% (Asturias) to 90.0% (Catalonia) (Table 2). Any of the populations from non-chemically treated orchards showed a R-PSMO significantly higher than for the laboratory SSp strain, whereas all the populations from CH or MD+CH, except one, showed a R-PMSO significantly higher than for the laboratory SSp strain one (Table 2).

Table 2. PSMO activity (pg of 7-hydroxycoumarine·insect^{-1}·min^{-1}), relative frequency of resistant individuals (R-PSMO), and resistance ratio (R/S) of 32 *C. pomonella* Spanish field populations and two susceptible laboratory strains. R/S = Ratio between the mean PSMO activity of each population and of SSp. n = no. codling moth adults. MD = mating disruption.

Spanish region	Population code	Orchard management	n	PSMO activity (mean ± SE)	PSMO activity range	R-PSMO (%)	R/S
Catalonia	SSp	Laboratory	34	9.1 ± 1.9	0 - 38.8	10.0	1.0
PACA (France)	Sv	Laboratory	37	13.6 ± 2,0	0 - 38.8	8.1 ns	1.5
Asturias	ABB	Organic	40	10.8 ± 2.1	0 - 52.3	7.5 ns	1.2
Asturias	ACE	Organic	40	14.1 ± 2.5	0 - 63.8	7.5 ns	1.6
Asturias	ANA	Organic	34	12.9 ± 2.1	0 - 46.7	2.9 * (<SSp)	1.4
Asturias	PAE	Organic	21	8.8 ± 2.0	0 - 31.6	0.0 * (<SSp)	1.0
Asturias	VEV	Organic	36	6.2 ± 1.2	0 - 30.8	0.0 * (<SSp)	0.7
Extremadura	BAD	Chemically treated	11	91.7 ± 24.7	6.8 - 279.7	63.6 ***	10.1
Aragon	CAS	Chemically treated	12	141.7 ± 39.6	7.7 - 414.3	66.7 ***	15.6
Aragon	EPI	Experimental treated	28	73.4 ± 10.2	0.7 - 179.0	64.3 ***	8.1
Aragon	GRI	Chemically treated	6	35.3 ± 11.6	5.7 - 78.3	16.7 ns	3.9
Aragon	TAM	Chemically treated	40	61.7 ± 10.2	0.8 - 294.2	50.0 ***	6.8
Aragon	VIL	Chemically treated	28	48.2 ± 13.9	0 - 307.8	32.1 **	5.3
Catalonia	COL	Organic	38	21.1 ± 3.2	0 - 82.6	18.4 ns	2.3
Catalonia	EAL	Organic	40	24.5 ± 3.9	0 - 103.7	19.0 ns	2.7
Catalonia	JAB	Abandoned	40	22.4 ± 5.2	0 - 116.3	17.5 ns	2.5
Catalonia	CAM	Chemically treated	40	135.6 ± 21.1	0 - 507.4	75.0 ***	14.9

Table 2 (cont). PSMO activity (pg of 7-hydroxycoumarine·insect^{-1}·min^{-1}), relative frequency of resistant individuals (R-PSMO), and resistance ratio (R/S) of 32 *C. pomonella* Spanish field populations and two susceptible laboratory strains. R/S = Ratio between the mean PSMO activity of each population and of SSp. n = no. codling moth adults. MD = mating disruption.

Spanish region	**Population code**	**Orchard management**	**n**	**PSMO activity (mean ± SE)**	**PSMO activity range**	**R-PSMO (%)**	**R/S**
Catalonia	PAR	Chemically treated	43	90.1 ± 14.7	0 - 365.6	60.5 ***	9.9
Catalonia	BUT	Chemically treated	41	122.6 ± 21.9	0 - 532.6	63.4 ***	13.5
Catalonia	LLI	Chemically treated	40	127.4 ± 23.6	0 - 744.2	62.5 ***	14.0
Catalonia	CML	Chemically treated	31	90.9 ± 14.5	0 - 311.98	74.2 ***	10.0
Catalonia	TRO	MD + Chemically	38	137.9 ± 23.3	1.26 - 788.9	84.2 ***	15.2
Catalonia	TOS	MD + Chemically	37	94.36 ± 15.3	2.0 - 362.8	67.6 ***	10.4
Catalonia	PGR	MD + Chemically	37	100.0 ± 23.2	0 - 691.1	56.8 ***	11.0
Catalonia	GAT	MD + Chemically	38	127.0 ± 17.3	5.8 - 488.4	84.2 ***	14.0
Catalonia	INT	MD + Chemically	36	166.8 ± 24.9	0.8 - 463.6	72.2 ***	18.3
Catalonia	TOR	MD + Chemically	37	87.5 ± 14.1	1.2 - 323.4	62.2 ***	9.6
Catalonia	ESC	MD + Chemically	40	171.8 ± 20.4	17.8 - 575.1	90.0 ***	18.9
Catalonia	BEL	MD + Chemically	40	102.3 ± 18.8	1.4 - 424.12	55.0 ***	11.2
Catalonia	LIN	MD + Chemically	40	108.9 ± 18.9	1.0 - 529.65	70.0 ***	12.0
Catalonia	JUN	MD + Chemically	42	102.3 ± 14.6	0 - 435.0	71.4 ***	11.2
Catalonia	MIR	MD + Chemically	40	121.4 ± 16.2	4.8 - 491.95	80.0 ***	13.3
Catalonia	SAS	MD + Chemically	32	34.2 ± 8.7	0 - 167.4	25.0 ***	3.8

The frequency of resistant individuals was compared with that of the susceptible strain using a χ^2 test: n.s = not significant; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$***. <SSp denotes a frequency of resistant individual lower than the SSp one.

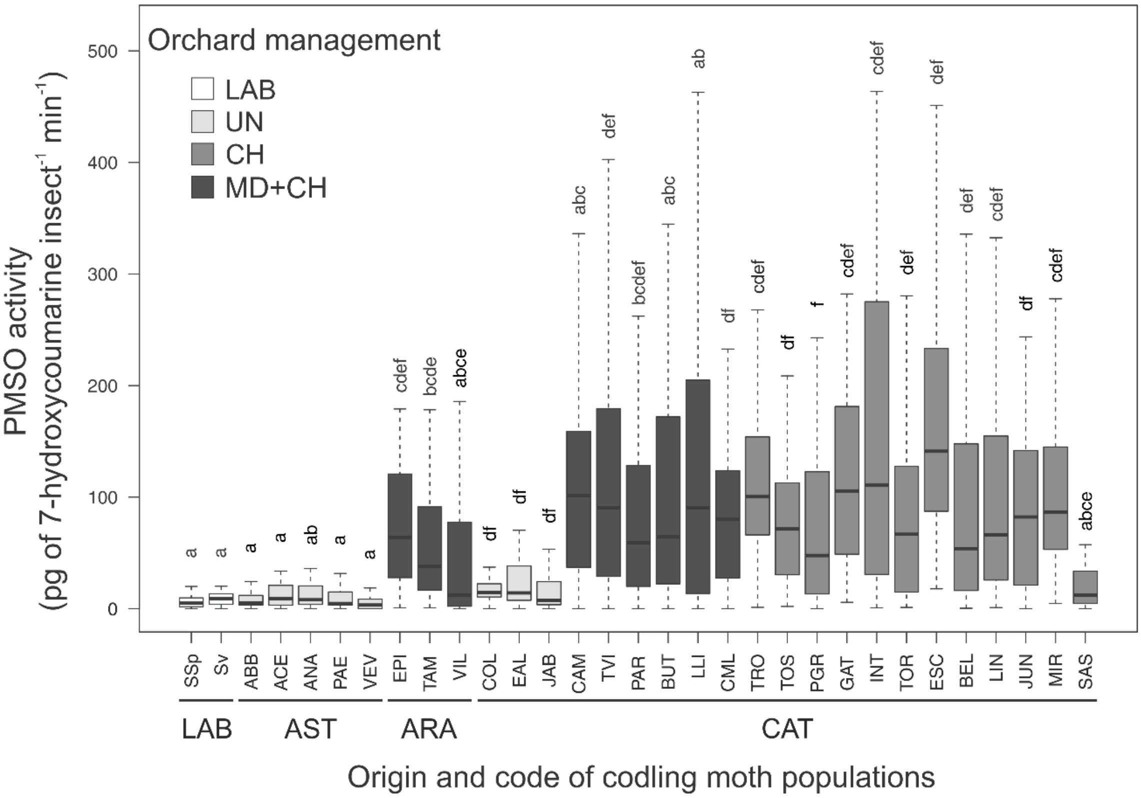

Figure 2. Box plot showing the PSMO activity of 29 Spanish codling moth field populations and two susceptible strains. Columns having the same letter were not significantly different ($p < 0.05$, Kruskal-Wallis multiple comparison test). Population origin: LAB = laboratory; AST = Asturias; ARA = Aragón; CAT = Cataluña. Population code, see Table 1. Orchard management: LAB = laboratory (SSp=Spain, Sv = (France); UN = non-chemically treated; CH = chemically treated; MD+CH = mating disruption + chemically treated.

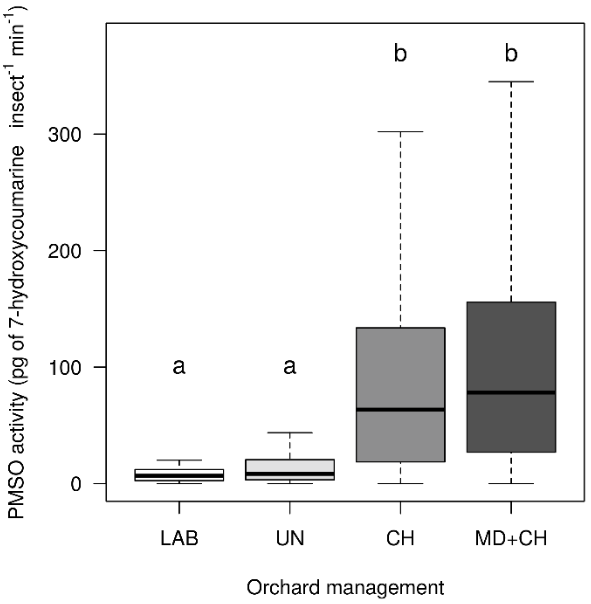

Figure 3. Box plot showing the PSMO activity of 29 Spanish codling moth populations and two susceptible strains grouped by the orchard management. Columns having the same letter were not significantly different ($p < 0.05$, Kruskal-Wallis multiple comparison test). Orchard management: LAB = laboratory (n = 2); UN = non-chemically treated (n = 8); CH = chemically treated (n = 9); MD+CH = mating disruption + chemically treated (n = 12).

AChE *and* kdr *mutations*

The *AChE* mutation, which confers resistance to organophosphate and carbamate insecticides, was detected in all populations from Catalonia (n=21), regardless of orchard management system, and in three populations from Aragon (CH) (Table 3). In populations from Asturias and from orchards with a low number of individuals sampled (BAD, from Extremadura; CAS and GRI, from Aragon), the *AChE* mutation was not detected (Table 3). The highest phenotype proportion of the *AChE* mutation (proportion of homozygotes + heterozygotes individuals) was 97.5%, in Catalonia (ESC, MD+CH), and it was 65% in Aragon (TAM, CH). For the *AChE* mutation, significant departure from the Hardy-Weinberg equilibrium was detected in 13 out of 21 field populations, all of them from Catalonia (COL, CAM, TVI, PAR, BUT, LLI, CML, TRO, GAT, INT, TOR, ESC and JUN; Fisher's exact test, $0.0001 < P < 0.015$) and in the field population TAM (CH) from Aragón (Fisher's exact test, $P = 0.023$).

The *kdr* mutation was detected in the recessive homozygosis condition (resistant phenotype, rr in Table 3) only in 12 populations from Catalonia. The lowest *kdr* proportion was found in TOR (2.6%), and the highest in TOS (56.8%), both orchards subject to MD+CH management systems (Table 3). In nine out of the 12 populations where the *kdr* mutation was detected, departures from the Hardy-Weinberg equilibrium was observed (COL, PAR, LLI, LIN, CML, TOS, PGR, INT, TOR; Fisher's exact test, $0.0008 < P < 0.044$). Although, *kdr* mutations give insecticide resistance to pyrethroid insecticides in the recessive homozygosis condition, in 18 field populations sampled the resistance allele was detected in the heterozygosis condition, in proportions that varied from 2.4% in MIR to 39.5% in GAT, MD+CH orchards (Table 3).

Relationship between R-PSMO and target site mutations

The R-PSMO was positively correlated to the proportion of individuals with the *AChE* phenotypic mutation ($r = 0.639$, $P = 4.5\ E^{-5}$) (Figure 4). A positive correlation between the R-PSMO and the proportion of individuals with the *kdr* phenotypic mutation ($r = 0.485$, $P = 0.0037$) and between the mutations *AChE* and *kdr* ($r = 0.508$, $P = 0.0022$) (Figure 4) were also found. The PCA (principal component analysis) was undertaken considering the 32 field populations and the two susceptible strains. The PC1 (65%) was mainly explained by *AChE* and R-PSMO, while the PC2 (24%) was explained by *kdr* (Figure 5). In general, the PCA representation showed a high degree of association between sprayed field populations (CH, MD+CH) and the frequency of R-PSMO and *AChE* mutations, especially among MD+CH orchards, all of them from Catalonia. However, this association was lower for CH orchards from Aragón (PC1). Untreated orchards, alongside susceptible strains, showed no association (Figure 5). The *kdr* relationships (PC2), in general only involved Catalana field populations. This representation also showed some Catalan field populations (COL, LLI, LIN and TOS) separated from the rest in the main clusters. The organic orchard COL showed a moderate percentage of *AChE* mutations compared to other non-chemically treated orchards (UN), but their *kdr* mutation propotion were comparable with chemicallu treated orchards (CH and MD+CH). The chemically treated orchards LLI (CH), LIN and TOS (MD+CH) displayed all the resistance mechanisms studied: *kdr* and *AChE* target site mutations and R-PSMO.

Table 3. Genotype frequency (%) of *AChE* and *kdr* loci in *C. pomonella* adults from two susceptible laboratory strains and 32 Spanish field populations. The resistant phenotypes are RR+RS (*AChE*) and rr (*kdr*).

Spanish region	**Population code**	**Orchard management**	**Orchard group**	**n**	**ACE1**			***kdr***		
					RR	**RS**	**SS**	**rr**	**rs**	**ss**
Catalonia	SSp	Laboratory	UN	34	0.0	0.0	100.0	0.0	0.0	100.0
PACA (France)	Sv	Laboratory	UN	37	0.0	0.0	100.0	0.0	0.0	100.0
Asturias	ABB	Organic	UN	40	0.0	0.0	100.0	0.0	0.0	100.0
Asturias	ACE	Organic	UN	40	0.0	0.0	100.0	0.0	0.0	100.0
Asturias	ANA	Organic	UN	34	0.0	0.0	100.0	0.0	0.0	100.0
Asturias	PAE	Organic	UN	21	0.0	0.0	100.0	0.0	0.0	100.0
Asturias	VEV	Organic	UN	36	0.0	0.0	100.0	0.0	0.0	100.0
Extremadura	BAD	Chemically treated	CH	11	0.0	0.0	100.0	0.0	0.0	100.0
Aragon	CAS	Chemically treated	CH	12	0.0	0.0	100.0	0.0	0.0	100.0
Aragon	EPI	Experimental treated	CH	28	3.6	3.6	92.9	0.0	0.0	100.0
Aragon	GRI	Chemically treated	CH	6	0.0	0.0	100.0	0.0	0.0	100.0
Aragon	TAM	Chemically treated	CH	40	40.0	25.0	35.0	0.0	0.0	100.0
Aragon	VIL	Chemically treated	CH	28	7.1	7.1	85.7	0.0	0.0	100.0
Catalonia	COL	Organic	UN	38	13.2	21.01	65.8	23.7	15.8	60.5
Catalonia	EAL	Organic	UN	40	10.0	25.0	65.0	0.0	20.0	80.0
Catalonia	JAB	Abandoned	UN	40	0.0	40.0	60.0	0.0	20.0	80.0
Catalonia	CAM	Chemically treated	CH	40	2.5	87.5	10.0	0.0	0.0	100.0

Table 3 (cont). Genotype frequency (%) of *AChE* and *kdr* loci in *C. pomonella* adults from two susceptible laboratory strains and 32 Spanish field populations. The resistant phenotypes are RR+RS (*AChE*) and rr (*kdr*).

