UNIVERSIDAD POLITÉCNICA DE VALENCIA
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TESIS DOCTORAL:

"Uso de semioquímicos en el control de plagas. Estudios básicos y de aplicación"

Presentada por:

Sandra Vacas González

Directores:

Vicente Navarro Llopis

Jaime Primo Millo

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Resumen

Los semioquímicos son sustancias implicadas en la comunicación entre seres vivos. En el caso de los insectos, pueden ser herramientas de gran utilidad en la lucha contra plagas, como alternativas a la aplicación de insecticidas convencionales. Son sustancias de gran selectividad y muy activas a baja dosis. Los semioquímicos (feromonas y aleloquímicos) se emplean en el control de plagas tanto para la detección y seguimiento de poblaciones como, de forma directa, en las técnicas de confusión sexual, trampeo masivo y atracción y muerte, entre otras.

En la presente tesis se describe la búsqueda y aplicación de semioquímicos para el control de diversas plagas de importancia agronómica, estudiando tres aspectos fundamentales. En primer lugar, el desarrollo y aplicación de la técnica de confusión sexual contra *Aonidiella aurantii* y *Tuta absoluta*. En el caso de *A. aurantii*, se trata de la primera aplicación eficaz de esta técnica contra una plaga de diaspinos y en el caso de *T. absoluta*, se establecen las condiciones para la aplicación de tratamientos eficaces de confusión sexual.

En segundo lugar, se ha estudiado la existencia de valores óptimos de emisión para sistemas basados en la atracción de insectos, aplicado a las plagas *Chilo suppressalis*, *Lobesia botrana*, *Bactrocera oleae* y *Ceratitis capitata*. En el caso de los lepidópteros *C. suppressalis* y *L. botrana* y del tefrítido *B. oleae* se demostró que la atracción de machos se ve afectada por el nivel de emisión de feromona, de forma que velocidades de emisión por encima y por debajo del nivel óptimo obtenido tienen menor poder atrayente. Sin embargo, respecto a *C. capitata*, y su paraferomona trimedlure, su respuesta es de tipo asintótico, no se encontró un nivel óptimo y por tanto, mayores dosis de paraferomona atraían al mismo número de machos. La obtención de emisores con velocidades de emisión óptimas es un aspecto poco estudiado en la literatura científica que, sin embargo, es esencial para el desarrollo de los métodos de control basados en la atracción.

Como tercera parte de esta tesis, se estudiaron detalladamente los compuestos emitidos por machos y hembras de *C. capitata* en diversos estadios, para intentar establecer compuestos y mezclas con posible poder atrayente. Para

este trabajo se ha utilizado la técnica de microextracción en fase sólida, no empleada hasta el momento para este problema, y la metodología estadística del análisis de componentes principales. La disponibilidad de atrayentes eficaces para hembras de *C. capitata* supondría una gran ventaja para el control de esta plaga.

Resum

Els semioquímics son substàncies implicades en la comunicació entre els éssers vius. Pel que respecta als insectes, poden ser eines de gran utilitat en mètodes de lluita contra plagues, alternatius als insecticides convencionals. Són substàncies selectives i molt actives a baixa dosi. Els semioquímics (feromones i aleloquímics) s'empren en el control de plagues tant per a la detecció i el seguiment de poblacions com, de forma directa, en les tècniques de confusió sexual, captura massiva i atracció i mort, entre altres. En aquesta tesi s'inclou la recerca i aplicació de semioquímics per al control de diverses plagues d'importància agronòmica, estudiant tres aspectes fonamentals. En primer lloc, el desenvolupament i aplicació del mètode de confusió sexual contra *Aonidiella aurantii* i *Tuta absoluta*. En el cas d' *A. aurantii,* és la primera aplicació eficaç d'aquesta tècnica contra diaspins i en el cas de *T. absoluta*, s'estableixen les condicions per als tractaments de confusió sexual eficaços.

En la segona part, s'estudia l'existència de valors òptims d'emissió per a sistemes basats en l'atracció d'insectes, aplicat a les plagues *Chilo suppressalis, Lobesia botrana, Bactrocera oleae* i *Ceratitis capitata*. En el cas dels lepidòpters *C. suppressalis* i *L. botrana* i del tefrítid *B. oleae*, es trobà que l'atracció dels mascles es veu afectada pel nivell d'emissió de feromona; de manera que, velocitats d'emissió majors i menors que el nivell òptim obtingut tenen menor poder atraient. En canvi, respecte a *C. capitata* i la seva paraferomona trimedlure, la resposta va ser de tipus asimptòtic, on les captures no augmenten significativament a partir d'un determinat valor d'emissió. L'obtenció d'emissors amb velocitats d'emissió òptimes és un aspecte poc estudiat en la literatura científica però essencial per al desenvolupament de mètodes basats en l'atracció.

Finalment, en la tercera part d'aquesta tesi, es van estudiar amb detall els compostos emesos per mascles i femelles de *C. capitata* en diversos estadis per intentar establir compostos i mescles amb possible poder atraient mitjançant la tècnica de microextracció en fase sòlida, no utilitzada fins al moment per a aquest problema, i la metodologia d'anàlisi de components principals. La disponibilitat d'atraients eficaços per a les femelles de *C. capitata* suposaria un gran avantatge per al control d'aquesta plaga.

Summary

Semiochemicals are substances involved in the communication between living beings. Regarding to insects, these could be useful tools in pest management, as alternative methods to conventional pesticides. They are both species-specific and active at low doses. Semiochemicals (pheromones and allelochemicals) are employed in pest management for detection and population monitoring and also for control methods such as mating disruption, mass trapping and lure and kill, among other techniques. Several studies on the application and the search for semiochemicals are included in the present thesis as a contribution to the control of some agronomic important pests. First, mating disruption technique was developed and applied to *Aonidiella aurantii* and *Tuta absoluta*. Regarding to *A. aurantii*, this is the first successful application of this technique to control a diaspidid pest. This thesis also describes the conditions for mating disruption to be effective against *T. absoluta*.

Secondly, the existence of optimum pheromone release rate values for attraction purposes was investigated for several pests: *Chilo suppressalis, Lobesia botrana, Bactrocera oleae* and *Ceratitis capitata*. For the lepidopterans *C. suppressalis* and *L. botrana* and the tephritid *B. oleae*, it was found that males' attraction is affected by the level of pheromone emission and the attractant power of the pheromone is reduced at release rates below and above the optimum values obtained. However, *C. capitata* males' response to its parapheromone trimedlure was asymptotic, so an optimum emission value was not found and higher quantities of parapheromone attracted the same number of flies. Scientific literature on obtaining pheromone dispensers with optimum emission rates is scarce and it is, however, essential to develop pest control methods based on attraction.

Finally, the third part of this thesis describes the detailed study on the volatiles emitted by different cases of males and females of *C. capitata*, to identify compounds and blends with potential attractancy by means of solid-phase microextraction (SPME), a technique not employed before for this matter, and the principal components analysis (PCA). Obtaining new effective attractants for *C. capitata* females would provide with a great advantage to control this fruit fly pest.

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LISTA DE ABREVIATURAS

Bt: *Bacillus thuringiensis* Berliner

CRS: California red scale

GC/FID: Gas Chromatography with Flame Ionization Detector

IGRs: *insect growth regulators* / RCIs: reguladores del crecimiento de insectos

IPM: Integrated Pest Management

LSD: least significant differences

MD: mesoporous dispenser

MDI: mating disruption index

MLR: multiple linear regression

MTD: moths per trap per day

MTW: moths per trap per week

NMR: *nuclear magnetic resonance* / RMN: resonancia magnética nuclear

PE: polietileno

PP: polipropileno

PRC: piojo rojo de California

PVC: *polyvinyl chloride*

SD: *standard dispenser*

SPME: *solid phase microextraction*

TDDA: acetato de (3*E*,8*Z*)-tetradecadienilo

TDTA: acetato de (3*E*,8*Z*,11*Z*)-tetradecatrienilo

TML: trimedlure

TLM: tomato leaf miner

INTRODUCCIÓN GENERAL

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1. Métodos de lucha contra plagas basados en semioquímicos

El valor de la lucha química convencional en la protección de los productos agrícolas es innegable, y ha sido una herramienta necesaria para la agricultura durante muchas décadas. El uso y abuso de los plaguicidas químicos convencionales ha dado lugar a graves consecuencias como residuos tóxicos, desarrollo de resistencias, explosiones de plagas secundarias, y en general, problemas de toxicidad. En respuesta a estos problemas surge la necesidad, tanto de cambiar las estrategias de aplicación de plaguicidas, como de buscar nuevos métodos de control de plagas más respetuosos.

Como alternativa, se comenzaron a desarrollar investigaciones sobre los llamados "métodos biorracionales", cuya estrategia de acción se basa en el conocimiento de los procesos fisiológicos y bioquímicos muy específicos, la patología de los insectos y los sistemas de comunicación intra e interespecífica, con el objetivo de obtener, agentes capaces de interferir en cualquiera de estos procesos (Vives de Quadras, 1988; Primo-Yúfera, 1991). La investigación se ha dirigido fundamentalmente hacia cuatro líneas:

- reguladores del crecimiento de insectos (RCIs)
- insecticidas de origen natural
- control biológico
- semioquímicos

De todos los métodos anteriormente citados, la presente tesis se centra en la búsqueda y aplicación de semioquímicos al control de plagas. Los semioquímicos son los compuestos químicos implicados en la comunicación entre insectos (Howse, 1998), y se pueden dividir en dos grupos (a su vez subdivididos en otros; ver **Tabla 1**), que son:

a) Feromonas: implicadas en la comunicación intraespecífica

b) Aleloquímicos: implicados en la comunicación interespecífica.

Semioquímicos	Tipos	
Feromonas	Inductoras	De alarma, sexuales, agregación…
	Primarias	De maduración sexual, desarrollo…
Aleloquímicos	Kairomonas	Benefician al receptor
	Alomonas	Benefician al emisor
	Sinomonas	Benefician a ambos
	Antimonas	No benefician a ninguno

Tabla 1 Categorías de semioquímicos

1.1 Las feromonas de insectos

Fue J.H Fabre (finales siglo XIX), pionero en la investigación sobre etología de los insectos, quien realizando una serie de ensayos con polillas y trampas rudimentarias, llegó a la conclusión de que las hembras emiten un olor sutil que los machos detectan con sus antenas plumosas (Jones, 1998). Pero no es hasta el siglo XX cuando fue posible detectar e identificar las minúsculas trazas de esos compuestos volátiles que atraían a los machos. Se trataba de compuestos orgánicos de tipo hidrocarburo de cadena lineal, con pesos moleculares comprendidos entre 180 y 300 uma.

En 1959, Karlson y Lüscher proponen el término “feromona”, palabra de raíz griega que significa portador de excitación. Con esta palabra se referían a aquellas sustancias que un animal segrega y que provocan una reacción o comportamiento específico en un miembro de la misma especie (Karlson y Luscher, 1959; Howse, 1998). En este mismo año, Butenandt y colaboradores (1959) son los descubridores de la primera feromona sexual de un insecto, la de la

mariposa de la seda, *Bombyx mori* L. Desde ese momento, se han ido identificando las feromonas propias de cientos de especies de insectos (Jutsum y Gordon, 1989). Según la clasificación creada por Wilson y Bossert en 1963, las feromonas pueden dividirse en: inductoras, aquellas que provocan un cambio inmediato en el comportamiento; o primarias, las que desencadenan cambios en el desarrollo (Wilson y Bossert, 1963; Howse, 1998).

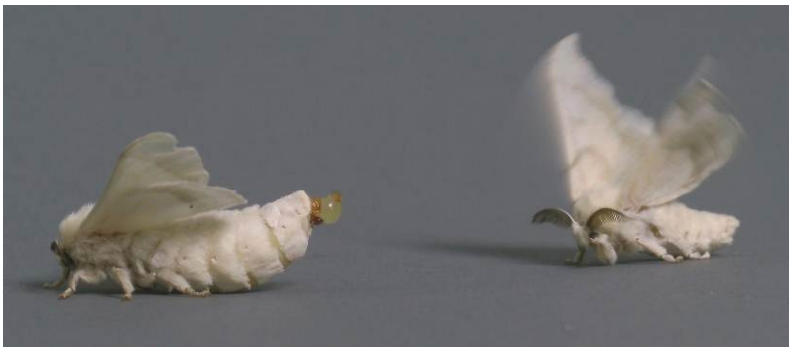

Figura 1.1 Imagen del apareamiento de la polilla de la seda, *Bombyx mori* L. (Fuente: Samuel Woo, UC Davies – Department of Entomology).

Las feromonas más estudiadas son las sexuales, que generalmente, emitidas por la hembra, provocan en el macho una respuesta de atracción y cópula. Las de lepidópteros son las más estudiadas y suelen ser cadenas largas (C10-C30), generalmente insaturadas, con grupos alcohol, éster acético o aldehído. Sin embargo, las estructuras de las feromonas sexuales de otras familias, como dípteros y coleópteros, son más variadas (Primo-Yúfera, 1991). Hasta el momento, se han aislado e identificado más de mil compuestos con actividad de feromona sexual para más de 60 familias de insectos (El Sayed, 2011).

1.2 Aislamiento e identificación de feromonas

La utilización de las feromonas en el control de plagas, ha requerido el desarrollo de técnicas analíticas muy sensibles para su detección e identificación, debido a que se trata de sustancias que se encuentran en los sistemas naturales en muy pequeña cantidad. Su aislamiento y purificación requiere técnicas físicas y cromatográficas adecuadas.

El primer paso para el aislamiento de una feromona es comprobar que existe respuesta a una señal química, mediante un ensayo biológico en el que se evalúe un comportamiento que puede ser de seguimiento de pista, agregación, alarma o estimulo sexual. Además, se ha de tener un buen conocimiento de la biología del insecto para, posteriormente, identificar la fuente de liberación del semioquímico en cuestión. Una vez identificado, se procede al aislamiento de la sustancia, por medio de una extracción con disolvente del insecto completo, de glándulas extirpadas del ovipositor; o una toma de muestras de los volátiles emitidos por el insecto en un momento determinado. La técnica de electroantenografía (EAG) permite detectar si los compuestos aislados provocan una respuesta en la antena del insecto. Sin embargo, la técnica de EAG ha de ir seguida por ensayos biológicos de comportamiento (en túnel de viento, olfatómetro…; **Figura 1.2**) para estudiar la respuesta específica de aquellos compuestos que hayan resultado biológicamente activos.

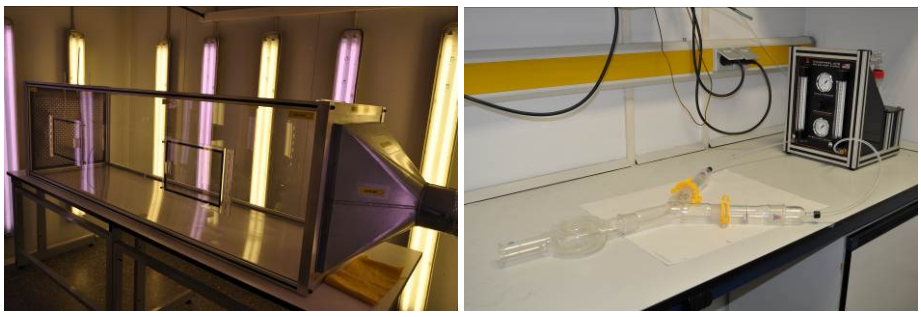

Figura 1.2 Túnel de viento (izq.) y olfatómetro en Y (dcha.) para ensayos biológicos de comportamiento.

Localizadas las sustancias activas, el esfuerzo en este momento se dirige hacia la elucidación estructural del compuesto. Para ello se dispone de diferentes técnicas de análisis estructural: la espectrometría de masas, acoplada a cromatografía de gases (EM-CG) o líquida (EM-HPLC), espectroscopia de infrarrojo, espectroscopia de ultravioleta-visible y la resonancia magnética nuclear (RMN). En algunos casos las técnicas físicas de determinación de estructuras pueden ser complementadas con algunas técnicas químicas, especialmente reacciones de derivatización o degradación.

1.3 Aplicación de las feromonas

El hecho de que las feromonas sean sustancias responsables de la comunicación entre insectos, las convierte en una potente herramienta para el control de los mismos. Las plagas provocan, cada año, importantes pérdidas para la agricultura, y por ello es importante lograr nuevos métodos de control, más eficaces, y sobre todo más respetuosos con el medio ambiente, que permitan el desarrollo de una agricultura sostenible.

La aplicación de feromonas en el control de plagas se dirige a la detección y seguimiento de poblaciones y a métodos directos de control (Campion y Nesbitt, 1981). Estos últimos se basan, principalmente, en dos modos de acción: la atracción hacia trampas y la confusión sexual, aunque existen otros métodos de control.

1.3.1 Detección y seguimiento de poblaciones

Se trata del uso de semioquímicos en trampas con cuatro propósitos fundamentales: detección de insectos plaga, establecimiento de periodos de emergencia de adultos, trazado de mapas de distribución y evaluación de abundancia de plaga (Howse, 1998). Además, estos datos de capturas proporcionan información para el establecimiento de calendarios para la aplicación de tratamientos insecticidas. Por ejemplo, en el caso de las polillas, las trampas de feromona resultan ventajosas frente a las trampas de luz convencionales por diversos motivos: son específicas, por lo que no se requieren grandes

conocimientos en entomología para identificar los individuos capturados y no precisan de una fuente de energía (Campion y Nesbitt, 1981).

1.3.2 Métodos directos de control

1.3.2.1 Captura masiva

La técnica de captura o trampeo masivo, consiste en el uso de un elevado número de trampas por hectárea para controlar la plaga por captura de una proporción de individuos de la población suficientemente elevada. Sin embargo, existen inconvenientes a su utilización: elevado coste por necesitar un elevado número de trampas con un diseño eficaz, la posibilidad de saturación de las mismas en casos de poblaciones muy elevadas o la captura únicamente de machos (en el caso de feromonas sexuales). La utilización de la captura masiva puede resultar ventajosa con trampas eficaces y potentes atrayentes, no solo para la captura de machos sino también de hembras.

1.3.2.2 Atracción y muerte/esterilización/infección

Esta técnica difiere de la captura masiva en que una vez el insecto es atraído por el semioquímico, no queda capturado en una trampa si no que es expuesto a un cebo tóxico, infeccioso o esterilizante que lo convierte en un vehículo de infección o esterilización intraespecífico o directamente produce su muerte.

1.3.2.3 Confusión sexual

La técnica se basa en bloquear la comunicación entre los insectos macho y hembra, mediante la saturación del medio con feromona sexual, para reducir o impedir las cópulas y, por lo tanto, evitar la reproducción de la especie.

Existen tres diferentes mecanismos por los que se puede conseguir la desorientación de los machos (Weatherston, 1990): adaptación/habituación, pistas falsas y camuflaje. El primer mecanismo tendría efectos neurofisiológicos directos sobre el insecto por la exposición constante a elevadas dosis de feromona, provocándose una adaptación de los receptores antenales y/o la habituación del

sistema nervioso central del insecto, impidiendo al macho responder a los niveles normales del estímulo de la feromona natural. El seguimiento de pistas falsas ocurre cuando el macho recibe estímulo desde muchos puntos emisores de feromona que compiten con las señales de las hembras en pauta de llamada. Por último, el mecanismo de camuflaje tiene lugar si la concentración de feromona en el ambiente es tal que la estela natural de feromona queda enmascarada o camuflada por la sintética.

1.4 Ventajas de su uso y dificultades para su desarrollo

El desarrollo de este tipo de métodos de control presenta varias ventajas:

- consiguen reducir poblaciones de la plaga por debajo del límite de daño comercial, evitando los inconvenientes de los insecticidas convencionales
- son métodos de control de plagas respetuosos con el medio ambiente y no dejan residuos
- tienen una elevada especificidad; los componentes feromonales no son productos activos frente a otras especies
- pueden usarse en programas de control integrado de plagas junto a otros métodos; por ejemplo permite su uso conjunto con el control biológico basado en enemigos naturales, al contrario de lo que ocurre con muchos productos insecticidas
- es poco probable la aparición de resistencias, aunque se encuentran algunos trabajos donde se estudia el potencial para la aparición de resistencias tras el uso reiterado de la confusión sexual (Evenden y Haynes, 2001; Shani y Clearwater, 2001; Mochizuki et al., 2002).

Sin embargo, su desarrollo presenta las siguientes dificultades:

- requieren un conocimiento exacto de la estructura de la feromona y, en caso de mezclas feromonales, de su composición exacta
- para los métodos basados en el trampeo, las feromonas empleadas han de ser de alta pureza; pequeñas impurezas en la mezcla pueden reducir de forma considerable la respuesta de los machos (Howse, 1998)

- muchos de los compuestos son muy inestables, descomponiéndose en pocos minutos en presencia de luz u oxígeno (Jutsum y Gordon, 1989)
- se requiere una síntesis química económica para tener una relación coste/beneficio favorable
- su actividad puede ser altamente dependiente de factores fisiológicos (madurez sexual) o factores climáticos (humedad, temperatura)
- su eficacia también depende del momento, tipo y emplazamiento correcto de las trampas (Robacker et al., 1990; Kondo y Tanaka, 1991; Oehlschlager et al., 1993; Suckling, 2000; Bacca et al., 2006).

1.5 Dispositivos emisores

Para cualquiera de las aplicaciones mencionadas en el apartado anterior es necesario disponer de un dispositivo que emita el atrayente, de forma constante y duradera en el tiempo. Dependiendo de la técnica que se vaya a emplear, existirán unas necesidades de emisión que el dispositivo tendrá que satisfacer, por lo que resulta esencial el diseño adecuado del mismo.

A la hora de diseñar un emisor de feromona, se han de tener en cuenta los siguientes aspectos:

a) *Naturaleza del dispositivo*: actualmente, gran parte de los emisores son fabricados con materiales poliméricos, como PVC, PE, etc., que no son biodegradables. La tendencia actual en el diseño de emisores se basa en el uso de materiales biodegradables, que no produzcan contaminación ambiental.

b) *Cinética de emisión*: para que el uso de las feromonas en el control de plagas sea eficaz, se ha de conseguir que el emisor tenga una cinética de emisión adecuada. La cinética ideal es la de orden cero (**Figura 1.5.1**), aquella en la que la velocidad de emisión es constante con el tiempo, evitando una emisión inicial elevada y una carga residual alta al final de tratamiento (Muñoz-Pallarés et al., 2001). En cualquier caso, la mayoría de los emisores comercializados tienen cinéticas de primer orden que con el tiempo pueden resultar inadecuadas para el funcionamiento de la técnica en cuestión. Las de tipo exponencial (**Figura 1.5.1**)

serían las menos adecuadas ya que proporcionarían elevados niveles de emisión iniciales, descendiendo bruscamente hasta velocidades de emisión que podrían ser insuficientes.

c) *Adaptabilidad a las necesidades de emisión*: las características del emisor deberían ser fácilmente modificables, de manera que pueda adaptarse a distintas aplicaciones y distintas condiciones climáticas.

d) *Sensibilidad a las condiciones climáticas*: la mayoría de los emisores comerciales son muy sensibles a las condiciones climáticas, y sobre todo a la temperatura. En algunos casos, el aumento de la temperatura en unos pocos grados puede incluso triplicar la velocidad de emisión (Leonhardt et al., 1989; Leonhardt et al., 1990; Bradley et al., 1995; McDonough, 1997; Domínguez-Ruiz, 2007). Es importante que la velocidad de emisión sea poco sensible a las variaciones climáticas como la temperatura, la humedad relativa o incluso la velocidad del viento.

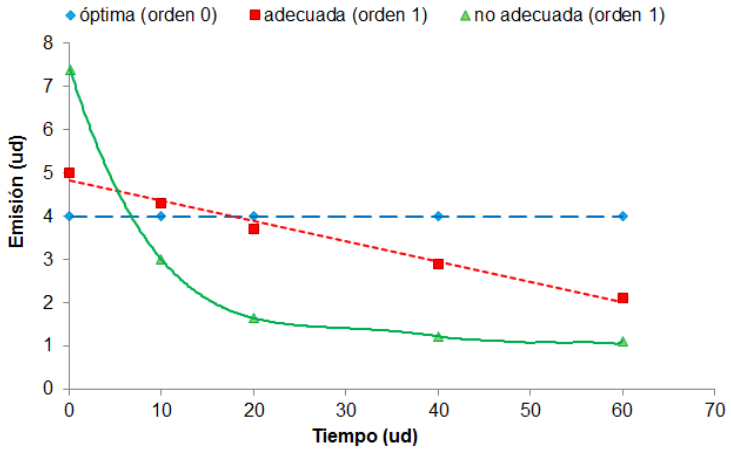

Figura 1.5.1 Tipos de cinéticas de emisión.

e) *Emisión de mezclas feromonales*: como algunas feromonas son mezclas de diversos componentes, estos deben emitirse en la proporción activa natural, lo cual no es fácil de conseguir.

f) *Rentabilidad*: su producción y aplicación deben ser económicamente viables.

En la actualidad, los emisores comercializados no cumplen a la vez todos los requisitos anteriormente citados, y en algunos casos resultan poco eficientes. Los tipos de emisores más importantes, disponibles en el mercado, son (Muñoz-Pallarés et al., 2001):

- Laminados: formados por una lámina de material adsorbente que contiene la feromona y cubierta a ambos lados por láminas de plástico semipermeables (estructura tipo "sandwich"). Las formas pueden ser variadas y algunas permiten la aplicación aérea.
- Fibras huecas: microcapilares de material polimérico llenos de feromona que se emite por capilaridad.
- Microcápsulas: pequeñas cápsulas de material semipermeable, con diámetros entre 1 y 1000 μm (Hall y Marrs, 1989), que contienen la feromona.
- Tubos de material polimérico: tubos de hasta 2 mm de diámetro y 20 cm de longitud, que realizan la emisión a través de sus paredes.
- *Rubber septa*: piezas de goma en forma de copa, con gran capacidad de absorción de feromona. Son muy baratos, de fácil suministro, pero tienen una vida útil muy corta y requieren muchas reposiciones.

Figura 1.5.2 Emisor de tipo *rubber septa.*

- Materiales poliméricos: de formas y tamaños variados, que llevan adsorbida la feromona
- Viales: que emiten la feromona a través de sus paredes semipermeables.

A la mayoría de estos emisores les afectan las condiciones ambientales, pueden ser contaminantes y no tienen una velocidad de emisión adecuada. Sin embargo, muchos de ellos han conseguido introducirse en el mercado y algunos, como los *rubber septa*, que son baratos y de fácil aplicación, son considerados emisores estándar.

1.6 Emisores basados en materiales porosos inorgánicos

Una de las líneas de trabajo en el Centro de Ecología Química Agrícola del Instituto Agroforestal del Mediterráneo (CEQA-IAM), de la Universidad Politécnica de Valencia (UPV), es el diseño y desarrollo de emisores biodegradables para la liberación controlada de semioquímicos.

Estos emisores se basan en la tecnología de tamices moleculares inorgánicos, que por su estructura, pueden actuar como soporte para la liberación de sustancias volátiles. Estos materiales poseen una compleja estructura formada por numerosos microporos y cavidades, y una elevada superficie específica, lo que les confiere gran capacidad de absorción y adsorción de los componentes feromonales. Dentro de estos tamices moleculares inorgánicos, los materiales estudiados en el CEQA-IAM son las zeolitas y los soportes tipo sepiolita y atapulgita. El primer uso de estos materiales como soporte de feromonas quedó reflejado en las siguientes patentes: “Production of semiochemical emitters having a controlled emission speed which are based on inorganic molecular sieves” (Corma et al., 1999) y “Emitter of semiochemical substances supported on a sepiolite, preparation process and applications” (Corma et al., 2000).

La sepiolita, de fórmula química $Mg_4Si_6O_{15}(OH)_2 \cdot 6H_2O$, es un mineral de arcillas, que aparece asociado a la serpentina, de color blanco grisáceo, amarillento o rosado. Se caracteriza por ser un material muy polar, con gran capacidad de absorción, y ser un mineral abundante y económico.

Estructuralmente, se trata de un filosilicato magnésico cristalino, cuya estructura está formada por láminas de tetraedros de sílice unidas mediante cationes Mg^{2+}, en coordinación octaédrica, formando capas tetraedro-octaedro-tetraedro (**Figura 1.6.1**). Estas láminas forman fibras que se unen entre sí mediante puentes Si-O-Si.

Su gran capacidad absorbente se debe a la rotación de las láminas de tetraedros, que da lugar a la formación de unas cavidades, donde pueden alojarse cationes, agua y compuestos orgánicos, como en este caso las moléculas de semioquímicos (Muñoz-Pallarés et al., 2001).

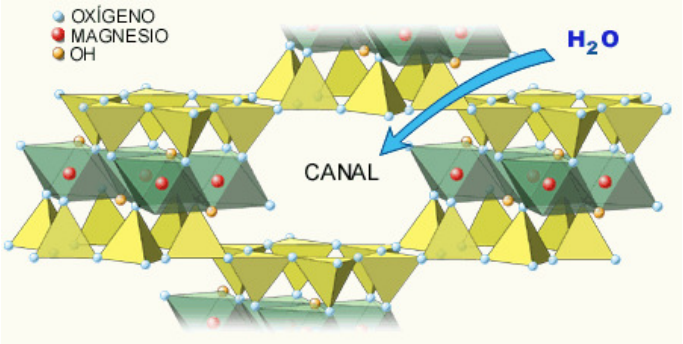

Figura 1.6.1 Estructura de la sepiolita.