Spanish region	**Population code**	**Orchard management**	**Orchard group**	**n**	**ACE1**			***kdr***		
					RR	**RS**	**SS**	**rr**	**rs**	**ss**
Catalonia	TVI	Chemically treated	CH	37	2.7	64.9	32.4	10.8	13.5	75.7
Catalonia	PAR	Chemically treated	CH	43	0.0	69.8	30.2	14.0	14.0	72.1
Catalonia	BUT	Chemically treated	CH	41	0.0	78.0	22.0	0.0	9.8	90.2
Catalonia	LLI	Chemically treated	CH	40	2.5	75.0	22.5	35.0	17.5	47.5
Catalonia	CML	Chemically treated	CH	31	0.0	67.7	32.3	6.5	6.5	87.1
Catalonia	TRO	MD + chemically	MD+CH	38	68.4	28.9	2.6	2.6	7.9	89.5
Catalonia	TOS	MD + chemically	MD+CH	37	18.9	45.9	35.1	56.8	13.5	29.7
Catalonia	PGR	MD + chemically	MD+CH	37	2.7	54.1	43.2	0.0	75.7	24.3
Catalonia	GAT	MD + chemically	MD+CH	38	47.4	15.8	36.8	21.1	39.5	39.5
Catalonia	INT	MD + chemically	MD+CH	36	11.1	83.3	5.6	8.3	19.4	72.2
Catalonia	TOR	MD + chemically	MD+CH	37	51.4	0.0	48.6	8.1	29.7	62.2
Catalonia	ESC	MD + chemically	MD+CH	40	5.0	92.5	2.5	17.5	20.0	62.5
Catalonia	BEL	MD + chemically	MD+CH	40	25.0	57.5	17.5	0.0	2.5	97.5
Catalonia	LIN	MD + chemically	MD+CH	40	60.0	32.5	7.5	35.0	30.0	35.0
Catalonia	JUN	MD + chemically	MD+CH	42	0.0	76.2	23.8	0.0	2.4	97.6
Catalonia	MIR	MD + chemically	MD+CH	40	5.0	57.5	37.5	0.0	0.0	100.0
Catalonia	SAS	MD + chemically	MD+CH	32	87.5	9.4	3.1	0.0	0.0	100.0

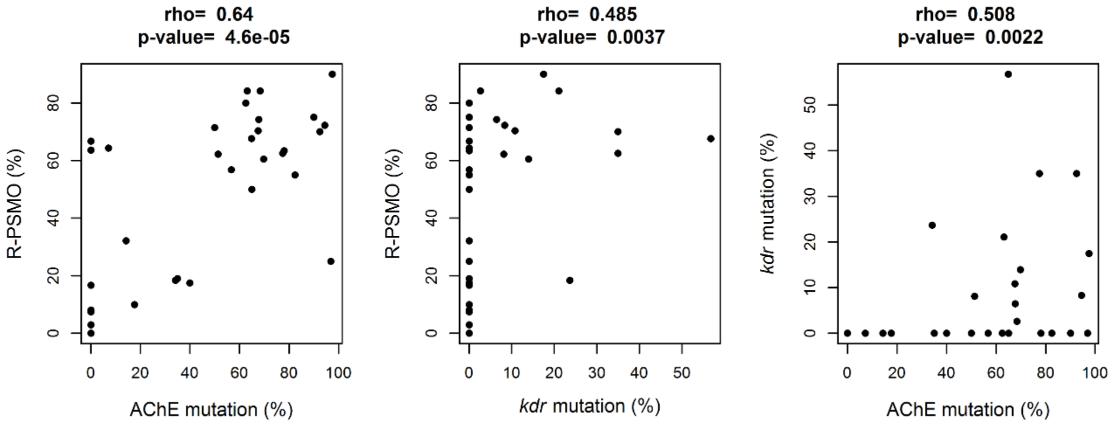

Figure 4. Pearson correlations between RPSMO (% frequency of resistant most), voltage-gated sodium channel, *kdr* mutation percentage and *AChE* mutation percentage, using 32 Spanish field populations and two susceptible strains.

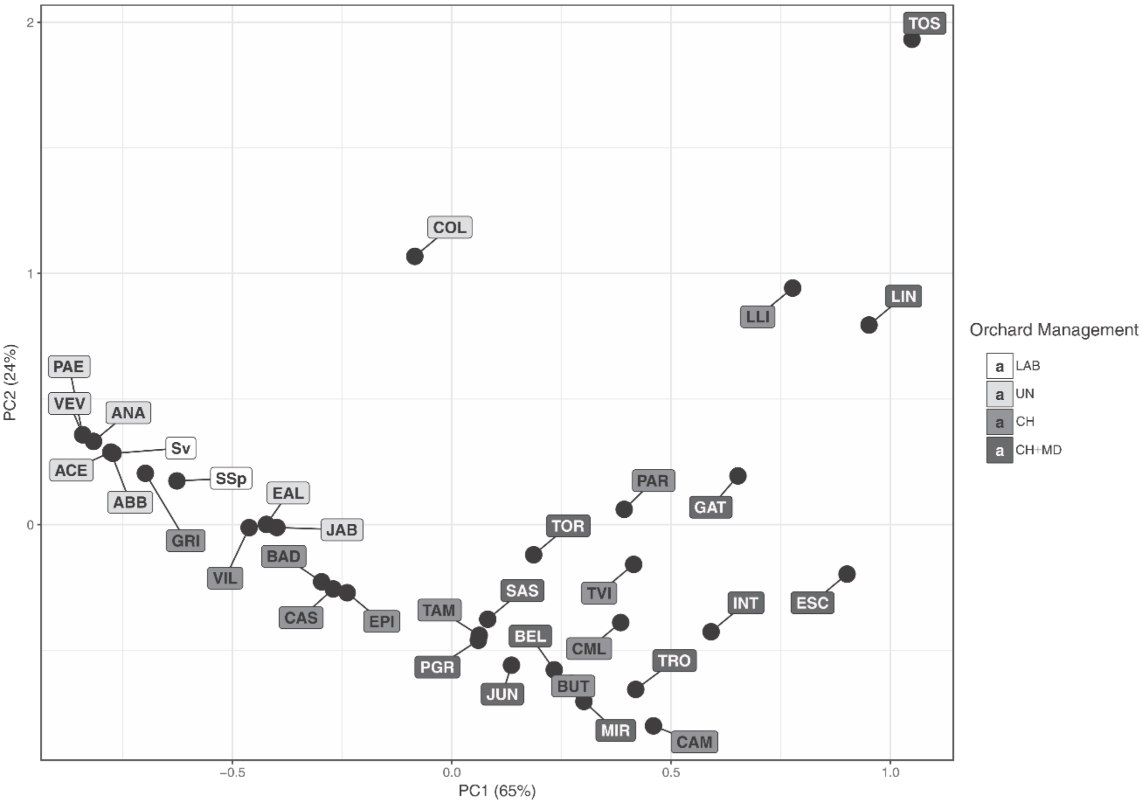

Figure 5. Principal components analysis for the frequency of resistant individuals, R-PSMO, and proportions of *AChE* and *kdr* target site mutations, from 32 Spanish field populations and two laboratory susceptible strains of *C. pomonella*.

Discussion

PSMO activity

The presence and extent of high PSMO activity in codling moth adults and larvae from field populations collected in chemically treated pome fruit orchards from Catalonia is well known (Bosch *et al*., 2016; Rodríguez *et al*., 2010, 2011a, 2012). However, in other Spanish regions, it has only been reported for one population from Aragón (Rodríguez *et al*., 2011b). The results of the present study confirm that the PSMO mechanism occurs widely in areas of Spanish in which codling moth populations are sprayed with insecticides (Catalonia and Aragón), although a more intensive survey in other Spanish pome fruit growing areas, such as Extremadura, should be performed. In contrast, there were no significant differences between the proportion of PSMO-resistant codling moth adults (R-PSMO) in laboratory strains and from UN orchards (Table 2), whether abandoned or organic orchards (mating disruption and carpovirusine treatment strategies). The R-PSMO occurrence was especially low, significantly lower than for the laboratory strain, in populations from Asturias, due to its pome fruit production system. Apples in Asturias are mainly grown for cider production and are generally organically cultivated. This situation has previously been found in Catalonia only in an isolated organic orchard (Rodríguez *et al.* 2011a). Interestingly, the R-PSMO proportions in populations from UN orchards in Asturias and Catalonia were different (from 0% to 7.5% in Asturias, and from 17.5% to 19% in Catalonia). A possible explanation of these results may be the presence of a high proportion of resistant moths in the vicinity of the Catalan UN orchards acting as a resistant moth reservoir, which indicates the importance of knowing the insecticide resistance levels in codling moth field populations at a local scale.

The R-PSMO was not different between CH and MD+CH orchards. As reported in Bosch *et al*. (2016), a pheromone mating disruption control system, used to reduce intensity of insecticide sprayed in the orchards, does not affect the proportion of R-PSMO adults found in the field with respect to CH orchards. Mating disruption used without the support of chemical treatments is not sufficiently effective in most of the Catalan orchards, due to the high population levels. Growers frequently install the mating disruption technique when they cannot control the pest using insecticides, while the optimum situation should be to install it at low population levels (Moffit & Westigard, 1984; Vickers & Rothschild, 1991). Although the application of chemical treatments in these orchards (MD+CH) is less extensive, this seems not to reduce the selection of resistant individuals, even when a resistance strategy management based on insecticide alternations is applied. Boivin *et al*. (2003) suggested the use of alternative methods to chemical treatments to try to reduce the frequency of resistant individuals in the field.

AChE *and* kdr *mutations*

In the present study, both *AChE* and *kdr* target site mutations were detected in Spanish field populations and were not detected in the two laboratory strains tested (Table 3). Occurrence of the *AChE* and *kdr* alleles was observed in samples collected in both chemically treated (CH, MD+CH) and non-chemically treated (UN) orchards. Only one of the 21 orchards from Catalonia (COL) was managed organically. In this case, the selection for the alleles of resistance must occur outside the orchard, in surrounding sprayed commercial orchards, as mentioned before for R-PSMO. The non-chemically treated orchards may be source or refuge of resistant codling moths only when a high proportion of resistant individuals exists in neighbouring areas. Reyes *et al*. (2009) obtained similar results for *kdr* mutation in two untreated field populations from France and Argentina.

Before our study, the *AChE* mutation in the codling moth was described by Cassanelli *et al.* (2006) in a laboratory azinphos-methyl resistant strain, reared from a population collected in an apple orchard of the Lleida region (Catalonia), and by Reyes *et al*. (2007) in a Spanish field population from the same area when they studied the diversity of resistance mechanisms in different countries worldwide. Reyes *et al*. (2015) and Voudouris *et al.* (2011) did not find these mutations in field populations from Chile and Greece, respectively. In our results, the *AChE* mutation was detected in all field populations from Catalonia, not only in 20 out of 20 field populations from the Lleida apple-growing area, but also in one field population from the Girona apple-growing area, 180 kilometres away from Lleida and near to the French border (approximately 62 km). We also found it in three field populations from the Aragón apple-growing area, a distance of from 34 km to 221 km aeay from the nearest Catalan population. Therefore it is likely that the area of Lleida is the source of the *AChE* mutation (five populations have more than 90% of phenotypically resistant individuals) and that it has spread to the other areas. We found the *AChE* mutation only in two geographically continuous areas (Catalonia and Aragón), but not in the Asturias area which is separated from the others by approximately 700 km.

The *AChE* mutation was already present in Catalonia in the 1990's, as the codling moth laboratory strain SSp, collected in 1992 from an abandoned orchard from Lleida apple growing area and reared in laboratory conditions without insecticide exposure since then, showed a low percentage of the mutation (Bosch *et al*., 2014). Nevertheless, this laboratory population did not show significant differences in susceptibility to different insecticidal products or different enzymatic activity levels (EST, GST, PSMO) in comparison to two other susceptible laboratory reference populations from France and Italy (Rodríguez *et al*., 2011b).

Moreover, our results show the occurrence of the *kdr* mutation in field populations from the Catalan apple-growing area. The voltage-gated sodium channel mutation, *kdr*, is widely distributed in many European populations (Reyes *et al*., 2007, 2009), and Franck *et al*. (2012) found independent origins for the *kdr* mutation from different codling moth populations worldwide. This could also be the case for Catalan populations, although Franck *et al*. (2012) did not find the *kdr* mutations in the Spanish field population they tested. We observed the occurrence of *kdr* resistant phenotype (recessive homozygote) in 12 field populations from Catalonia, but 18 field populations showed the resistant allele. In the organic orchard from Catalonia (COL) not treated with pyrethroids, we found the *kdr* mutation in a proportion comparable to the *kdr* proportions found in CH and MD+CH orchard. This could be due to the low fitness cost of the codling moth *kdr* mutation, as suggested by Boivin *et al*. (2001) when they explained the high proportion of *kdr* mutations found in French orchards not treated with pyrethroids.

As with Catalonia, Aragón field populations have been subjected to pyrethroid treatments, but in Aragón the *kdr* mutation was not detected. It is possible that metabolic resistance mechanisms, as enhanced by PSMO activity, play a more important role in pyrethroid detoxification in neonate larvae from Aragon field populations, as previously found by Rodríguez *et al*. (2011b).

Asturias field populations were free of both mutations, but we attribute this result to the management system of the orchards (all under organic production and with regular use of granuloviruses), concurrent with a restricted gene-flow between Asturias and the other Spanish apple-growing areas, due to long geographical distance and low codling moth dispersion capability. The same argument was mooted by Voudouris *et al*. (2011) to explain the lack of the *kdr* mutation in Greece in relation to European codling moth field populations.

Nevertheless, in Spanish field populations we believe that dispersion by adult codling moth played an important role in the spread of the *kdr* mutation. Fragmentation of the continuous resource, orchard type, landscape, and tree canopy structure increase or decrease the adult codling moth mobility at a local scale (Mazzi & Dorn, 2012; Ricci *et al*., 2009, 2011; Stoeckli *et al*., 2008). The codling moth is regarded as a relatively sedentary pest (Chen & Dorn *et al*., 2010; Margaritopoulos *et al*., 2012; Voudouris *et al*., 2012); thus its dispersal ability is rather limited. Several studies aimed at examining its dispersal behaviour using mark-release-recapture and immunomarking methodologies have found that adults largely disperse within 60-80 m (Basoalto *et al*., 2010; Keil *et al*., 2001; Mani & Wildbolz, 1977; Margaritopoulos *et al*., 2012), although a small proportion (7.4% to 20.0%) are able to fly up to several km (Margaritopoulos *et al*., 2012). Immigration of susceptible individuals has been suggested as an important mechanism of insecticide resistance mitigation (Fuentes-Contreras *et al*., 2007, 2014; Reyes *et al*., 2009), and the preservation of susceptible individuals in refuges, within productive growing areas could also be a determinant factor in delaying the increase in the proportion of insecticide-resistant individuals (Caprio *et al*., 2004; Soleño *et al*., 2012; Yu, 2008). Moreover, differences in body mass and wing morphology between sexes are indicative of different dispersal capabilities (Torres *et al*. 2015) and also of different detoxification insecticide capabilities (Fuentes-Contreras *et al*. 2007; Reyes *et al*., 2004, 2015; Varela *et al*., 1993). Therefore, our results suggest that in addition to knowing the resistance status at local scale, the study of the landscape is very relevant, due to its influence on codling moth dispersion.

Nonetheless, movement due to human commercial activities should also be considered, especially in continuous geographical areas. The control of the presence of pests in fruits intended for export is strict, sometimes requiring the European Phytosanitary passport, whereas there is no control for the internal market. This difference in control can affect global codling moth dispersion and consequently, the dispersion of mutations. The codling moth has become a serious quarantine pest for apple exports to some countries and regions, for example Taiwan, Western Australia and China, among others (Washington State University, 2017; Western Australia Government, 2017).

An interesting contribution from this present study to the knowledge concerning codling moth resistance mechanisms in Spanish field populations is the association found between the three resistance mechanisms studied. High R-PMSO and a high proportion of *AChE* and *kdr* mutations were observed in the field populations from Catalonia, and three the three mechanisms of resistance were detected even in a single individual. The combination of different resistance mechanisms has been found in some species in laboratory selected strains (*Musca domestica* L. (Diptera. Muscidae) (Williamson *et al*., 1996), *Drosophila melanogaster* Meigen (Diptera. Drosophilidae) (Mutero *et al*., 1994), *Aedes aegypti* (L.) (Diptera. Culicidae) (Vaughan *et al*., 1997), *Plutella xylostella* (L.) (Lepidoptera. Plutellidae) (Lee *et al*., 2007)), but in few species in field conditions (*Culex pipiens* L. (Diptera. Culicidae) (Gazave *et al*., 2001). The presence and frequency of codling moth mutations found in the field in this present study was surprisingly wide and makes us ponder their ability to persist in natural populations and the cost of their maintenance. Ffrench-Constant *et al*. (1999), in a review of *AChE* mutation studies, said that the acetylcholinesterase enzyme was so efficient that perhaps the resistance associated with the mutations has little effect on its function, and therefore it can be readily maintained in natural populations. In addition, in *D. melanogaster* (Mutero *et al*., 1994) and *A. aegypti* (Vaughan *et al*., 1997), the level of resistance conferred by a single mutation was low, but increased when there were combined mutations. This could be the reason why the low mutation percentage in the codling moth susceptible laboratory strain, SSp, seemed not to produce any resistance effect, because there was no other resistance mechanism activated in it. Conversely, the codling moth field populations had enhanced enzymatic mechanisms combined with the site action mutation. Another possibility to consider is pre-existing polymorphisms in the field populations before the introduction of the insecticides (ffrench-Constant & Bass, 2017). These polymorphisms could become unbalanced due to the use of insecticides. Departure from the Hardy-Weinberg equilibrium was detected in our study in 14 populations for *AChE* and in 9 populations for *kdr*. The insecticide selection was probably the reason for this disequilibrium and the presence of the mutations could also have contributed to it (Wigginton *et al*., 2005).

Since the use of azinphos-methyl (2008) and chlorpiryfos-ethyl (2015) were banned in Europe, the use of chlorpiryfos-methyl and pyrethroids has progressively increased, which implies that the selection of resistant individuals will continue. Although as yet undetected, the presence of unidentified mutations other than *AChE* or *kdr*, wichy may also confer insecticide insensitivity, as seen in other insect species, cannot be ruled out in future codling moth studies (ffrench-Constant, 1999; Pang, 2006; Villatte *et al*., 2000,; Voudouris *et al*., 2011).

The factors that can influence the appearance of resistance mechanisms are complex and variable. Several studies have shown that codling moth resistance mechanisms vary according to the geographical origin of codling moth field populations (Reyes *et al*., 2009; Rodríguez *et al*., 2012), even when the resistance is towards a single insecticide. Furthermore, Li *et al*. (2015) found different invasion processes between codling moth field populations from northeastern and northwestern China. Even biotic (sex) and abiotic (temperature) factors can influence the fitness-cost of resistance and therefore its evolution (ffrench-Constant & Bass, 2017).

Conclusions

Codling moth field populations from the Ebro Valley (Catalonia and Aragón), which was the area most sampled, exhibited a wide range of defence responses to insecticide applications, as evidenced by the high frequency of insects with enhanced PSMO activity and the two identified target-site mutations, *kdr* and *AChE*. In contrast, the field populations from Asturias did not present any of these mechanisms. The Ebro Valley is the only area globally with an extensive distribution of the codling moth *AChE* mutation. In the past, other areas of apple production have also received intensive insecticide treatments with organophosphates and carbamates to control the pest but *AChE* mutation has not been detected, perhaps because it has not been researched sufficiently intensively. The rapid increase in molecular biological information will probably lead to the discovery of as yet unidentified mutations in the most important pest species, the codling moth among them.

Studies on the genetic structure of the codling moth Spanish field populations are needed to investigate the possibility of different codling moth origins in the different Spanish regions, so as to explain the presence of the *AChE* mutation in just one area, and also to explore the variability of fitness costs in the field populations, as dependent upon the geographical origin, the management system and the resistance mechanisms activated.

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CAPÍTULO 3

Determination of the baseline susceptibility of European populations of *Cydia pomonella* (Lepidoptera: Tortricidae) to chlorantraniliprole and the role of cytochrome P450 polysubstrate monooxygenases

Determination of the baseline susceptibility of European populations of *Cydia pomonella* (Lepidoptera: Tortricidae) to chlorantraniliprole and the role of cytochrome P450 polysubstrate monooxygenases

Bosch, D.[1]; Rodríguez, M.A.[2]; Depalo, L.[3]; Avilla, J.[4]

[1] Institut Recerca i Tecnologia Agroalimentària (IRTA), Av. Alcalde Rovira Roure, 191. 25198 Lleida, Spain. *Corresponding autor.*

[2] Universidad de Concepción, Castilla 160-C. Concepción, Chile.

[3] University of Bologna, Viale G. Fanin, 42. 40127 Bologna, Italy.

[4] University of Lleida, Av. Alcalde Rovira Roure, 191. 25198 Lleida, Spain.

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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 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 polysubstrate monooxygenases (PSMO) in the possible decrease of the susceptibility of field populations to the insecticide.

Ten field populations from Spain were used to calculate the baseline response of larvae to chlorantraniliprole incorporated into the diet. A pooled probit line was calculated and three DC concentrations were established: 0.3 mg a.i./kg (close to the LC_{50}), 1.0 mg a.i./kg (close to the LC_{90}), and 10 mg a.i./kg diets (three-fold the LC_{99}). The DC were used to test the susceptibility to chlorantraniliprole of 27 coldling moth 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 PSMO activity. However, the mortality caused by the $DC_{0.3}$ decreased as the mean PSMO activity increased. Field populations resistant to other insecticides were susceptible to chlorantraniliprole. The determined baseline codling moth susceptibility to chlorantraniliprole is a valuable reference for tracking possible future alterations in the efficacy of the insecticide.

Introduction

The codling moth, *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 (Ioriatti & Lucchi, 2016; Witzgall *et al.*, 2008). The existence of codling moth 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: 1A – carbamates, 1B – organophosphates, 3A – pyrethroids, 4A – neonicotinoids, 5 – spinosyns, 6 – avermectins, 7B – phenoxy-ethyl carbamates, 15 – benzoylureas, 18 – diacylhydrazines and 22A oxadiazines (Dunley & Welter, 2000; Fuentes-Contreras *et al.*, 2007; Grigg-McGuffin *et al.*, 2015; Ioriatti *et al.*, 2007; Iscy & Ay, 2017; Knight, 2010; Reuveny & Cohen, 2004; Rodríguez *et al.*, 2010, 2011, 2012; Sauphanor *et al.*, 1998, 2000; Stará & Kocourek, 2007; Voudouris *et al.*, 2011).

Codling moth resistance to insecticides is mainly metabolic, due to three enzymatic complexes (cytochrome P450 polysubstrate monooxygenases (PSMO), 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, when compared to non-chemically treated orchards, an increase of the frequency of resistant codling moth individuals has been detected in many chemically treated orchards in the tree fruit area of Lleida, and enhanced PSMO activity was the main enzymatic mechanism involved (Bosch *et al.*, 2016; Rodríguez *et al.*, 2010, 2011). However, enhanced GST and EST activity was also detected (Rodríguez *et al.*, 2011), as the presence of *AChE* and *kdr* mutations (Bosch *et al.*, 2014). Metabolic cross-resistance, a big concern in any management resistance strategy, may be occur either at the level of interaction with the various chemical families or at a geographical level (Dunley & Welter, 2000; IRAC Codling Moth WG, 2013; Reyes *et al.*, 2007; Voudouris *et al.*, 2011). New pesticides with new modes of action and with an environmentally safe toxicological profile are then necessary to control resistant codling moth 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 IRAC (Insecticide Resistance Action Committee) 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 potent and 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 non-target arthropods (DuPont™, 2008; Lahm *et al.*, 2009). Chlorantraniliprole is active on codling moth, 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 to consider the state of the insecticide resistance in every area.

The aims of this study were to determine the baseline susceptibility of European codling moth 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 mixed function oxidases 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, and the results were pooled to calculate a common probit line. These data, along with others obtained from populations in Italy, France, the Netherlands and Belgium and tested since 2005 in Italy, were used by DuPont® to calculate the DC. Values close to the LC_{50}, LC_{90}, and to three times the LC_{99} were chosen as the DC. These DC were then applied to 27 European populations collected from 2008 to 2015 to test their susceptibility to chlorantraniliprole. Two more concentration – response lines were later determined for two more Spanish populations collected in 2010. The derived DC were whitin the range of the ones previously selected, and, consequently, the data from the 10 Spanish populations 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 PSMO was measured for 12 field populations and for the laboratory susceptible strain.

Insects

The list of the codling moth field populations used in the bioassays is shown in Table 1. The 10 Spanish populations used to adjust the 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 yet 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 codling moth 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 non diapausing 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 codling moth strain, S-Spain, was collected from an abandoned apple orchard in Lleida in 1992, and it has been reared since then using a semi-artificial dehydrated apple diet at the joint IRTA and UdL laboratory (Lleida, Spain). Its response to many insecticides, its PSMO, GST and EST enzymatic activity and the presence of the *AChE* mutation are well known (Bosch *et al.*, 2014; Rodríguez *et al.*, 2010; 2011)

Table 1. Population name, origin, year of collection, state of the insects collected (DP = diapausing) and generation tested of the field codling moth populations treated with chlorantraniliprole to obtain either the probit line (Probit) or the mortality produced by the discriminant concentrations (DC). The PSMO activity was calculated for the populations reported in column PSMO activity.

Population	Country / County	Collection year	Insect collection state	Tested laboratory generation	Bioassay done	PSMO 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 & DP larvae *	F2	Probit	Yes
Cal-4 (SP) (08)	Spain / Aragón	2008	Non DP & DP larvae	F2	Probit	
Torref 15-3 (SP) (08)	Spain / Catalunya	2008	Non DP & DP larvae *	F2	Probit	Yes
Torreg 11-160 (SP) (08)	Spain / Catalunya	2008	Non DP & DP larvae	F2	Probit	
Bal 2-480 (SP) (08)	Spain / Catalunya	2008	Non DP & 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 & DP larvae *	F1	Probit	Yes
Tamarite (SP) (10)	Spain / Aragón	2010	Non DP & 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 & DP larvae *	F2	DC	Yes
Malpartit (SP) (09)	Spain / Catalunya	2009	Non DP & 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	

*Adults caught in pheromone traps

Table 1 cont. Population name, origin, year of collection, state of the insects collected (DP = diapausing) and generation tested of the field codling moth populations treated with chlorantraniliprole to obtain either the probit line (Probit) or the mortality produced by the discriminant concentrations (DC). The PSMO activity was calculated for the populations reported in column PSMO activity.

Population	Country / County	Collection year	Insect collection state	Tested laboratory generation	Bioassay done	PSMO activity
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 & 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) 2015	France / Provence-A-C.A.	2015	DP larvae	F1	DC	
Meckenheim (DE) 2015	Germany	2015	DP larvae	F1	DC	
Orsingen (DE) 2015	Germany	2015	DP larvae	F1	DC	
Ravenna (IT) (15)	Italy	2015	DP larvae	F1	DC	

*Adults caught in pheromone trapsStudies of codling moth insecticide resistance to the main active ingredients used in its control have been performed on some of the Spanish and on one French field populations: Tamarite (10), PuigvertB (11), PuigvertC (11), SAS (11) and Le Thor (FR) (12). The studies include the use of chlorpyrifos-ethyl (organophosphate), lambda-cyhalothrin (pyrethroid), thiacloprid (neonicotinoid), methoxyfenozide and tebufenozide (diacylhydrazines), indoxacarb (oxadiazine), spinosad and spinetoram (spinosyns), chlorantraniliprole (diamide) and emamectin benzoate (avermectin) (see Chapter 1 of the present thesis).

The PSMO activity in codling moth adults was determined for 12 field populations from the Ebro Valley (Spain). Codling moth adults from the first flight were captured from seven orchards in pheromone traps in 2009 and 2010 (Table 1). The 2009 and 2010 1st adult flight catches correspond to the 2008 and 2009 diapausing larvae, respectively, collected in corrugated cardboard. As for the other five populations, collected in 2010 and 2011, the PSMO activity was measured on adults emerging from the collected diapausing larvae.

Insecticide and bioassay experimental procedure

Chlorantraniliprole was used as DPX-E2Y45 20SC (Coragen® 20SC, DuPont de Nemours France SAS., Nambsheim, France) and a Stonefly Premix® (Stonefly Industries LTD) lyophilized diet was used as feeding substrate in the bioassays, following the IRAC susceotibility test method 017 (www.irac-online.org). To content 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. Codling moth neonate larvae (less than 24-h old) were exposed to chlorantraniliprole in diet incorporated assays.

To calculate the baseline probit lines, 20 grams of diet were mixed with 60 g of the insecticide solution at a concentration ranging from 0.01 to 1.0 mg a.i./kg of diet at approximately 3x 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 ± 1 ºC, 16:8 h (L:D) and 45% humidity. Larval mortality was assessed after four days. Larvae were considered dead when they did not 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 the control mortality was < 20% were analyzed Missing larvae were subtracted from the initial number.

Once the DC were decided, the same procedure was carried out to test the susceptibility to chlorantraniliprole of 27 European populations.

PSMO enzymatic activity.