Su utilización comporta, entre otras, las siguientes ventajas:

- Es una materia prima abundante y fácilmente extraíble.
- Se adapta a las necesidades de emisión. Las propiedades químicas de la sepiolita son fácilmente modificables, mediante la variación del pH y la proporción de cationes de intercambio, de manera que se puede regular la fuerza de absorción de los semioquímicos, para controlar la cantidad de feromona emitida y, por tanto, el tiempo de vida útil del emisor.
- Los emisores son fáciles de preparar y aplicar y admiten diversas formas de presentación.
- No es contaminante. Por su naturaleza química, cuando los emisores quedan agotados pueden incorporarse al suelo agrícola sin contaminar.

Los emisores fabricados a base de estos materiales porosos, se formulan junto con sustancias antioxidantes y polímeros, para proteger la feromona de la degradación y conseguir que la emisión de la misma se haga de forma gradual y duradera; además de conferir resistencia al emisor.

Tomando como base esta tecnología, el CEQA-IAM ha desarrollado con éxito diversos tipos de emisores con la sepiolita como soporte: emisores para atrayentes de machos y hembras de la mosca Mediterránea de la fruta, *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) (Domínguez-Ruiz, 2007) y emisores para la confusión sexual de *Lobesia botrana* Den. & Schiff. (Lepidoptera: Tortricidae) y *Cydia pomonella* L. (Lepidoptera: Tortricidae) (Femenia, 2011).

Figura 1.6.2 Imágenes de emisores mesoporosos. Emisor para confusión sexual de *Lobesia botrana* (izq.; fuente: B. Femenia.) y emisor de acetato amónico para *Ceratitis capitata* (dcha.; fuente: J. Domínguez-Ruiz)

2. EL PIOJO ROJO DE CALIFORNIA (*Aonidiella aurantii*)

2.1 Importancia y distribución

El piojo rojo de California, *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae), es una de las plagas más importantes que afectan a los cítricos a nivel mundial. Originario del sudeste asiático, se ha convertido en una plaga cosmopolita, extendiéndose prácticamente a todas las zonas citrícolas del mundo. Aunque su hospedero preferido sean los cítricos, *A. aurantii* es una plaga polífaga y se cita también sobre peral, manzano, viña, especies de acacias, rosáceas, algarrobo, olivo, viña (Beardsley y González, 1975; University of California, 1991).

Su clasificación sistemática es la siguiente:

Orden Hemiptera
Suborden Sternorrhyncha
Familia Diaspididae
Subfamilia Diaspidinae
Tribu Aspidiotini
Genero *Aonidiella*
Especie *A. aurantii* (Maskell 1879)

En España, apareció por primera vez en 1955, en Aspe (Alicante); pero no es hasta 1985 cuando se detectan más focos en otras zonas de la Comunidad Valenciana y sobre todo en la Comunidad Andaluza (Alfaro et al., 1999a). Actualmente, se encuentra en todas las regiones citrícolas españolas en menor o mayor intensidad (Pina, 2007). Respecto a la Comunidad Valenciana, el piojo rojo está ampliamente distribuido por su centro y sur, y se ha convertido en la plaga más importante en todas las comarcas citrícolas valencianas (Sorribas et al., 2008).

2.2 Biología de la plaga

La principal característica morfológica de esta especie de insecto es la presencia en su parte dorsal de una cubierta protectora, llamada escudo, que protege el cuerpo del insecto de las agresiones físicas y los agentes climáticos (Dickson, 1951). *A. aurantii* presenta el típico ciclo biológico de los diaspinos (Tashiro y Beavers, 1968): comenzando por el huevo, al eclosionar emergen las llamadas ninfas móviles (*crawlers*), el único de los estadios ninfales con capacidad de locomoción, gracias a unas pequeñas patas (Ben-Dov, 1990). Estas ninfas se fijarán a cualquier órgano de la planta, en un periodo máximo de 4-5 horas, y empezarán a desarrollar el escudo céreo. En un primer momento, la apariencia de la ninfa fijada es la de un punto blanco (w*hite cap*), pero seguirá desarrollando el escudo hasta llegar a la primera muda, constituyendo el primer estadio ninfal (NI). Hasta este momento, el desarrollo de machos y hembras es paralelo, pero a partir de este estadio la evolución es diferente; las hembras mudan dos veces, mientras que los machos completan su desarrollo en cuatro mudas (Howell y Tippins, 1990).

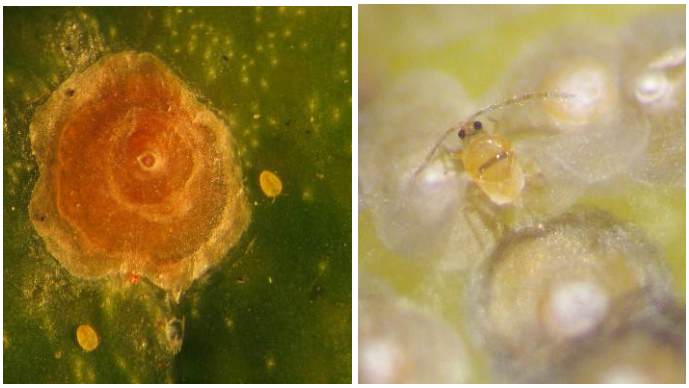

Figura 2.2.1 Hembra adulta junto a dos *crawlers* (izq.) y macho adulto (dcha.) de *Aonidiella aurantii*.

En esta especie existe un dimorfismo sexual muy marcado. En el caso de los machos, su desarrollo sigue de la siguiente manera: segundo estadio ninfal

(NII) (con un escudo más alargado que el de las hembras), prepupa, pupa y adulto. El macho adulto es alado y de vida libre; no se alimenta y únicamente se desplaza en busca de la cópula. Generalmente, la mortalidad de los machos comienza a las 2 horas desde la emergencia (Tashiro y Beavers, 1968), aunque otros autores señalan que los machos pueden llegar a vivir de 1 a 3 días (Beardsley y González, 1975; Koteja, 1990).

Tras el estadio NI, las hembras de *A. aurantii* realizan únicamente dos mudas para formar los estadíos NII, y posteriormente NIII o hembra joven. A continuación se produce una elongación del margen del escudo de la hembra, que indica que está receptiva sexualmente, coincidiendo con la emergencia de los machos adultos. La hembra emitirá una feromona sexual para atraer a los machos a la cópula. A diferencia del macho, la hembra de *A. aurantii* es ápoda y áptera, pasa toda su vida bajo el escudo que desarrolla, y dispone de aparato bucal, para succionar la savia de los tejidos de la planta (Koteja, 1990).

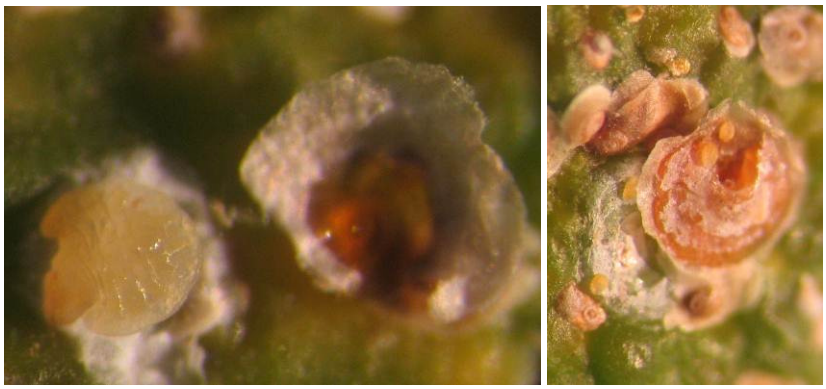

Figura 2.2.2 Hembra joven con pigidio extendido (izq.) y hembra grávida con *crawlers* (dcha.).

2.3 Daños

Se puede encontrar piojo rojo colonizando cualquier parte del árbol, pero prefiere las hojas y frutos (Alfaro et al., 1999a). Se puede considerar dos tipos de daño infligidos por este insecto: el daño directo producido por la succión de savia,

que con niveles de infestación muy elevados pueden causar amarilleo de hojas, defoliación e incluso la muerte del árbol (Grafton-Cardwell y Reagan, 1995); y el daño indirecto, llamado cosmético, por la presencia de escudos en la superficie del fruto, que provoca la depreciación e incluso el rechazo del producto, por lo que se considera el principal daño de esta plaga.

Figura 2.3.1 Imagen de fruto atacado por piojo rojo de California.

2.4 Métodos de lucha

2.4.1 Métodos convencionales

Tradicionalmente, los productores han empleado el control químico para prevenir los daños de esta plaga. Sin embargo, está ampliamente demostrado que estos diaspinos han sido capaces de desarrollar resistencias contra numerosos insecticidas utilizados para su control, tales como organofosforados y carbamatos (Yust et al., 1943a; Yust et al., 1943b; Collins et al., 1994; Grafton-Cardwell y Vehrs, 1995; Grafton-Cardwell et al., 1998). Debido a este fenómeno, el uso de aceites minerales como estrategia de control está ampliamente expandido, a pesar de ser potencialmente fitotóxicos (Grout y Richards, 1991a; Grafton-Cardwell y Reagan, 1995; Tan et al., 2005; Urbaneja et al., 2008). La presencia de compuestos acidificantes en los aceites de forma natural, o los productos de oxidación de moléculas insaturadas pueden causar efectos agudos de fitotoxicidad, tales como: quemaduras, necrosis o caída de hojas y frutos (Tan et al., 2005). Pero los problemas derivados del empleo de aceites también pueden

ser originados por una aplicación de forma incorrecta o unas condiciones no adecuadas de temperatura, humedad o dosis. Por estos motivos, el manejo adecuado de las aplicaciones de aceites es esencial para prevenir efectos fitotóxicos y asegurar la eficacia de los tratamientos. En cualquier caso, también es sabido que las aplicaciones de aceites no son tan efectivas en el control del piojo rojo, comparado con la eficacia de los tratamientos químicos convencionales (Grafton-Cardwell y Reagan, 1995). Es por ello que continúa la búsqueda de métodos alternativos de lucha contra esta plaga.

El control químico ha evolucionado hacia la inclusión de los RCIs, de entre los cuales hay que mencionar el buprofezin (Grout y Richards, 1991a; Ishaaya et al., 1992) y el piriproxifen (Alfaro et al., 1999b; Grafton-Cardwell et al., 2006; Eliahu et al., 2007), como productos efectivos contra el piojo rojo de California. Sin embargo, el efecto de estos RCIs sobre los enemigos naturales aún no es bien conocido (Grafton-Cardwell y Gu, 2003; Lauziere y Elzen, 2007; GIP CÍTRICOS, 2011).

Por otro lado, el piojo rojo de California ha sido objeto de numerosos proyectos sobre control biológico, siendo su enemigo natural más efectivo el parasitoide *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae) (Hare y Luck, 1994; Murdoch et al., 2006; Pina, 2007; Sorribas y García-Marí, 2010). Sin embargo, el control biológico clásico no ha demostrado hasta ahora ser capaz de controlar efectivamente, por sí solo, las poblaciones de piojo rojo (Luck, 1981; Furness et al., 1983; Moreno y Luck, 1992; Bedford, 1996; Jacas y Urbaneja, 2010). En estudios realizados en la Comunidad Valenciana, el parasitismo natural raramente excede del 30% (Pina, 2007; Vanaclocha et al., 2009; Pekas et al., 2010; Sorribas et al., 2010), por lo que se están llevando a cabo estudios sobre la implementación de las sueltas aumentativas de *A. melinus* como control adicional. La eficacia en el uso de estos agentes biológicos depende de un seguimiento minucioso de la plaga, para así establecer con precisión las fechas de suelta de los parasitoides y controlar los tratamientos insecticidas que han de ser selectivos y no afectar a los mismos.

2.4.2 Feromonas

Desde la identificación de la feromona sexual de *A. aurantii* por Roelofs en 1977, el seguimiento de poblaciones mediante el uso de estos semioquímicos ha sido ampliamente utilizado para esta plaga. Esta feromona fue descrita como la mezcla de dos compuestos: acetato de 3-metil-6-isopropenil-9-decenilo (I) y acetato de (*Z*)-3-metil-6-isopropenil-3,9-decadienilo (II) (Roelofs et al., 1977). El compuesto (I) contiene dos centros asimétricos (en los carbonos 3 y 6), mientras que el compuesto (II) tiene un carbono asimétrico en posición 6 y un doble enlace con posibilidad de isomería *Z-E* en posición 3. Todos los isómeros geométricos y ópticos posibles fueron sintetizados y probados por Gieselmann en 1980. Los resultados dieron a conocer que sólo un isómero de cada compuesto era significativamente más activo: (3S,6R)-(I) y (3Z-6R)-(II), y que la presencia de otros isómeros en la mezcla no tenía efecto ni influía negativamente en los resultados de capturas (Tashiro et al., 1979; Gieselmann et al., 1980).

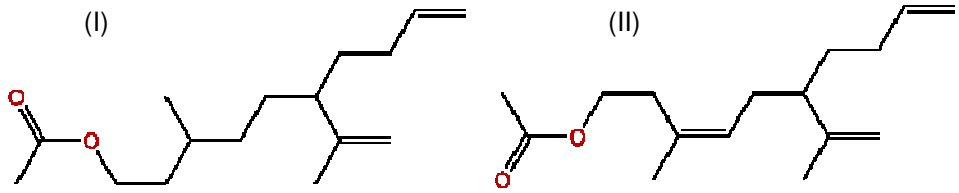

Figura 2.4.2.1 Moléculas componentes de la feromona de *Aonidiella aurantii*: 3-metil-6-isopropenil-9-decenilo (I) y acetato de (Z)-3-metil-6-isopropenil-3,9-decadienilo (II).

Estos descubrimientos son la base para el desarrollo de un método de control basado en la confusión sexual. Además, al requerir solo un componente, el coste final de aplicación de la técnica se podría ver claramente reducido. Sin embargo, en la literatura disponible solo se han podido encontrar referencias de experiencias muy preliminares sobre confusión sexual en piojo rojo (Barzakay et al., 1986; Hefetz et al., 1988), por lo que han sido necesarios estudios más

extensos, con diferentes dosis de feromona, para establecer el potencial de esta técnica en el control de *A. aurantii*.

Figura 2.4.2.2 Imagen de trampa pegajosa para el seguimiento de poblaciones de *Aonidiella aurantii.*

Por otro lado, sólo existe un producto comercial, TCB-RSD (Red Scale Down®), fabricado en Estados Unidos, comercializado como "atrayente para la interrupción del apareamiento del Piojo Rojo de California" y registrado en 2004 por la Agencia de Protección Ambiental norteamericana (United States EPA). Se trata de emisores de baja carga de feromona, que necesitan una reposición antes del inicio de cada vuelo del insecto y se colocan a una densidad de 200 emisores por ha. Estudios llevados a cabo con este emisor en la Región de Murcia han demostrado que es un producto de muy baja eficacia en la lucha contra el piojo rojo (Lucas-Espadas, comunicación personal).

Figura 2.4.2.3 Imagen del emisor TCB-RSD (Red Scale Down®).

3. EL BARRENADOR DEL ARROZ (*Chilo suppressalis*)

3.1 Importancia y distribución

Originario del Extremo Oriente (Ramoneda, 1988), el barrenador del arroz, o *Chilo suppressalis* Walker (Lepidoptera: Pyralidae), es una plaga cosmopolita y destructiva en todas las zonas del mundo en las que se cultiva el arroz (*Oryza sativa* L.) (Batalla, 1999a). Además, también ataca a la caña de azúcar, sorgo y mijo (Batalla, 1999b). El arroz es un alimento básico para más de la mitad de la población mundial, por lo que mantener un cultivo libre de plagas y enfermedades es importante para la economía y la población de numerosos países.

La clasificación sistemática de este barrenador es la siguiente:

Orden Lepidoptera
- Suborden Glossata
  - Familia Pyralidae
    - Subfamilia Crambinae
      - Tribu Chilini
        - Genero *Chilo*
        - Especie *Chilo suppressalis* (Walker 1863)

Se encuentra afectando a las zonas arroceras más templadas de Asia: Bangladesh, Camboya, India, Filipinas, Vietnam, Japón, Corea y China), encontrándose también en Irán y en la zona sur de Rusia (Casagrande, 1993). En Europa, es plaga principalmente en España, donde se detectó por primera vez en 1933 sobre plantas de arroz en Benifaió (Valencia) (Gómez-Clemente, 1940). No es hasta 1935 cuando se encuentra con carácter de plaga en la mayoría de términos municipales de la Ribera Alta y en algunos de la Ribera Baja del Júcar. Hacia 1939 se encontró en las zonas arroceras de Murcia y Albacete (Hellín y Calasparra, respectivamente). También se encuentra con carácter de plaga en las provincias de Tarragona, Huesca y Girona; y muy ocasionalmente se presenta en los arrozales de Badajoz y Sevilla (Ramoneda, 1988). En este momento, es la

plaga más importante del arrozal valenciano (Alfaro, 2006). Dentro de las parcelas, su distribución es aleatoria y agregada (Alfaro, 2006; Zibaee et al., 2009).

3.2 Biología de la plaga

El barrenador del arroz es un lepidóptero heterócero, y como miembro de la familia Pyralidae, tiene hábitos crepusculares y nocturnos, y presenta fototropismo positivo (García-Marí et al., 1994).

El insecto adulto es una polilla de color amarillento. La envergadura alar de los machos adultos es de entre 20 y 24 mm, mientras que las hembras son más grandes y pueden alcanzar los 28 mm (Ramoneda, 1988). El dimorfismo sexual en esta especie se manifiesta en diversos rasgos: el tamaño, como ya se ha mencionado; la coloración de las alas, siendo las de los machos de un color ocre más oscuro que las hembras; y los últimos segmentos abdominales. Estos segmentos 9 y 10 en los machos y 8, 9, 10 en las hembras están modificados por las estructuras internas que forman la genitalia (Ramoneda, 1988).

Los huevos son elípticos y aplastados, de color amarillo claro y 0,9 mm de longitud (Gómez-Clemente, 1940). Las larvas pueden tener hasta 2 cm de longitud; también son amarillentas y con cinco líneas longitudinales (3 dorsales y 2 laterales) más oscuras, de color violáceo, que permanecen durante todas las mudas y edades de su desarrollo. Su aparato bucal es masticador y tienen 3 pares de patas torácicas y 4 pares de patas abdominales (Batalla, 1999b). Las larvas se desarrollan totalmente sobre el cultivo y mudan cinco veces, transcurriendo entre una y otra de unos 5 a 6 días.

Figura 3.2.1 Imágenes del adulto, larva y puestas (Fuente: K.E. Mueller) de *Chilo suppressalis* (de izq. a dcha.)

El número de generaciones que desarrolla este lepidóptero puede ser variable, dependiendo de las condiciones ambientales. Se cita a *C. suppressalis* desarrollando de una a cinco generaciones en las zonas más cálidas de Asia (Hou et al., 2010). En los arrozales de la Comunidad Valenciana se pueden producir hasta 3 generaciones del barrenador: la 1ª que aparece, gradualmente, desde últimos de Abril hasta finales de Junio; la 2ª, en Julio-Agosto; y una 3ª, parcial, de finales de Agosto a mediados de Septiembre (Casagrande, 1993; Batalla, 1999b). Al finalizar la campaña del cultivo del arroz, con la llegada de las bajas temperaturas y la reducción del fotoperiodo, el barrenador inverna en forma de larva (procedentes de la última generación) en el interior de los tocones y en las cañas colindantes, que darán lugar a los adultos de la primera generación. La puesta de huevos se realiza sobre el limbo de las hojas superiores, más frecuentemente en el envés (y raramente en el tallo), en grupos alargados de 1-2 cm (Batalla, 1999b). Entre Mayo y Junio, se produce la eclosión de los huevos y las larvas comienzan a alimentarse, en primer lugar de las hojas, desde donde se dispersan fácilmente descolgándose con hilos de seda y siendo arrastradas por el viento. Posteriormente, penetran en el tallo y viven en su interior, pudiendo trasladarse de una caña a otra.

3.3 Daños

Las larvas crecen al mismo tiempo que el arroz, y son las causantes del daño en las plantas debido a sus hábitos alimenticios y a su desarrollo en el interior del tallo de sus hospederos. Los daños que van a causar dependen del momento del ciclo biológico del cultivo; así, las larvas de primera generación (primavera) afectan principalmente al ahijamiento de las plantas. Las de segunda generación afectarán al resto de estados fenológicos del arroz (encañado, espigado, floración y granazón), por lo que provocan los daños más importantes en la cosecha. En caso de que las condiciones ambientales permitan el desarrollo de una tercera generación completa, su acción se produciría una vez ya se ha formado el grano, por lo que las larvas de tercera generación no suelen causar daños que afecten a la cosecha. Sin embargo, será importante tener en cuenta el nivel de población de esta generación, ya que será indicador del nivel de población de plaga al año siguiente (Ramoneda, 1988).

Figura 3.3.1 Daños en espiga de segunda generación de *Chilo suppressalis* (izq.) y larva en el interior de una caña (dcha.)

3.4 Métodos de lucha

3.4.1 Control químico

El caso de la Comunidad Valenciana es un buen ejemplo de la evolución que ha sufrido el control de *C. suppressalis*. Esta plaga viene afectando a los arrozales valencianos desde la década de los 30 y hasta 1950 no se dispuso de

insecticidas apropiados para la eliminación directa del barrenador. Por tanto, el único modo de lucha contra la plaga era el empleo de acciones físicas y culturales: destrucción del insecto en sus refugios invernales, por medio de la destrucción de la paja-rastrojo y los tocones; o quema de la paja, junto con la inundación de los tocones, para reducir la población de orugas invernantes (Gómez-Clemente, 1940). Durante esta misma etapa, a mediados de los años 40, se ensayaron por primera vez los nuevos insecticidas organoclorados: DDT (dicloro-difenil-tricloroetano) y HCH (hexacloro-ciclohexano). Sus resultados fueron satisfactorios, pero su puesta en práctica resultó un fracaso, ya que en ese tiempo no se conocía perfectamente la evolución de la plaga, y por tanto, las aplicaciones no se realizaban en el momento en que las larvas pueden ser afectadas fuera de los tallos.

También en los años 40 ya se recomendaba la captura de los adultos mediante trampas luminosas, con cebos o mangas (Gómez-Clemente, 1940). Pero no es hasta 1953 cuando se colocan trampas luminosas en zonas afectadas y se logra un seguimiento de las poblaciones de la plaga en campo, observando las tres generaciones durante el periodo de cultivo del arroz, y las fechas de vuelo máximo de adultos. Estas observaciones sirvieron como guía para la determinación del momento más adecuado para la realización de los tratamientos insecticidas. A partir de 1965, gracias a la determinación de las fechas del vuelo de adultos, se comenzaron a practicar los tratamientos aéreos con insecticidas fosforados sobre la mayor parte de los arrozales valencianos (Batalla, 1999a), reduciendo el número de tratamientos individuales. Sin embargo, persistía el problema del desequilibrio ecológico que provoca este tipo de insecticidas de síntesis, especialmente por el hecho de que el arroz se cultiva en humedales protegidos de alto valor ecológico.

Hasta 2007, aún se aplicaban en China insecticidas organofosforados para el control del barrenador, como metamidofos, monosultap y triazofos, sobre los que este insecto ha creado importantes niveles de resistencia (He et al., 2007). Debido a su toxicidad estos insecticidas fueron prohibidos y existen numerosos estudios para encontrar nuevas sustancias a las que sea susceptible *C.*

suppressalis, como piretroides e IGRs (He et al., 2007; He et al., 2008). Entre estos últimos, el tebufenocida muestra gran actividad para el control de larvas de lepidópteros en el arroz, y también en otros cultivos frutales, viñedos y forestales (Smagghe y Degheele, 1994).

Volviendo a la Comunidad Valenciana, el hecho de que la mayor parte de la superficie cultivada de arroz pertenezca al Parque Natural de la Albufera de Valencia, ha hecho que, en los últimos 15 años, la superficie de arrozal controlada mediante tratamientos químicos convencionales disminuyese a favor del desarrollo y aplicación de nuevos métodos de control basados en aplicaciones aéreas con tebufenocida y la introducción de las feromonas sexuales.

3.4.2 Feromonas

La feromona sexual de la hembra de *C. suppressalis*, fue identificada por primera vez como una mezcla de los aldehídos (*Z*)-11-hexadecenal (Z11C16al) y (*Z*)-13-octadecenal (Z13C18al) (Nesbitt et al., 1975; Ohta et al., 1976), pero una vez probada su eficacia para atraer a los machos, esta mezcla resultaba menos eficaz que la atracción que ejercían las hembras vírgenes vivas (Beevor et al., 1990; Tatsuki, 1990). Este hecho hizo reexaminar la composición de la feromona, sugiriendo la presencia en la mezcla de otros componentes sinérgicos y encontrando cuatro compuestos adicionales a los dos iniciales, que fueron: hexadecanal, (*Z*)-9-hexadecenal (Z9C16al), octadecanal y (*Z*)-11-hexadecen-1-ol. Tras diversos ensayos, se determinó que de estos 4 nuevos compuestos identificados, sólo el Z9C16al, isómero de posición del doble enlace del Z11C16al, era el que tenía actividad feromonal (Beevor et al, 1990; Tatsuki, 1990). La mezcla de este último compuesto con los Z11C16al y Z13C18al aumentó enormemente la eficacia de atracción, y desde entonces ha sido la base para el desarrollo de las formulaciones comercializadas (Casagrande, 1993). Tatsuki (1990) ha determinado una proporción natural en la emisión de la hembra de *C. suppressalis* para la mezcla Z11C16al, Z13C18al y Z9C16al, de aproximadamente 48:6:5, respectivamente. En este caso, como en otros, las variaciones en las proporciones de los distintos compuestos que forman una feromona influyen en su poder atrayente (Tatsuki et al., 1990).

Figura 3.4.2.1 Molécula del (Z)-11-hexadecenal, componente mayoritario de la feromona de *Chilo suppressalis.*

Kanno et al. (1978-1980), pioneros en la investigación sobre confusión sexual en el barrenador del arroz, demostraron que su feromona sexual y otros compuestos relacionados estructuralmente con ella, interferían en la atracción de los machos. Posteriormente, se llevaron a cabo, también en Japón, ensayos con los emisores de tipo tubo de polietileno, desarrollados por la empresa Shin-Etsu Chemical Co., Ltd (Tokio, Japón). En ellos, Kanno (1982) confirmó que se puede obtener un índice de inhibición de capturas del 90% con emisores cargados con Z11C16al separados 16 m entre sí y emitiendo a un nivel de ~50 mg por ha y día. Además, se podía conseguir un buen nivel de confusión tanto con sólo Z11C16al, como con la combinación de los tres componentes (Tanaka et al., 1987).

Figura 3.4.2.2 Emisor para confusión sexual de *Chilo suppressalis* Selibate®CS.

En España, de 1987 a 1990 se realizaron diversos experimentos en la zona del Parque Natural de la Albufera de Valencia para diseñar la estrategia de confusión sexual más adecuada (CAPA, 1988; Beevor et al., 1990; Serrano et al., 1998; Batalla, 1999a), reduciendo progresivamente el número de difusores por ha, hasta llegar un marco de colocación de 10×10, con los emisores de PVC, Selibate®CS (Agrisense BCS Ltd., Pontypridd, UK) (Casagrande, 1993). Así que desde 1991 se aplicaban 100 emisores por ha, cargados con 400 mg de feromona, que proporcionaban una concentración de 40 g por ha. Serrano y sus colaboradores (1998) describieron los ensayos de eficacia que se desarrollaron en 1997 para reducir el número de difusores por ha; pero no fue hasta 2003 cuando se realizan experimentos durante tres años consecutivos y se recomendó que se podía reducir la densidad a 39 emisores por ha en zonas ya tratadas por confusión (Alfaro, 2006).

El uso del seguimiento de poblaciones de este barrenador está generalizado, tanto para detección como para la racionalización de los tratamientos químicos. El trampeo masivo también aparece como una alternativa viable (Jian-wei et al., 2003), pudiendo proporcionar el mismo nivel de control que los tratamientos químicos pero sin los inconvenientes ecológicos. Esta técnica se encuentra en pleno funcionamiento en el Delta del Ebro (Tarragona) desde el año 2000, y se emplea de forma combinada con tratamientos de tebufenocida, en toda la superficie cultivada de arrozal, que es de unas 21.000 ha (Ramoneda et al., 2006).

Numerosas empresas han desarrollado difusores de feromona para el seguimiento de poblaciones y trampeo masivo de *C. suppressalis* basados en polímeros plásticos, que aunque son efectivos, no son biodegradables y su funcionamiento puede no estar optimizado.

3.4.3 Otros

Respecto al control biológico, en los años 50 se intentó la lucha biológica en la Comunidad Valenciana, con colonias del himenóptero calcídido, *Trichogramma minutum* Riley (Hymenoptera: Trichogrammatidae), que parasita los huevos del barrenador; sin embargo, los resultados no fueron demasiado satisfactorios (Batalla, 1999a). También se pueden encontrar algunas citas más recientes sobre ensayos en países asiáticos con *Trichogramma japonicum* Ashmed parasitando huevos de *C. suppressalis* (Chen et al., 2010).

Otra línea de investigación sobre el control del barrenador iniciada en los países asiáticos es el desarrollo de variedades de arroz transgénicas. Algunos cultivos modificados genéticamente con *Bacillus thuringiensis* Berliner (Bt) expresan genes de proteínas con actividad insecticida, principalmente contra lepidópteros y coleópteros. Los cultivos transgénicos maíz-Bt y algodón-Bt están comercialmente disponibles desde 1996; sin embargo, aunque se han desarrollado docenas de genotipos para el arroz-Bt desde 1993, aún no son comerciales debido a la preocupación sobre los potenciales impactos ecológicos (Wang et al., 2010). Los ensayos de campo con arroz-Bt comenzaron en 1998 y aún no se han descrito impactos negativos sobre organismos no-objetivo (Chen et al., 2007), ni el desarrollo de resistencias en el barrenador (Chen et al., 2010).