The adult PSMO 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-ethoxycoumarin-O-desethylation (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 pg of 7-ethoxycoumarin (7OH)$\cdot$insect$^{-1}\cdot$min^{-1}.

Data analysis

To calculate the baseline, a probit analysis (using the program POLO Plus, LeOra Software, 1987) was performed, and the LC_{50}, the LC_{90} and their 95% fiducial limits were calculated. Two LC_{50} 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-F_{50}) and the resistance ratio relative to the susceptible laboratory strain (RR-L_{50}) were calculated for the LC_{50}. Values close to the LC_{50}, to the LC_{90}, and to three times the LC_{99} were chosen as the DC. The higher DC was expected to kill approximately 100% of the susceptible population, but a small percentage when applied to a resistant insects (ffrench-Constant & 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 resistance ratio (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 the field populations were calculated to estimate the dispersion of the data.

The frequency of PSMO-resistant individuals in every codling moth field population analysed was compared to the susceptible S-Spain using a Pearson chi-square ($\chi2$) test.

Moths were classified as resistant if their PSMO enzyme activity exceeded the highest activity value corresponding to 90% of S-Spain individuals (Reyes *et al*., 2007). Regression lines between the mean PSMO activity (pg 7OH·insect^{-1}·min^{-1}) and the LC_{50}, and between the PSMO 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 LC_{50} values for the codling moth 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, Torreg 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 six of the ten tested field populations. The $RR\text{-}F_{50}$, calculated comparing each LC_{50} value with the LC_{50} 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 $RR\text{-}L_{50}$, calculated comparing each LC_{50} value with the LC_{50} 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 LC_{50} and LC_{90}. The same happened with Riud (SP) (10), S-Spain and Tam (SP) (10) that had parallel slopes to SAS (SP) (09) ($\chi 2 = 3.34$, d.f. = 3, $P < 0.343$). Torreg 11-160 (SP) (08) had the lowest slope (1.371 ± 0.168) compared to the next smallest one, Torref 15-3 (SP) (08) ($\chi 2 = 4.80$, d.f. = 1, $P < 0.028$). Due to its low slope, Torreg 11-160 (SP) (08) had a very wide interval of concentrations between LC_{50} and LC_{90} and, thus, a high intrapopulation variability and it did not present significant differences with any other field population.

The LC_{50} value of the pooled data was 0.250 mg a.i./kg diet, the LC_{90} value was 0.888 mg a.i./kg diet, and the LC_{99} value was 3.323 mg a.i./ kg of diet.

Table 2. Baseline susceptibility of *Cydia pomonella* Spanish field collected populations to chlorantraniliprole. n = number of individuals tested. RR-F_{50} = resistance ratio calculated by dividing the LC_{50} of the strain tested by the LC_{50} of the most susceptible field population (Riud (SP) (10)). RR-L_{50} = resistance ratio calculated by dividing the LC_{50} of the strain tested by the LC_{50} of the susceptible laboratory population (S-Spain). Heterogeneity factor (HF = $\chi 2$/dF) is provided as a curve fit value. Values of the LC are mg a.i./kg diet. LC_{50} followed by the same letter are not significantly different (LC_{50} are considered significantly different when their CI do not overlap).

Population (year)	n	Probit analyses parameters							RR-F_{50}	RR-L_{50}
		Intercept	Slope ± SE	LC_{50}	CI 95%	LC_{90}	CI 95%	HF		
S-Spain	571	4.039	3.796 ± 0.293	0.086	0.070-0.105	0.188	0.147-0.287	4.296	-	-
Bal 2-371 (SP) (07)	275	1.606	2.220 ± 0.396	0.189 ab	0.124-0.254	0.714	0.488-1.502	1.057	1.2	2.2
Jun 14-16 (SP) (08)	572	1.102	2.183 ± 0.260	0.313 bc	0.220-0.426	1.209	0.786-2.861	2.503	1.9	3.6
Cal-4 (SP) (08)	612	1.974	2.800 ± 0.269	0.197 ab	0.160-0.243	0.566	0.427-0.867	1.661	1.2	2.3
Torref 15-3 (SP) (08)	400	0.684	1.954 ± 0.260	0.446 c	0.309-0.665	2.021	1.173-6.365	2.068	2.8	5.2
Torreg 11-160 (SP) (08)	262	0.671	1.371 ± 0.168	0.324 abc	0.182-0.592	2.788	1.302-11.396	1.793	2.0	3.8
Bal 2-480 (SP) (08)	436	0.809	2.079 ± 0.248	0.408 c	0.312-0.536	1.687	1.114-3.531	1.667	2.5	4.7
SAS (SP) (09)	380	2.235	4.860 ± 0.557	0.347 c	0.251-0.431	0.636	0.503-1.051	3.978	2.2	4.0
Jun 3-63 (SP) (09)	283	2.041	2.722 ± 0.317	0.178 ab	0.137-0.230	0.526	0.383-0.849	1.130	1.1	2.1
Tam (SP) (10)	684	2.624	3.748 ± 0.486	0.199 ab	0.162-0.248	0.438	0.332-0.749	1.830	1.2	2.2
Riud (SP) (10)	554	3.139	3.951 ± 0.337	0.161 a	0.136-0.185	0.339	0.258-0.431	1.797	1.0	1.9
Pooled	3220	1.187	2.184 ± 0.100	0.286	0.253-0.321	1.104	0.921-1.388	2.386	1.6	2.9

Based on these results, and others carried out by DuPont de Nemours in different countries, the DC selected to test the susceptibility of codling moth field populations to chlorantraniliprole were 0.3 and 1.0 mg a.i./kg diet, approximately the LC_{50} and the LC_{90}, respectively, and 10 mg a.i./kg diet, which corresponds to three-fold the LC_{99} 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 codling moth 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 were 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 $RR\text{-}F_{0.3}$ ranged between 1.1 and 4.8, and between 1.0 and 1.5 for the other two DC.

The PSMO enzymatic activity of codling moth adults and the relative frequency of resistant individuals (those whose PSMO activity was higher than 23.92 pg $7OH\cdot adult^{-1}\cdot min^{-1}$, the highest PSMO activity measured in 90% of S-Spain individuals) are shown in Table 4. The mean PSMO activity ranged from 10.52 to 74.61 pg $7OH\cdot insect^{-1}\cdot min^{-1}$, 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 PSMO mean activity of the field populations ($R^2 = 0.95$), which implies that there are few individuals with a very high resistance level that strongly influence the mean PSMO value (Figure 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 ($R^2 = 0.89$, Figure 2). When the regression line was adjusted between the PSMO 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 (Figure 3). The regression lines obtained with the higher DC (1.0 and 10 mg a.i./kg diet) had not good adjustments due to the high mortality obtained in all the field populations treate

Table 3. Susceptibility, expressed as corrected mortality (%), of *Cydia pomonella* European field collected populations to discriminant concentrations of chlorantraniliprole (0.3, 1.0 and 10.0 mg a.i./kg diet). 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. Mean ± SEM of the corrected mortality produced by the DCs and coefficient of variability (CV) across all the field populations were calculated. Numbers in brackets are the number of individuals tested.

Population	**Control mortality (%)**	**Discriminant concentration (mg a.i./kg diet)**					
		0.3	**$RR\text{-}F_{0.3}$**	**1.0**	**$RR\text{-}F_{1.0}$**	**10.0**	**$RR\text{-}F_{10.0}$**
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

Table 3 cont. Susceptibility, expressed as corrected mortality (%), of *Cydia pomonella* European field collected populations to discriminant concentrations of chlorantraniliprole (0.3, 1.0 and 10.0 mg a.i./kg diet). 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. Mean ± SEM of the corrected mortality produced by the DCs and coefficient of variability (CV) across all the field populations were calculated. Numbers in brackets are the number of individuals tested.

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$_{10.0}$**
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	

Table 4. Enzymatic PSMO activity, expressed as frequency of resistant insects (%) and mean activity ± SEM (pg 7OH·insect^{-1}·min^{-1}), of *Cydia pomonella* field adults captured with pheromone traps. The value of the PSMO threshold obtained using the S-Spain population was 23.92 pg 7OH·insect^{-1}·min^{-1}. The frequency of resistant individuals was compared using a Pearson $\chi 2$ test (df = 1; *p = 0.05; **p = 0.01; ***p = 0.001).

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

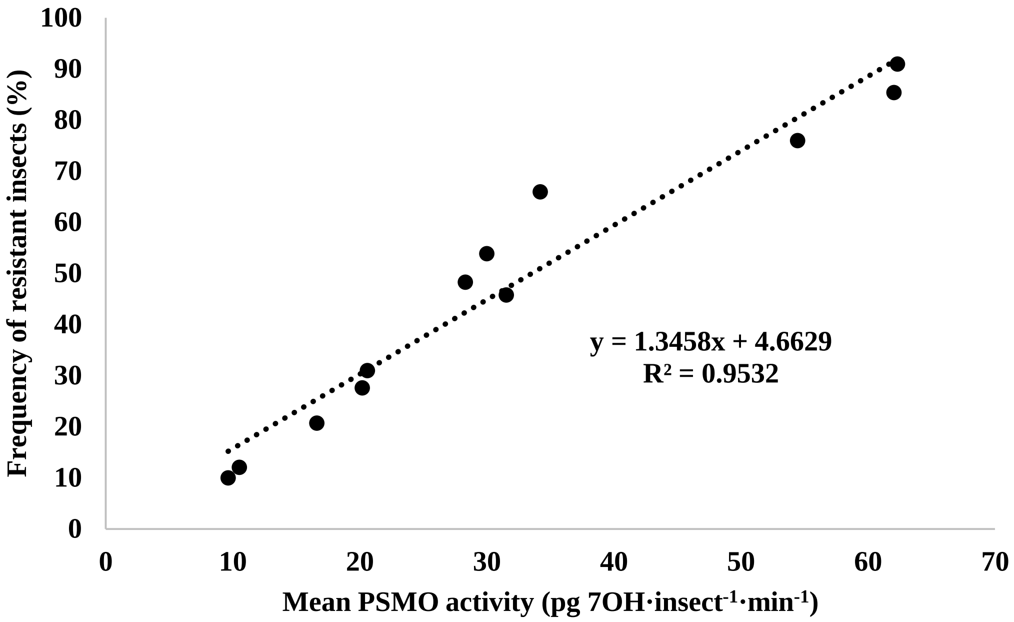

Figure 1. The frequency of resistant codling moth adults in 11 Spanish field populations and the laboratory strain S-Spain as explained by the mean enzymatic PSMO activity.

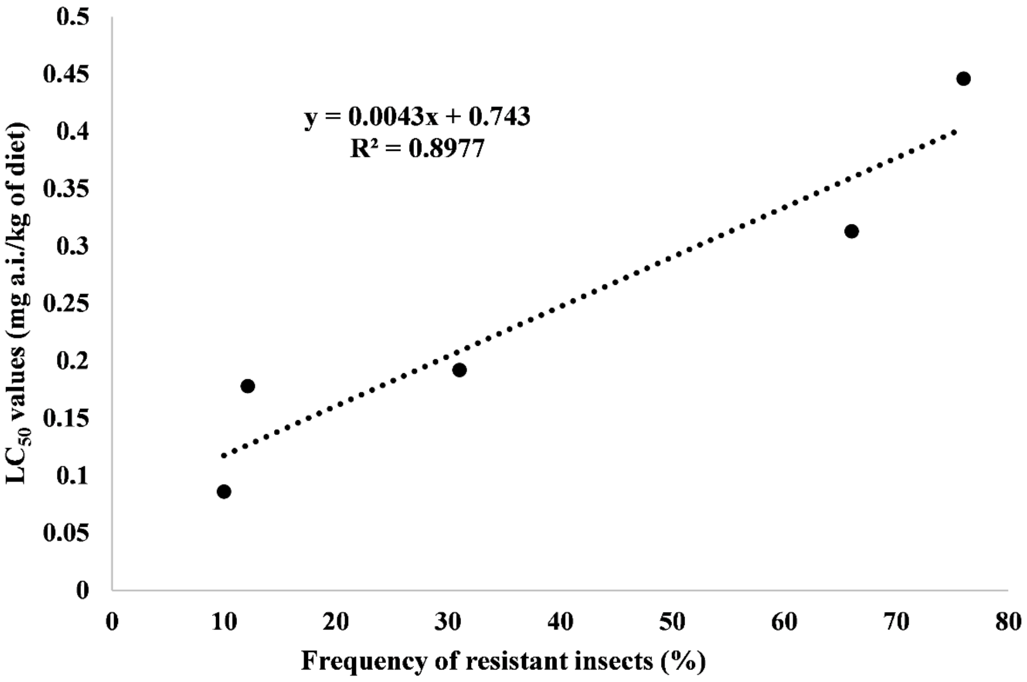

Figure 2. The LC_{50} of chlorantraniliprole for codling moth adults of four Spanish field populations and the laboratory strain S-Spain as explained by the frequency of resistant insects (%).

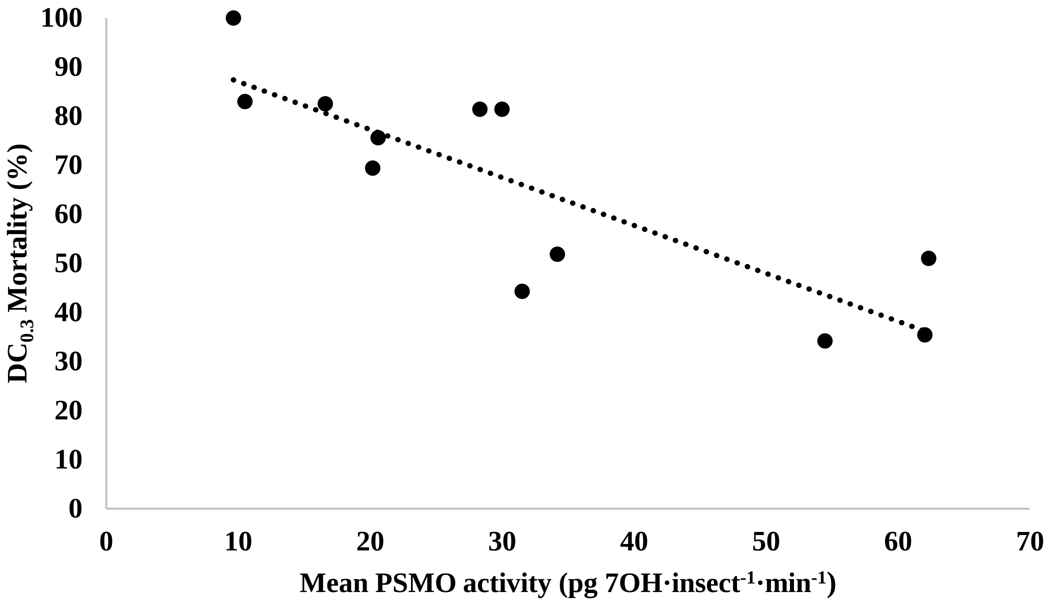

Figure 3. The mortality caused by the chlorantraniliprole $DC_{0.3}$ on larvae of 11 Spanish field populations and the laboratory strain S-Spain as explained by the mean enzymatic PSMO activity in adults.

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. The product was registered in Spain in 2011. The codling moth Spanish field populations showed a high and relatively uniform susceptibility to chlorantraniliprole. This was likely because the RR relative to the most susceptible field populations (the RR-F_{50}) was lower than 2.8, even though significant differences on the LC_{50} 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 was. Ffrench-Constant & 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 the field, the susceptibility of insect populations might reduce over time due to potential resistance evolution (ffrench-Constant & Roush, 1990). In this case, susceptible strains can be helpful as an invariable mortality reference point, which are commonly used (Caballero *et al*., 2013; da Silva, 2012; Lai *et al*., 2011; Zheng *et al*., 2011).

The LC_{50} 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 mg a.i./L and 0.59 mg a.i./L, respectively. The same insecticide diet incorporation bioassay obtained an LC_{50} value range in different laboratory susceptible strains of different species of Lepidoptera: 0.014 mg a.i./L in *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) (Lai *et al*., 2011) and 0.28 mg a.i./L in *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae) (Sial & Brunner, 2012). Different species respond in a different way to the same insecticide, independently of the body mass differences. For example, codling moth is heavier than *Lobesia botrana* (Denis & Schiffermüller) (Lepidoptera: Tortricidae) but females of codling moth were 115 times more susceptible to thiacloprid than females of *L. botrana* were (Navarro *et al*., 2017). In the laboratory, where the 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 ($RR\text{-}L_{50}$) was 5.2 (Table 2) which is a low value compared to 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 LC_{50} 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 LC_{50} 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 $RR\text{-}F_{0.3}$ obtained were similar to the $RR\text{-}F_{50}$, demonstrating its reliability in comparing populations. The $DC_{1.0}$ produced a mean mortality of 96.44%, and 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 Spanish market, but the population had a very high mean PSMO activity (Table 4), which may play a role in the detoxification of the active ingredient, as it 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 (two years), Isle SLS (three years) and Noves P (five 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% to 93.3%, except in 2012, when the efficacy of the $DC_{0.3}$ decrease to 20.8% (obtaining a $RR_{0.3}$ 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 codling moth 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 (Chapter 1 of this thesis; Rodríguez *et al*., 2010, 2011). Insecticide bioassays (Chapter 1 of this thesis) 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 pyretroid 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) was tolerant to different active ingredients, but with RRs always lower than 4.4. None of these populations was resistant to chlorantraniliprole, with SAS (11) the only one that had a RR slightly higher than 2 (RR = 2.2). Despite being multi-resistant 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 (Selvy *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 PSMO, 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 (PSMO) 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 codling moth field populations is PSMO (Rodríguez *et al*., 2010, 2011), and in the studied populations, the frequency of PSMO 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 LC_{50} was used instead of the mortality of the $DC_{0.3}$ (R^2 = 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, PSMO seems not to be the main mechanism implied in the detoxification of the product although it may have a certain role in it.

Cydia 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 PSMO enzymatic activity and the mortality produced by the approximate LC_{50} used as a DC. As many insecticides can induce an PSMO 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 codling moth baseline susceptibility data established provides a valuable reference for tracking possible future alterations in the efficacy of the product.

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CAPÍTULO 4

Captures of MFO-resistant *Cydia pomonella* adults as affected by lure, crop management system and flight

Captures of MFO-resistant *Cydia pomonella* adults as affected by lure, crop management system and flight

Bosch, D.[1]; Rodríguez, M.[2]; Avilla, J.[3]

[1] Institut Recerca i Tecnologia Agroalimentària (IRTA), Av. Alcalde Rovira Roure, 191.
25198 Lleida, Spain. *Corresponding autor.*

[2] Universidad de Concepción, Castilla 160-C, Concepción, Chile

[3] University of Lleida, Av. Alcalde Rovira Roure, 191. 25198 Lleida, Spain

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Abstract

The main resistance mechanism of codling moth (*Cydia pomonella*) in the tree fruit area of Lleida (NE Spain) is multifunction oxidases (MFO). We studied the frequency of MFO-resistant adults captured by different lures, with and without pear ester, and flights in orchards under different crop management systems. The factor year affected codling moth MFO-resistance level, particularly in the untreated orchards, highlighting the great influence of codling moth migration on the spread of resistance in field populations. Chemical treatments and adult flight were also very important but mating disruption technique showed no influence. The second adult flight showed the highest frequency, followed by the first flight and the third flight. In untreated orchards there were no significant differences in the frequency of MFO-resistant individuals attracted by Combo^{TM} and BioLure^{TM}. Red septa lures baited with pear ester (DA) captured sufficient insects only in the first generation of 2010, obtaining a significantly lower proportion of MFO-resistant adults than Combo^{TM} and BioLure^{TM}. In the chemically treated orchards, in 2009 BioLure^{TM} caught a significantly lower proportion of MFO-resistant adults than Combo^{TM} during the first and third flight, and than DA during the first flight. No significant differences were found between the lures or flights in 2010.

These results cannot support the idea of a higher attractiveness of the pear ester for MFO-resistant adults in the field but do suggest a high influence of the response to the attractant depending on the management of the orchard, particularly with regard to the use of chemical insecticides.

Introduction

The codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), the main pest for apple, pear and walnut crops worldwide, has usually been controlled with insecticides. In Spain, organophosphates have been widely used for more than thirty years, but since the late 90's, control of the pest through the use of insecticides has become more difficult due to the development of resistance (Bosch *et al*., 1999). At present, the control strategy for codling moth relies on the combined use of chemicals and mating disruption, especially in problematic orchards. Mating disruption has been applied extensively in the Ebro Valley since approximately 2007 and was applied in 2009 on more than 10,000 ha, of which, according to the distributors, 5,450 ha were in Lleida, accounting for 68% of the production area. These figures were approximately the same in 2011 (Agriculture Department, personal communication). Pest population monitoring is a crucial component of any integrated pest management program. To monitor the phenology of codling moth in the field, the use of traps baited with 1 mg (in chemical orchards) or 10 mg (in mating disruption orchards) of (*E, E*)-8,10-dodecadien-1-ol (codlemone) has been extensive. However, in some cases, the lack of catches in the traps was unreliable (false negative), and particularly in mating disruption orchards it is necessary to check fruits to discard fruit infestations and not rely only on the trap catches. In 2001, Light *et al*. described a pear-derived kairomone (pear ester), ethyl (2*E*, 4*Z*)-2,4-decadienoate, which attracted *C. pomonella* male and female adults. Recently, the use of a synthetic lure baited with a combination of 3 mg of this kairomone and 3 mg of codlemone, Pherocon CM-DA Combo^{TM}, has been quickly replacing the use of 10 mg codlemone lures in mating disruption orchards because it catches a higher number of moths (Torà *et al*., 2009; Joshi, 2011).

Cydia pomonella has developed resistance to a wide range of insecticides in almost all productive apple areas in the world (Sauphanor *et al*., 1998, 2000; Reuveny & Cohen, 2004; Fuentes-Contreras *et al*., 2007; Ioratti *et al*., 2007; Stará & Kocourek, 2007; Soleño *et al*., 2008, 2012; Knight, 2010; Rodríguez *et al*., 2010, 2012). A high frequency of resistant codling moth individuals has been detected in most of the problematic orchards in the tree fruit area of Lleida (NE Spain) (Rodríguez *et al*., 2011). Insect resistance may be due to the detoxification of the insecticides by three enzymatic complexes (mixed function oxidases [MFO], glutation transferases [GST] and esterases [EST]), or to structural changes (mutations) in the insecticide target protein that make the protein less sensitive to the insecticide (the acetylcholinesterase [*AChE*] mutation in the case of organophosphates and carbamates, described by Cassanelli *et al.* [2006], and the knockdown resistance [*kdr*] mutation in the voltage-gated sodium channel in the case of pyrethroids, described by Brun-Barale *et al*. [2005]). An increased production of MFO enzymes in adults and larvae was involved in the insecticide detoxification in all the field populations of the area tested (Rodríguez *et al*., 2010, 2012). GST were also detected, and EST were detected only in larvae (Rodríguez *et al*., 2011). These results cannot be generalized to all the codling moth populations in the area, but confirm that it is a real and increasing problem that may interfere with the management of the orchards.

Negative pleiotropic effects of insecticide resistance in codling moth, such as different development rates (Boivin *et al*., 2001, 2003a) and critical photoperiod for inducing diapause (Boivin *et al*., 2005), may affect the proportion of resistant insects present in the field during the different generations. These alterations have also been reported in other Lepidoptera species such as *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) and *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) (Han *et al*. 2012; Ribeiro *et al*., 2014). Negative effects on sexual communication, such as low pheromone production by females and lower ability of males to detect the pheromone source, have also been detected (Poulot *et al*., 2001, Trimble *et al*., 2004). Nevertheless, Frérot *et al*. (1999) and Poulot *et al*. (2001) found that the level of attraction of the codling moth sex pheromone in the traps for released susceptible and resistant males in the orchards and in a wind tunnel was the same. Sauphanor *et al*. (2007) demonstrated a higher attraction of MFO-resistant male moths in pure kairomone–baited traps in apple orchards.

The aim of this work was to determine the frequency of MFO-resistant *C. pomonella* adults captured by different lures, with and without kairomone, and during the different flights in orchards under different crop management systems. The general state of the resistance in the area with regard to MFO activity is also noted.

Material and methods

Insects and attractants

Field codling moth catches were obtained in 20 and 25 field orchards in 2009 and 2010, respectively. Five orchards were classified as chemically untreated (UN, abandoned or organic orchards), eight as chemically treated (CH) and 32 as pheromone mating disruption orchards supplemented with chemical insecticides (MD + CH). The plots were distributed throughout the production area of Lleida (Catalonia, NE Spain), with a maximum distance of 60 km between them. The Spanish susceptible strain (S_Spain) was used as a reference to determine the level above which an insect was recorded as resistant. This threshold was the highest 7-ethoxycoumarin-O-deethylation (ECOD) activity value corresponding to 90% of the S_Spain–analyzed individuals. S_Spain was collected from an abandoned apple orchard in Lleida in 1992, and it has been reared since then using a semi-artificial dehydrated apple diet at the Sustainable Plant Protection Laboratory of the UdL-IRTA Centre for R&D (University of Lleida, Institute for Food and Agricultural Research and Technology, Lleida, Spain).

The three codling moth attractants compared were BioLure^{TM} CM 10X (Suterra) (named BioLure, loaded with 10 mg of (*E*, *E*)-8,10-dodecadien-1-ol [codlemone]), Pherocon CM-DA Combo^{TM} (named Combo, a mixture of 3.0 mg of codlemone and 3.0 mg of ethyl (2*E*, 4*Z*)-2,4-decadienoate) and red septa lures baited with pear ester (named DA, 3.0 mg of pear ester). The traps were revised once a week and well maintained and the attractants were changed approximately every eight weeks. The maximum period of time in which adults were analyzed was 4 weeks for each generation to avoid catching and analyzing adults from different generations in the same trap. The moment to begin the analysis was decided on the basis of the phenological model used in the area and was approximately one week after the prediction of the beginning of each adult flight. During these periods the adult field catches were taken to the laboratory twice a week in order to obtain as many live adults as possible.

Enzymatic activity

The adult MFO activity was determined with an in vivo protocol (Rodríguez *et al.*, 2012) using 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 a 465 nm emission filters and was expressed as pg of 7-ethoxycoumarin (7OH)$\cdot$adult$^{-1}\cdot$min^{-1}.

Data analysis

The differences between the absolute frequencies of resistant individuals according to the different attractants, adult flights, management systems and years were compared using a Pearson chi-square (χ^2) test. Moths were classified as resistant if their MFO enzyme activity exceeded the highest activity value corresponding to 90% of S_Spain individuals (Reyes *et al*., 2007)

Results

The three periods of codling moth trap catches in 2009 were 6 to 26 May, 30 June to 30 July, and 18 August to 1 September. It was considered that the adults caught in these periods belonged to the first, second and third flights, respectively. In 2010 the three periods were 5 to 31 May, 13 July to 16 August, and 23 August to 20 September (Figure 1). BioLure- and Combo-baited traps tracked in a similar way the moth flights during the two seasons in mating disruption and non-mating disruption orchards. In 2009 the number of catches was in general higher than in 2010 and the three flight peaks were also more evident in the flight curve. The flight curve obtained with DA is not represented in Figure 1 due to the low number of captures. The maximum number of catches per trap and week was recorded both years in non-mating disruption orchards, in Bio traps in 2009 and Combo traps in 2010, resulting in 38.3 and 9.5 moths, respectively. The second flight always showed a lower number of catches than the first, and the third flight was always very short, especially in 2009.

The percentages of MFO-resistant insects obtained in 2009 and 2010 in the orchards with different management systems using the attractants BioLure and Combo are shown in Table 1. The value of the MFO threshold obtained using the S-Spain population was 23.92 pg $7OH\cdot adult^{-1}\cdot min^{-1}$. In both years and with both attractants the UN orchards obtained a significantly lower proportion of resistant insects than the CH and the MD + CH orchards, and the CH orchards obtained the same proportion of resistant insects as the MD + CH orchards (BioLure 2009, UN-CH, dF = 1; $\chi^2 = 7.24$ and $p = 0.007$; UN–MD + CH, dF = 1; $\chi^2 = 10.00$ and $p = 0.002$; CH-MD + CH, dF = 1; $\chi^2 = 0.09$ and $p = 0.762$; BioLure 2010, UN-CH, dF = 1; $\chi^2 = 65.57$ and $p = 5.60 \times 10^{-16}$; UN-MD + CH, dF

= 1; χ^2 = 121.96 and p = 2.35 x 10^{-28}; CH-MD + CH, dF = 1; χ^2 = 0.14 and p = 0.705; Combo 2009, UN-CH, dF = 1; χ^2 = 33.39 and p = 7.5 x 10^{-9}; UN-MD + CH, dF = 1; χ^2 = 28.55 and p = 9.15 x 10^{-8}; CH-MD + CH, dF = 1; χ^2 = 3.68 and p = 0.055; Combo 2010: UN-CH, dF = 1; χ^2 = 84.21 and p = 4.46 x 10^{-20}; UN-MD + CH, dF = 1; χ^2 = 103.80 and p = 2.23 x 10^{-24}; CH-MD + CH, dF = 1; χ^2 = 1.16 and p = 0.282). It can also be seen in Table 1 that there was always numerically higher number of MFO-resistant moths in 2009 than in 2010. These differences were significant in the UN orchards for BioLure attractant and in the chemically treated orchards (CH and MD + CH) for Combo attractant (BioLure, UN orchards 2009-2010, dF = 1; χ^2 = 9.50 and p = 0.002; CH orchards 2009-2010, dF = 1; χ^2 = 0.04 and p = 0.839; MD + CH orchards 2009-2010, dF = 1; χ^2 = 1.99 and p = 0.158; Combo, UN orchards 2009-2010, dF = 1; χ^2 = 2.04 and p = 0.153; CH orchards 2009-2010, dF = 1; χ^2 = 9.37 and p = 0.002; MD + CH orchards 2009-2010, dF = 1; χ^2 = 18.84 and p = 1.41 x 10^{-5}).