4. LA POLILLA DEL RACIMO (*Lobesia botrana*)

4.1 Importancia y distribución

La polilla del racimo, *Lobesia botrana* (Denis y Schiffermüller) (Lepidoptera: Tortricidae), es una plaga clave para el cultivo de la vid (*Vitis vinifera* L.), por los daños que ocasiona y la necesidad de aplicar tratamientos de forma sistemática para su control (Coscollá, 1997; Pérez-Moreno et al., 2000). Es una de las plagas más graves para la viña a nivel mundial. Se extiende por las principales áreas de cultivo de vid en Europa, Asia, África, y especialmente en la cuenca Mediterránea. Se describió por primera vez en Austria en 1776. En España no se localizó hasta 1879 y se fue extendiendo por la Península hasta alcanzar carácter de plaga. Actualmente, tiene escasa incidencia en el Norte peninsular y variable en el interior, pero afecta de forma importante al cultivo de la viña en toda la costa del Mediterráneo, el Atlántico, Extremadura y Aragón (Coscollá, 1997). En el continente americano, *L. botrana* fue detectada por primera vez en Chile en 2008, mientras que en Septiembre de 2009 ya se obtuvieron las primeras larvas de esta polilla en el condado de Napa (California, Estados Unidos) (Gilligan et al., 2011).

Su clasificación sistemática es la siguiente:

Orden Lepidoptera
- Suborden Glossata
  - Familia Tortricidae
    - Subfamilia Olethreutinae
      - Tribu Olethreutini
        - Género *Lobesia*
        - Especie *Lobesia botrana* (Den. & Schiff., 1776)

Se trata de un insecto polífago, que se puede encontrar afectando a multitud de especies pertenecientes a 27 familias (Ioratti et al., 2011); entre ellas: Vitaceae, Oleaceae, Rosaceae, Grossulariaceae, Berberidaceae y Cornaceae (Stavridis y Savapoulou-Soultani, 1998; Thiéry y Moreau, 2005).

4.2 Biología de la plaga

Como insecto holometábolo, en su desarrollo pasa por los estadíos de huevo, larva, crisálida y adulto. Las hembras de *L. botrana* realizan puestas de 50 a 80 huevos, de forma aislada en la superficie de las inflorescencias y de las bayas en crecimiento (Coscollá, 1997). Las larvas que surgen de los huevos eclosionados tienen una coloración verdosa y su longitud varía desde 1 mm al nacer, hasta 10-15 mm cuando completan el quinto estadio de su desarrollo. En ese momento, buscan un lugar adecuado en los troncos, el suelo o el interior de los racimos para formar la crisálida de la que emergerá el adulto. La envergadura alar del adulto será de 10-13 mm y tendrá una longitud de 6-8 mm. El dimorfismo sexual no es muy marcado, aunque los machos son un poco más pequeños que las hembras.

El número de generaciones que puede desarrollar es variable: en las zonas más septentrionales desarrolla dos generaciones, y en las más meridionales llegan a completarse tres. En las regiones más cálidas de Murcia y Almería puede llegar a tener lugar una cuarta generación parcial (Coscollá, 1997).

Figura 4.2.1 Imágenes del adulto (Fuente: Bayer CropScience) y larva de *Lobesia botrana* (Fuente: Corbis Images).

4.3 Daños

Los daños son producidos por las fases larvarias de la polilla. Las larvas de la primera generación destruyen botones florales, flores y frutos recién cuajados. Sin embargo, los daños en esta fase no suelen causar importantes pérdidas y la propia planta puede compensar la pérdida de hasta el 50% de las flores (Coscollá et al., 1982; Coscollá, 1998).

Las larvas de la segunda y tercera generación son las que producen importantes pérdidas cuantitativas y cualitativas, ya que se alimentan de las bayas en crecimiento y favorecen su pudrición por la proliferación de hongos, como *Botrytis cinerea* Persoon.

Figura 4.3.1 Daños de primera y tercera generación provocados por *Lobesia botrana* en racimos.

4.4 Métodos de lucha

4.4.1 Control químico

Por lo que respecta a las larvas de primera generación, no se suelen realizar tratamientos contra ellas porque su ataque no tiene repercusión en la cosecha y solo se hacen con niveles poblacionales muy elevados. Las aplicaciones de insecticidas más importantes se realizan contra los huevos y larvas de la segunda y tercera generaciones, antes de que penetren en las bayas

(Coscollá, 1997). Los productos más utilizados, tradicionalmente, han sido organofosforados, carbamatos y piretroides; y más concretamente metil-paratión, clorpirifos, acefato, diazinón, fenitotrión, fosalona y malatión. En cuanto a los IGRs, tebufenocida y metoxifenocida son los más utilizados. Otros productos registrados actualmente para su uso contra la polilla del racimo son: metil-clorpirifos, clorpirifos, indoxacarb, azadiractina, flufenoxuron y spinosad (MARM, 2011).

El uso reiterado del control químico puede derivar en la aparición de resistencias a diversos productos organofosforados, piretroides e IGRs (Ioriatti et al., 2002), explosiones de plagas secundarias (como *Planococcus* sp., *Sparganothis pilleriana* Schiffermüller o *Frankliniella occidentalis* Persoon) y problemas de contaminación ambiental y salud alimentaria. En los últimos años, el uso de insecticidas se ha racionalizado gracias a la utilización de las trampas de feromona, que dan información sobre el ciclo biológico y la dinámica poblacional de la plaga.

4.4.2 Control biológico

El control biológico de *L. botrana* se ha centrado en la utilización de himenópteros del género *Trichogramma*, *T. evanescens* Westwood y *T. cacoeciae* Marchal, que actúan como parasitoides de huevo. El nivel de parasitismo natural no suele ser muy alto, pero mediante las sueltas aumentativas de *T. evanescens* se podría conseguir un control más eficaz (El-Wakeil et al., 2009). También se cita el parasitismo larvario por parte de icneumónidos, bracónidos y eulófidos, pero con porcentajes normalmente bajos (Izquierdo-Casas, 2000; Pérez-Moreno et al., 2000). Algunos trabajos, destacan los himenópteros parasitoides de larvas *Dibrachys affinis* Masi y *D. cavus* Walker (Coscollá, 1997; Coscollá, 1998; Aronson y Shai, 2001).

Por lo que respecta al control mediante microorganismos entomopatógenos, las formulaciones más utilizadas contra *L. botrana* son las de la bacteria *Bacillus thuringiensis* (Bt) (Aronson y Shai, 2001). También, los hongos entomopatógenos de géneros como *Spicaria*, *Beauveria* o *Aspergillus*, entre otros, pueden infectar una gran proporción de pupas hivernantes (Ioratti et al., 2011).

4.4.3 Feromonas

Fue en los años 70 cuando se describió el componente mayoritario de la feromona sexual de *L. botrana* como el acetato de (*E,Z*)-7,9-dodecadienilo (Roelofs et al., 1973). Posteriormente, se identificaron dos nuevos compuestos, (*E,Z*)-7,9-dodecadien-1-ol y acetato de (*Z*)-9-dodecenilo, con efecto sinérgico en cuanto a la captura de machos (Arn et al., 1988; El Sayed et al., 1999).

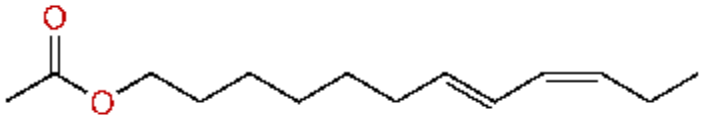

Figura 4.4.3.1 Molécula del acetato de (*E,Z*)-7,9-dodecadienilo, componente mayoritario de la feromona de *Lobesia botrana*

Estos descubrimientos fueron clave para la aplicación de las técnicas de control basadas en feromonas y el seguimiento y detección que llevan a una gestión de la plaga más dirigida. Aunque algunos de los componentes minoritarios encontrados en la mezcla natural de la feromona mejoran el nivel de atracción en ensayos de túnel de viento, solo el componente mayoritario de la feromona de *L. botrana* se utiliza en campo tanto para el seguimiento de poblaciones como para los métodos de control directo. De hecho, la confusión sexual es el método más eficaz y extendido para el control de *L. botrana* en Europa (Ioratti et al., 2011). Desde la identificación de la feromona, ésta se lleva utilizando durante más de dos décadas en Alemania, Suiza y el norte de Italia; pero en otras regiones europeas, la introducción de estos métodos ha sido más lenta (Witzgall et al., 2010).

Figura 4.4.3.2 Emisor para confusión sexual de *Lobesia botrana* del tipo tubo de polietileno (Fuente: B. Femenia).

Como ya se ha mencionado las trampas de feromona para el seguimiento de poblaciones juegan un papel muy importante en la detección de plaga y la programación de tratamientos. Los emisores más utilizados en estas trampas son los del tipo *rubber septa*, pero en muchos casos su funcionamiento no está optimizado y tienen una emisión muy elevada durante la primera semana, que luego desciende de forma acusada. Dada la importancia de estos sistemas de trampeo, es necesario disponer de un emisor de feromona con una velocidad de emisión adecuada para conseguir una buena eficacia y extender el uso de las feromonas en los programas de control de plagas.

5. LA POLILLA DEL TOMATE (*Tuta absoluta*)

5.1 Importancia y distribución

La polilla del tomate, *Tuta absoluta* (Povolny) (Lepidoptera: Gelechiidae), es una de las plagas más importantes del cultivo del tomate *Lycopersicon sculentum* (L.). Es considerada plaga endémica del tomate en muchos países de América del Sur (Torres et al., 2001); entre ellos, Argentina, Bolivia, Brasil, Colombia, Chile, Ecuador, Paraguay, Perú, Uruguay, Venezuela (EPPO, 2005).

Su clasificación sistemática es la siguiente:

Orden Lepidoptera
Suborden Glossata
Familia Gelechiidae
Subfamilia Gelechiinae
Genero *Tuta*
Especie *Tuta absoluta* Povolny (1994)

Es un pequeño lepidóptero considerado plaga para el tomate y también de la patata y otras solanáceas, tanto silvestres como cultivadas (Picanço et al., 1998; EPPO, 2005). Parte de la importancia de esta plaga recae en el hecho de que puede afectar a lo largo de todo el ciclo de cultivo, llegando a causar daños de hasta el 100% (EPPO, 2005) como minador de hojas y de otras partes de la planta como flores, frutos, brotes y tallos.

Fue descrita por primera vez a principios de los años 80 en el sureste de Brasil (Souza y Reis, 1986). Más recientemente, fue detectada en España en el año 2006, concretamente en la provincia de Castellón y durante el año 2007 se extendió a otras regiones de la costa mediterránea causando importantes daños (Urbaneja et al., 2007). En 2008, se describió su presencia en Marruecos (EPPO, 2008a), Argelia (EPPO, 2008b), Francia (EPPO, 2009a) y Portugal (EPPO, 2009b). En el resto de Europa ha sido detectada en Reino Unido, diversas

regiones centrales de Italia y en Sicilia (EPPO, 2009a), Malta, Suiza (EPPO, 2009a), Alemania, Chipre, Hungría, Bulgaria, Albania y Kosovo (EPPO, 2010). En su gran dispersión geográfica, también se han descrito las primeras invasiones de *T. absoluta* en Turquia (Kiliç, 2010) e Israel (Seplyarsky et al., 2010).

5.2 Biología de la plaga

El ciclo biológico de *T. absoluta* incluye cuatro estados de desarrollo: huevo, larva, pupa y adulto. El adulto hace la puesta en el envés de las hojas o en los tallos, y en menor proporción en los frutos. Tras la eclosión, las larvas penetran en hojas, frutos o tallos, formando galerías donde se alimentarán y desarrollarán, pasando por cuatro estadios larvarios. Completado su desarrollo, las larvas salen de la galería para pupar en el suelo y también en hojas. Los adultos emergidos miden 6-7 mm de longitud, presentan antenas filiformes y escamas grisáceas y presentan hábitos principalmente nocturnos.

La duración de su ciclo biológico es altamente dependiente de las condiciones ambientales (Barrientos et al., 1998) y el número de generaciones desarrolladas es, por tanto, variable. Las larvas no entran en diapausa siempre que tengan alimento disponible, por lo que pueden llegar a desarrollar hasta 10-12 generaciones por año (EPPO, 2005).

Figura 5.2.1 Estadios larvarios (izq.) y pupas (dcha.) de *Tuta absoluta.*

5.3 Daños

T. absoluta puede afectar a las plantas de tomate en cualquier estado de su desarrollo, produciéndose la puesta, preferentemente, en hojas (73%) y después en tallos (21%), sépalos (5%) o frutos verdes (1%) (Estay, 2000). En hojas, el daño está causado por la formación de galerías por parte de las larvas, lo que afecta la capacidad fotosintética y el rendimiento final de la planta. En los frutos, la formación de galerías conlleva la colonización de la planta por patógenos secundarios y la podredumbre del fruto.

Figura 5.3.1 Insectos adultos (izq.; fuente: Phytoma) y planta de tomate y frutos gravemente atacados por *Tuta absoluta* (dcha.).

5.4 Métodos de lucha

5.4.1 Control químico

La primera estrategia a la que se recurre para controlar a este lepidóptero es el control químico. El control de *T. absoluta* requiere la aplicación de plaguicidas con efecto translaminar o aplicaciones repetidas de productos para afectar a los estadios más sensibles de la plaga; momento en que las larvas salen de las galerías. Por este motivo, es tan importante la cuidadosa determinación de estos momentos de aplicación y para ello se estudia la dinámica poblacional mediante muestreos en campo o el seguimiento de las poblaciones a través de

captura de adultos en trampas con feromona. Debido a este protocolo de repetidas aplicaciones de plaguicidas, por ejemplo de 15 a 17 tratamientos en Chile (Salazar y Araya, 2001), se encuentran numerosas citas bibliográficas que documentan el desarrollo de resistencias en este lepidóptero a productos como: deltametrín, abamectina, esfenvalerato, λ-cihalotrín, cartap (Siqueira et al., 2000; Salazar y Araya, 2001; Siqueira et al., 2001; Lietti et al., 2005). También encontramos en la literatura estudios en los que se evalúa la aplicación del neonicotinoide imidacloprid (Collavino y Giménez, 2008) y extractos de plantas (da Cunha et al., 2005; da Cunha et al., 2006; Gonçalves-Gervasio y Vendramim, 2007; da Cunha et al., 2008). Los productos autorizados actualmente en España contra *T. absoluta* son: abamectina, emamectina, spinosad, indoxacarb y etofenprox (MARM, 2011).

5.4.2 Control biológico

En cuanto al control biológico, se han descrito gran variedad de parasitoides y depredadores que atacan los huevos, larvas o pupas de *T. absoluta* (Miranda et al., 1998; Blaeser et al., 2004; Urbaneja et al., 2009; Desneux et al., 2010). Para promover estos sistemas es necesario el desarrollo e introducción de nuevos métodos de control compatibles con el control biológico como técnicas culturales, biotecnológicas, métodos biológicos, como la introducción de hongos entomopatógenos y nematodos (Rodríguez et al., 2006; Batalla-Carrera et al., 2010) o tratamientos con cepas de la bacteria *B. thuringiensis*, cuya eficacia ya ha sido demostrada (Giustolin et al., 2001; Theoduloz et al., 2003; Niedmann y Meza-Basso, 2006; González-Cabrera et al., 2010) y su uso se encuentra autorizado en España.

5.4.3 Feromonas

La utilización de métodos basados en el uso de feromonas aparece como una alternativa viable desde el descubrimiento de la emisión de una feromona sexual por parte de las hembras vírgenes de *T. absoluta* (Quiroz, 1978). Posteriormente, esta feromona fue caracterizada por diversos investigadores como

el acetato de (3*E*,8*Z*,11*Z*)-tetradecatrienilo (al que llamaremos TDTA) (Attygalle et al., 1995; Attygalle et al., 1996). Esta sustancia representa alrededor del 90% del material volátil encontrado en la glándula sexual de las hembras en estado de llamada. También fue identificado un compuesto minoritario (~10%) como el acetato de (*3E,*8*Z*)-tetradecadienilo (TDDA) (Griepink et al., 1996; Svatos et al., 1996). Estos descubrimientos han permitido el desarrollo de emisores de feromona para la aplicación de las técnicas de seguimiento de poblaciones, atracción y muerte y confusión sexual, que se resumen a continuación.

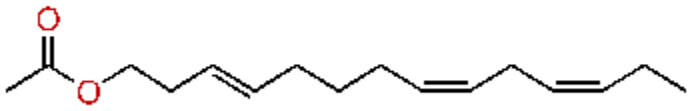

Figura 5.4.3.1 Molécula del acetato de (3E,8Z,11Z)-tetradecatrienilo, componente mayoritario de la feromona de *Tuta absoluta*.

Se han publicado varios trabajos en los que se ha estudiado el desarrollo de nuevos tipos de trampas y formulaciones de feromonas, adecuadas para el seguimiento de la plaga (Ferrara et al., 2001; Salas, 2004; Salas, 2007). En campo, se han ensayado varias formulaciones de la feromona de *T. absoluta*, incluyendo el TDDA, el acetato de (3*E*,11*Z*)-tetradecadien-1-ilo y el acetato de (8*Z*,11*Z*)-tetradecadien-1-ilo, isómeros diénicos como componentes minoritarios. En ningún caso se mejoró la eficacia atrayente del TDTA, componente principal de la feromona (Michereff et al., 2000a). Varias empresas han desarrollado emisores de feromona para la detección y seguimiento poblacional de *T. absoluta*. Estos dispositivos emisores son de tipo *rubber septa* y tienen una cinética de emisión muy deficiente, liberando la feromona en los primeros días en campo y perdiendo su eficacia rápidamente. Por ello, las capturas que se obtienen con este tipo de emisor son muy irregulares, capturando más durante las primeras semanas de

vida del emisor, lo que impide valorar correctamente la verdadera población que existe en el campo a lo largo del tiempo.

Hasta ahora, la confusión sexual contra la polilla del tomate solo se había ensayado en Suramérica, con dosis de TDTA entre 10 y 80 g ha^{-1}, en parcelas de cultivo al aire libre de apenas 200 m^2, sin buenos resultados y sin estudios de emisión de feromona (Michereff et al., 2000b). En la única experiencia descrita en España sobre confusión sexual de *T. absoluta* se ensayaron dosis entre 0.15 y 2 g ha^{-1}, también con resultados poco favorables (Martí et al., 2010).

6. LA MOSCA DEL OLIVO (*Bactrocera oleae*)

6.1 Importancia y distribución

La mosca del olivo, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), es un díptero monófago de los frutos del género *Olea*, especialmente *O. europea* L. tanto cultivado como silvestre. El origen más probable de este tefrítido se encuentra en el África subsahariana, donde se encuentran las variedades de las que provienen los cultivares domesticados. Sin embargo, su mayor abundancia y repercusión tiene lugar en los olivos de la cuenca Mediterránea. Actualmente, *B. oleae* ha invadido las zonas olivareras de California y México, y se cita su presencia en África, Pakistán y Oriente Medio (Nardi et al., 2005; Daane y Johnson, 2010).

Su clasificación sistemática es la siguiente:

Orden Diptera
Suborden Brachycera
Familia Tephritidae
Subfamilia Dacinae
Tribu Dacini
Género *Bactrocera*
Especie *B. oleae* (Rossi, 1790)

6.2 Biología de la plaga

Se trata de un insecto holometábolo que pasa por las fases de huevo, larva, pupa y adulto. Las hembras adultas ponen los huevos bajo la superficie de la oliva, perforando con su oviscapto y dejando una incisión en forma triangular. De esta forma, al eclosionar el huevo, la larva neonata tendrá acceso directo al alimento (Fletcher, 1987). La larva es ápoda, alargada, de color amarillento y pasa por tres estadíos, viviendo como barrenadoras en el mesocarpo de la oliva (Daane y Johnson, 2010). Completado el último estadio se aproximan a la superficie de la

oliva y forman la pupa. El desarrollo de *B. oleae* es altamente dependiente de la temperatura, y con las condiciones óptimas, el desarrollo de huevo, larva y pupa puede completarse hasta en 1, 8 y 9 días, respectivamente (Daane y Johnson, 2010). La mosca adulta mide entre 4-5 mm de longitud y presenta coloraciones pardo o anaranjado, sobre la que destacan una serie de placas de color negro, con el borde posterior del tórax de color amarillo. El dimorfismo sexual de la especie viene dado principalmente por la presencia del oviscapto en la hembra.

Figura 6.2.1 Pupas de *Bactrocera oleae* en el interior del fruto (izq.) e insecto adulto (dcha.).

6.3 Daños

El ataque de *B. oleae* reduce la producción de forma cuantitativa y cualitativa, ya que las larvas se alimentan de la pulpa de los frutos. Por lo que respecta a la oliva de mesa, los frutos atacados caen antes de completar la maduración, dejándolas inservibles para su comercialización. Y para la oliva dedicada a la producción de aceite, el ataque de *B. oleae*, además de reducir el rendimiento en aceite, afecta a su calidad, ya que aumenta su nivel de acidez (Daane y Johnson, 2010).

6.4 Métodos de lucha

6.4.1 Control químico

Durante las cuatro últimas décadas, el control de la mosca del olivo se ha basado en pulverizaciones de insecticidas organofosforados, especialmente dimetoato y fentión. Ya en los años 70 se comienzan a citar casos de resistencias al dimetoato (Kakani et al., 2010), que añadido a la problemática del uso de estos insecticidas de amplio espectro, llevan a la búsqueda e introducción de los programas de gestión integrada de plagas que reduzcan o eliminen el uso de insecticidas. Durante los últimos años se ha introducido el uso de algunos piretroides, pero su uso está limitado (Kakani et al., 2010) y ya se han observado casos de resistencias a la cipermetrina (Margaritopoulos et al., 2008). Más recientemente, el producto spinosad ha sido introducido en algunos países, aplicado como pulverización en cebo, que aumenta la eficacia con menos cantidad de ingrediente activo (Daane y Johnson, 2010). Sin embargo, existen trabajos que documentan el aumento en la tolerancia de *B. oleae* a spinosad en zonas donde se ha utilizado de forma extensiva (Kakani et al., 2010).

6.4.2 Atrayentes y feromonas

Los métodos de trampeo han tenido siempre una especial consideración para el control de *B. oleae* por la disponibilidad de atrayentes alimenticios eficaces y atrayentes sexuales, que pueden llevar a los insectos hacia trampas (Haniotakis et al., 1991; Broumas y Haniotakis, 1994). Algunos de los cebos utilizados incluyen hidrolizados de proteína, levadura de Torula, y sales amónicas (bicarbonato amónico, sulfato amónico y fosfato biamónico). Los cebos utilizados varían según regiones y el tipo de trampa utilizada (Daane y Johnson, 2010).

Las hembras de *B. oleae* son las únicas hembras de tefrítidos conocidas que emiten feromona sexual, siendo el 1,7-dioxaspiro[5.5]undecano (spiroacetal) su componente mayoritario (Baker et al., 1980; Jones et al., 1983; Haniotakis y

Pittara, 1994). Los machos también producen este compuesto para atraer a otros machos como feromona de agregación. Sin embargo, las hembras no son atraídas, en ningún caso, por el spiroacetal (Haniotakis y Pittara, 1994).

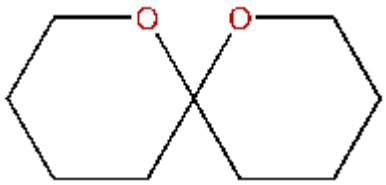

Figura 6.4.2.1 Molécula del 1,7-dioxaspiro[5.5]undecano (spiroacetal), feromona de la hembra de *Bactrocera oleae.*

El descubrimiento de la feromona ha sido muy importante para el seguimiento de las poblaciones de la mosca del olivo. Normalmente, se utilizan trampas pegajosas cebadas con emisores de spiroacetal del tipo vial de polietileno para el seguimiento de las poblaciones de machos, mientras que el seguimiento de hembras se realiza con trampas tipo McPhail u Olipe, cebadas con una mezcla de bicarbonato amónico y bifosfato amónico.

Figura 6.4.2.2 Trampa pegajosa amarilla para seguimiento de poblaciones de *Bactrocera oleae.*

6.4.3 Control biológico

En la literatura se encuentran numerosas especies de himenópteros, como enemigos naturales de la mosca del olivo (Daane y Johnson, 2010), pero el parasitoide más ampliamente utilizado en estos programas de control biológico ha sido el bracónido *Psyttalia concolor* (Szépligeti). Resultados de control biológico clásico contra la mosca del olivo han proporcionado resultados variables (Daane y Johnson, 2010). Algunos estudios sugieren que este parasitoide se ha establecido en muchas regiones, pero raramente contribuye a un control eficaz de la plaga (Yokoyama et al., 2011). En cambio, Miranda y colaboradores han registrado parasitismo estacional mayor del 20% en olivares ecológicos (Miranda et al., 2008).

7. LA MOSCA DEL MEDITERRÁNEO (*Ceratitis capitata*)

7.1 Importancia y distribución

La mosca del Mediterráneo, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) es una de las plagas más perjudiciales en el mundo. Se encuentra distribuida en los países del Mediterráneo, África Oriental y Suboccidental, América, Australia y en muchas islas del Atlántico y del Océano Pacífico (Enkerlin et al., 1989). Se han citado un total de 350 especies de plantas, pertenecientes a 67 familias diferentes, que pueden ser atacadas por *C. capitata*. La mayoría de especies son frutales de zonas templadas y subtropicales (Liquido et al., 1990; Liquido et al., 1991; Batkin, 1995). Por tanto, este elevado número de hospederos, con frutas maduras en diferentes estaciones, permite a *C. capitata* mantener su actividad prácticamente de forma continuada a lo largo de todo el año. La zona del Levante español es un claro ejemplo de continuidad de cultivos susceptibles a *C. capitata.*

Su clasificación sistemática es la siguiente:

Orden Diptera
- Suborden Brachycera
  - Familia Tephritidae
    - Subfamilia Trypetinae
      - Tribu Trypetini
        - Genero *Ceratitis*
        - Especie *C. capitata* (Wiedemann 1824)

7.2 Biología de la plaga

La mosca del Mediterráneo pertenece al grupo de los insectos holometábolos y presenta las siguientes fases de desarrollo: huevo, larva, pupa y adulto. Los huevos son de color blanco, de forma ovoide alargados de no más de 1 mm de longitud. Al eclosionar, las larvas miden escasamente 2 mm, pero llegan

a alcanzar los 8 mm tras realizar dos mudas. Se caracterizan por tener un aparato bucal masticador con el que destruyen los tejidos de la pulpa y se abren camino en el interior del fruto. Alcanzada la madurez salen al exterior para pupar en el suelo. Los puparios son de forma cilíndrica, de 5 mm de longitud, superficie lisa y ligera segmentación. En condiciones favorables (~26ºC) después de 10 días emerge el insecto adulto de 5 mm de longitud, presentando colores amarillo, blanco y negro. Las alas son transparentes, de color claro, con tres líneas anaranjadas, una longitudinal y dos transversales, con numerosas manchas negras. Existe un claro dimorfismo sexual en esta especie, el cual viene dado por el oviscapto agudo de forma triangular de las hembras y la terminación romboidal en las antenas de los machos. Una característica importante del comportamiento sexual de la mosca del Mediterráneo es la formación de *leks* por parte de los machos (Arita y Kaneshiro, 1985; Kaspi y Yuval, 1999). El sistema *lek* es aquel en el que los machos forman grupos y emiten señales visuales, acústicas y químicas, para atraer a las hembras y aparearse. Este sistema de apareamiento es común en la familia Tephritidae.

Figura 7.2.1 Dimorfismo sexual en *Ceratitis capitata* (macho izq., hembra dcha.).

7.3 Daños

El daño producido por *C. capitata* se debe, principalmente, a la picadura de la hembra sobre los frutos, al insertar su oviscapto para realizar las puestas. El

posterior desarrollo de las larvas en el interior de los mismos provoca la pudrición y devaluación del producto. En la Comunidad Valenciana el coste presupuestario destinado al control de *C. capitata* durante el año 1996 no llegaba al millón de euros (GVA, comunicación personal), en años sucesivos se incrementó hasta superar los 10 millones de euros.

7.4 Métodos de lucha

7.4.1 Control químico

La plaga se ha controlado, tradicionalmente, protegiendo los frutos con tratamientos insecticidas (organofosforados y piretroides), aunque es conocido que estas aplicaciones tienen efectos negativos sobre la fauna auxiliar y aumentan el riesgo de presencia de residuos en la cosecha; además de que no siempre son capaces de controlar satisfactoriamente la plaga. El tratamiento que más se utilizó contra *C. capitata* durante las décadas de los años 70, 80, 90 y principios del 2000 fue la aplicación, terrestre o aérea, de insecticidas organofosforados como el fentión y el malatión. El fentión fue retirado en los años 90 por su perfil toxicológico, aunque en tratamientos aéreos ya había sido reemplazado por el malatión una década antes. Estas aplicaciones, que van acompañadas de un cebo proteico, tienen los siguientes efectos negativos: afección de la fauna útil por su amplio modo de acción, aparición de resistencias (Ortego et al., 2005), contaminación medioambiental y residuos en cosecha. Por estos motivos, la búsqueda de nuevos métodos de control alternativos era necesaria, y los insecticidas de nueva generación, cuyo ejemplo más relevante es el spinosad, ha sido una primera alternativa. Este producto consiste en dos toxinas (spinosin A y spinosin D) procedentes de la bacteria *Saccharapolyspora spinosa* (Actionomycetes). Presenta actividad frente a varios insectos, incluidos los dípteros, y actúa por ingestión o contacto mediante la activación de los receptores de nicotín-acetilcolina, siendo éste un mecanismo nuevo entre los insecticidas conocidos actualmente (Salgado, 1998). Su degradación en el ambiente es bastante rápida y ha demostrado ser tan eficaz como el malatión en aplicaciones cebo (Adán et al., 1996; Burns et al., 2001).