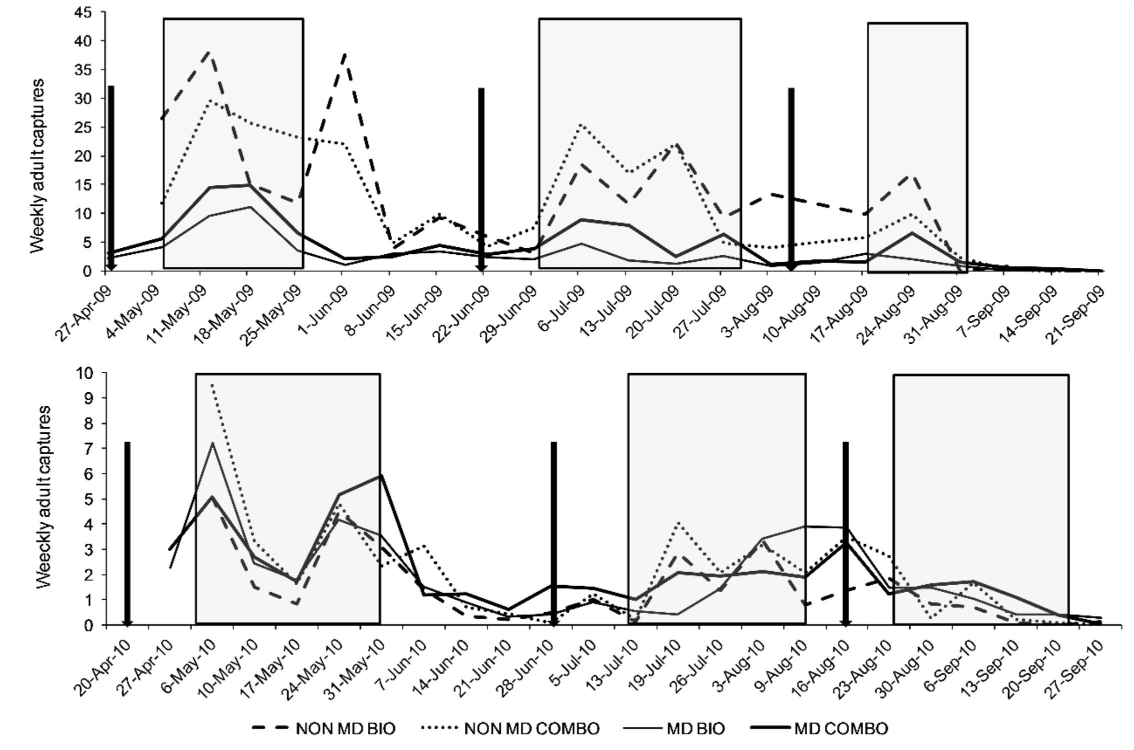

Figure 1. Mean weekly codling moth adults caught in mating disruption (MD) and non-MD orchards using BioLure^{TM} CM 10X and Pherocon CM-DA Combo^{TM} lures and period of time (rectangle area) when the MFO activity was measured in each flight (gray area). Number of traps = 30 (2009) and 58 (2010). Solid bars show the beginning of each generation.

The frequencies of resistant individuals caught during the three adult flights in the orchards with different management systems during the two years of the assay are shown in Table 2. There were no significant differences between the frequency of MFO-resistant adults captured in the different flights in the UN orchards, in spite of the higher proportion of resistant adults in the second flight in both years (2009, 1st – 2nd flight, dF = 1; χ^2 = 1.30 and p = 0.255; 2010, 1st – 2nd flight, dF = 1; χ^2 = 2.86 and p = 0.091; 1st – 3rd flight, dF = 1; χ^2 = 0.11 and p = 0.745; 2nd – 3rd flight, dF = 1; χ^2 = 3.32 and p = 0.069). In the CH and MD + CH orchards the second adult flight was the one with the highest proportion of MFO-resistant adults and the differences from the first flight were always significant. The third flight was the one with the lowest frequencies of MFO-resistant adults in 2009 but in 2010 it was the first flight which had the lowest frequency. (2009, CH orchards, 1st – 2nd flight, dF = 1; χ^2 = 9.99 and p = 0.002; 1st – 3rd flight, dF = 1; χ^2 = 6.35 and p = 0.012; 2nd – 3rd flight, dF = 1; χ^2 = 24.83 and p = 6.27×10^{-7}; 2009, MD + CH orchards: 1st – 2nd flight, dF = 1; χ^2 = 5.85 and p = 0.016; 1st – 3rd flight, dF = 1; χ^2 = 4.99 and p = 0.025; 2nd – 3rd flight, dF = 1; χ^2 = 13.22 and p = 2.77×10^{-4}; 2010, CH orchards, 1st – 2nd flight, dF = 1; χ^2 = 4.04 and p = 0.045; 1rst – 3rd flight, dF = 1; χ^2 = 1.18 and p = 0.277; 2nd – 3rd flight, dF = 1; χ^2 = 0.01 and p = 0.922; 2010, MD + CH orchards, 1st – 2nd flight, dF = 1; χ^2 = 8.78 and p = 0.003; 1st – 3rd flight, dF = 1; χ^2 = 5.03×10^{-4} and p = 0.982; 2nd – 3rd flight, dF = 1; χ^2 = 1.85 and p = 0.174).

Table 1. Percentage of MFO-resistant codling moth adults caught in 2009 and 2010 in orchards with different management systems in the area of Lleida in traps baited with BioLure™ CM 10X and Pherocon CM-DA Combo™.

Lure	Management system	2009			2010		
		Nº orchards	Nº insects	Resistance frequency (%)	Nº orchards	Nº insects	Resistance frequency (%)
BioLure	UN	2	36	47.2 a A	3	215	22.8 a B
	CH	2	135	71.1 b A	6	103	69.9 b A
	MD+CH	16	367	72.5 b A	16	478	68.0 b A
Combo	UN	2	32	34.4 a A	3	211	22.7 a A
	CH	2	123	84.6 b A	6	178	69.1 b B
	MD+CH	16	583	76.7 b A	16	492	64.6 b B

UN, untreated orchards; CH, chemically treated orchards; MD + CH, mating disruption orchards supported by chemical treatments. For each attractant, numbers followed by the same lower case letter on the same column are not significantly different (P<0.05). For each attractant and management system, numbers followed by the same upper case letter on the same line are not significantly different (P<0.05)

Table 2. Percentage of MFO-resistant codling moths adults caught in the three flights of the years 2009 and 2010 in orchards with different management systems in the area of Lleida.

Year	Management system	Nº orchards	1st flight		2nd flight		3rd flight	
			Nº insects	Resistance frequency (%)	Nº insects	Resistance frequency (%)	Nº insects	Resistance frequency (%)
2009	UN	2	40	35.0 a	31	48.4 a	2	-
	CH	2	140	76.4 b	100	92.0 a	44	56.8 c
	MD+CH	16	727	75.0 b	214	82.2 a	108	64.8 c
2010	UN	3	189	19.6 a	138	27.5 a	127	18.11 a
	CH	6	194	63.4 b	100	75.0 a	27	74.1 ab
	MD+CH	16	797	64.1 b	242	74.4 a	42	64.3 ab

UN, untreated orchards; CH, chemically treated orchards; MD + CH, mating disruption orchards supported by chemical treatments. For each year and management system, numbers followed by the same lower case letter on the same line are not significantly different (P<0.05).

As the year, the adult flight and the application of treatments in the orchards influenced the amount of MFO-resistant codling moths in the field, all these factors were taken into account to study the influence of the lure in the frequency of MFO-resistant individuals captured in the traps (Figures 2 and 3). From this moment the CH and MD + CH orchards were considered as chemically treated orchards.

There were no significant differences in the frequency of MFO-resistant individuals attracted by Combo and BioLure in the UN orchards in either of the two years. The attractant DA captured enough insects to compare with the other two attractants only in the first flight of 2010, obtaining a significant lower proportion of MFO-resistant adults than Combo and BioLure (2009, 1st flight, Combo-BioLure, dF = 1; χ^2 = 1.39 and p = 0.238; 2nd flight, Combo-BioLure, dF = 1; χ^2 = 0.89 and p = 0.345; 2010, 1st flight, Combo-BioLure, dF = 1; χ^2 = 0.08 and p = 0.784; Combo-DA, dF = 1; χ^2 = 6.83 and p = 0.009; BioLure-DA, dF = 1; χ^2 = 6.24 and p = 0.012; 2nd flight, Combo-BioLure, dF = 1; χ^2 = 0.42 and p = 0.518; 3rd flight, Combo-BioLure, dF = 1; χ^2 = 0.38 and p = 0.540). In the 2009 chemically treated orchards BioLure caught a significantly lower proportion of MFO-resistant adults than Combo during the first and third flight and also than DA during the first flight. There were no significant differences between the frequency of MFO-resistant insects attracted by the different lures during the three flights in 2010 (2009, 1rst flight, Combo-BioLure, dF = 1; χ^2 = 4.27 and p = 0.039; Combo-DA, dF = 1; χ^2 = 0.53 and p = 0.466; BioLure-DA, dF = 1; χ^2 = 3.90 and p = 0.048; 2nd flight, Combo-BioLure, dF = 1; $\chi^2 = 0.49$ and $p = 0.482$; Combo-DA, dF = 1; $\chi^2 = 0.16$ and $p = 0.688$; BioLure-DA, dF = 1; $\chi^2 = 0.003$ and $p = 0.956$; 3rd flight, Combo-BioLure, dF = 1; $\chi^2 = 3.96$ and $p = 0.047$; 2010, 1st flight, Combo-BioLure, dF = 1; $\chi^2 = 0.29$ and $p = 0.593$; Combo-DA, dF = 1; $\chi^2 = 0.05$ and $p = 0.831$; BioLure-DA, dF = 1; $\chi^2 = 0.32$ and $p = 0.572$; 2nd flight, Combo-BioLure, dF = 1; $\chi^2 = 0.21$ and $p = 0.650$; Combo-DA, dF = 1; $\chi^2 = 2.55$ and $p = 0.110$; BioLure-DA, dF = 1; $\chi^2 = 2.06$ and $p = 0.152$; 3rd flight, Combo-BioLure, dF = 1; $\chi^2 = 0.02$ and $p = 0.883$).

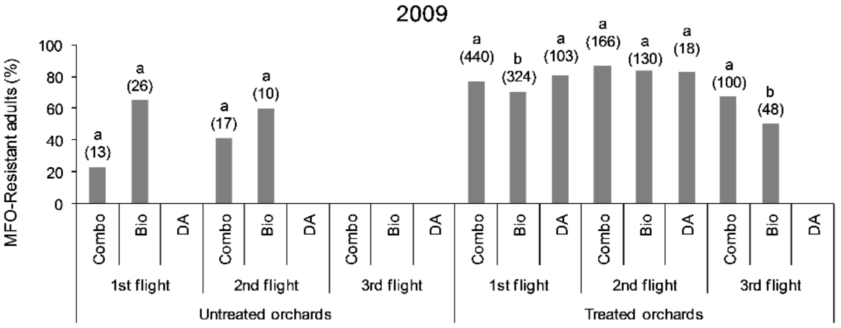

Figure 2. Percentage of MFO-resistant codling moth adults in the three field flights captured by delta traps lured with Pherocon CM-DA Combo™, BioLure™ CM 10X and the pear ester DA (3.0 mg of pear ester) in two untreated and 18 treated orchards of the production area of Lleida during the year 2009. Numbers within the brackets represent the number of insect processed.

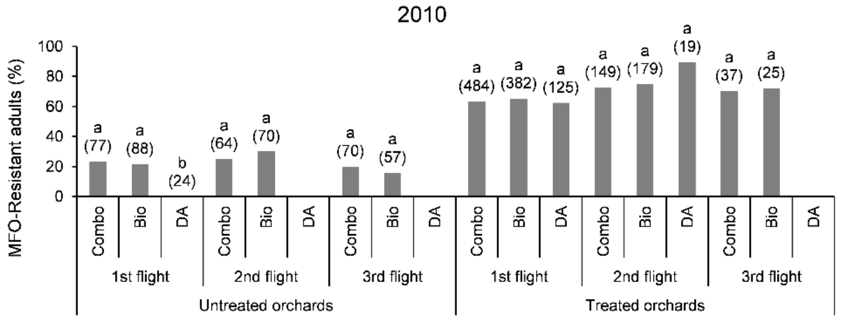

Figure 3. Percentage of MFO-resistant codling moth adults in the three field flights captured by delta traps lured with Pherocon CM-DA Combo™, BioLure™ CM 10X and the pear ester DA (3.0 mg of pear ester) in three untreated and 22 treated orchards of the production area of Lleida during the year 2010. Numbers within the brackets represent the number of insect processed.

Discussion

The MFO-resistance level of codling moth in the field was affected by the year. In general, the frequency of MFO-resistant adults was higher in 2009 than in 2010. A higher number of catches obtained during the year usually led to a higher number of chemical treatments to control the pest and consequently a higher selection of insecticide-resistant individuals. The UN orchards, in this case abandoned and organic orchards, did not use insecticides to control codling moth and the organic orchards based their strategy on mating disruption and carpovirusine treatments. The frequency of MFO-resistant codling moth adults was higher in 2009 and lower in 2010 in the UN orchards. The lower number of captures obtained in 2009 may influence this result, but this result may also point out the great influence that can have the codling moth migration in the spread of resistance in field populations. Several dispersal behavior studies using mark-release-recapture and immunomarking methodologies found that the main proportion of adults dispersed within 60-80 m (Keil *et al.*, 2001; Margaritopoulos *et al.*, 2012), although a small proportion (7.4% to 20.0%) was able to fly up to several kilometers as an adaptive trait, in order to survive in case of habitat deterioration (Schumacher *et al.*, 1997a, 1997b; Keil *et al.*, 2001). This capacity of dispersion may be responsible for the fluctuations in the presence of resistant adults in the untreated orchards that, in our area, were surrounded by treated orchards.

Presence or absence of chemical treatments in the orchards is the main factor affecting the frequency of MFO-resistant adults. No difference was found between orchards using codling moth mating disruption and those not using it. Mating disruption used without the support of chemical treatments is not sufficiently effective in the area of Lleida to control the pest because of an important constraint of this technique: the need for low population levels (Moffit & Westigard, 1984; Vickers & Rothschild, 1991). Therefore, the use of insecticides combined with mating disruption did not in general lead to a significant reduction in resistance selection.

The phenology of codling moth in the field depends on major abiotic factors such as temperature and day length (Shel'Dova, 1967; Riedl & Croft, 1978; Steimberg *et al.*, 1992) and biotic factors such as food availability (Brown *et al.*, 1979; Riedl, 1983). However, the application of insecticides may condition its phenology and cause behavioral and/or physiological damage to insects besides death (Buckingham *et al.*, 2005; Davies *et al.*, 2007; Casida, 2009; Tan *et al.*, 2014). The different moth adult flights showed a significantly different proportion of MFO-resistant adults in chemically treated orchards (CH and MD + CH). The second adult flight had the highest frequency of resistant individuals. These adults came from the surviving neonate larvae affected by the chemical treatments applied in the orchards during the entire first adult flight. The first flight in the area of the assay lasted approximately 9 weeks in 2009 and 11 weeks in 2010, while the second and third flights lasted approximately 6-7 weeks and 4-5 weeks, respectively. The longer duration of the first flight usually means, in case of a medium or high population in the orchard, that a higher amount of insecticide treatments may be applied. The frequency of MFO-resistant first adult flight depends on the frequency of the second and third generation diapausing larvae of the previous year, and on their proportion, which in turn depends on the annual variation in climate conditions. Boivin *et al*., (2004) stated that if the third generation in the field was not successfully completed, due to a sudden shorter summer or harvest, the second generation diapausing cohorts were the only source for the genetic pool of the next spring generation. In addition, in winter negative pleiotropic effects on the codling moth diapausing larvae associated with enhanced MFO levels may be expected (Boivin *et al*., 2001). Deleterious effects in MFO-resistant pupae during diapause were also proved in *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) (Daly, 1993). The third adult flight showed the lowest frequency of MFO-resistant adults. Homozygous MFO-resistant individuals have slower developmental rates and earlier diapause timing than susceptible homozygotes and heterozygotes (Boivin *et al*., 2003b; Boivin *et al*., 2004). We may therefore assume that the third flight was mainly formed by these more susceptible individuals that were able to produce an additional generation. Data from 2010 may also support this conclusion. The proportion of MFO-resistant individuals in 2010 was lower than in 2009, therefore a lower number of homozygous individuals can be expected in the field justifying the lack of significant differences against the three adult flights.