En el campo de los IGRs, se ha estudiado e introducido el uso del lufenurón, una benzofenilurea que produce la quimioesterilización de *C. capitata*. En estudios iniciales, se comprobó que anulaba la descendencia en la fase de huevo cuando se administraba en la comida a dosis de 1.000 ppm en hembras y a 5.000 ppm en machos (Casaña-Giner et al., 1999). A partir de estos resultados se planteó el diseño de un sistema de lucha que aprovechase este efecto esterilizante, y se desarrolló la aplicación del lufenurón con un cebo que permitiera la ingestión del mismo en aplicaciones de campo (Navarro-Llopis, 2001; Navarro Llopis et al., 2004, 2007). Actualmente, es un método de control disponible comercialmente, bajo el nombre Adress® (Syngenta AG, Basilea, Suiza) (ver **Figura 7.4.3.1**).

7.4.2 Técnica del insecto estéril (TIE)

Consiste en la producción masiva de machos que posteriormente son esterilizados en su fase de pupa, mediante radiación gamma, y liberados al campo para competir con los insectos salvajes. Los insectos estériles copulan con las hembras salvajes y les transfieren esperma estéril, esto provoca la infertilidad de los huevos, lo que conlleva una reducción de la población. Es un método muy ventajoso debido a su alta especificidad, pero requiere una gran infraestructura.

El primer programa contra *C. capitata* data de 1970, evitando la invasión de la plaga desde América Central al sur de México. Además, se ha llevado a cabo con éxito en Chile, Israel, Sudáfrica y Tailandia (Klassen y Curtis, 2005). En la Comunidad Valenciana se viene desarrollando desde 2007 y se aplica sobre una superficie de 152.500 ha.

7.4.3 Trampeo masivo

En el caso de *C. capitata*, la técnica de trampeo masivo no emplea la feromona sexual del insecto al no encontrarse perfectamente descrita. Esta técnica se inició a principios del siglo XX, utilizándose una botella de vidrio llamada

mosquero McPhail cargada con proteínas o sustancias azucaradas en fermentación (Newell, 1936; McPhail, 1939). Esta técnica se empleó masivamente en la zona citrícola de Valencia (Gómez-Clemente y Planes, 1952). Las trampas y atrayentes utilizados actualmente, tanto para trampeo masivo como para realizar el seguimiento de la población de *C. capitata,* han evolucionado notablemente. En cuanto a atrayentes, se han conseguido formulaciones más eficaces que combinan acetato amónico, trimetilamina y putrescina (Heath et al., 1997). Esta mezcla ha demostrado ser más eficaz en la capturas de machos y hembras de *C. capitata* que las proteínas hidrolizadas (Katsoyannos et al., 1999). La utilización de estos atrayentes se ha extendido con éxito por todo el mundo (Ros et al., 1999; Cohen y Yuval, 2000; Miranda et al., 2001). Pese a todo, el trampeo masivo sigue siendo un sistema de lucha caro, con un coste variable entre 100 y 200 € por ha, en función de la densidad de trampas utilizada.

Figura 7.4.3.1 Mosquero para captura masiva de *Ceratitis capitata* (izq.) y sistema de quimioesterilización Adress®.

7.4.4 Feromonas

El sistema de comunicación mediante feromonas en *C. capitata* es complejo y no está bien establecido. Hace casi cinco décadas que Féron (1962) observó la existencia de compuesto emitidos por los machos que atraían a las hembras; y desde entonces, se ha estudiado ampliamente el comportamiento sexual de este díptero (Eberhard, 2000). Una década después del descubrimiento de Féron, se describe la feromona sexual de *C. capitata* como una mezcla de 15 compuestos, incluyendo ácidos carboxílicos y los compuestos metil (*E*)-6-nonenoato y (*E*)-6-nonen-1-ol (Jacobson et al., 1973). Según Ohinata, estas mezclas atraían a machos y hembras en laboratorio, y sólo a machos en ensayos de campo. Se realizaron formulaciones con (*E*)-6-nonenoato de metilo que parecía ser tan atrayentes como el TML (Ohinata et al., 1973). Más tarde, la tarea de identificación de los compuestos que forman las emisiones volátiles de los machos de *C. capitata*, sigue de la mano de Baker y sus colaboradores, que describen una mezcla de nueve compuestos, incluyendo la imina 3,4-dihidro-2H-pirrol (1-pirrolina), a la que se atribuye un papel muy importante en la atracción sexual (Baker et al., 1985). En el trabajo de Jang, se detectaron 69 compuestos en emisiones de machos, mientras que en las emisiones de hembras solo se detectaron algunos aldehídos de cadena corta y a nivel de trazas (Jang et al., 1989). Posteriormente, Flath (1993) revisaría la composición de las emisiones de machos, incluyendo en su estudio los factores de edad y momento de emisión durante el día. Estos últimos estudios coinciden en los tres componentes mayoritarios de las emisiones de machos vírgenes de *C. capitata*: (*E*)-3-octenoato de etilo, acetato de geranilo y (*E,E*)-α-farneseno. Se han realizado formulaciones de estos compuestos para su ensayo en campo (Heath et al., 1991; Flath et al., 1993; Jang et al., 1994; Light et al., 1999), pero no se ha conseguido una buena actividad atrayente con ellos. Es posible que la ausencia de alguno de los compuestos minoritarios, no incluidos en la mezcla, sea clave para conseguir un buen poder atrayente. Además, puede que el cortejo y la agrupación de machos al inicio del mismo (*leks)* sea esencial para la atracción final a corta distancia, lo que explicaría que no se haya encontrado una mezcla feromonal eficaz.

7.4.5 Control biológico

Existe bastante literatura sobre lucha biológica en tefrítidos, pero los enemigos naturales de *C. capitata* utilizados con más éxito han resultado ser *Diachasmimorpha longicaudata* (Ashmed) y *Diachasmimorpha tryoni* (Cameron) (Wong et al., 1984). Varias especies de parasitoides han sido introducidas en distintas partes del mundo con el objetivo de realizar control biológico clásico, pero con resultados diferentes según la zona. En la Comunidad Valenciana se han criado dos especies exóticas, *D. tryoni* y *Fopius arisanus* (Sonan) para evaluar su capacidad como parasitoides de *C. capitata* (Beitia et al., 2002).

A parte de los parasitoides también se ha descrito un importante efecto depredador, de hasta el 25 % de las larvas, por parte de la hormiga *Solenopsis geminata* (Fabricius) (Eskafi y Kolbe, 1990). En general, en los meses cálidos, los depredadores más activos son las hormigas, y en los más fríos la depredación la realizan básicamente las arañas, coleópteros estafilínidos y otros depredadores (Urbaneja et al., 2006).

Otra alternativa es la aplicación de hongos entomopatógenos, de las especies *Metarhizium anisopliae* (Metschnikoff) Sorokin, *Beauveria bassiana* (Balsamo) Vuillemin y *Isaria fumosoroseus* (Wize) Brown & Smith, cuya eficacia para infectar adultos de *C. capitata* ha sido demostrada en ensayos de laboratorio (Castillo et al., 2000) y de campo (Moya et al., 2003). Además, existen experiencias, tanto de laboratorio como de campo, con nematodos entomopatógenos, sobre todo del género *Steinernema* (Lindegren y Vail, 1986; Gazit et al., 2000).

JUSTIFICACIÓN Y OBJETIVOS

JUSTIFICACIÓN Y OBJETIVOS

La presente tesis doctoral describe los trabajos realizados sobre estudios básicos y de aplicación de semioquímicos para el control de plagas, centrándose en tres objetivos:

(1) Desarrollo del método de confusión sexual contra plagas de importancia: *Aonidiella aurantii y Tuta absoluta*.

(2) Optimización de emisores para sistemas de atracción de plagas importantes: *Chilo suppressalis, Lobesia botrana, Bactrocera oleae* y *Ceratitis capitata*.

(3) Estudio de volátiles emitidos por *Ceratitis capitata*, con el objetivo de buscar nuevos semioquímicos para esta plaga.

1. Estudios sobre confusión sexual

La confusión sexual, como método de control altamente específico y respetuoso con el medio ambiente, se presenta como una importante alternativa para el control de una plaga dañina para la citricultura, el piojo rojo de California (*A. aurantii*), y también para una plaga de reciente introducción como es la polilla del tomate (*T. absoluta*).

Por lo que respecta al piojo rojo de California, el objetivo principal de este estudio ha sido evaluar la confusión sexual como posible método de control y para ello se han desarrollado emisores biodegradables de la feromona sexual de *A. aurantii* utilizando un material inorgánico poroso como soporte. Se han elaborado diversas formulaciones de emisores con diferentes cargas feromonales, que se ensayaron en condiciones de campo, para elegir el emisor más adecuado, evaluar la eficacia de la técnica de confusión sexual y proponer la estrategia de aplicación más idónea.

En cuanto a *T. absoluta*, debido a la reciente introducción de esta plaga que causa tan importantes pérdidas en la producción de tomate, se hace indispensable la búsqueda de métodos alternativos al control químico convencional. Como en el caso anterior, el principal objetivo de esta parte de la tesis ha sido el desarrollo y evaluación de la técnica de confusión sexual como posible método de control. Para ello, se han elaborado también formulaciones de emisores utilizando soportes inorgánicos porosos para evaluar la eficacia de la técnica en invernaderos con distintos grados de aislamiento.

2. Optimización de emisores para sistemas de atracción

La atracción eficaz de los insectos hacia las trampas es clave tanto para la detección y el seguimiento de poblaciones, como para los métodos de control basados en el trampeo. La mayoría de los emisores que se encuentran en el mercado para estos fines no reúnen las características adecuadas. Los principales problemas de estos emisores son una gran tasa de emisión inicial, elevados valores residuales de feromona no emitida al final del periodo útil y una alta sensibilidad a los factores ambientales. Todo ello puede producir que el efecto atrayente sea muy diferente a lo largo del periodo de uso y que además la feromona, que supone la mayor parte del coste de los emisores, no se aproveche al máximo. Por ello, el objetivo de esta parte de la tesis ha sido estudiar la relación nivel de emisión-capturas de distintos emisores, intentando determinar la existencia de un valor de emisión óptimo para obtener el máximo número de capturas. Este estudio se ha realizado para las feromonas sexuales de los lepidópteros *C. suppressalis* y *L. botrana*, para el díptero *B. oleae* y también para *C. capitata*, en relación a la emisión de su paraferomona Trimedlure.

3. Estudio de los volátiles emitidos por *C. capitata*

Los atrayentes para *C. capitata* han sido ampliamente estudiados en la literatura y no se ha determinado claramente la existencia de una feromona sexual que emitida por la hembra cause una respuesta en los machos conspecíficos,

como es habitual en lepidópteros. Sin embargo, en la literatura se documenta que los machos son los responsables de emitir una mezcla de sustancias atrayentes para la hembra. La composición de esta mezcla también ha sido estudiada pero sin resultados relevantes en la captura de hembras en condiciones de campo. La disponibilidad de atrayentes eficaces para las hembras de *C. capitata* supondría una gran ayuda para el control de la plaga y para la integración de los sistemas de trampeo con otros métodos, como la técnica del insecto estéril. La presente tesis, en su tercera parte, aborda la composición de las emisiones volátiles, tanto de machos como de hembras de *C. capitata*, mediante la técnica de microextracción en fase sólida (SPME), no utilizada previamente en estudios de este tipo con especies de tefrítidos. Posteriormente, aplicando la metodología de análisis de componentes principales (PCA) se consiguió la discriminación y clasificación de las sustancias identificadas en las emisiones, todo ello para su posterior uso en ensayos de comportamiento del insecto y evaluar su papel como semioquímicos de *C. capitata*.

Capítulo I

"The first account of the mating disruption technique for the control of California red scale, *Aonidiella aurantii* Maskell (Hemiptera: Diaspididae) using new biodegradable dispensers"

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The first account of the mating disruption technique for the control of California red scale, *Aonidiella aurantii* Maskell (Hemiptera: Diaspididae) using new biodegradable dispensers

Published in Bulletin of Entomological Research (Vacas S., C. Alfaro, V. Navarro-Llopis and J. Primo. 2009)

Abstract. Semiochemical-based pest management programs have been increasingly used to provide environmentally friendly methods for the control of major insect pests. The efficacy of the mating disruption technique has been demonstrated for several moth pests. Unfortunately, not many experiments on mating disruption to control diaspididae species have been documented. In this work, biodegradable dispensers for mating disruption with increasing pheromone loads were used in order to study the potential of this technique for the control of *Aonidiella aurantii* Maskell. Field trial results demonstrated that dispensers loaded with 50 mg (a.i.) (20 g ha^{-1}) and 100 mg (a.i.) (40 g ha^{-1}) of sex pheromone were the most suitable, achieving significant reductions in male catches, compared to an untreated plot. In treated plots, virtually a 70% reduction in damaged fruit was recorded. Pheromone release profiles of all the dispensers were also studied under field conditions. We found that emission values >250 µg day^{-1} were the most suitable. This study suggests a new biodegradable dispenser capable of interfering with normal *A. aurantii* chemical communication. The use of mating disruption as a control method against *A. aurantii* is discussed.

I.1 Introduction

California red scale (CRS), *Aonidiella aurantii* (Maskell), is one of the most important citrus pests occurring worldwide and is an important economic pest in Spain. Damage caused by this armored scale, which can be considered as cosmetic, lead to downgrading or rejection of the product at the packing house. Moreover, heavy scale infestations may lead to yellowing of leaves, defoliation, branch dieback and possible tree death (Grafton-Cardwell and Reagan, 1995).

The female CRS can give birth from 100 to 150 active crawlers. They emerge from under the female cover in a day or two, depending on the temperature. These crawlers travel short distances and settle onto twigs, leaves or fruits, so as they are the only immature instars capable of movement (Bodenheimer, 1951). During the second instars, females and males begin to develop differently. Adult male emergence coincides with the development of third instar females, which then mate and produce the next generation. Virgin females attract males by releasing a pheromone. Males may crawl to nearby females or fly to other trees (University of California, 1991). The number of generations of CRS that could develop in orange fruits range from three to five, influenced by the degree-day accumulation (Kennett and Hoffmann, 1985; Grout et al., 1989). Under our environmental conditions, CRS shows three complete generations with three male flights, the first of which takes place between mid-April and mid-May, the second between mid-June and late July and the third from mid-August to early-September.

Traditionally, chemical control has been used by growers in order to prevent such damage to citrus. However, since the development of resistances to many insecticides was documented for CRS (Grafton-Cardwell and Vehrs, 1995), other control techniques have been introduced. The use of oil sprays has been expanded although these can be potentially phytotoxic (Grout and Richards, 1991a; Grafton-Cardwell and Reagan, 1995; Tan et al., 2005). Thus, satisfactory

management of oil applications is essential in order to prevent these effects and to ensure the efficacy of the treatment. In search of alternative methods of CRS control, the use of insect growth regulators (IGR), such as buprofezin (Grout and Richards, 1991a; Ishaaya et al., 1992) and pyriproxyfen (Alfaro et al., 1999b; Grafton-Cardwell et al., 2006; Eliahu et al., 2007; GIP CITRICOS, 2011), was included even though the effect of these IGR on natural enemies is still not clear (Grafton-Cardwell and Gu, 2003; Lauziere and Elzen, 2007). Classical biological control also offers an alternative to CRS, being the most successful natural enemy the parasitoid *Aphytis melinus* DeBach (Hare and Luck, 1994; Moreno and Luck, 1992; Murdoch et al., 2006). However, its effectiveness depends on careful monitoring, in order to establish the exact release date and the use of selective insecticides for other pests which do not affect *A. melinus*.

In recent years, semiochemical-based pest management programs, such as mass-trapping, have been increasingly used to provide environmentally friendly methods for the control of major insect pests (El Sayed et al., 2006). The production of sex pheromone was demonstrated in CRS years before the chemicals were first reported by Roelofs et al. in 1977. Since then, synthetic sex pheromone traps have been widely employed as a management and detection tool for CRS populations (Moreno et al., 1972; Gardner et al., 1983; Kennett and Hoffmann, 1985; Moreno and Kennett, 1985; Samways, 1988; Grout et al., 1989; Grout and Richards, 1991b). The CRS sex pheromone was described as 3-methyl-6-isopropenyl-9-decen-1-yl acetate (I) and (*Z*)-3-methyl-6-isopropenyl-3,9-decadien-1-yl acetate (II) (Roelofs et al., 1977). All possible geometrical and optical isomers of the two compounds were synthesized and tested by Gieselmann in 1980. The results showed that only one isomer from each compound was significantly more active: (3S,6R)-I and (3*Z*-6R)-II and the presence of other isomers in the mixture had no effect on trap catches (Tashiro et al., 1979; Gieselmann et al., 1980). These findings can lead to the development of new methods of control based on pheromones, such as mating disruption. As a pure isomeric pheromone composition is not required, the cost of the pheromone can be reduced to bearable levels. Unfortunately, not many experiments on mating

disruption to control CRS have been documented (Barzakay et al., 1986; Hefetz et al., 1988), and more extensive studies with different pheromone dosages are necessary in order to establish the potential of this method in controlling *A. aurantii*.

The final aim of our experiments is to evaluate mating disruption as a possible control method against CRS. The efficacy of mating disruption for this pest has not yet been demonstrated and published in scientific literature; moreover, commercial dispensers are not currently available in Spain. Therefore, the main aim of this work is to develop a new biodegradable dispenser for this purpose. Two new formulations for dispensers with several CRS sex pheromone loads were tested during two years of field trials. These trials were designed to check the efficacy of the dispensers and to select the most suitable one. Further field trials will be carried out to evaluate the mating disruption technique as a CRS control method using the dispenser obtained in this study.

I.2 Material and Methods

I.2.1 Field trials

During the years 2006 and 2007, field trials were carried out in a citrus orchard in Alicante, Spain.

I.2.1.1 First trial year

An initial study, to evaluate our CRS mating disruption dispensers, was conducted in a twenty-year-old orchard in 2006 using sweet oranges from the Navel group, *Citrus sinensis* Osbeck. The trees were spaced 6×4 m apart. Two mesoporous dispensers based on the technology of inorganic molecular sieves (Corma et al., 1999, 2000) with 8 and 20 mg (a.i.) pheromone loads (which will be called D8 and D20 throughout the paper) were tested in two plots, both approximately 1.5 ha in size. A third 1.5 ha plot was left without treatment as an untreated plot. Separation between plots was 50 m, using a *Cupressus sempervirens* L. barrier as a boundary. On 7 April 2006, before the occurrence of red scale second flight, dispensers were hung with a density of one dispenser per

tree (nearly 400 dispensers per ha). The number of pheromone point sources employed in our trials was decided upon the biology of the pest, concentrating on CRS dispersion characteristics, as diaspidids maintain a relatively intimate relationship with a single host-plant (McClure, 1990). Mesoporous dispensers were placed inside small micro-perforated polyethylene (PE) bags, supplied by Ecología y Protección Agrícola S.L. (Valencia, Spain), hanging from internal branches at a height of 1.5–2.0 m. These mesoporous dispensers inside the bags were not replaced through the season.

I.2.1.2 Second trial year

In a second trial in 2007, dispensers were developed using a new formulation with 50 and 100 mg (a.i.) pheromone loads (which will be called D50 and D100 throughout the paper). D50 and D100 dispensers were tested under the same field conditions as in the first trial year, and they were effective during the whole season without replacement. On this occasion, on 14 March 2007, dispensers were placed inside small perforated cotton bags and hung one per tree. The cotton bags were manufactured using a cotton mesh (1×1 mm), supplied by Bi-Medica (Barcelona, Spain), and were 5 cm long by 3.5 cm wide. The change in dispenser bag material from PE to cotton was made in order to obtain a totally biodegradable pheromone release device. Previous laboratory studies demonstrated that there were no differences in emission between dispensers placed in micro-perforated polyethylene and cotton bags.

I.2.2 Evaluation of treatment efficacy

In order to evaluate the efficacy of mating disruption, three commercial white sticky pheromone traps (PHEROCON® V Scale Trap), supplied by Trécé Inc. (Adair, OK, USA), were placed in each treated and untreated plot. This evaluation was made by comparing CRS male trap catches from the untreated plots with those obtained from the treated plots. Sticky traps were revised and replaced weekly, whereas the PHEROCON® monitoring lures (Trécé Inc., Adair, OK, USA), loaded with 250 µg pheromone, were replaced every 40 days. The absence of trap

catches during mating disruption treatment is a good indication of the technique effectiveness, but crop damage assessment provides the ultimate proof (Howse, 1998). To assess crop damage, 40 fruits per tree were evaluated, ten fruits per orientation. Ten trees per plot were randomly selected and evaluated. Infestation levels of zero, 1–2 and >2 scales per fruit were recorded. Treatment efficacy results were given as a percentage of damaged fruit. We considered a fruit to be damaged when more than two scales were present.

The assessment of fruit damage was carried out both in the inner and buffer areas of each treated plot. We considered the buffer area to be 15 m from the plot border.

I.2.3 Mesoporous pheromone dispenser

New mesoporous pheromone dispensers were developed for the field trials carried out in 2006 and 2007, described above. These were elaborated based on a mesoporous material (Corma et al., 1999, 2000). The dispensers were cylindrical tablets 9 mm in diameter, with several sizes and loads (D8, D20 and D50) and 15 mm in diameter for the 100 mg pheromone load (D100). The formulations contained the diastereomeric mixture (3S,6R and 3S,6S) of the 3-methyl-6-isopropenyl-9-decen-1-yl acetate compound from the *A. aurantii* sex pheromone (74% purity). This mixture was supplied by Ecología y Protección Agrícola S.L. (Valencia, Spain).

I.2.4 Pheromone release profiles

In parallel with the field trials, all dispensers were simultaneously aged in a nearby area, over a period of 150 days in 2006 and 210 days in 2007. Residual pheromone content was extracted at different ageing times: 0, 7, 15, 30, 45, 60, 90, 120, 150, 180 and 210 days of ageing, and then quantified by Gas Chromatography using a flame ionization detector (GC/FID). Three replicates for

ageing time were extracted by solvent-extraction, at 40ºC, with a 3:2 methanol and dichloromethane mixture.

Red scale pheromone content was measured by GC/FID analyses (Clarus®500 gas chromatograph from PerkinElmer, Wellesley, MA, USA) of the extracts using 1-pentanol as the internal standard. All injections were made onto a ZB-5MS (30×0.25 mm×0.25 µm) column, held at 160ºC for 5 min and then programmed at 2ºC min^{-1} up to 180ºC, where it was held for 1 min, and then programmed at 45ºC min^{-1} up to 250ºC. The carrier gas was helium at 1.2 ml min^{-1}. The amounts of pheromone and the responses were connected by fitting a linear regression model, $y = a + bx$, where y is the ratio between pheromone and 1-pentanol responses and x is the amount of pheromone remaining in the dispensers.

Pheromone release for each dispenser type was represented by fitting an exponential model, $y=a \times e^{bx}$, where y is the remaining pheromone load and x represents the ageing days.

Pheromone emission values were obtained with the following formula: $x= (e_1–e_2) / (t_2–t_1)$, where x is the pheromone emission value for a time period, e is the residual amount of pheromone in a dispenser for a given aging time and t is the number of aging days.

I.2.5 Statistical analysis

Male catches in pheromone-baited traps, per trap per week, were analyzed using data from the entire study period. In a second analysis, data from the three different flights were used: the first flight included trap catches from one to 48 days, the second flight from 49 to 130 days and the third flight from 131 days to the end of the CRS season. The log-transformed total male counts per trap per week for each type of pheromone dispenser, ln (N+1), was analyzed using a one-way ANOVA model, followed by an LSD test at P=0.05, to assess the significance of differences in male captures among treatments.

In order to test the significant differences in fruit damage between pheromone treatments and untreated plots, a one-way ANOVA model was employed. The Statgraphics 5.1 package was used for all the statistical analyses (StatPoint Technologies, Warrenton, VA, USA).

I.3 Results

I.3.1 Dose-response trial: 2006

I.3.1.1 Male catches

Figure I.1 shows the male flights throughout the season from the untreated plot compared to those obtained from the pheromone treated plots. When analyzing data from the entire study period, male catches decreased significantly if we compare D20 treatment (20 mg dispenser) to the untreated plot, but there were no statistical differences between D8 (8 mg dispenser) treatment and the untreated plot ($F=3.41$; $df=179,2$; $P=0.035$). Male catches analyzed using data from the first and second flights gave the same result (flight 1: $F=4.22$; $df=58,2$; $P=0.020$; flight 2: $F=3.18$; $df=51,2$; $P=0.049$) while in the third flight any differences were found between untreated and both treatments ($F=1.32$; $df=64,2$; $P=0.27$). As a consequence of these results, we observed a preliminary male disorientation effect in line with the reduction in captures with the D20 treatment. However, during the third flight this effect disappeared. This may be due to the fact that the pheromone release rate was diminishing at the end of the season.

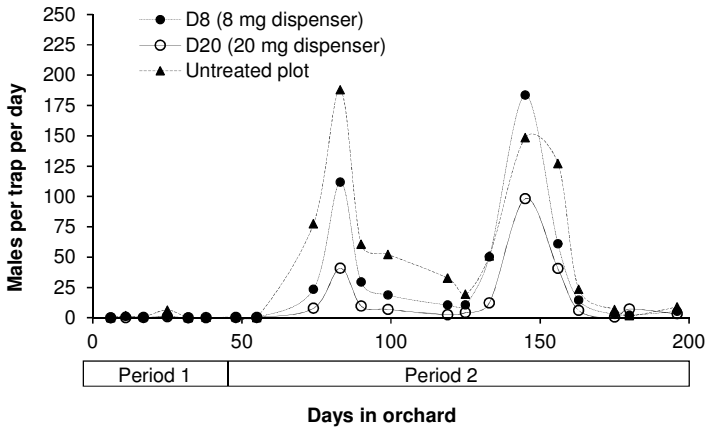

Figure I.1 Male CRS catches per trap per day during the 2006 trial for pheromone treated plots, D8 and D20, and the untreated plot.

I.3.1.2 Fruit damage

Fruit damage assessment was made at the end of the season in order to evaluate the performance of the dispensers. Data from trees located inside the plot (inner area) and from trees in the buffer area (15 m from the plot border) were analyzed separately. Fruit damage is shown in **Figure I.2**. The results indicated that the percentage of damaged fruit from the D8 treatment was significantly higher than that of the D20 and the untreated plot in the inner area ($F=3.86$; $df=15,2$; $P=0.044$). The D20 treatment displayed similar levels of damage to those returned by the untreated plot. However, in the buffer area, there was no sign of any mating disruption effect ($F=6.16$; $df=9,2$; $P=0.020$), so fruit damage in both treated plots (D8 and D20) was higher than in the untreated plot. As observed in these field trial results, D8 dispensers were not capable of interfering with normal CRS chemical communication. In contrast, D20 dispensers had a preliminary effect on CRS male flight disruption; but, unfortunately, this treatment was not capable of reducing damage on fruits.

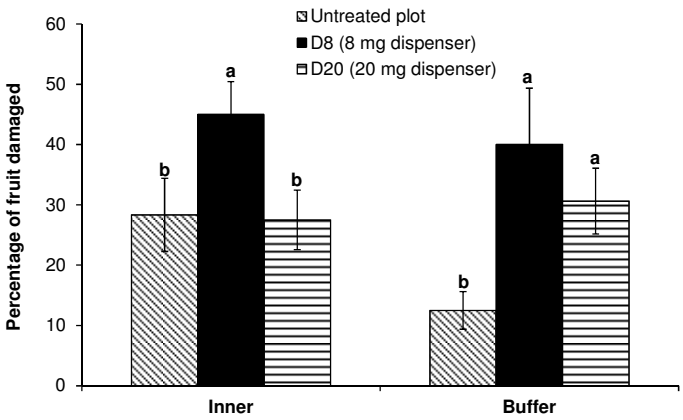

Figure I.2 Mean percentage of damaged fruits (with more than 2 scales) observed inside the untreated and pheromone treated plots, D8 and D20, (inner area) at the end of the 2006 season. Fruit damage in the buffer area (15 m from the plot border) is also represented. Bars with the same letter do not differ significantly (ANOVA test, $P>0.05$)

I.3.1.3 Pheromone release profiles

During the first year, we developed two types of pheromone dispensers with 8 and 20 mg pheromone loads (D8 and D20). **Figure I.3** gives the pheromone release profiles for both dispensers. From this data, we calculated the mean pheromone emission values for each type of dispenser, and it was observed that the D20 dispensers released CRS pheromone at a faster rate (90 µg day^{-1}) than the D8 dispensers (40 µg day^{-1}).

However, field trial results revealed that none of the dispensers emitted adequate amounts of pheromone to enable a viable mating disruption treatment. Furthermore, both types of pheromone dispensers had a high residual pheromone amount at the end of the season. Because male catches seem to decrease significantly under the D20 treatment, a new formulation with two pheromone loads was developed to improve the dispensers, achieving a better pheromone release rate. These new dispensers were put to use in the 2007 field test.

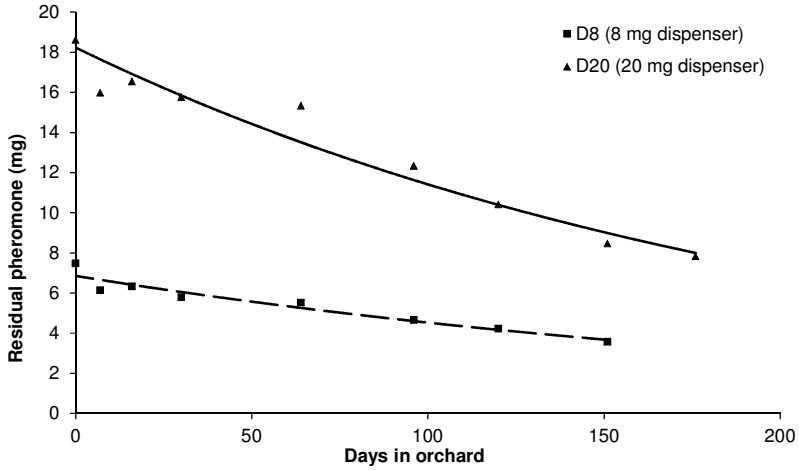

Figure I.3 Relation between the amount of residual pheromone (in mg) and days of field exposure for the two types of dispensers (D8 and D20) tested in the 2006 trial. Signification of the exponential model for the D8 treatment was R^2=0.9511 and R^2=0.9564 for the D20 treatment.

I.3.2 Dose response trial: 2007

In 2007, a new formulation was tested under field conditions with pheromone loads of 50 mg (a.i.) (D50) and 100 mg (a.i.) (D100) to control CRS by mating disruption. These new dispensers were formulated in order to increase the mean pheromone release rate, avoiding the replacement of the dispensers along the season.

I.3.2.1 Male catches

The biological effects of the treatments applied during the 2007 trial are shown in **Figure I.4**, and the existence of statistical differences can be seen in **Table I.1**. The analysis with average male CRS catches, per trap per week, during the whole season showed statistical differences between the untreated plot and both treatments, but no differences were observed between pheromone treated plots ($F=46.48$; $df=2,195$; $P<0.001$). The same occurred for captures during the first flight (Flight 1: $F=6.61$; $df=2,51$; $P<0.001$). When focussing on the second and

third flights (Period 2 on **Fig I.4**), the main male flights, it was noted that the depression of trap catches, in both the D50 and D100 treated plots, was significant compared to the untreated plot (Flight 2: F=69.57; df=2,87; P<0.001. Flight 3: F=25.78; df=2,51; P<0.001).

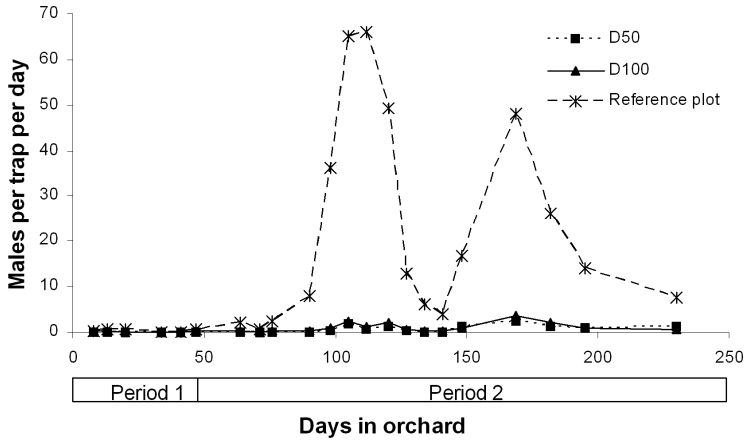

Figure I.4. Male CRS catches per trap per day during the 2007 trial for the pheromone treated plots, D50 and D100, and the untreated plot.

Table I.1 Mean±SE males per trap per week for each dispenser during the whole season and over separate flights in 2007 trial. Means in a column followed by the same letter are not significantly different (ANOVA test, *P*>0.05)*

Treatment	Males per trap per week			
	Whole season	Flight 1	Flight 2	Flight 3
D50	7.52±1.95 a	0.38±0.14 a	3.40±0.88 a	21.50±5.90 a
D100	10.11±2.47 a	1.11±0.28 a	6.57±1.22 b	25.00±7.89 a
Untreated	174.85±31.91 b	2.55±0.77 b	189.57±34.38 c	322.61±88.68 b

I.3.2.2 Fruit damage

A damage survey was conducted at the end of the season in order to test the operation of the dispensers. The level of fruit damage reduction achieved in the treated plots is illustrated in **Figure I.5**. In both the inner and buffer areas, we observed a reduction of almost 70% in damaged fruit without significant differences between treated plots D50 and D100 (inner area: F=24.69; df=15,2; *P*=0.000; buffer area: F=11.28; df=12,2; *P*=0.002). Given that both treated plots returned the same values for damage reduction, dispensers with loads greater than 50 mg would not be necessary given the CRS densities recorded in these trials.

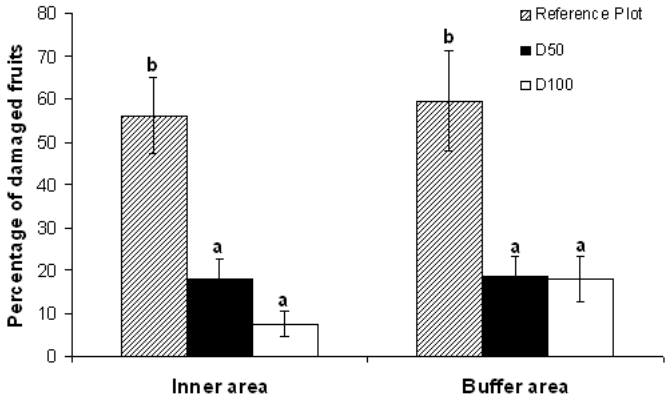

Figure I.5. Mean±SE percentage of damaged fruits (with more than 2 scales) observed inside the untreated and pheromone treated plots, D50 and D100, (inner area) at the end of the 2007 season. Fruit damage in the buffer area (15 m from the plot border) is also represented. Bars in each group labelled with the same letter do not differ significantly (ANOVA test, *P*>0.05).

I.3.2.3 Pheromone release profiles

The pheromone profiles studied for the two dispensers tested in 2007 are shown in **Figure I.6**. If we combine the results obtained in the field trials with the pheromone release rate of the dispensers employed, we can observe that an emission value approximating that obtained with the D50 and D100 dispensers (above 250 µg day^{-1}) is suitable for conducting an effective CRS control using the

mating disruption technique. This emission value was set according to the mean emission value of D50 dispenser along the season. The mean emission value of D100 dispenser was 390 µg day^{-1}. As seen on the efficacy results, we consider that emission values above 250 µg day^{-1} are adequate to maintain a suitable amount of pheromone in the atmosphere.

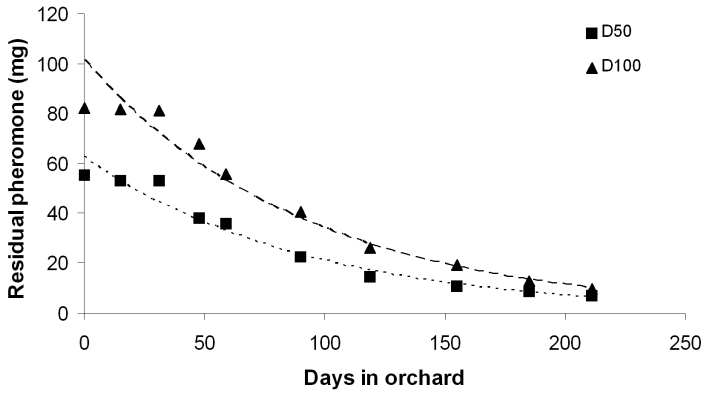

Figure I.6. Relation between the amount of residual pheromone (in mg) and days of field exposure for the two types of dispensers (D50 and D100) tested in the 2007 trial. Signification of the exponential model for the D50 treatment was R^2=0.9826 and R^2=0.9846 for the D100 treatment.

The D50 and D100 treatments provided hopeful results in the field trials. In addition, both types of dispensers contained low amounts of residual pheromone at the end of the CRS season. This is a key factor in dispenser cost and, consequently, in the cost of the control method. It is also an essential feature of a good pheromone dispenser (Muñoz-Pallarés *et al*., 2001; Stelinski *et al*., 2005).

I.4 Discussion

In the first experiment, we examined two new CRS pheromone dispensers (D8 and D20). None of these pheromone dispensers were efficient to control scale populations. Despite the poor performance of 8 and 20 mg treatments, a slight

reduction in male catches was noticed. This result encouraged us to continue testing new dispensers capable of disrupting chemical communication between scales. For successful mating disruption to occur, sufficient quantities of the artificial pheromone must be present in the air for the entire sexually active period of these insects (Cardé et al., 1975; Howse, 1998). Therefore, a dispenser that remains releasing sufficient amounts of pheromone during the seven months on which the CRS reproductive cycle takes place is needed. According to the results for the first year trial, it was observed that these dispensers had high levels of residual pheromone at the end of the trial. Moreover, we considered that the poor efficacy achieved by D8 and D20 treatments was mainly due to insufficient pheromone emission levels. During the second and third flights, the amount of pheromone emitted was very low and the inhibition of male catches was not achieved. Male catches were higher in the treated plots during the third flight, which may be due to the possible cumulative effect of the population, so in the second flight there was not a satisfactory disruption. Considering these two factors, low emission and high residual pheromone, we decided to change the initial pheromone load and the formulation used for the dispensers.

In the second year, we developed a new formulation with two different pheromone loads, 50 and 100 mg (D50 and D100). The pheromone profiles of the two dispenser types were studied under field conditions. We found that both dispenser types achieved very low residual pheromone amounts. This goal has been successfully accomplished using the mesoporous dispenser technology described by Corma in 1999 and 2000 (Corma et al., 1999, 2000).

The reduction in trap catches obtained with the D50 and D100 treatments was an indicator of the possibility of achieving a satisfactory control of CRS using the mating disruption technique. Fruit damage assessment in the 2007 trial indicated that chemical communication was greatly affected by both treatments. The level of scale control was similar for both D50 and D100. Therefore, both dispensers would be operationally viable in ensuring an efficient treatment procedure. Due to the high cost of the pheromone and the need for economical

pest treatments to improve the cost-effectiveness of the fruit growers, we selected the D50 dispenser for enhancement in further trials.

An ideal pheromone-release device should remain effective for a prolonged period and not waste active ingredients. In addition, production and implementation costs should be low and the device should be non-toxic (Stelinski et al., 2005). The D50 dispenser, proposed in this paper, meets all the aforementioned requirements. Furthermore, this dispenser has been developed based upon a biodegradable matrix, giving it added value, as this feature contributes towards providing an environmentally friendly control method.

In our opinion, both larger and isolated orchards are needed mainly to avoid pest intrusion from neighbouring orchards and, thereby, to guarantee an efficient mating disruption treatment. However, this is not a key factor for CRS. As the *A. aurantii* crawlers are the only stage capable of locomotion, other than males which cannot initiate new infestations by themselves, the crawlers represent the principal stage enabling the dispersal of the pest (Greathead, 1990). After emergence, crawlers wander until they settle, but the total distance travelled is no greater than 50 cm. This could explain their dispersal into the tree canopy but not at longer distances (Bodenheimer, 1951). In compliance with the biology of this pest (McClure, 1990), our trials were designed in 1–2 ha plots, although for other pests, such as moths, larger plots must be selected. The extent to which aerial dispersal takes place is unclear (Greathead, 1990) although it has been demonstrated that the wind affects aerial dispersal of the pest. For this reason, we evaluated the fruit damage in the buffer and inner areas separately; and we tried to use natural barriers, such as *C. sempervirens*, in order to avoid any possible intrusion of the pest.

Previous works tried to perform the mating disruption technique for the control of CRS using rubber pheromone dispensers. The results showed a male catch reduction, but the effectiveness of the technique was not clearly demonstrated (Barzakay et al., 1986; Hefetz et al., 1988). In these trials, rubber septa were loaded with six or less mg per dispenser. This pheromone amount was clearly insufficient to reach a good efficiency, as it was demonstrated in our first

year of trials with the dose of 8 mg. Moreover, the rubber dispensers release high amounts of pheromone in the first weeks, showing worse performances than the mesoporous dispensers during long periods. Rubber septa emission is highly temperature dependent (McDonough et al., 1989), whereas the mesoporous dispenser release rate is rather temperature independent (Domínguez-Ruiz et al., 2008), wasting a high amount of pheromone in the warmer part of the day and therefore losing efficacy, as CRS males flight activity occurs in the afternoon or evening, depending on temperature among other factors (University of California, 1991; Gieselmann, 1990). In addition, the rubber septa were not biodegradable and needed to be replaced every two months throughout the season. We consider that the availability of a non-replacement, biodegradable dispenser is essential in reducing the economic, as well as the ecological, cost of the treatment; the D50 dispenser, developed for this work, has these two key features.

Currently, this type of mesoporous dispenser is being commercially developed by Syngenta as part of the Adress System® against *Ceratitis capitata* (Wiedemann) (Navarro-Llopis et al., 2007), and D50 dispensers are being tested for product registration. We are of the opinion that mating disruption to control CRS could become a potentially beneficial method, providing citrus growers with an alternative in the integrated pest management of CRS.

Acknowledgements

We are grateful to Francisco Cuenca, Juan Franch and Rogelio Serrano (Servei de Sanitat Vegetal, Conselleria d'Agricultura, Pesca i Alimentació, Generalitat Valenciana) for assisting with the collection of trapping data. This research has been supported by 'Fundación José y Ana Royo', 'Conselleria d'Agricultura, Pesca i Alimentació, Generalitat Valenciana' and the Agroalimed Grant program. We would like to thank the R&D&I Linguistic Assistance Office, Universidad Politécnica de Valencia (Spain), for granting financial support for the linguistic revision of this paper.

Capítulo II

"Mating disruption of California red scale, *Aonidiella aurantii* Maskell (Hemiptera: Diaspididae), using biodegradable mesoporous pheromone dispensers"

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Mating disruption of California red scale, *Aonidiella aurantii* Maskell (Hemiptera: Diaspididae), using biodegradable mesoporous pheromone dispensers

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Abstract. The control of California red scale (*Aonidiella aurantii* Maskell) has encountered many difficulties, which have raised interest in alternative control methods. Up to now, the *A. aurantii* sex pheromone has been used only for monitoring. In a previous work we have described a biodegradable mesoporous pheromone dispenser for mating disruption. To verify the efficacy of these dispensers, three field trials were conducted and the results are shown in this paper. The study of the release profile of these dispensers revealed a mean pheromone emission value of 269 µg day^{-1} and levels of residual pheromone of 10% at the end of 250 days. During the second flight, an *A. aurantii* male catch reduction of 98% was achieved in the mating disruption plot of trial 1, 93.5% in trial 2 and 76.7 % in trial 3. During the third flight, reductions were 94.1, 82.9 and 68.1% in trials 1, 2 and 3 respectively. Considering damaged fruit with more than 5 scales, it was obtained a reduction of about 80% and 60% in the mating disruption plot of trial 2 and 3 respectively compared to an untreated plot, and a reduction of about 70% in trial 1 compared to an oil treated plot. Mating disruption has been found to be an efficient technique to control this pest, working equally well as a correctly sprayed oil treatment. Further studies are needed to improve the determination of the time of the dispensers' application and evaluate the effects of the pheromone on natural enemies.

II.1 Introduction

Worldwide citrus orchards are greatly affected by diaspidid pests, specially *Aonidiella aurantii* (Maskell), known as California red scale (CRS), which is listed as the most important species causing economic damage and crop losses, to whom its life cycle has been extensively studied (Tashiro and Beavers, 1968; Kennet and Hoffmann, 1985; Koteja, 1990; University of California, 1991). Adult male emergence coincides with the development of third instar females (virgin females) (Tashiro and Beavers, 1968; Kennet and Hoffmann, 1985; University of California, 1991), which then mate and produce the next generation. Virgin females attract males by releasing a pheromone, and then males may crawl to nearby females or fly to other trees (Koteja, 1990; University of California, 1991). The number of generations of CRS that could develop in citrus ranges from three to five and it is influenced by temperature (Kennet and Hoffmann, 1985; Grout et al., 1989). Under the climatic conditions of Spanish citrus areas, CRS shows three complete generations with three male flights, the first of which takes place between mid-April and mid-May, the second between mid-June and late July and the third from mid-August to late-September, with little variation between regions. Cosmetic damage caused by this armored scale leads to downgrading or rejection of the fruit at the packing house. Moreover, heavy scale infestations may lead to yellowing of leaves, defoliation, branch dieback and possible tree death (Grafton-Cardwell and Reagan, 1995).

The economic importance of this armoured scale is due to the fruit damage, the cost of the management tools to defeat it, as well as the difficulty in efficiently applying insecticide treatments. Traditional chemical control for CRS has been affected by the development of resistances to insecticides, including fumigation with hydrocyanic acid (HCN) in the beginning of the last century (Yust et al., 1943a,b), and the use of organophosphate and carbamate insecticides (Collins et al., 1994; Grafton-Cardwell and Vehrs, 1995; Grafton-Cardwell et al., 1998). Consequently, growers have had to rely on new integrated and biological control programs. The use of mineral oils appeared to be a good alternative to conventional pesticides, having low residual toxicity for beneficial insects. But these

can potentially be phytotoxic (Grafton-Cardwell and Reagan, 1995; Tan et al., 2005), requiring certain precautions to avoid negative effects and to ensure the efficacy of the spray. Moreover, oil treatments require an accurate determination of the timing of the treatment, to be applied when the target pest is in its most vulnerable first instar stage (University of California, 1991). The use of insect growth regulators (IGR) such as buprofezin (Yarom et al., 1988; Grout and Richards, 1991a; Ishaaya et al., 1992) and pyriproxyfen (Alfaro et al., 1999b; Grafton-Cardwell et al., 2006; Eliahu et al., 2007; Rill et al., 2007), provided an important alternative to replace traditional insecticides. These IGR were classified as reduced risk insecticides, but their role in the conservation of some natural enemies groups was dubious (Grafton-Cardwell and Gu, 2003; Grafton-Cardwell et al., 2006; Lauziere and Elzen, 2007). Augmentative releases of the aphelinid parasitoid *Aphytis melinus* DeBach are competitive with conventional insecticide treatments in California (Moreno and Luck, 1992), South Africa (Bedford, 1996), and Australia (Furness et al., 1983). *A. melinus* was introduced into the citrus-growing region of Eastern Spain in 1976 from Antibes (France) (Rodrigo et al., 1996; Pina and Verdú, 2007). Since then, it has been mass-reared at the Insectary of the Plant Protection Service in Almazora, (Castellón, Spain), and released in several areas of the Valencian Community (region of Eastern Spain). Rodrigo et al., in 1996, published that these parasitoids did not provide an economic level of control of the pest. CRS control by augmentative releases of this parasitoid is currently under study in Spain. Sorribas et al. indicated in 2008 that augmentative releases of *Aphytis sp.* could be helpful to the naturally-occurring parasitism. On the other hand, its effectiveness depends on careful monitoring, in order to establish the exact release date and the use of selective insecticides for other pests which do not affect natural enemies.

The production of sex pheromone was demonstrated in CRS years before the chemical structures were reported by Roelofs et al. in 1977 (Tashiro and Chambers, 1967; Roelofs et al, 1977). Since then, synthetic sex pheromone traps have been widely employed as a management and detection tool for CRS populations (Moreno et al, 1972; Gardner et al., 1983; Kennet and Hoffmann,

1985; Moreno and Kennet, 1985; Samways, 1988; Grout et al., 1989; Grout and Richards, 1991b). The CRS sex pheromone was described as 3-methyl-6-isopropenyl-9-decen-1-yl acetate (I) and (*Z*)-3-methyl-6-isopropenyl-3,9-decadien-1-yl acetate (II) (Roelofs et al, 1977). All possible geometrical and optical isomers of the two compounds were synthesized and tested by Gieselmann in 1980. The results showed that only one isomer of each compound was significantly more active: (*3S,6R*)-I and (*3Z-6R*)-II and the presence of other isomers in the mixture had no effect on trap catches (Tashiro et al., 1979; Gieselmann et al., 1980). These findings may lead to the development of new methods of control based on pheromones, such as mating disruption. Some researchers attempted to perform mating disruption for CRS using rubber pheromone dispensers. The results showed a male catch reduction, but the effectiveness of the technique was not clearly demonstrated (Barzakay et al., 1986; Hefetz et al., 1988). However, a new mesoporous pheromone dispenser was described in a previous work (Vacas et al., 2009a). In the current study, the duration and efficacy of mesoporous mating disruption dispensers to control *A. aurantii* were verified in three citrus orchards in Spain. The mating disruption treatment was compared with untreated plots, oil treatments and the combination of mating disruption+oil spray. This paper describes the first effective mating disruption treatment against a diaspidid pest.

II.2 Material and Methods

II.2.1 Mesoporous dispenser and device

Mesoporous dispensers are cylindrical tablets 9 mm in diameter and 10 mm in length, made of a mesoporous material (Corma et al., 1999, 2000). The initial load of dispensers was 50 mg (a.i) of the CRS sex pheromone, and the formulation contained the diastereomeric mixture (*3S,6R* and *3S,6S*) of the 3-methyl-6-isopropenyl-9-decen-1-yl acetate (74% of purity). This mixture was supplied by Ecología y Protección Agrícola (Valencia, Spain).

Dispensers were hung inside polypropylene (PP) baskets, supplied by Ecología y Protección Agrícola. The PP baskets are 50 mm wide and 90 mm in length. The pheromone is released through a 6 x 5 mm mesh. The pheromone basket has a hanger at the top for attachment to branches.

II.2.2 Experimental design

Three mating disruption trials were conducted in > 10 years old citrus orchards, 1 trial located in Rio Tinto (Huelva, Spain) and 2 trials in Picasent (Valencia, Spain), to test the efficacy of the mesoporous mating disruption dispensers. To choose the orchards, the population level of *A. aurantii* during the previous season was monitored based on the flight of males. The maximum of male catches in Picasent was approximately 1100 males per trap per week and around 400 males per trap and week in Rio Tinto. For the mating disruption treatment, devices were hung at a height of about 2 m, inside the tree canopy, at a density of one dispenser per tree.

Oil treatments were timed for the presence of crawlers. Plant Protection Service of the local government carried out the crawler assessments and the oil treatments timing was defined according to their data. The crawlers were monitored according to the sampling method suggested by the Valencian Community IPM program. Twenty five 2 to 3 year old infested branches were randomly sampled in each trial, each week from the date of first flight, and taken to the laboratory. Leaves and twigs were removed from those branches and they were cut into 10 cm long pieces. One hundred live scales were identified as first, second and third instars, adult females and adult females with crawlers, using a binocular scope. The oil treatment was applied when first and second instars represented 70% of live scales and more than 90% of adult females had crawlers. The decision to treat the second and third generation was based on the percentage of infested fruit. The treatment threshold was established in 2% of infested fruit according to Valencian Community IPM guidelines. Ten trees and 10 fruits per tree (8 outer and 2 inner) were randomly collected and the percentage of fruit with more

than 3 scales was recorded. All paraffinic oil (10 g l^{-1}) (Argenfrut RV, Gulf Oil Argentina, S.A., Argentina) applications were made with an M1500 speedsprayer (Marisan, Valencia, Spain) calibrated to deliver 2500-3500 l ha^{-1} at 150 psi with the tractor driven at 1.55 km h^{-1}.

II.2.2.1 Trial 1

This trial was carried out in Rio Tinto (Huelva, Spain) in a navelina *Citrus sinensis* Osbeck orchard with trees spaced at 7 by 5 m. It is an orchard with a steep slope, with a difference in high of almost 3 m between rows. The orchard was divided by roads into three plots, as follows: (1) 5 ha were treated with the combination mating disruption-oil spray (MD+Oil). (2) The second plot with 10 ha was only treated with an oil spray (Oil Control plot). (3) Inside the MD+Oil plot, 0.3 ha were left oil-free and were treated only with mating disruption (MD plot). Separation between plots was approximately 30 m, using roads as boundaries. In this trial, it was not possible to have an untreated plot because of the high cost of the potential crop loss. MD dispensers were applied on 5 March 2008, before the beginning of the first CRS male flight and they were not replaced throughout the season. Oil sprays were applied on 7 June 2008, timed for the presence of crawlers. After assessing the fruit, no more oil treatments were needed.

II.2.2.2 Trial 2 and 3

In Picasent, two trials were carried out in an organic clemenules *Citrus reticulata* Blanco orchard (trial 2) and a late maturing Valencia *Citrus sinensis* Osbeck orchard (trial 3) with trees spaced 6 by 4 m. Both trials were designed with four plots, as follows: (1) 1.5 ha MD+Oil plot, (2) 1 ha Oil Control plot, (3) inside the MD+Oil, 0.15 ha were left oil-free as an MD plot and (4) inside the Oil Control plot, 0.15 ha were left oil-free as an untreated plot. MD dispensers were applied on 21 February 2008, before the beginning of the first CRS male flight and they were not replaced throughout the season. The oil treatment was applied on 25 May, timed for the presence of crawlers. After assessing the fruit, no more oil treatments were needed.

II.2.3 Evaluation of treatment efficacy

In order to evaluate the efficacy of mating disruption, three commercial white sticky pheromone traps (PHEROCON® V Scale Trap), supplied by Trécé Inc. (Adair, OK, USA), were placed across the diagonal of each plot, at least 30 m apart. CRS male trap catches between plots treated with pheromone and plots without pheromone dispensers were compared. Sticky traps were replaced weekly, whereas the PHEROCON® monitoring lures (Trécé Inc., Adair, OK, USA), loaded with 250 µg sex pheromone, were replaced every 42 days.

Male flights could not be monitored in the untreated plots because of their small area in trials 2 and 3. So, oil plots were considered as control plots to compare male catches between plots with and without pheromone dispensers (mating disruption plot vs. oil plot). Therefore, traps in control plots should catch males, while in pheromone treated plots an inhibition of male catches should be observed. The absence of trap catches during mating disruption treatment is a good indication of the technique effectiveness, but crop damage assessment provides the ultimate proof (Howse, 1998). To know the percentage reduction in males captured in pheromone traps between MD and control plots, the mating disruption index (MDI) was calculated, according the following formula, $MDI=(1-(x/y))\times100$, where x was the number of males captured in MD plots and y was the number of males captured in control plots. MDI for each flight was the average of the weekly MDI during the flight period.

Damage was assessed on 10 November 2008 in trial 1, 11 September 2008 in trial 2 and 6 November 2008 in trial 3. Ten trees per plot were randomly selected and evaluated for crop damage assessment. Forty fruits per tree were evaluated, with 10 fruits per orientation. The treatment threshold published in the Valencian Community IPM guidelines is 3 scales per fruit. However, fruit with up to 5 scales is generally accepted by the market (market threshold). As our goal is to test the mating disruption treatment efficacy to obtain marketable fruit, this threshold of 5 scales per fruit was employed to assess the fruit damage. However, the percentage of fruit with 1 to 5 scales was also recorded to perform a sensitivity

analysis. Treatment efficacy results were given as a percentage of damaged fruit. In trial 1, MD efficacy was compared to the efficacy of an oil treatment and also relative to an untreated region of the orchard in trials 2 and 3.

Degree-days (Dd) were calculated according the following formula, $Dd=((T_{max} + T_{min})/2)-T_{critical}$, where T_{max} and T_{min} were the maximum and minimum temperature of the day, respectively and $T_{critical}$ was considered 11.7ºC (Kennet and Hoffmann, 1985). Temperature data were provided by the agro-climate stations of each location.

II.2.4 Pheromone release profiles

In parallel with the field trials, 40 additional dispensers were simultaneously aged over 250 days in a citrus orchard in Picasent, 500 m away from trials 2 and 3. The dispensers were aged from 21 February to 2 November 2008. Residual pheromone content was extracted at different ageing times: 0, 7, 15, 30, 45, 60, 90, 120, 150, 180, 210 and 250 days, and then quantified by Gas Chromatography using a flame ionization detector (GC/FID). Three dispensers were taken from the field and analyzed in the laboratory for each ageing time period. These dispensers were extracted by solvent-extraction, at 40ºC, with a 3:2 methanol/dichloromethane mixture.

Red scale pheromone content was measured by GC/FID analyses (Clarus®500 gas chromatograph from PerkinElmer, Wellesley, MA, USA) of the extracts using 1-pentanol as internal standard. All injections were made onto a ZB-5MS (30m×0.25mm×0.25 µm) column, held at 160ºC for 5 min and then programmed at 2ºC min^{-1} up to 180ºC, where it was held for 1 min, and then programmed at 45ºC min^{-1} up to 250ºC. The carrier gas was helium at 1.2 ml min^{-1}. The amounts of pheromone and the responses were connected by fitting a linear regression model, $y=a+bx$, where y is the ratio between pheromone and 1-pentanol responses and x is the amount of pheromone remaining in the dispensers.

Pheromone release for each dispenser type was represented by fitting an exponential model, $y=a \times e^{bx}$, where y is the remaining pheromone load and x represents the ageing days.

II.2.5 Statistical analysis

To normalize the distributions and homogenize variance, male catches in pheromone-baited traps, per trap per week, were transformed by log (N+1) before analysis of variance (ANOVA) using data from the period belonging to the 2nd flight (from 25 June to 13 August in trial 1 and from 19 June to 14 August in trials 2 and 3) and 3rd flight (from 20 August to 5 November in trial 1 and from 21 August to 8 October in trials 2 and 3). An LSD test at $P=0.05$ was used to assess the significance of differences in male captures among plots treated with pheromone and those without.

In order to test for significant differences in percentages of fruit injured between treatments, a one-way ANOVA model was employed with square root-transformed data (LSD test at $P=0.05$). The Statgraphics 5.1 package was used for all the statistical analyses (StatPoint Technologies, Warrenton, VA, USA).

II.3 Results

II.3.1 Efficacy trials

II.3.1.1. Male catches

A slight first flight took place in both locations during April (**Figures II.1, II.2** and **II.3**), from 23 April to 15 May in trial 1 (maximum 38 males per trap per week), and from 20 March to 24 April in trials 2 and 3 (maximum 9 males per trap per week). The second flight began in mid-June (19 June and 25 June, respectively), with the maximum number of males caught in mid-July. The first male catches corresponding to the third flight were obtained at the end of August and third flight

ended at the beginning of October in all locations. Male catches from 14 October were considered to be a partial fourth flight, as only 194ºC degree-day were accumulated up to December and 593ºC degree-day are needed for the development of one generation (University of California, 1991).