In 2009 treated orchards, BioLure was less attractive than the other lures containing pear ester during the first and the third generation, but these results were not confirmed in 2010, when all the attractants captured the same frequency of MFO-resistant adults. Conversely, in UN orchards BioLure always caught the same frequency of MFO-resistant individuals as Combo, but in 2010 in first flight —the only flight with sufficient captures— DA caught the lowest frequency of resistant insects. Sauphanor *et al*. (2007) found significantly higher mean MFO activity in first flight male adults captured in field traps baited with 40 mg of pear ester than in those captured in traps baited with 10 mg of codlemone. The differences remained in the chemical and organic orchards in both years. However, some differences in the tests may have influenced the results. Sauphanor *et al.* (2007) used the mean pg of 7OH formed per adult and minute to compare the level of resistance of the different orchard populations, whereas we considered that some high values in a few insects may significantly modify the average and preferred to use the frequency of resistant individuals as an indicator of the level of resistance in the orchards. Furthermore, in our study previous results led us to include females in the analysis. Rodríguez *et al*. (2010), in field adults from different orchards in the same production area, found no clear tendency in the enzymatic activity between sexes. The differences appeared at random and were due to higher values for males or for females depending on the orchards. Therefore, due to the low number of captures traditionally obtained with the pear ester alone in our area (Bosch & Avilla, 2005), we decided to include the females to increase the number of captured adults analyzed in the DA attractant.

The opposite tendency in the attraction of MFO-resistant adults of pear ester in treated and untreated orchards may be influenced by the sublethal effect of insecticides. A list of identified effects of insecticide sublethal doses on the olfactory system of agricultural insect pests can be found in Tricoire-Leignel *et al*. (2012). Field treatments with the ecdysteroid agonists tebufenozide and methoxyfenozide decrease male responses to pheromone in the tortricid moths *Choristoneura fumiferana* (Clemens), *C. rosaceana* (Harris), *Argyrotaenia velutinana* (Walker), *C. pomonella* and *Grapholita molesta* (Busck) (Hoelscher & Barrett, 2003; Dallaire *et al*., 2004; Barrett, 2008). Barret (2010) found that methoxyfenozide also disrupted responses of codling moth males to pear ester. Treatments with the organophosphate malathion decrease the ability of corn borer *Ostrinia furnacalis* (Gueneé) (Lepidoptera: Crambidae) males to respond to the female pheromone (Zhou *et al*., 2005) and some pyrethroids disrupt the male attraction to the sex pheromone in the pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), the corn borer *O. furnacalis* and the noctuid *Trichoplusia ni* Hübner (Haynes & Baker, 1985; Haynes *et al*., 1986; Moore, 1988; Clark & Haynes, 1992; Wei & Du, 2004). In addition, pesticides could affect in a different way the chemical communication of susceptible and resistant insects. The alteration of sexual communication, a fitness component, imposed by insecticide resistance in some moth species is expected to contribute to the decline of resistance in the absence of insecticide (Delisle & Vincent, 2002). Sauphanor *et al*. (2007) found that pear ester produced an enhanced or altered response in wind tunnel in codling moth adults with MFO and *kdr* resistance mechanisms. However, this response of *kdr*-resistant genotipes was not confirmed in the field. The enzymatic system mainly involved in insecticide detoxification in Spanish codling moth field populations, monooxygenases (Rodríguez *et al*., 2012), is also involved in the recognition of the host-plant (Feyereisen, 1999). Trimble *et al.* (2004) found that the proportion of azinphos-methyl resistant males of obliquebanded leafrollers (*C. rosaceana*) that located a synthetic pheromone source in the orchard was 32% lower than the proportion of susceptible males, while in the flight tunnel a similar proportion of susceptible and resistant males located the source. According to Sauphanor *et al*. (2007), the sensory or behavioral response to a semiochemical compound is more likely to be altered by a mutation affecting the nervous system than by a metabolic resistance.

Summarizing, in the field populations of the study area a general enhanced enzymatic MFO activity in all the chemically treated orchards was demonstrated. The frequency of MFO-resistant codling moth adults was more stable in these orchards than in the untreated orchards that showed a higher variability depending on the general frequency of MFO-resistance in the year. These results proved the importance of dispersion of resistant adults in a neighboring production area. The role of the chemically untreated orchards as a reservoir of insecticide susceptible individuals must to be demonstrated. Our results cannot support the idea of a higher attractiveness of the pear ester for MFO-resistant adults in the field and point to a different response depending on the management of the orchards. In addition, the target mutations in sodium channel gene (*kdr*) and AChE1 proteins (*AChE*), respectively responsible for pyrethroid and organophosphate resistance, have been detected extensively in the Ebro Valley production area (Bosch *et al*., 2014). Further research is needed to clarify the possible incidence of a higher proportion of individuals in the field with the mutations (*kdr* and *AChE*) in response to different semiochemical compounds.

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DISCUSIÓN GENERAL

Discusión general

Diversidad y extensión de la resistencia a insecticidas de C. pomonella *en España (Objetivos a y b).*

La resistencia a los insecticidas se considera distinta a la tolerancia a los insecticidas. La tolerancia es considerada como la habilidad natural de una población a resistir el efecto tóxico de un insecticida (Yu, 2008). Se puede desarrollar en una generación como resultado de una adaptación fisiológica, pero se pierde cuando los insectos no están expuestos al tóxico. Por el contrario, la resistencia implica un cambio genético heredable como respuesta a un proceso de selección por un tóxico (Sawicki, 1987), y por lo tanto, es un proceso evolutivo. Por convención, el término resistencia se aplica a situaciones en que el ratio de resistencia (RR) es como mínimo de 10 (Mota-Sánchez *et al.*, 2008).

En los bioensayos realizados en las poblaciones de la zona del Valle del Ebro se obtuvo una baja susceptibilidad, comparada con la de la población susceptible, en un 37 % de las combinaciones población-insecticida ensayadas (Capítulo 1). Sin embargo, en la mayor parte de esos casos (70 %), esa baja susceptibilidad implicaba ratios de resistencia (RR) bajos, entre 1,1 y 4,4, lo que podría considerarse dentro de los niveles de tolerancia naturales de cada población. Se obtuvieron RR similares en los bioensayos realizados en poblaciones de campo de la misma zona con chlorantraniliprole (Rynaxypyr®) (Capítulo 3), materia activa que nunca había sido aplicada, y en bioensayos realizados por otros autores con el mismo producto en otras especies de Lepidoptera (Campos *et al.*, 2015; Wang *et al.*, 2010; Zheng *et al.*, 2011) los cuales concluyeron que las poblaciones de campo presentaban una baja variabilidad en su respuesta.

En los bioensayos de la presente tesis doctoral, un 7 % de las combinaciones población-insecticida resultaron resistentes a los insecticidas. Todas las combinaciones que resultaron resistentes se dieron en tres poblaciones de Catalunya (PuigverdB, PuigverdC y Mir7/84) y con tres insecticidas (lambda-cihalotrin, metoxifenocide y tiacloprid) destacando los elevados RR obtenidos con lambda-cyhalotrin. Los casos de resistencia documentados a productos insecticidas de amplio espectro, principalmente organofosforados y piretroides, en *C. pomonella* han sido frecuentes (Bush *et al.*, 1993; Dunley & Welter, 2000; Mota-Sánchez *et al.*, 2008; Voudouris *et al.*, 2011). Mota-Sánchez *et al*. (2008) en Michigan, zona con un historial de insecticidas similar al de nuestra zona de estudio, basado en los organofosforados, detectó resistencia a metil-azinfos, lambda-cihalotrin y metoxifenocide. La resistencia a lambda-cyhalotrin de las poblaciones de Catalunya y Aragón está justificada por su amplio uso, junto a otros piretroides, tanto para el control de carpocapsa como de otras plagas, debido a su corto plazo de seguridad y sus bajos precios, mientras que la resistencia a metoxifenocide es debida, probablemente, a una resistencia cruzada, tal y como ya había sido anteriormente documentada en carpocapsa (Mota-Sánchez *et al.*, 2008) y en otros tortrícidos (Ahmad *et al.*, 2002; Waldstein *et al.*, 1999). Metoxifenocide resultó ser la materia activa menos efectiva en el conjunto de las poblaciones obteniendo una mortalidad siempre significativamente inferior a la de la población susceptible. Los RR obtenidos con este producto oscilaron entre 3,2 y 15,9 en cinco de las siete poblaciones del ensayo, siendo superiores a 10 en dos de ellas.

La reducción de la susceptibilidad de la plaga a estos insecticidas, aunque sea obteniendo unos RR inferiores a 10, puede tener importantes implicaciones en el control de la misma en campo ya que las exigencias del mercado en lo referente a la presencia de daños son muy elevadas. En Israel, RR entre 2 y 7 obtenidos con organofosforados en larvas neonatas supusieron un importante incremento de frutos atacados en campo por carpocapsa (Reuveny *et al.*, 2001) y un RR de 14 en un bioensayo con metoxifenocide en una población de campo en Michigan supuso un elevado número de penetraciones en fruto en un ensayo de actividad residual insecticida (Mota-Sánchez *et al.*, 2008).

La población PuigverdC fue la única que resultó resistente a tiacloprid. El resto de las poblaciones resultó con RR inferiores a 3,1. El uso de tiacloprid en ese momento (2010-12) no era extensivo para el control de carpocapsa, sin embargo, sí que se utilizaba en el control de la psila del peral y cabe la posibilidad de que el flujo de poblaciones entre fincas vecinas contribuyera en este resultado. A pesar de que esta población resultó multiresistente, (a metoxifenocide (RR = 14,6) y a lambda-cyhalotrin (RR = 876,0)), no se pensó en una posible resistencia cruzada de tiacloprid con alguno de estos productos ya que otras poblaciones del ensayo, también resistentes a los mismos insecticidas, obtuvieron con tiacloprid RR inferiores a 2,5.

Etil-clorpirifos resultó ser muy efectivo en todas las poblaciones de campo. Su eficacia no había disminuido respecto a la que había demostrado en años anteriores (Rodríguez *et al.*, 2011) a pesar de ser el organofosforado más utilizado. El resto de productos ensayados, tebufenocide, spinosad, spinetoram, indoxacarb, clorantraniliprole o emamectine, resultaron efectivos en el control de dichas poblaciones.

Los resultados de los bioensayos en las poblaciones asturianas fueron muy distintos a los de las poblaciones del Valle del Ebro, ya que resultaron muy similares a la población susceptible de referencia. Estos resultados eran los esperados debido a las diferencias en el manejo de las poblaciones (Miñarro & Dapena, 2001; Miñarro & Jaques, 2011). Las manzanas producidas en Asturias se dedican casi exclusivamente a la producción de sidra (4.470 ha para la producción de sidra y 180 para la producción de manzana de mesa (MAPAMA, 2013)), por lo que las exigencias en la comercialización del producto son distintas, además de soportar una incidencia de carpocapsa y otros lepidópteros mucho menor que en la zona del Valle del Ebro.

Para determinar los mecanismos de detoxificación enzimática causantes de la resistencia encontrada en las poblaciones de campo de la zona del Valle del Ebro el uso de sinergistas no resultó efectivo (Capítulo 1). Las PSMO y las EST están implicadas en el 63 % y 64 % de los casos de detoxificación en lepidópteros, mientras que las GST únicamente en un 36 % de los casos (Navarro-Roldán, 2017). Las poblaciones de campo españolas, en las que se realizan tratamientos fitosanitarios, presentan unos niveles de PSMO altos de forma generalizada (Capítulo 2, Bosch *et al.*, 2016; Rodríguez *et al.*, 2011), sin embargo, esto no quedó reflejado en nuestros resultados con sinergistas en las poblaciones que resultaron resistentes en los bioensayos insecticidas.

Los resultados de la aplicación de sinergistas dependen de varios factores. Uno de ellos es la dosis a aplicar, que es variable en función del sinergista y de la población a tratar. Un inconveniente importante para resolverlo es la dificultad de obtener una elevada progenie de una población de campo para poder realizar el cálculo de las dosis apropiadas tanto de los sinergistas como de los insecticidas. Otro factor es el tiempo transcurrido entre la aplicación del sinergista y la aplicación del insecticida, ya que en la bibliografía se pueden encontrar distintos planteamientos. Y por último, el tiempo transcurrido entre la aplicación del tratamiento y la lectura del nivel enzimático en las poblaciones (Navarro-Roldán, 2017). Estos resultados implican que es necesario mejorar la metodología para poder obtener información fiable a partir del uso de sinergistas.

En cuanto a los mecanismos de resistencia por insensibilidad en el sitio de acción (Capítulo 2), la mutación de la acetilcolinesterasa o acetilcolinesterasa insensitiva (*AChE*) está presente de forma generalizada en las fincas del Valle del Ebro (96 %), mientras que la insensibilidad nerviosa o *knockdown resistance* (*kdr*) está presente en la mayoría de las fincas en Catalunya (57 %). Se ha muestreado únicamente una población procedente de

Extremadura, por lo que no podemos considerar que dicha zona productiva esté suficientemente estudiada para descartar la presencia de mutaciones, mientras que el número de poblaciones de Asturias fue de cinco y en ninguna de ellas se encontró la presencia de alelos resistentes, resultado que era esperable teniendo en cuenta el resultado de los bioensayos y el histórico de tratamientos de la zona de producción.

Así pues, podemos afirmar que las poblaciones españolas de carpocapsa del Valle del Ebro, que fue la zona con una mayor intensidad de muestreo, presentan una amplia gama de mecanismos para defenderse de la toxicidad de los insecticidas, tanto a nivel de mecanismos de detoxificación enzimática como de insensibilidad en el punto de acción. Al constatar la gran diferencia con las poblaciones asturianas, parece claro que esta diversidad de mecanismos ha sido provocada por la intensa selección con insecticidas a la que han sido sometidas las poblaciones. Sin embargo, las poblaciones del Valle del Ebro son las únicas en el mundo que presentan la mutación *AChE*, a pesar de que en la mayoría de las zonas productivas de manzana de mesa las poblaciones de carpocapsa se han visto sometidas de forma más o menos intensiva a aplicaciones de insecticidas (Charmillot *et al*., 2002; Ioratti *et al*., 2007; Reyes *et al*., 2004; Sauphanor & Bouvier, 1995; Stará *et al*., 2006; Varela *et al.*, 1993), y a tratamientos con organofosforados y/o carbamatos en concreto, como en nuestro caso (Croft & Riedl, 1991; Fuentes-Contreras *et al*., 2007; Parra-Morales *et al*., 2017). La causa podría ser un muestreo insuficiente, ya que el estudio de la presencia de mutaciones se ha realizado fundamentalmente en algunos países en Europa y América del Sur (Reyes *et al*., 2007, 2009; Voudouris *et al*., 2011), aunque también podría ser debido a un distinto origen de las poblaciones de cada zona, y por lo tanto, una distinta base genética que ha evolucionado de distinta forma ante la selección con insecticidas.