Pheromone trap catches of CRS males in mating disruption plots were low throughout the entire season and differed significantly with catches obtained in their respective control plots, according to the statistical values of **Table II.1**. Thus, a male disruption effect was achieved with the mesoporous dispensers, obtaining MDI values ranging from 98.1-94.1% in trial 1, 93.5-82.9% in trial 2 and 76.7-68.1% in trial 3, for the two main flights.

From 15 August to 20 October (third male flight), mean male catches per trap and week were increased in trials 2 and 3 MD plots in comparison to trial 1 MD plot. Moreover, the MDI (**Table II.1**) was significantly lower in trials 2 and 3 than in trial 1 during the third flight (F=10.60; df=2,22; P<0.01). Both results suggested a loss in disruption during this period in trials 2 and 3.

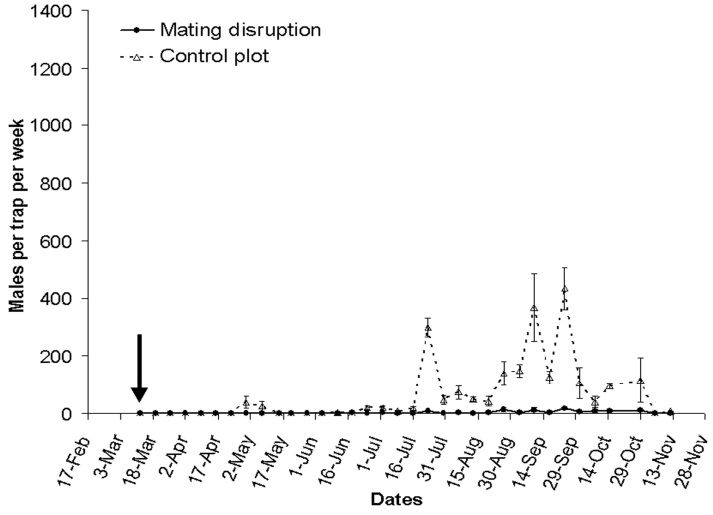

Figure II.1 Male CRS catches per trap per week, in monitoring sticky traps, for mating disruption treated plots and control plots in Trial 1 (Rio Tinto, var. Navelina). Oil plot (without pheromone dispensers) was considered as control plot. The arrow points out the date of dispensers' application.

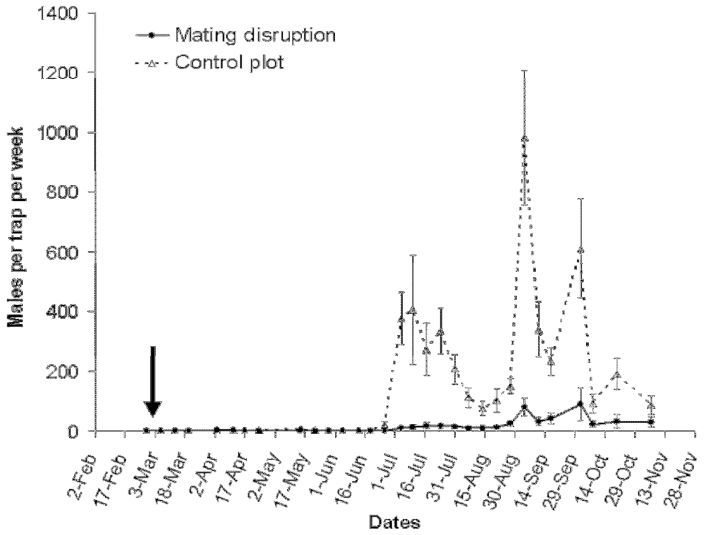

Figure II.2 Male CRS catches per trap per week, in monitoring sticky traps, for mating disruption treated plots and control plots in Trial 2 (Picasent, var. Clemenules). Oil plot (without pheromone dispensers) was considered as control plot. The arrow points out the date of dispensers' application.

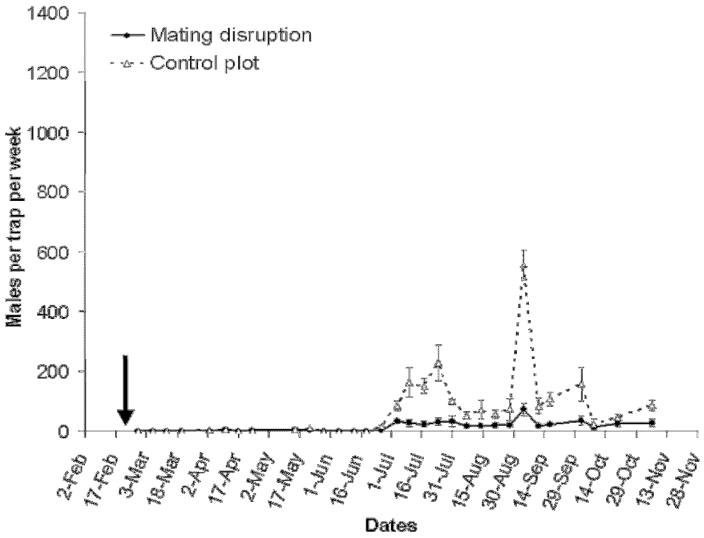

Figure II.3 Male CRS catches per trap per week, in monitoring sticky traps, for mating disruption treated plots and control plots in Trial 3 (Picasent, var. Valencia). Oil plot (without pheromone dispensers) was considered as control plot. The arrow points out the date of dispensers' application.

Table II.1 Mean and SE males per trap per day, mating disruption index (MDI) and statistical parameters obtained by analysis of variance (ANOVA), to assess the significance of differences in total male captures among plots treated with pheromone and those without, during 2nd and 3rd flights. Means in a row followed by the same letter are not significantly different (ANOVA test, *P>0.05*). MDI explain the mean percentage reduction in male catches per flight for each trial.

		MD plot Mean ± SE	Control plot Mean ± SE	MDI	F	df	*P*
2nd flight	Trial 1	1.36 ± 0.51 a	52.50 ± 12.98 b	98.1	56.29	1,82	<0.001
	Trial 2	9.35 ± 1.08 a	180.13 ± 29.11 b	93.5	36.65	1,68	<0.001
	Trial 3	17.69 ± 2.08 a	85.57 ± 13.76 b	76.7	26.51	1,67	<0.001
3rd flight	Trial 1	6.82 ± 1.38 a	144.09 ± 23.66 b	94.1	53.89	1,75	<0.001
	Trial 2	40.36 ± 4.39 a	308.96 ± 58.11 b	82.9	25.70	1,61	<0.001
	Trial 3	27.81 ± 2.91 a	114.92 ± 31.50 b	68.1	7.45	1,60	0.008

II.3.1.2. Fruit damage

In trial 1, the efficacy of the mating disruption treatment was checked relative to the efficacy of an oil treatment (**Figure II.4**). No significant differences were found between treatments for 1 to 5 scales (F=1.58; df=2,26; *P*=0.226). But the percentage of fruit with > 5 scales was significantly reduced in MD and MD+Oil plots (less than 7% damaged fruit), compared to 20% scale-infested fruit in the Oil control plot with no significant differences between the MD and MD+Oil plots (F=12.31; df=2,26; *P*<0.001). If we focus on trial 2 results (**Figure II.5**), significant differences were observed between the percentage of fruit with 1 to 5 scales (F=9.06; df=3,41; *P*<0.001). But for > 5 scales per fruit damage was highly reduced in MD plot (less than 10% damage), compared to the 45% of fruit with > 5 scales in the untreated plot. In trial 2, there were no significant differences between MD and oil control plots. The best results were obtained with the combination of mating disruption and oil treatment, applied in the first generation, for this early variety of citrus (F=24.18; df=3,41; *P*<0.001). In trial 3 (**Figure II.6**), significant differences in

fruit injured with 1 to 5 scales were observed between the combination MD+Oil and the untreated plot (F=1.97; df=3,36; *P*=0.138). In addition, damage assessment in trial 3, showed that any of these treatments was effective in reducing damage compared to the untreated plot (F=8.79; df=3,36; *P*<0.001) when the threshold of more than 5 scales was set, although assessment was performed long before harvest and this time may have allowed the development of a new CRS generation on fruit.

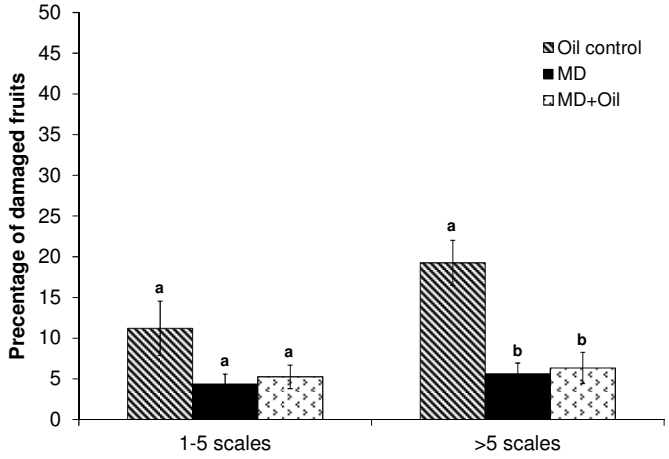

Figure II.4 Mean percentage of damaged fruits observed inside the different plots: oil control, mating disruption (MD) and MD+oil treatment, for Trial 1 (Rio Tinto, var. Navelina). Bars labelled with the same letter do not differ significantly (ANOVA test, *P*>0.05)

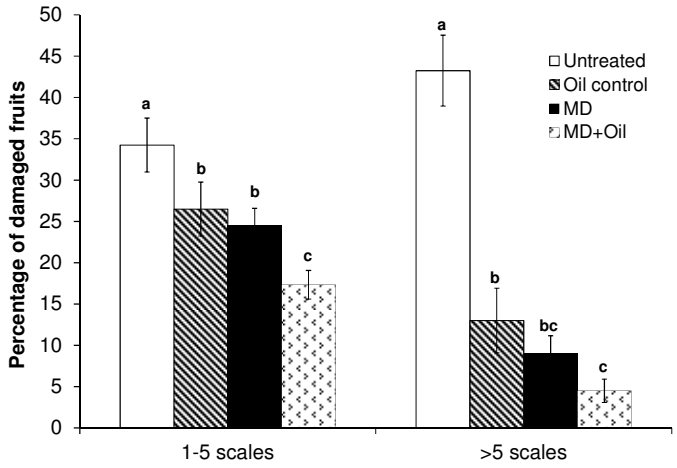

Figure II.5 Mean percentage of damaged fruits observed inside the different plots: untreated, oil control, mating disruption (MD) and MD+oil treatment, for Trial 2 (Picasent, var. Clemenules). Bars labelled with the same letter do not differ significantly (ANOVA test, *P*>0.05)

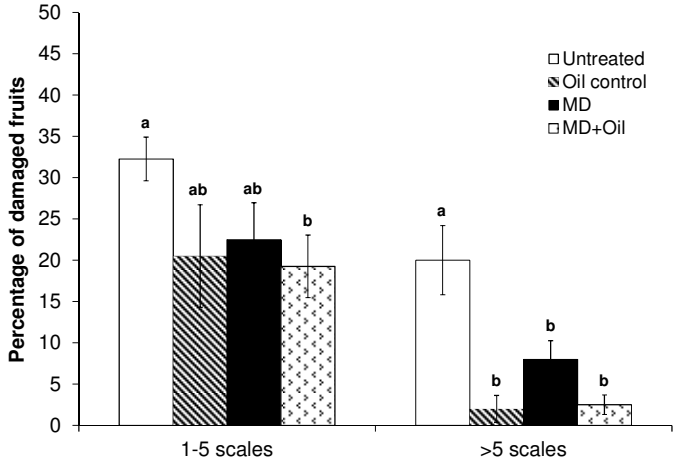

Figure II.6 Mean percentage of damaged fruits observed inside the different plots: untreated, oil control, mating disruption (MD) and MD+oil treatment, for Trial 3 (Picasent, var. Valencia). Bars labelled with the same letter do not differ significantly (ANOVA test, *P*>0.05)

II.3.2. Pheromone release profiles

Figure II.7 shows the residual pheromone versus time for the mesoporous dispensers. The residual pheromone load fits the exponential model

$y = 49{,}668 \times e^{-0{,}0087x}$, resulting R^2=0.98. This study also showed that the mesoporous dispenser emitted approximately 90% of its pheromone load during the test period. The low content of residual pheromone in the dispenser is a key parameter for the cost of the treatment.

The mean amount of pheromone emitted per day from this dispenser was 269 µg day^{-1}. This value is consistent with data published by our group, determining the minimum mean release value (> 250 µg day^{-1}) to obtain disruption effect in CRS males (Vacas et al, 2009a). This mesoporous dispenser has a regular pheromone release during the first 150 days, which decreases significantly from that moment on.

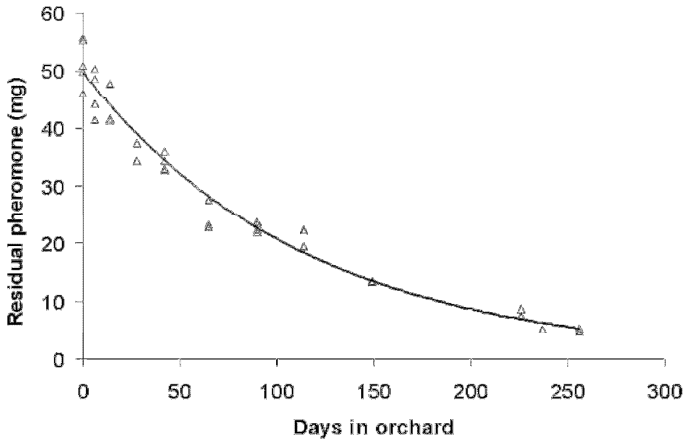

Figure II.7 Relationship between the remaining amount of pheromone in the mesoporous dispensers (mg) and the corresponding days of field exposure. Signification of the exponential model was R^2=0.98.

II.4 Discussion

The level of male CRS captures in trials 2 and 3 MD plots increased significantly in September in comparison with trial 1, with lower MDI values during the third flight (September-October) in trials 2 and 3. This could be due to the pheromone application, which was dispensed 2 weeks earlier in trials 2 and 3. Comparing this increase of captures to the pheromone release profile, this period coincides with the end of the life-span of these dispensers, which is 6 months (mean pheromone release rate < 250 µg day^{-1}) (Vacas et al., 2009a). These results indicated that after 6 months of field application, the disruption effect decreased because the emission of pheromone was not high enough to disrupt the CRS third flight. Not considering the pheromone release profile, this increase of captures could be attributed to higher density of scales at that time of year. However, results of damage assessment showed that the percentage of damaged fruit was significantly lower in MD plot compared to an untreated plot. This means that the disruption effect took place and therefore a higher density of scales was not likely before September. Additional trials are needed to adjust the timing of dispenser application and promote pheromone release until the CRS generational cycles are completed. This research could alter the date of application of the dispensers or suggest a higher pheromone dosage. But the increase of the pheromone load has some drawbacks, because the pheromone represents approximately the 95% of the price of the dispenser.

Our results in trials 2 and 3 showed that any of the control methods employed in these trials was effective against CRS compared to an untreated plot. We have found that mating disruption treatment alone could reduce damage of *A. aurantii* in fruit by 80 and 60 % in trials 2 and 3, respectively. Also, trials 2 and 3 demonstrated that mating disruption worked equally well as a correctly sprayed oil treatment. Correct timing of the oil application, a good calibration of the sprayer and a good coverage of all the above-ground parts of the tree are key factors to ensure the efficacy of the treatment. The fruit injury obtained in Oil plot of trial 1 was significantly higher than in MD plot, which was not the case in trials 2 and 3.

As the timing of the oil application was well defined in concordance with the number of crawlers, the low efficacy of oil treatment in trial 1 could be explained by the particular slope of Rio Tinto orchard.

CRS is widely distributed and, although the host susceptibility is related to the number of oil glands in the leaves (McClure, 1990; Asplanato and García-Marí, 1998), all citrus varieties are sensitive to its attack. According to this, our trials showed satisfactory results for mid-season varieties during the life-span of the pheromone dispensers and the three CRS generations, which generally take place in Spain. For late season varieties, like Valencia oranges, it is possible that the first generation of the following year could affect the non-harvested fruit, so it should be treated with new application of pheromone dispensers before the first flight or other effective treatment.

The PP device employed was a prototype to conduct the trials. The final device should be made of a biodegradable material, which could be left in the field without threatening the environment and could be resistant to weather conditions for almost seven months.

The pheromone device is still in development, however, we have estimated that the cost of this treatment will be approximately 200 € ha^{-1}, which is economically competitive with a conventional oil spray (266 € ha^{-1} including oil and speedsprayer). In addition, in a mating disruption treatment, an accurate determination of the moment of application is not necessary, while for an oil treatment it is essential and assumes an added cost which is not often considered.

As well as oil sprays, the majority of growers have adopted the use of IGRs as a part of integrated pest management programs. The effect of buprofezin and pyriproxyfen on life stages of natural enemies has been extensively studied and they appear to be compatible with augmentative releases of *A. melinus* (Rill et al., 2008) but they are incompatible with other agents like *Rodolia cardinalis* (Mulsant) (Grafton-Cardwell and Gu, 2006). It must be added that European Directives regulating the use of insecticides are becoming more and more severe. In fact, the Commission Decision of the European Communities (2008/771/EC) of 30

September 2008, concerning the non-inclusion of buprofezin in Annex I to Council Directive 91/414/EEC, states that authorizations for plant protection products containing buprofezin were withdrawn by 30 March 2009. So mating disruption could be a good alternative to settle this matter.

In conclusion, CRS mating disruption achieved control equal to conventional oil sprays and could provide growers with a method for controlling a key citrus pest without using insecticides. Mating disruption could also be highly conducive to conservation of natural enemies. However, it is necessary to evaluate the possible effect of a high concentration of CRS sex pheromone on the behaviour of *A. melinus* and other parasitoids and predators of *A. aurantii*, as well as the influence of the pheromone on natural enemies of other pests. In this way, we are studying the influence of the mating disruption treatment in the behaviour of some CRS parasitoids. In addition, we should consider that the reduction of a wide range of insecticide treatments, due to the implementation of mating disruption as *A. aurantii* control method, could potentially increase secondary pest populations. Mating disruption technique could replace the use of oil spray for CRS control, but these oil treatments could be occasionally necessary for the control of other scale pests.

Acknowledgements

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Capítulo III

"Different strategies to apply mating disruption for the control of *Aonidiella aurantii* Maskell (Hemiptera: Diaspididae)"

Version of the article submitted to
***Crop Protection* (2011)**

Different strategies to apply mating disruption for the control of *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae)

Submitted to Crop Protection 2011 (Vacas, S., C. Alfaro, J. Primo and V. Navarro-Llopis)

Abstract. Infestations of the California red scale, *Aonidiella aurantii* (Maskell), raise concerns about its management and these concerns have led to the introduction of new integrated control methods. These methods include the implementation of techniques based on pheromones for monitoring and detection, and more recently, mating disruption. Previous works described efficient mating disruption pheromone dispensers to control *A. aurantii*. The main aims of these works were to adjust the timing of dispenser applications and study the importance of controlling the early first generation of *A. aurantii* by testing two different application dates: before (March) and after (May) the first generation. The combined strategy that included mating disruption with oil spray was also evaluated. Male captures in pheromone monitoring traps showed that every mating disruption strategy achieved more than 80% flight disruption compared with an untreated plot – as well as mean fruit damage reductions of about 80%, without significant differences between the different application dates. The need for controlling the first generation of CRS, and the combination of mating disruption with conventional oil treatments are discussed.

III.1 Introduction

Infestations of California red scale (CRS), *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae) pose a serious problem for citrus grove managers, as CRS may lead to a reduction in tree vigor and the downgrading or commercial rejection of fruits. The economic importance of this armored scale is due to the fruit damage and the cost of the management tools used to defeat it. The difficulties in efficient insecticide applications against CRS have been widely described: traditional chemical control is affected by the development of resistances to insecticides, such as hydrocyanic acid, chlorpyrifos, or methidation (Yust et al., 1943a; Collins et al., 1994; Grafton-Cardwell and Vehrs, 1995; Levitin and Cohen, 1998). Consequently, efforts have been directed to the introduction of new integrated and biological control programs. The use of mineral oils appeared as a good alternative to conventional pesticides, although requiring certain precautions in order to avoid phytotoxic effects (Grafton-Cardwell and Reagan, 1995; Tan et al., 2005) and an accurate determination of the treatment timing (University of California, 1991). Another important alternative to replace traditional insecticides has been the use of insect growth regulators (IGR), such as buprofezin (Yarom et al., 1988; Grout and Richards, 1991a) and pyriproxyfen (Grafton-Cardwell et al., 2006; Eliahu et al., 2007; Rill et al., 2007), and these products have provided good control results. However, it has been reported that these substances can affect the conservation of the natural enemies (Grafton-Cardwell and Gu, 2003; Grafton-Cardwell et al., 2006; Lauziere and Elzen, 2007). Considerable work has been devoted to studies and explorations for the biological control of *A. aurantii*. The main enemies of CRS are species of the aphelinid parasitoids *Aphytis* (DeBach, 1959; DeBach & Argyriou, 1967) and biological control has been successful in many places, such as California (Moreno and Luck, 1992), Greece (DeBach and Argyriou, 1967), Israel (Avidov et al., 1970), South Africa (Bedford, 1996) or Australia (McLaren and Buchanan, 1973; Furness et al., 1983). *Aphytis melinus* DeBach was introduced into the citrus growing region of eastern Spain in 1976 from Antibes (France) (Rodrigo et al., 1996), and the control of CRS by

augmentative releases of this parasitoid is currently under study in Spain (Sorribas et al., 2008). Its effectiveness depends on careful monitoring to establish the exact release date, and, as mentioned before, the use of selective insecticides for other pests that do not affect natural enemies.

Integrated pest management programs include the implementation of control methods based on pheromones. Tashiro and Chambers (1967) demonstrated the production of a sex pheromone in CRS, whose chemical structures were reported by Roelofs et al., as 3-methyl-6-isopropenyl-9-decen-1-yl acetate (I) and (Z)-3-methyl-6-isopropenyl-3,9-decadien-1-yl acetate (II) (Roelofs et al., 1977). Since then, synthetic sex pheromone traps have been widely employed as a detection tool for CRS populations (Gardner et al., 1983; Kennett and Hoffmann, 1985; Moreno and Kennett, 1985; Grout et al., 1989; Grout and Richards, 1991b). The efficacy of mating disruption technique against CRS was not clearly demonstrated in the first experiments using rubber pheromone dispensers (Barzakay et al., 1986; Hefetz et al., 1988). However, by studying different pheromone doses, Vacas et al. (2009a) described a new mesoporous dispenser capable of interfering with normal *A. aurantii* chemical communication. The efficacy of these mesoporous dispensers was further verified in 2010, when significant flight disruptions and fruit damage reductions were obtained by applying doses of about 40 g pheromone per ha for six months (Vacas et al., 2010). It was found that CRS mating disruption achieved control equal to conventional oil sprays. However, research had shown the need for additional trials to adjust the timing of dispenser application to cover all the CRS generational cycles. In the present study, the importance of controlling the early first generation of *A. aurantii* has been investigated and the efficacy of mating disruption applied before and after the first generation has been examined.

III.2 Materials and methods

III.2.1 Mesoporous dispenser and device

The pheromone dispensers applied in the mating disruption treatments are based on a mesoporous material (Corma et al., 1999; Corma et al., 2000). These dispensers only differ in the initial pheromone load with respect to those described by Vacas et al. (2010), and contained 70 mg (a.i.) of the CRS sex pheromone as the diastereomeric mixture (3S,6R and 3S,6S) of the 3-methyl-6-isopropenyl-9-decen-1-yl acetate (75% purity). This mixture was supplied by Ecología y Protección Agrícola SL (Valencia, Spain).

Dispensers were attached to tree branches inside polypropylene baskets 50 mm wide and 90 mm long with a hanger at the top. Pheromone is released through a 6 × 5 mm mesh. These devices were also provided by Ecología y Protección Agrícola SL (Valencia, Spain).

III.2.2 Experimental design

The field trial was conducted in a 3 ha *Citrus reticulata* Blanco (var. Ortanique) orchard located in Denia (Alicante, Spain). Trees were >10 years-old and spaced 6 m by 4 m. The trial was designed with 11 plots alternately arranged (**Figure III.1**) to test three different procedures for the application of mating disruption: pheromone dispensers were applied on 29 March 2009 in three plots of 0.3 ha (March1, March2, and March3, respectively) before the appearance of the first CRS generation. Another three plots, with the same aforementioned areas, had dispenser applications on 28 May 2009, before the CRS second generation, and these plots are referred to as May1, May2 and May3 plots. The combination of May dispenser application and an oil treatment was tested on other three plots (May+Oil 1, 2 and 3), where dispensers were also placed on 28 May 2009 and oil treatments were applied on 28 May in only these three plots. Pheromone dispensers in every plot were placed at a density of one per tree. Finally, two plots of 0.1 ha were left without any treatment as Untreated plots 1 and 2.

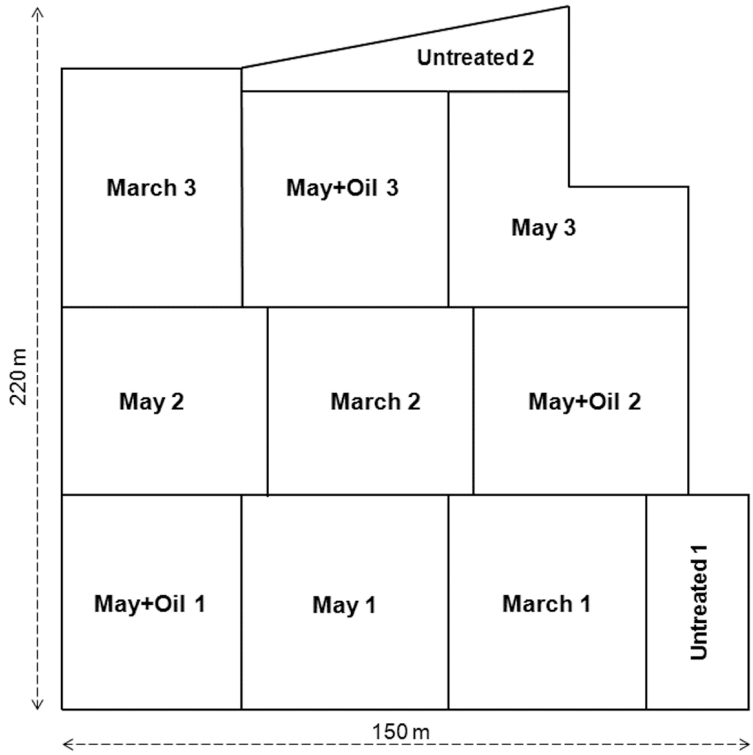

Figure III.1 Sketch showing the arrangement of the 11 plots in the field with their corresponding strategies. Plots with mating disruption dispensers applied on 29 March 2009, shown as March 1, March 2, and March 3. Dispensers were applied on 28 May 2009 in plots May 1, May 2, and May 3; and mating disruption was applied in May together with an oil treatment in plots May+Oil 1, 2 and 3.

Oil treatments were timed for the presence of crawlers, which were monitored according to the sampling method suggested by the Valencian regional IPM program (Resolution 27 October 2008 of Conselleria de Agricultura, Pesca y Alimentación; DOCV no. 5901, 26 November 2008). A total of 25 infested branches (2–3 years old) were randomly sampled each week from the date of the first flight and taken to the laboratory. Leaves and twigs were removed from the branches and cut into 10 cm long pieces. Using a binocular scope, a total of 100 live scales were identified as first, second and third instars, adult females, and adult females with crawlers. The oil treatment was applied when first and second instars

represented 70% of live scales and more than 90% of adult females had crawlers. Paraffinic oil (10 g l^{-1}) (Argenfrut RV; GulfOil Argentina SA, Argentina) applications were made with an M1500 speed sprayer (Marisan, Valencia, Spain) calibrated to deliver 2500–3500 l ha^{-1} at 150 psi with the tractor driven at 1.55 km h^{-1}.

III.2.3 Evaluation of treatment efficacy

The efficacy of the different application strategies was evaluated according to the flight disruption of the males and fruit damage assessment. One commercial white sticky pheromone trap (Pherocon® V Scale Trap; Trécé Inc., Adair, OK, USA) was placed in each plot to compare male catches between the different control strategies every two weeks. Note that sticky traps were replaced every two weeks, whereas the Pherocon® monitoring lures (Trécé Inc., Adair, OK, USA), loaded with 250 µg sex pheromone, were replaced every 42 days.

To measure the inhibition of male catches that occurred in pheromone-treated plots, the mating disruption index (MDI) for each strategy was calculated as an indicator of the treatment efficacy using the following formula (Vacas et al., 2009a): MDI = (1-(x/y))×100; where x is the number of males captured in MD plots and y is the number of males captured in untreated plots.

Eight central trees were randomly selected in each plot and evaluated for crop damage on 21 September 2009. Forty fruits per tree were evaluated, with ten fruits per orientation. A fruit was considered to be damaged when it had more than three scales on its surface, as suggested by the treatment threshold published in the Valencian regional IPM guidelines. The percentage of fruits with more than ten scales was also recorded to perform a sensitivity analysis. The percentage of damaged fruit obtained with the three strategies (March, May and May + Oil) was compared with the results from the untreated plots.