Posibilidades de control y manejo de las poblaciones resistentes
(Objetivos c y d)

Los insecticidas ejercen una presión de selección en los insectos que ha comportado la evolución de la resistencia a la mayoría de los modos de acción insecticida del mercado (Tabashnik *et al*., 2014) y esto ha hecho muy necesaria la búsqueda de estrategias de manejo de dichas resistencias.

El concepto de manejo de la resistencia consiste en prevenir el incremento de la frecuencia de genes resistentes en una población intentando no seleccionarla de forma importante mediante el uso de insecticidas. Para ello IRAC (http://www.irac-online.org/about/resistance/management/) recomienda el uso de tantas estrategias de control como sean posibles, no utilizar únicamente insecticidas sintéticos y biológicos, sino también métodos de control culturales, biológicos y biotecnológicos. Existen distintas estrategias de manejo de resistencias como por ejemplo actuaciones para reducir la frecuencia génica de individuos resistentes, el uso de mezclas, secuencias y rotaciones de insecticidas, el uso de nuevos insecticidas o bien el uso de depredadores y parasitoides resistentes, todo ello combinado con un buen monitoreo de la plaga en campo (Yu, 2008). Sin embargo, la valoración de dichas estrategias es muy difícil y compleja. En campo existen muchas perturbaciones en la interpretación de resultados (Parker *et al.*, 2006) y en laboratorio el tamaño de población evaluada es poco representativo de la realidad en campo. Esto ha hecho que se realicen estudios de la evolución de la resistencia a partir de modelos matemáticos que simulen un amplio rango de sistemas de manejo. Se han realizado estudios con estrategias de uso de distintas dosis de insecticida (Roush & Tabashnik, 1990; Tabashnik & Croft, 1982), de uso de mezclas de insecticidas (Comins, 1986; Mani, 1985) o de uso alterno de insecticidas (Mani, 1989) para ver la reducción del desarrollo de los niveles de resistencia en poblaciones. Helps *et al.* (2017) concluyeron, a partir de un estudio mediante un modelo matemático, que reduciendo la dosis de insecticida se obtenía con frecuencia un retraso en el desarrollo de la resistencia. Sin embargo, en determinadas situaciones esto no se cumplía: cuando en la población había una importante entrada de población susceptible, cuando la resistencia se debía a un gen recesivo o cuando se utilizaba un insecticida de elevada eficacia.

Así pues, conocer la eficacia del insecticida a utilizar es uno de los puntos básicos en el manejo de la resistencia a insecticidas y el estudio de la línea base de susceptibilidad del mismo es una herramienta muy útil que servirá para detectar los cambios de susceptibilidad del producto en un futuro y detectar una posible resistencia al mismo de forma temprana.

En el Capítulo 3 se realizó el estudio de la línea base de susceptibilidad de la materia activa clorantraniliprole (Rynaxypyr®), producto desarrollado por DuPont (USA) y que fue registrado para su uso en Europa los años 2008-09. Es una diamida y su modo de acción ha sido clasificado dentro de un nuevo grupo por IRAC (grupo 28, moduladores del receptor de la rianodina) (Nauen, 2006). Para ello se calcularon unas concentraciones discriminantes próximas a la CL_{50}, CL_{90} y a tres veces la CL_{99}. Los RR obtenidos en veintisiete poblaciones de origen europeo oscilaron entre 4,8 y 1,1, demostrando una baja variabilidad de la respuesta de las poblaciones al producto en una amplia zona geográfica. La eficacia insecticida de clorantraniliprole fue buena incluso en aquellas poblaciones donde se había encontrado resistencia a lambda-cihalotrin, a metoxifenocide o a tiacloprid (Capítulo 1), demostrando que no existía resistencia cruzada entre estas materias activas. Sin embargo, se observó que la frecuencia de individuos con una elevada actividad PSMO estaba relacionada con el porcentaje de mortalidad obtenido con la concentración discriminante próxima a la CL_{50}. Esto resaltaba la importancia del uso, de forma estricta, de los criterios de manejo de resistencia recomendados por la casa comercial, ya desde el inicio del registro del producto en el mercado (http://www.irac-online.org): realizar dos tratamientos consecutivos a la dosis recomendada durante la primera generación de carpocapsa, separados entre 12-14 días y utilizar insecticidas con distinto modo de acción para controlar la segunda y tercera generación (DuPont, 2014).

A partir de esta línea base se podrá realizar un seguimiento de la respuesta de distintas poblaciones de *C. pomonella* al producto a lo largo de sus años de aplicación en el campo y detectar cualquier cambio en la eficacia del producto.

Como hemos visto en la primera parte de la discusión, los insecticidas no registrados en España emamectin y spinetoram resultaron también muy efectivos en el control de las poblaciones resistentes de carpocapsa a los insecticidas más convencionales al igual que spinosad, tebufenocide e indoxacarb (Capítulo 1). En una estrategia de manejo de resistencias los agricultores necesitan disponer de diversos insecticidas efectivos para poder controlar la plaga, así como saber situarlos en campo en el momento correcto tanto para controlar la plaga como para obtener un nivel de residuos aceptable en el momento de la recolección. Actualmente, las exigencias del mercado son muy estrictas a nivel de residuos estando, en algunos casos, muy por debajo de las autorizadas legalmente lo que complica de sobremanera las estrategias de control de plagas.

Por otro lado, en cualquier programa de control de carpocapsa, el seguimiento y el muestreo de las poblaciones en campo, tanto mediante capturas en trampa como con muestreos visuales, es crucial para decidir el momento y la necesidad de realizar un tratamiento insecticida. Siendo la confusión sexual una técnica muy necesaria para reducir el nivel poblacional, uno de los principales problemas que comporta es el poder realizar un seguimiento fiable del vuelo de los adultos. Para ello, se han estado utilizando trampas cebadas con difusores de 10 mg de feromona o bien, cada vez con más frecuencia, con difusores Pherocon CM-DA Combo® (3 mg de feromona y 3 mg de cairomona, el éster de pera (2E, 4Z)-decadienoato de etilo). Este atrayente captura un elevado número de adultos, principalmente machos, en nuestras condiciones (Joshi *et al.,* 2011, Torà *et al*. 2009) y posibilita el seguimiento del vuelo, siempre y cuando la trampa esté bien situada y mantenida: en la parte más alta del árbol y cambiando los difusores y las bases engomadas cuando sea necesario.

En poblaciones resistentes de laboratorio se determinó que la resistencia producía efectos pleiotrópicos en el desarrollo y la fenología de carpocapsa (Boivin *et al*., 2001, 2003, 2005) y que esto podía afectar a la proporción de individuos resistentes presentes en cada generación. Estos resultados, sin embargo, realizados con poblaciones seleccionadas en laboratorio no pueden decir mucho acerca de los costos reales de la resistencia en el campo (Helps *et al*., 2017). Por su parte, Sauphanor *et al*. (2007) demostró que la cairomona pura producía una elevada atracción en campo de machos de carpocapsa con elevados niveles de PSMO. Dados los elevados niveles de PSMO en las poblaciones de campo de la zona productiva de Lleida, este hecho podía implicar la posibilidad de mejorar el monitoreo en las fincas con una elevada presencia de insectos resistentes utilizando el mejor atrayente para su monitoreo.

Según nuestros resultados la mayor proporción de adultos resistentes en campo se capturó durante el segundo vuelo y la menor durante el tercer vuelo (Capítulo 4; Bosch *et al*., 2016). Atribuimos estas diferencias a que la mayor incidencia de tratamientos insecticidas se produce durante el primer vuelo, por tener una mayor duración en el tiempo. Sin embargo, el hecho de tener una menor proporción de individuos PSMO-resistentes en el tercer vuelo sí que podía deberse a un efecto pleiotrópico de la resistencia, indicando una entrada en diapausa de forma más temprana de los individuos homocigotos resistentes. No se obtuvo un mayor nivel de capturas de individuos PSMO-resistentes en los atrayentes que contenían la cairomona, ni en los difusores Pherocon CM-DA Combo® ni en difusores cebados únicamente con la cairomona. Estos últimos atrayentes capturaron un número extremadamente bajo de capturas por lo que no fue posible obtener las curvas de vuelo ni analizar suficiente número de individuos.

Por todo ello, en una estrategia de manejo o de prevención de la resistencia de las poblaciones españolas de carpocapsa es necesario el uso de confusión sexual y de insecticidas selectivos de bajo riesgo como los mencionados en la primera parte de la discusión, cuando fuera necesario, ya que se han demostrado eficaces en el control de poblaciones de campo resistentes a insecticidas convencionales ampliamente utilizados. Además, la utilización de insecticidas biológicos como el virus de la granulosis debería incorporarse a la estrategia y ser ampliamente utilizado, tanto en las fincas con problemas ya establecidos, por su capacidad de control y/o reducción de las poblaciones, como en las fincas con una baja incidencia de la plaga (Bosch, 2015). Boivin *et al*. (2003) afirmaron que una estrategia de manejo de resistencias únicamente basada en la alternancia de productos químicos no sería suficiente para reducir la selección de individuos resistentes y apuntaban la necesidad de utilizar métodos de control alternativos a los químicos.

Cuando las aplicaciones insecticidas en campo fallan, en muchos casos, ya es demasiado tarde para implementar estrategias de resistencia debido a la presencia de una alta frecuencia de individuos resistentes. Por ello, es importante realizar de forma rutinaria bioensayos insecticidas y enzimáticos, y determinar las posibles resistencias genéticas presentes en las zonas de producción en estudio. Sería también interesante el estudio del origen de las poblaciones en las distintas zonas productivas de España así como el estudio de la variabilidad de los costos adaptativos de las poblaciones de campo en función del sistema de manejo y de los mecanismos de resistencia presentes en dichas poblaciones.

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CONCLUSIONES

Conclusiones

Objetivo a. *Evaluar la resistencia a insecticidas de* C. pomonella *en 3 áreas de producción españolas con dos sistemas de manejo de plagas completamente distintos, determinando la eficacia insecticida de nuevos productos sobre larvas neonatas de poblaciones que presenten, en algún caso y dependiendo de la zona, resistencia a los insecticidas tradicionalmente utilizados, como son los organofosforados y piretroides.*

- El sistema de manejo de las fincas es el factor determinante de la presencia de resistencias a insecticidas. Por ello, las poblaciones Asturianas, dedicadas a la producción de sidra y con baja incidencia de tratamientos, resultaron susceptibles a todos los insecticidas ensayados mientras que las poblaciones del Valle del Ebro presentaron resistencia en algún caso o una menor susceptibilidad.
- Las materias activas que resultaron menos eficaces fueron metoxifenocida, lambda-cyhalotrin y tiacloprid. Todas las poblaciones de las zonas con tratamientos insecticidas presentaron tolerancia o resistencia a metoxifenocida. Lambda-cyhalotrin fue el insecticida con el que se alcanzaron unos RR más elevados.
- Etil-clorpirifos y el resto de productos ensayados, tebufenocida, spinosad, spinetoram, indoxacarb, clorantraniliprol y emamectina, resultaron efectivos en el control de las poblaciones, incluso en las que presentaban elevados niveles de resistencia alguna de las materias activas.
- No se detectó resistencia cruzada entre los insecticidas convencionales y los nuevos insecticidas de bajo riesgo.
- El uso de sinergistas no resultó efectivo para determinar los mecanismos enzimáticos de resistencia activos en las poblaciones estudiadas. El ajuste de las dosis necesarias en las poblaciones de campo resultaría necesaria para poder obtener una información fiable así como la estandarización de una metodología.

Objetivo b. *Determinar la extensión de las mutaciones* AChE *y* kdr *en poblaciones españolas de* C. pomonella.

- La mutación de la acetilcolinesterasa (*AChE*) está presente de forma generalizada únicamente en las fincas del Valle del Ebro, mientras que la *knockdown resistance* (*kdr*) está presente en la mayoría de las fincas en Catalunya. En las poblaciones de Asturias y la población de Extremadura analizada no se detectaron mutaciones.

- Es necesario un muestreo más intensivo de otras zonas productivas de manzanos y/o perales para descartar la presencia de estas mutaciones en España.

Objetivo c. Realizar el estudio de una línea base de resistencia insecticida de carpocapsa con chlorantraniliprole (Rynaxypyr®), materia activa de una nueva familia insecticida, en poblaciones españolas y europeas, así como el seguimiento de la evolución de la resistencia al mismo a lo largo de los años.

- Los niveles de tolerancia natural de las poblaciones europeas de carpocapsa ensayadas determinaron una baja variabilidad en la respuesta a clorantraniliprol, similar a la respuesta al producto en otras especies de insectos plaga.
- La mortalidad obtenida en las poblaciones ensayadas con las concentraciones letales a lo largo de los años demuestra que, por el momento, no hay resistencia al producto.
- A pesar de no implicar resistencia, existe una relación entre la frecuencia de individuos PSMO-resistentes con el porcentaje de mortalidad en la aplicación de la concentración discriminante próxima a la CL_{50}.

Objetivo d. Estudiar la frecuencia de adultos resistentes (con un elevado nivel de enzimas de detoxificación PSMO) de C. pomonella *capturados en trampas con distintos difusores, con y sin la presencia de cairomona, durante las distintas generaciones y en fincas con distintos sistemas de manejo.*

- Los difusores cebados con la cairomona no ejercieron una mayor atracción en los adultos de carpocapsa con mayor actividad de PSMO, ni los difusores combinando el producto con feromona, ni los difusores con cairomona pura, que capturaron un número muy bajo de individuos.
- La presencia de tratamientos químicos en el sistema de manejo fue determinante en el nivel de adultos PSMO-resistentes presentes en las fincas, mientras que la utilización de la confusión sexual no tuvo ninguna incidencia.
- El flujo de poblaciones entre fincas tiene una incidencia importante en la extensión de la resistencia.
- El nivel de resistencia varía entre generaciones, siendo la segunda generación la que presenta un mayor porcentaje de adultos PSMO-resistentes y la tercera la que lo

presenta menor. Esto refleja la existencia de efectos pleiotrópicos en los individuos resistentes en campo.

Objetivo general. Determinar el estado general de los niveles de resistencia de C. pomonella *en poblaciones de campo españolas para determinar su influencia en el seguimiento y control de la plaga en campo y establecer una estrategia de manejo de la resistencia.*

- Las poblaciones españolas de carpocapsa de la zona del Valle del Ebro presentan una amplia gama de mecanismos de resistencia, tanto metabólicos (PSMO, EST y GST) como de insensibilidad en el punto de acción (*AChE* y *kdr*) y, en este punto, son completamente distintas a las poblaciones asturianas.
- El manejo de las poblaciones resistentes es posible mediante el uso de nuevos insecticidas de bajo riesgo, pero es completamente necesario seguir una estrategia de manejo de resistencias para preservar su eficacia. Esto implica una limitación en su uso con la aplicación de criterios técnicos específicos para cada producto.
- En esta estrategia de manejo de resistencias la combinación con otros métodos de control, como la confusión sexual, el uso de insecticidas biológicos o los métodos culturales, es imprescindible.