III.2.4 Pheromone release profiles

Thirty additional dispensers were simultaneously aged over 220 days in an orchard at least 500 m away from the trial orchard. Dispensers were aged from 29 March 2009 to 8 December 2009 in order to extract their residual pheromone content at different aging times (0, 30, 60, 90, 120, 150, 170, 190, 220, and 253 days). Three replicates per aging period were taken from the field and the pheromone content was extracted at 40ºC with dichloromethane: methanol (2:3 by volume). Pheromone was then quantified by gas chromatography with flame ionization detector (GC/FID; Clarus® 500 gas chromatograph; PerkinElmer Inc., Wellesley, MA, USA) using 1-dodecanol as the internal standard. All injections were made onto a ZB-5ms (30m×0.25 mm×0.25 µm) column (Phenomenex Inc., Torrance, CA, USA), held at 160ºC for 5 min, and then programmed at 2ºC min^{-1} up to 180ºC, where it was held for 1 min, and then programmed at 45ºC min^{-1} up to 250ºC. The carrier gas was helium at 1.2 ml min^{-1}. The pheromone amount was estimated according to the ratio between the pheromone and 1-dodecanol responses by means of a linear regression model.

Multiple linear regression was used to study the evolution of the residual pheromone load (mg) versus time (days) for the dispenser employed. To determine whether the emission was constant during the time under study, the significance of the quadratic effect was statistically checked.

III.2.5 Statistical analysis

Analysis of variance (ANOVA) was carried out to study the significance of differences in CRS male captures among the different pheromone-treated plots and the Untreated plot (LSD test at P=0.05). Prior to analysis, male catch data, per trap per week (MTW), was transformed by log(x+1) to normalize the distributions and homogenize the variance. Data from the first male flight was discarded as the May application of MD dispensers was not carried out until 28 May 2009. Thus, ANOVA was applied with data belonging to the second flight, from 11 June to 9 July 2009, and the third flight, from 7 August to 12 November 2009.

Fruit damage level differences were assessed by one-way ANOVA with the log transformed data of the percentage of damaged fruit at the end of the trial for each strategy (with LSD test at *P*=0.05).

Statgraphics Centurion XVI v16.1 software (StatPoint Technologies Inc., Warrenton, VA, USA) was used for all the statistical analyses.

III.3 Results

III.3.1 Efficacy of the different strategies

III.3.1.1 Male catches

Population dynamics of *A. aurantii* in the area of study can be observed by the data obtained with traps located in the untreated plots (**Figure III.2**). First flight took place during May with a maximum of 6.93 CRS males per trap and day (MTD). The second flight began at the end of June with the maximum number of males captured in mid-July. The population of *A. aurantii* increased from the first week of August. It reached a maximum on 31 August and began to decrease slowly up to the beginning of November, when only 0.4 MTD were registered in the untreated plots. Therefore, *A. aurantii* had three complete generations during the 2010 season in the area under study.

Male catches in plots treated with pheromone remained low throughout the entire season, and only slight peaks were registered according with the three described flight peaks (**Figure III.2**). To study the differences among treatments, data from the first flight was discarded, as this period covered April and the beginning of May, when MD strategies applied in May were not yet established. ANOVA found statistical differences between all the plots treated with pheromone and the untreated plots, and the statistical values are shown in **Table III.1**. Thus, a disruption effect took place during the period under study with average male flight inhibitions of about 86% with any of the three tested mating disruption strategies, and without significant differences between them.

Table III.1 Mean ± SE males per trap per day (MTD), mating disruption index (MDI), and statistical parameters obtained by analysis of variance (ANOVA) in order to assess the significance of differences in male captures among the different strategies tested: dispenser application on March, dispenser application in May, the combination of May application with an oil treatment and the untreated plots. Rows labeled with the same letter did not significantly differ (ANOVA test, P>0.05).

Strategy	Mean ± SE	MDI	F	df	P
March	1.05 ± 0.43 a	81,5			
May	0.70 ± 0.26 a	87,7	15.72	3,71	<0.001
May + Oil	0.63 ± 0.26 a	88,9			
Untreated	5.68 ± 1.87 b	-			

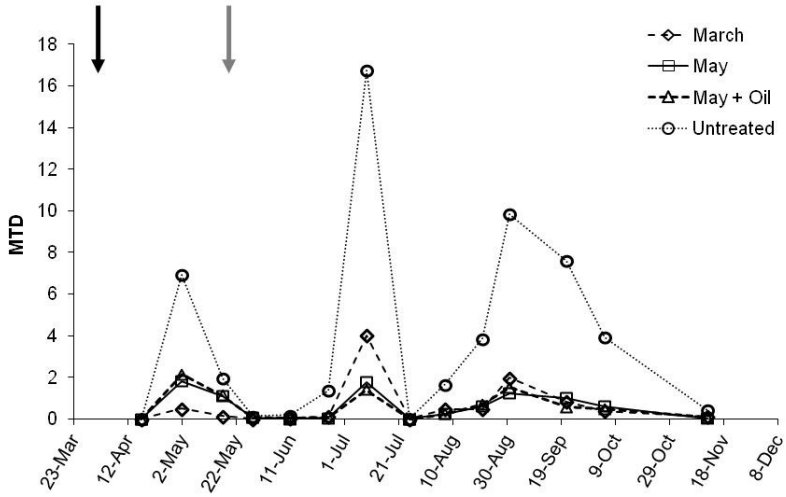

Figure III.2 Population dynamics of *Aonidiella aurantii* shown as males per trap per day (MTD) captured on the different mating disruption plots: dispenser application in March, dispenser application in May, the combination of May application with an oil treatment, and the untreated plots. Black arrow indicates dispenser application in March strategy (29 March 2009).The grey arrow points out pheromone application in May strategy (28 May 2009) and oil application in the May+Oil plots.

III.3.1.2 Fruit damage

Thirty-one percent of fruits were damaged with more than three scales on the surface when no treatment was applied in the orchard (Untreated plot in **Figure III.3**). All of the mating disruption strategies achieved a reduction in injured fruit compared to the Untreated plot: 81% damage reduction for MD applied in March; 95% for May application of MD; and 96% by the combination May application + oil spray. In summary, results of every MD treatment differed significantly from the absence of treatments in the ANOVA test for both damage levels recorded (more than three scales: $F=24.79$; $df=3,36$; $P<0.001$; more than 10 scales: $F=29.58$; $df=3,27$; $P<0.001$). Although damage was significantly reduced by March application, it differed significantly from the May strategies (**Figure III.3**). Damage observed in March plots did not exceed 6%, whereas less than 1.5% of fruit was found to be damaged in the May plots. Moreover, the addition of oil spray to the mating disruption strategy did not significantly improve the results obtained using just the May application of pheromone dispensers.

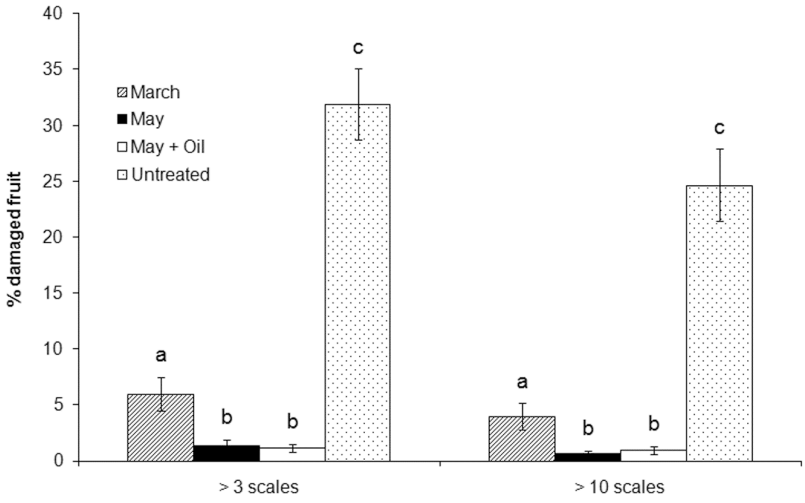

Figure III.3 Mean percentage of damaged fruits observed in the different plots: Untreated, March application of MD, May application of MD, and the combination of May application with oil spray. Bars labeled with the same letter do not differ significantly (ANOVA test, $P>0.05$).

III.3.2 Pheromone release profiles

The quantification of the residual pheromone amounts reveal that 18.7% of the initial pheromone load remained in the mesoporous dispensers after 253 days of field exposure. The complete pheromone release profile is depicted in **Figure III.4**, which was fitted to an exponential model (solid line in **Figure III.4**; eq. 1) resulting in $R^2 = 0.95$.

$$y = 71.322 \times e^{-0.008x} \qquad \text{(eq. 1)}$$

However, statistical analysis showed that the curvature of this model was due to data from the last three months of the dispenser life-span, with the release profile fitted to a linear model (discontinuous line in **Figure III.4**; eq. 2), and resulting $R^2 = 0.99$.

$$y = 70.241 - 0.334x \qquad \text{(eq. 2)}$$

Multiple linear regression of data from 0 to 154 days of aging confirmed the absence of curvature in the model given by equation 2 (quadratic effect not significant, P=0.31). This means that emission is assumed to be constant from 0 to 154 days, and the mean release rate given by the slope of the linear model is 334 µg day^{-1}. From this date on, emission level decreased below 100 µg day^{-1} during the last month of the period under study.

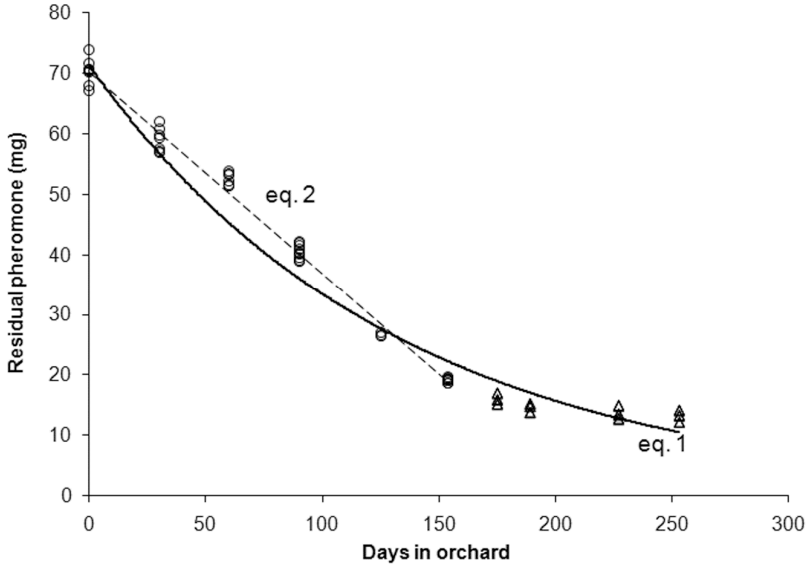

Figure III.4 Evolution of the remaining load of pheromone (mg) on the mesoporous dispensers versus time (days in orchard). The complete release profile was fitted to an exponential model (eq. 1; R^2 = 0.95), although pheromone release rate was constant until 154 days of field exposure and fitted a linear model (eq. 2; R^2 = 0.99).

III.4 Discussion

The present study further confirms the efficacy of the mating disruption technique against CRS infestations, previously demonstrated by Vacas and coworkers (Vacas et al., 2009a, 2010) and suggests a strategy to apply this control technique.

CRS is able to develop from three to five generations per year, mainly influenced by temperature (Kennett and Hoffmann, 1985; Grout et al., 1989). Under the climatic conditions of Spanish citrus areas, CRS shows three complete generations and a partial fourth generation during warmer autumns. The first male flight takes place between mid-April and mid-May, and is usually not too abundant.

The importance of controlling the first generation of *A. aurantii* was investigated by the application of mating disruption at two different moments: March, before development of the first generation, and May, before the second generation. According to CRS male flight monitoring, both applications achieved more than 80% flight disruption. However, the fruit damage assessment revealed that the May application results were significantly better than the March strategy, despite reducing damage by around 80%. This could be explained by the release profile and life span of the pheromone dispensers. Extraction and quantification of the pheromone remaining in the aged dispensers showed that release rate was constant for 154 days and equal to 334 µg day^{-1}, and thereafter decreasing. If the mesoporous dispensers are applied in March, this period would protect the crop until the end of August, but not cover the whole third flight of CRS males. In this way, the flight could not be properly disrupted, allowing the mating and development of a partial generation. Dispensers applied on May would cover the whole third flight with suitable emission levels and so protect the crop. Therefore, with medium infestation levels, the control of the first generation is not crucial and damage can be controlled by establishing mating disruption before the second flight with pheromone dispensers releasing 334 µg day^{-1} constantly for at least five months.

Several authors demonstrated that early pheromone applications prevent mid-season increases in Lepidoptera populations (Staten et al., 1987; Kehat et al., 1995; Lykouressis et al., 2005); these populations being responsible for high yield losses. Therefore, mating disruption of the first emerging moths is crucial for the development of the subsequent generations throughout the season in Lepidoptera species. By contrast, the control of the first CRS generation is not so essential for achieving a good efficacy, as this generation does not usually have available fruit. The second annual crawler generation, which takes place in the summer, is generally considered to be mainly responsible for the infestation of fruit (Rodrigo et al., 2004). In this study, the increase in fruit damage observed when mating disruption was applied in March, was probably due to the lack of dispenser efficacy after five months of field exposure. Moreover, the application of mating disruption

before the second CRS generation gave significantly better efficacy. Therefore, the key factor to obtain a good control is to release pheromone at a suitable level during the most harmful generations. The development of pheromone dispensers with longer lifespans would achieve good results if mating disruption treatment is applied before the first flight. However, this would not be cost-effective, as the initial pheromone load must be increased – so increasing the cost of the treatment.

The combined strategy of mating disruption in May with oil treatment was included in this trial to check whether the delayed pheromone application needs an oil spray to control the susceptible instars of the first generation. According to the results, oil treatment did not significantly improve disruption efficacy in May, as damage was equally controlled with just the May mating disruption. However, the oil spray, as a wide range treatment, could be affecting other secondary scale insects, such as *Parlatoria pergandii* Comstock, *Lepidosaphes beckii* Newman, or *Chrysomphalus dictyospermi* (Morgan), and so preventing the proliferation of their populations. For this reason, it is worthwhile studying whether the total suppression of these oil sprays can cause breakouts of these insects. In that case, the oil treatments should be reduced, for example, to one application every two years, rather than being completely suppressed.

Acknowledgments

The authors want to thank Ecología y Protección Agrícola SL for the pheromone supply. The translation of this paper was funded by the Universidad Politécnica de Valencia (Spain).

Capítulo IV

"Studies on the development of a mating disruption system to control the tomato leaf miner, *Tuta absoluta* Povolny (Lepidoptera: Gelechiidae)"

Version of the article published in
***Pest Management Science* (2011) online**

Studies on the development of a mating disruption system to control the tomato leaf miner, *Tuta absoluta* Povolny (Lepidoptera: Gelechiidae)

Published online in Pest Management Science (Vacas S., C. Alfaro, J. Primo Millo and V. Navarro Llopis. 2011. DOI: 10.002/ps.2202).

Abstract. The tomato leaf miner (*Tuta absoluta* Povolny) has rapidly colonized the whole Mediterranean and South-Atlantic coasts of Spain, and it has become a key problem in both outdoor and greenhouse crops. New control methods compatible with biological control are required and mating disruption appears to be a perfect method in current agriculture as it is an environmentally-friendly and residue-free technique. IPM packages tested have included the use of pheromones to detect populations, but there has not been much previous research on mating disruption of *T. absoluta*. In this work, pheromone doses varying from 10 to 40 g ha^{-1}, emitted at a constant rate over four months, were tested in greenhouses with different levels of containment, in order to evaluate the efficacy of mating disruption on *T. absoluta*. Trials on containment level revealed that the flight of *T. absoluta* was satisfactorily disrupted with an initial pheromone dose of 30 g ha^{-1}, and levels of damage did not significantly differ from those in reference plots with insecticide treatments. Later efficacy trials confirmed our previous experiences, and release studies showed that control of damage and flight disruption were taking place when releasing, at least, 85 mg pheromone per ha per day. Effective control using pheromone application against *T. absoluta* can be achieved, in greenhouses with high containment levels, for 4 months, with initial doses of 30 g ha^{-1}. Further research must be conducted in order to evaluate the prospect of outdoor application of mating disruption systems.

IV.1 Introduction

Since the first detection in Spain of *Tuta absoluta* Povolny (Lepidoptera: Gelechiidae) in 2006 (Castellón, Eastern Spain), the tomato leaf miner (TLM) has invaded the whole Mediterranean and South-Atlantic coasts of the Iberian Peninsula and many other interior regions of Spain (Urbaneja et al., 2009). Due to its rapid colonization and the high levels of damage it wreaks, intervening in the control of *T. absoluta* has become a key issue for tomato production, both in outdoor and greenhouse crops. Being a moth of the Gelechiidae family, *T. absoluta* is a leaf miner that attacks tomato plants in all stages of development, damaging the stems, apices, flowers and fruits, in addition to mining the leaves (Miranda et al., 1998). In high densities, TLM is able to cause significant production losses in tomato crops (Picanço et al., 1998).

The control of this pest requires expensive treatments with translaminar active chemicals, or the repeated application of chemicals in order to affect larvae outside the galleries. However, some investigators have proven the development of resistance to some insecticides in this moth (Siqueira et al., 2000; Siqueira et al., 2001; Lietti et al., 2005) and the repeated use of the few authorized active ingredients could hasten the appearance of such resistance. It must be taken into account that, in many cases, these insecticides could also affect natural enemies, making the consolidation of biological control systems impossible. In fact, a broad variety of parasitoids and predators have been reported attacking egg, larval or pupal stages of *T. absoluta* (Miranda et al., 1998; Pratissoli & Parra, 2000; Parra & Zucchi, 2004; Luna et al., 2007; Urbaneja et al, 2009; Desneux et al., 2010). Spanish tomato producers have been encouraged to control virus vector insects, such as thrips and whiteflies, by augmentative releases and conservation of beneficial insects in greenhouses, as these vector insects have become resistant to many insecticides (van Houten et al., 1995; Gerling et al., 2001; Blaeser et al., 2004). Thus, alternate means of suppressing TLM populations are needed in order to prevent the deleterious side effects of repeated applications of insecticides. IPM

packages could include other cultural, biotechnological and biological methods, such as application of entomopathogenic fungi and nematodes (Rodriguez et al., 2006; Batalla-Carrera et al., 2010), and treatments with *Bacillus thuringiensis*, whose efficacy has already been demonstrated (Giustolin et al., 2001; Theodoluz et al., 2003; Noedmann & Meza-Basso, 2006; Gonzalez-Cabrera et al., 2011).

Pest management tactics could also include the introduction of techniques based on pheromones. Since virgin TLM females release a sex pheromone that strongly attracts males (Quiroz, 1978), efforts were directed towards identifying it. *T. absoluta* pheromone was characterized as (3*E*,8*Z*,11*Z*)-tetradecatrienyl acetate (TDTA hereafter) (Attygalle et al., 1995,1996). This component represents about 90% of the volatile material found in the sex gland of calling males, but a minor component (~10%) was identified as (3*E*,8*Z*)-tetradecadienyl acetate (TDDA) (Griepink et al., 1996; Svatos et al., 1996). These findings permitted the development of pheromone dispensers in order to test attract and kill (Michereff et al., 2000a) or mating disruption (Michereff et al., 2000b) control methods against TLM. Up until now, mating disruption has been tested in South America with doses ranging from 10 to 80 g ha^{-1}, in outdoor plots of under 200 m^2, without success in controlling TLM and without studies of pheromone release. Several companies have developed pheromone dispensers to detect and monitor *T. absoluta* populations but there is only one reported experiment of mating disruption in Spain, testing doses from 0.15 to 2 g ha^{-1}, with unsuccessful results (Martí et al., 2010).

Our present work shows the results of mating disruption field trials testing pheromone doses from 10 to 40 g ha^{-1}, emitted at a constant rate over four months. Mating disruption was tested in minimum-containment mesh greenhouses, as well as in high-containment glass greenhouses. Efficacy and requirements in order for mating disruption to be successful are discussed.

IV.2 Materials and Methods

IV.2.1 Mesoporous pheromone dispensers

New mesoporous pheromone dispensers were developed for the field trials carried out in the present work. All of them were formulated based on a mesoporous material (Corma et al., 1999, 2000), supplied by Ecología y Protección Agrícola SL (Valencia, Spain). The dispensers were cylindrical tablets 9 mm in diameter, of various lengths and initial loads: 20, 60 and 80 mg (T20, T60 and T80, respectively). The formulations contained the main compound of the *T. absoluta* sex-pheromone 3,8,11-tetradecatrienyl acetate (TDTA), supplied by Ecología y Protección Agrícola SL (Valencia, Spain).

The dispensers were hung inside polypropylene (PP) baskets, also supplied by Ecología y Protección Agrícola SL (Valencia, Spain). The PP baskets were 50 mm wide and 90 mm long, and the pheromone was released through a 6×5 mm mesh. The pheromone basket had a hanger at the top to attach it to the trellis strings.

IV.2.2 Containment level trials

IV.2.2.1 Low-containment trial

An initial study was conducted to evaluate the prospects of mating disruption applied to *T. absoluta* in three commercial mesh greenhouses growing tomatoes (*L. sculentum*, var. Valenciano), located in El Perelló (Valencia, Spain). The trial covered the whole summer cycle of the crop, from 4 June to 26 August 2008. Plots were arranged in two 500m^2 and one 300m^2 minimum-containment mesh greenhouses (9×6 threads cm^{-2}) as follows: each 500 m^2 greenhouse was divided, with the same mesh, into two 250 m^2 plots in order to apply two different pheromone doses in four separate plots: three 250 m^2 plots with 10 g ha^{-1} and the fourth to test a higher dose of 40 g ha^{-1}. To obtain the aforementioned doses per ha, two different mesoporous mating disruption dispensers were developed, with loads of 20 mg and 80 mg TDTA (T20 and T80, respectively). Mating disruption

treatment was installed following transplantation on 4 June, together with monitoring pheromone traps. In all plots, pheromone dispensers were installed at a density of 500 dispensers ha^{-1}, distributed inside the greenhouse attached to trellis strings, at a height of at least 1.8 m. The 300 m^2 mesh greenhouse was the reference plot with conventional insecticide treatments.

Insecticide treatments were applied in accordance with weekly assessments, when the percentage of live stages of *T. absoluta* exceeded 10%. The reference plot had the conventional treatments used by the grower: indoxacarb (Steward® Indoxacarb 30% (Du Pont Ibérica SL, Barcelona, Spain) at 100 g ha^{-1}, applied on 25 June, 12 July and 1 August) alternated with spinosad (Spintor* 480 SC (Dow AgroSciences Ibérica, Madrid, Spain) at 300 g ha^{-1} applied on 7 July). In view of the results, the mating disruption plots were also treated with indoxacarb on 15 July and 1 August, as successful control of the pest had not been achieved.

IV.2.2.2 High-containment trial

In a second experiment in 2009, a dose of 30 g ha^{-1} (with T60 mesoporous dispensers, loaded with 60 mg of TDTA) was tested in a 1000 m^2 plastic greenhouse, property of Fundación Ruralcaja, located in Paiporta (Valencia, Spain). This was a high-containment greenhouse, which included a mesh cover (10×14 threads cm^{-2}) on ventilation windows and double doors. The crop was tomato, var. Valenciano, in hydroponic substrate, begun in January 2009. Mating disruption dispensers were applied on 4 March 2009, at the same density and position described in the low-containment trial above. The trial was conducted on the crop until 20 July (20 weeks). Monitoring pheromone traps were placed on 19 January to obtain population data prior to pheromone application.

A second 1000 m^2 greenhouse, with the same crop and containment features as the first, was used as reference plot, using the conventional chemical control applied by the grower. Unlike the first trial, treatments with *Bacillus thuringiensis* Ber., var. *Kurstaki*; (Bt hereafter) at 0.13% (Costar, Syngenta Agro SA, Madrid, Spain), were applied to combat any presence of *T. absoluta* live

stages, in accordance with weekly assessments. It was applied on 16 and 30 March, 20 April and 8 June 2009 in the Reference plot. In addition, indoxacarb (100 g ha^{-1}) was applied when the threshold of 15-20% of plants with live stages was exceeded (8 May and 20 May in the Reference plot).

IV.2.3 Efficacy trials

After performing preliminary trials and having checked the influence of greenhouse containment level, two new efficacy trials were carried out in order to precisely evaluate the mating disruption technique with the T80 mesoporous dispensers.

IV.2.3.1 First trial: 2009

The first efficacy trial was conducted in a high-containment greenhouse property of the company Tomspring, located in Alicante (Spain). Four plots of tomato (var. Valenciano) were arranged inside a 2600 m^2 mesh greenhouse (16×10 threads cm^{-2}) with a plastic cover. Following plantation, plastic sheets were used to divide up the plots, as shown in **Figure IV.1**.

Pheromone treatment began on 8th October 2009, and the trial lasted the entire crop cycle, which was 20 weeks (until February 2010). Pheromone dispensers were applied at 500 dispensers ha^{-1} (dose of 40 g ha^{-1}), with the same method and positioning as described for the previous experiments. Monitoring pheromone traps were also installed on 8 October.

Chemical treatments in the Reference plot were applied in accordance with the weekly assessment of live stages of *T. absoluta*: one treatment with indoxacarb (100 g ha^{-1}) on 13 October, one with etofenprox (Trebon 30 LE (Certis, Alicante, Spain) at 0.1% applied on 24 November) and seven applications of *B. thuringiensis* (Bt at 0.05% applied on 13 and 27 October, 3, 10, 17, 24 November, and the 22 December).

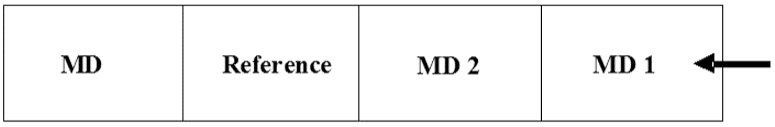

Figure IV.1 Arrangement of the different plots inside the greenhouse for the 2009 efficacy trial (Alicante, Spain). The arrow indicates the entrance of the greenhouse. Mating disruption plots are referred to as 'MD1', 'MD2', 'MD3', and 'Reference' is the control plot with conventional chemical treatments.

IV.2.3.2 Second trial: 2010

The mating disruption efficacy trial was performed in one 1000 m^2 and three 250 m^2 high-containment glasshouses. The crop was tomato, var. Valenciano in hydroponic substrate. The trial consisted of three MD plots and two reference plots, arranged as follows: the 1000 m^2 glasshouse was divided by a thermal blanket into two plots of 500 m^2 each, in order to set up a mating disruption plot and a reference plot for conventional treatments. Two of the other three 250 m^2 glasshouses were set up for mating disruption treatment, and the remaining glasshouse also acted as a reference plot. Monitoring pheromone traps were placed on 25th January 2010, while MD pheromone dispensers were applied on 8th February, at 500 dispensers ha^{-1} (40 g ha^{-1}) density according to the usual method and placing. This trial covered the tomato cycle up to the harvest, which was after 23 weeks.

The 500 m^2 reference plot had four *B. thuringiensis* treatments (Bt at 0.13%, applied on 3rd May, 24th May, 26th June and 2nd July), in accordance with the weekly assessment of live stages. Meanwhile, the remaining plots only received the final treatment, applied on 2nd July.

IV.2.4 Evaluation of treatment efficacy

In all cases, in order to evaluate the efficacy of mating disruption, three commercial Delta traps, with sticky bases, supplied by Biagro SL (Valencia, Spain), were placed in each untreated or pheromone treated plot. Each trap was baited with commercial pheromone monitoring lures from Pherobank™ (Wageningen, The Netherlands). The evaluation was made by comparing the moth trap catches from the mating disruption plots with those obtained from the reference plots. Captures on sticky bases were recorded and replaced weekly, whereas the monitoring lures were replaced every 40 days. The absence of trap catches during mating disruption treatment is a good indication of the effectiveness of the technique, but crop damage assessment provides the final proof (Howse, 1998). In order to assess the percentage reduction in males captured in pheromone traps between the MD and reference plots, the mating disruption index (MDI) was calculated according to the following formula: $MDI = (1 - (x/y)) \times 100$; where x is the number of males captured in the MD plots and y is the number of males captured in the control plots.

To assess crop damage, 40 plants from the central area of each plot were randomly evaluated weekly, and the number of galleries and live stages of *T. absoluta* (eggs, pupae and larvae) were recorded. Treatment efficacy results were given as a percentage of plants with live stages. For efficacy trials, the percentage of damaged fruits was also recorded by revising every fruit from the 40 selected plants in the first week of harvest.

IV.2.5 Pheromone release profiles

In parallel with the greenhouse trials, additional dispensers were simultaneously aged in nearby areas inside greenhouses of the same type, for 90 days in 2008, 124 days in 2009 and 164 in 2010 trial. Residual TDTA content was extracted at different ageing times. Three dispensers for ageing time were extracted by solvent-extraction, at 40ºC during 2 h, with magnetic agitation and dichloromethane as solvent.

TDTA content was measured by gas chromatography with flame ionization detector (GC/FID) using a Clarus®500 gas chromatograph (PerkinElmer Inc., Wellesley, MA, USA). Extracts were analyzed and quantification was made using dodecane as internal standard. All injections were made onto a ZB-5 (30m× 0.25mm×0.25 mm) column (Phenomenex Inc., Torrance, CA), held at 120ºC for 2 min and then, raised at 20ºC/min^{-1} up to 260ºC, maintained for 3 min. The carrier gas was helium at 1.5 ml min^{-1}. The amounts of pheromone and the responses were connected by fitting a linear regression model, $y = a+bx$, where y is the amount of pheromone and x is the ratio between pheromone and dodecane responses.

The residual pheromone load, called P (µg), for each dispenser was fitted by polynomial regression. The independent variable was the number of days that dispensers had been installed in the plot, which was called t (days).

IV.2.6 Statistical analysis

Moth catches in pheromone baited traps, per trap and week, were analyzed using a one-way ANOVA model, followed by an LSD test (P<0.05), to assess the significance of differences observed in captures between treatments.

Contingency tables and the Pearson's chi-square (χ^2) test were used to test the correlations between treatments in regard to the number of damaged plants with live stages of *T. absoluta*. A significant chi-square statistic (P<0.05) is evidence for the existence of differences. Analysis was performed with SPSS 16.0.1 software (SPSS Inc., Chicago, IL, USA).

IV.3 Results

IV.3.1 Low-containment level trial: El Perelló 2008

Figure IV.2a shows population dynamics of *T. absoluta*, as number of moths captured per trap and day (MTD), for the different plots set up in the 2008 trial. For statistical analysis, data from weeks 1 to 5 were grouped into a single period in order to homogenize the data, together with that from weeks 9 and 10. One-way ANOVA performed with sqrt-transformed catch data showed that there were no statistical differences among catches obtained in the Reference plot and those obtained in any of the mating disruption plots ($F=0.52$; df=2,53; $P=0.52$). Therefore, none of the pheromone treatments (T20 and T80) had achieved a reduction in moth population in comparison with the Reference plot with chemical control, and average MDI values were around 40% during some periods.

The most important issue is the evolution of the percentage of plants with live stages throughout the different assessments, which is depicted in **Figure IV.2b**. From March to June, no live stages of *T. absoluta* were found in any plot. Up to the beginning of July, chemical treatments were only applied to the Reference plot (indicated by black arrows in **Figure IV.2b**), in the 3rd July assessment it was found that around 20% and 10% of the plants in the mating disruption plots (T20 and T80, respectively) had been attacked by *T. absoluta*. Thus, the presence of live stages had increased in the pheromone treated plots but not in the chemical Reference plot. According to the level of damage, it was decided to perform indoxacarb treatments in all the plots, which happened on 12 July and 1 August. The 12 July treatment managed to restrain the increase of population as observed in the 17 July assessment (**Figure IV.2b**), with no significant differences between treatments (χ^2, $P=0.822$). However, at the end of the cycle (31 July assessment in **Figure IV.2b**), the damage level in the Reference plot was significantly lower than that in the pheromone treatment plots ($P=0.018$), which means that pheromone dispensers had not achieved the disruption of *T. absoluta* population, with any of the pheromone doses tested.

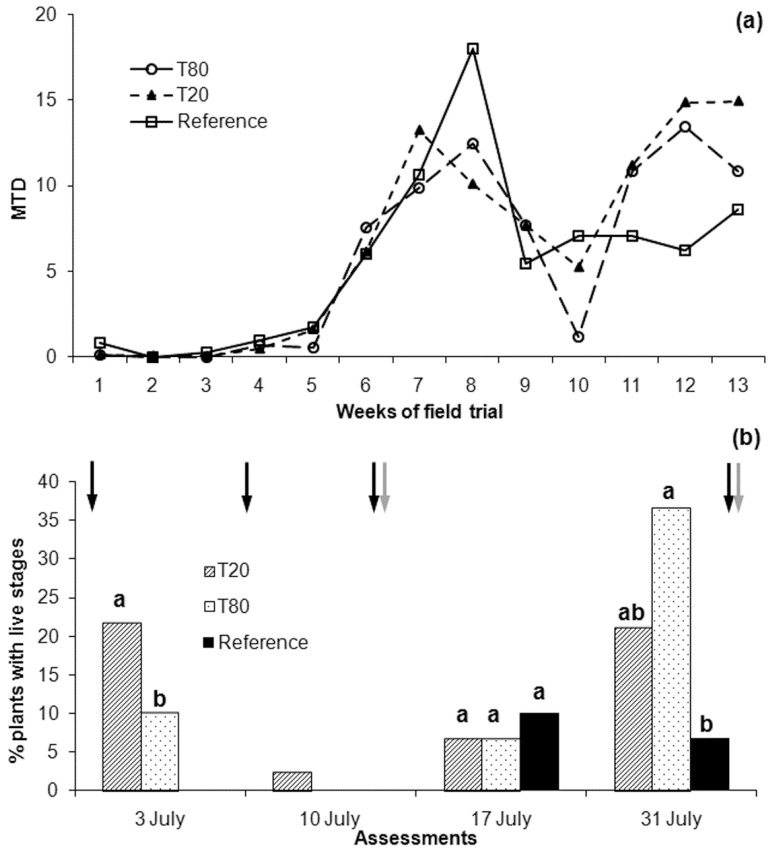

Figure IV.2 Results from low-containment level trial conducted in El Perelló (Valencia, Spain) in 2008. **(a)** Captures of *Tuta absoluta*, as moths per trap and day (MTD), in commercial monitoring traps for pheromone treated plots (T80 and T20) and the Reference plot. The arrow indicates the date when pheromone dispensers were applied. **(b)** Damage level obtained in the mentioned plots, as percentage of plants with *T. absoluta* live stages (eggs, pupae or larvae). Black arrows indicate the dates when indoxacarb and spinosad were applied to the Reference plot and grey arrows indicate indoxacarb applications on the pheromone treated plots.

IV.3.2 High-containment level trial: Paiporta 2009

Before commencing MD treatment in the 6th week, 5 and 29 moths were captured in MD and Reference plots, respectively, over 5 weeks. However, data up to 14th week were not employed for statistical analysis, as population levels were not high enough to provide reliable information (**Figure IV.3a**). Therefore, statistical analysis was performed with data from the 15th to 25th weeks, when TLM populations had increased sufficiently in order to detect the differences between plots, which were statistically significant in the mentioned period ($F=98.89$; $df=1,58$; $P<0.001$). Average catches in the Reference plot were significantly higher, which means that the pheromone dispensers installed had on this occasion a presumptive disruptive effect on the moth's flight, obtaining an average MDI of 94.4%.

Regarding damage assessment, the presence of live stages of TLM in both pheromone-treated and Reference plots is shown in **Figure IV.3b**. During March no recordings of live stages were obtained in any of the plots. Damage significantly increased in the Reference plot in April, but the applications of indoxacarb, on 8 and 20 March, together with the application of indoxacarb+Bt on 8 June, reduced the presence of TLM. Regarding the mating disruption plot (MD), the percentage of damaged plants only exceeded 5% at certain times and the same level of attacked plants was recorded in the Reference plot during June. Damage levels ranged from 2–5% in the MD plots during the last month without any additional chemical treatment, being significantly lower in comparison with the Reference plot ($P=0,006$).

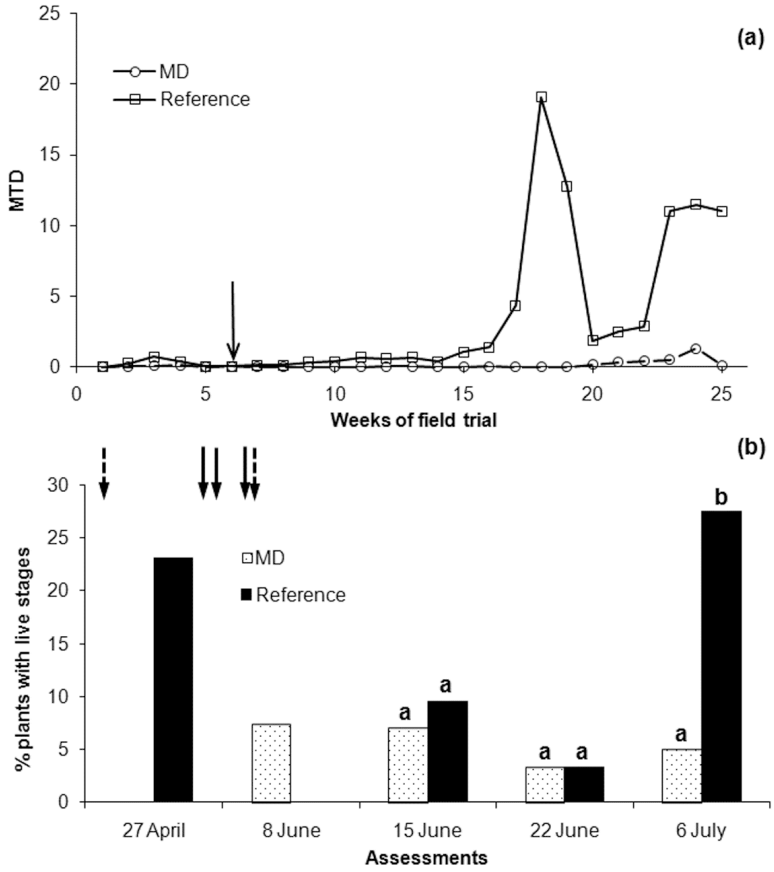

Figure IV.3 Results from high-containment level trial conducted in Paiporta (Valencia, Spain) in 2009. **(a)** Captures of *T. absoluta*, as moths per trap and day (MTD), in commercial monitoring traps for the pheromone treated plot (MD) and the Reference plot with conventional chemical treatments. **(b)** Damage level obtained in the mentioned plots, as percentage of plants with TLM live stages (eggs, pupae or larvae). Black arrows indicate the dates when indoxacarb treatments were applied and discontinuous arrows indicate Bacillus *thuringiensis* (Bt) applications, all of which in the Reference plot.

IV.3.3 Efficacy trial: Alicante 2009

TLM population levels were different both during and following November (9th week in **Figure IV.4a**), so they were compared separately. Mean captures from the three MD plots differed significantly from those obtained in the Reference plot (F=34.37; df=1,75; *P*<0.001) up to the 9th week (**Figure IV.4a**), obtaining an average MDI of 84.6%.

During November, the Reference plot received one treatment with etofenprox and four with Bt. These treatments affected larval instars and achieved a population reduction in the Reference plot. As a consequence, differences between the MD and Reference plots were not so evident at the end of the cycle (10th to 21st weeks; F=4.03; df=1,108; *P*=0,047). However, captures in MD Plots were higher than expected during the last week, which could signify the dispenser having reached the end of its lifespan.

Damage assessments showed that no live stages of *T. absoluta* were detected in the Reference plot throughout the period under study (**Figure IV.4b**), and any presence of *T. absoluta* in the MD plots was only detected after several assessments. However, live stages never exceeded the threshold of 5% presence on plants and the differences between the MD and Reference plots were not significant in any case according to χ^2 test (*P*>0.05). No damaged fruit was observed throughout the trial in either the pheromone-treated or Reference plots.

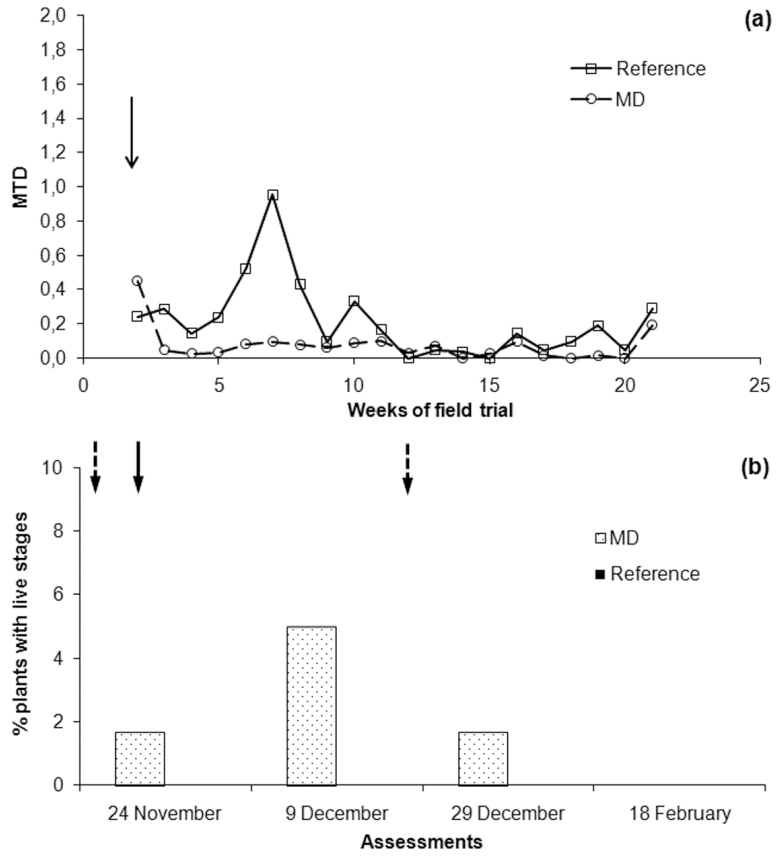

Figure IV.4 Results from the efficacy trial conducted in Alicante (Spain) in 2009. **(a)** Average captures of *T. absoluta*, as moths per trap and day (MTD), in commercial monitoring traps for the pheromone treated plots (MD) and the Reference plot with conventional chemical treatments. **(b)** Damage level obtained in the mentioned plots, as percentage of plants with TLM live stages (eggs, pupae or larvae). The black arrow indicates etofenprox application in the Reference plot and discontinuous arrows indicate the dates when Bt was applied, all of which in the Reference plot.

IV.3.4 Efficacy trial: Paiporta 2010

TLM populations were virtually zero, both in the 500 m^2 and 250 m^2 plots, up to the 14th week (**Figure IV.5a**). Twenty-one and 49 moths were captured in 14 weeks in the MD and Reference plots, respectively. From this date, statistical analysis showed that average captures obtained in MD plots differed significantly from those of Reference plots (F=31.96; df=1,148; P<0.001) up to the date of harvest. Thus, a flight disruption effect was taking place in the pheromone treated plots, and the average MDI was 83.2%. However, a changing trend was observed in all the MD plots, as captures were higher than expected during the two last weeks of the trial. This could signify the dispenser having reached the end of its lifespan.

The average percentage of plants with live stages of *T. absoluta* in the MD plots was lower than that in the Reference plots up until June. Specifically, the 26 April assessment (**Figure IV.5b**) showed no significant differences in the percentage of plants with live stages between the pheromone treated and reference plots (P=0.174). From April up until the end of May, the percentage of damaged plants did not exceed 8% in the plots with mating disruption treatments, and differed significantly from the plot with conventional control in the May recordings (P=0.008). By contrast, from mid-June to the end of the cycle, plant damage in the MD plots increased, and damage did not differ from that in the Reference plots. In the same way, at the end of the trial, an average of 1.94% of tomatoes was damaged in the Reference plots, whereas 2.89% was recorded in the MD plots. The results of the July assessment would also indicate the dispenser having reached the end of its lifespan, with a consequent loss of efficacy.

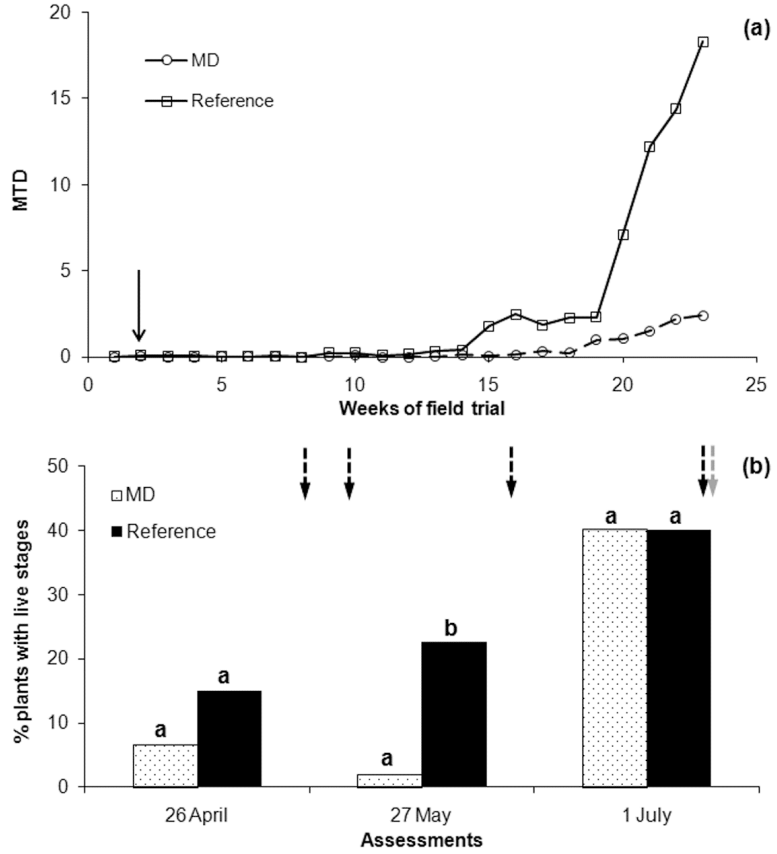

Figure IV.5 Results from the efficacy trial conducted in Paiporta (Valencia, Spain) in 2010. **(a)** Average captures of *T. absoluta*, as moths per trap and day (MTD), in commercial monitoring traps for the pheromone treated plots (MD) and the Reference plots with conventional chemical treatments. **(b)** Damage level obtained in the mentioned plots, as percentage of plants with TLM live stages (eggs, pupae or larvae). Black discontinuous arrows indicate the dates when Bt was applied in the Reference plot. The grey arrow indicates Bt application on the MD plots at the end of the trial.

IV.3.5 Pheromone release profiles

Release profiles of TDTA were studied for the different dispensers tested each year. The residual pheromone load was fitted by linear regression in all cases (**Figure IV.6a**). For T80 dispensers in 2008, a linear equation was obtained (eq. 1). Data at t=0 were not fitted properly, which is explained by the higher emission rate during the first few days, until the dispenser reaches an equilibrium with the environment. So these values were disregarded, along with three outliers, resulting in a coefficient of determination of R^2=0.974. Data considered outliers were disregarded according to normal probability plots and residuals of regression analysis.

$$P_{T80} = 59{,}715 - 0{,}4913x \qquad (1)$$

Residual pheromone load of T20 dispensers was also fitted to a linear model (eq. 2 in **Figure IV.6b**), disregarding data at t=0, resulting R^2=0.901.

$$P_{T20} = 15{,}275 - 0{,}1322x \qquad (2)$$

In view of the results from containment-level trials, it was decided to employ the high load dispensers with some changes in the formulation. In the case of the dispensers employed in 2009, the linear model (eq. 3 in **Figure IV.6c**) resulted in R^2=0.859, with three discarded outliers. However, these dispensers still contained 65.8% of their initial TDTA load after 124 days of trial.

$$P_{T60} = 61{,}953 - 0{,}1716x \qquad (3)$$

Finally, for the T80 employed in 2010, it was observed that these dispensers stopped emitting after 121 days of ageing, so average pheromone contents at 143 and 164 days were the same as at 121 days. Thus, these final data were discarded and release profile was fitted to a linear model (eq. 4 in **Figure IV.6d**), resulting in a coefficient of determination of R^2=0.915.

$$P_{2010} = 81{,}738 - 0{,}2427x \qquad (4)$$

Thus, equations 1 to 4 fitted to linear models, which means that pheromone is released at a constant rate during the period under study and mean emission rates are assumed to be the value of their slopes: 491.3 and 132.2 µg day^{-1} for T80 and T20, respectively, in the 2008 trial, while 171.6 and 242.7 µg day^{-1} were the release values for T60 in 2009 and T80 in the 2009-2010 efficacy trials, respectively.

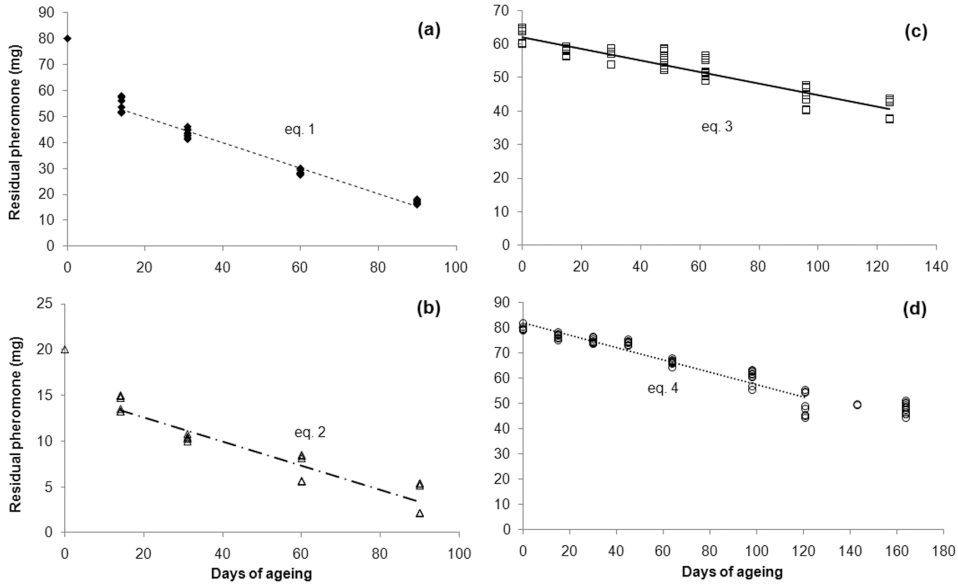

Figure IV.6 Release profiles of (3E,8Z,11Z)-tetradecatrienyl acetate (TDTA), the major *T. absoluta* pheromone component, from the four dispensers employed for the different trials: **(a)** T80 dispenser from low-containment trial 2008, **(b)** T20 dispenser from low-containment trial 2008, **(c)** T60 dispenser from high-containment trial 2009, **(d)** T80 dispenser from efficacy trials 2009-2010. Fitted lines describe the TDTA content of the dispenser (mg) versus time (number of days of ageing).

The percentage of residual pheromone load at the end of the study period must be taken into account. Dispensers T80 and T20 had residual percentages of 21.5 and 18.7% respectively. By contrast, formulations employed in 2009 and 2010 still contained more than 60% of their initial pheromone loads after 124 days, which would be a poor feature for an efficient dispenser.

IV.4 Discussion

The interruption in pheromone communication of *T. absoluta* in pheromone treated plots has been demonstrated by Michereff and coworkers (Michereff et al., 2000b), but they highlighted the need for more detailed pheromone studies to achieve a more effective mating disruption in TLM. In this previous experiment, doses up to 80 g a.i. ha^{-1} were tested, with dispensers being replaced every 4 weeks and a density of application greater than the 500 dispensers ha^{-1} employed in this study. Our present work contributes new efficacy trials for mating disruption in greenhouses applied to TLM and a new mesoporous pheromone dispenser for this purpose.

The main conclusion drawn from our containment-level trials was that mating disruption could not be achieved, with the tested pheromone doses, when a tomato crop is grown outdoors or in low-containment greenhouses. Low isolation meshes or the existence of holes in greenhouse covers allow the immigration of moths from outside the pheromone-treated areas, maintaining high population densities and increasing the likelihood of casual mating. Immigration of mated females is the reason of mating disruption failures in open field environments and the need of treatments in huge areas to avoid edge effect. Few studies have focused on the use of mating disruption in indoor facilities (such as greenhouses and storages), even though indoor applications provide natural boundaries that limit outdoor constraints (van der Pers & Minks, 1998; Ryne et al., 2001). Nowadays, the application of outdoor mating disruption for *T. absoluta* is not affordable due to the need of high pheromone amounts to be applied in wide areas and its excessive cost. Currently, the cost of pheromone synthesis is crucial for this technique to be applied. Commercial price of the pheromone is 900 € g^{-1} but this cost can be reduced to 30 € g^{-1} when it is synthesized at industrial scale (Ecología y Protección Agrícola SL pers. comm.). Tomato crop conducted in greenhouses allows mating disruption to be applied using lower pheromone doses in small plots.

In the first years of our field trials, there was not enough quantity of pheromone available to have true replicates. This was the case of Paiporta 2009

trial, where three different locations inside the same greenhouse were considered for statistical analysis. Once this preliminary test showed efficacy against TLM, and due to the big impact caused by this pest in Spain, true replicates were conducted in 2009-2010 in two different locations, Alicante and Paiporta.

According to our results, in low-containment greenhouses, damage was not reduced in plots treated with two different pheromone doses (10 and 40 g ha^{-1}), in comparison with a reference plot with four chemical treatments. However, when containment level increased, as in the case of Paiporta 2009 trial, damage did not significantly differ from that in the reference plot with four Bt and three indoxacarb treatments and male flight was satisfactorily disrupted with about 30 g ha^{-1} of TDTA. Therefore, MD treatment was as effective as chemical control, with the mesoporous dispensers, for at least 4 months, when applied in greenhouses protected with a double door and a more closely-woven mesh. This would confirm the importance of the degree of containment on the success of pheromone treatment on the TLM, as it prevents the migration of pests. The following efficacy trials conducted in Alicante and Paiporta, in 2009 and 2010 respectively, achieved good control of damage at the end of the cycle, what finally supported results of flight disruptions. An average MDI of 84% was obtained in Alicante when moth population peaked at the beginning of November, before Bt and etofenprox treatments were applied in the Reference plot. In Paiporta, average MDI was 83% from the 14^{th} week until the end of the experiment.

As stated by other authors, it is difficult to link a reduction in moth catches with an equal reduction in damage (van Steenwyk & Oatman, 1983; Lykouressis et al., 2005). For many moth species, it is especially difficult to obtain a relationship between male catches and plant damage (McNeil, 1991). Regarding plant damage in our present work, control of *T. absoluta* with MD was as effective as nine conventional treatments applied to the reference plot in Alicante, and damage was controlled in the 2010 Paiporta MD plots up to June, as observed in the 1 July assessment (**Figure IV.5b**). Due to the high containment level of these glasshouses in Paiporta, moth migrations did not occur, so the depletion of the

pheromone dispensers, reaching the end of their lifespan, could be the reason for this reduction in efficacy.

Focusing our attention on the dispensers' release profiles, in 2008, formulations T80 and T20 did not prove effective under the particular conditions of the field trial, but release profiles were fitted to linear models and residual pheromone amounts were of about 20%. T60 in Alicante disrupted moth's flight and controlled damage for at least 120 days, with a linear release profile, emitting an average of 171.6 µg day^{-1}, despite having a high residual level of pheromone (65.8% after 124 days). In 2010, T80 also had a lifespan of 4 months (121 days) with a residual pheromone load of 61.6% and an average release value of about 240 µg day^{-1}. However, this 4 month period was not sufficient to control TLM in Paiporta 2010 up to the harvest, which is evidenced by the increase in damage from June onwards (**Figure IV.6b**). Therefore, some key changes should be made to dispenser formulation to ensure the disruption of pheromone communication. The percentage of residual pheromone load must be reduced, as it is known that the pheromone synthesis is the main cost for mating disruption implementation and pheromone must not be wasted. As it has been proven that TLM flight and damage could be controlled by releasing at least 170 µg day^{-1}, the lifespan should be extended in order to achieve this level of emission for almost 6 months, to cover longer crop cycles and maintain pheromone doses of at least 30 g ha^{-1}. This value is consistent with Michereff et al. (2000b) who found interruption in male orientation in plots treated with 35 to 50 g ha^{-1} of sex pheromone for *T. absoluta*. There are some experiences on mating suppression to manage other Gelechiidae species, including *Pectinophora gossypiella* (Saunders), *Tecia solanivora* Povolny, *Sitotroga cerealella* (Olivier) and the tomato pinworm *Keiferia lycopersicella* (Walsingham) (van Steenwyk & Oatman, 1983; Fadamiro & Baker, 2002; Lykouressis et al., 2005; Bosa et al., 2008). Specifically, *P. gossypiella* has been widely controlled by mating disruption in cotton fields with 78 g ha^{-1} of gossyplure, and tests with *T. solanivora* have been successful by applying 86 g ha^{-1}.

Regarding treatment application procedure, pheromone dispensers were hung a few days after plantation in all cases, when the tomato plants were 10-15

cm high. With the proper formulation in the dispensers, this early application was sufficient to protect the crop throughout the season. Several authors have demonstrated that early pheromone applications prevent Lepidoptera populations from increasing at mid-season (Staten et al., 1987; Kehat et al., 1995; Lykouressis et al., 2005), which can result in high-yield losses. Therefore, mating disruption of the first emerging moths is crucial in order to affect development of the subsequent generations throughout the season.

In conclusion, this study revealed that effective pheromone application against *T. absoluta* can be achieved, in high containment greenhouses for 4 months, with doses of 30 g TDTA per ha employing new mesoporous dispensers. These dispensers were not replaced throughout the season and provided suitable emission rates, but formulations must be improved to avoid high residual loads and the subsequent waste of active ingredients. On the other hand, under the current conditions of pheromone synthesis and the affordable pheromone doses, the application of mating disruption needs high-containment degree greenhouses in order to succeed and be competitive with insecticide control. Therefore, research must be directed at reducing the price of pheromone synthesis and to evaluate the prospects of the outdoor application of mating disruption systems to control *T. absoluta* damage in outdoor tomato.

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Capítulo V

"Study on the optimum pheromone release rate for attraction of *Chilo suppressalis* (Lepidoptera: Pyralidae)"

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Study on the optimum pheromone release rate for attraction of *Chilo suppressalis* Walker (Lepidoptera: Pyralidae)

Published in Journal of Economic Entomology (Vacas S., C. Alfaro, V. Navarro-Llopis, M. Zarzo and J. Primo. 2009)

Abstract. Traditional chemical control against *Chilo suppressalis* Walker is currently being replaced in Spain by new methods based on pheromones. A key step to improve the efficacy of these methods is the determination of the optimum pheromone release rate, which is still uncertain for this pest. In this work, the pheromone release profile and the field performance of a new mesoporous dispenser was compared with a standard commercial dispenser. For this purpose, pheromone loads were extracted from field-aged dispensers and quantified by GC/FID. In addition, a field trial was carried out with traps baited with one, two or three mesoporous dispensers per trap, as well as with traps containing one standard dispenser. We obtained that the highest number of field catches did not correspond to the highest pheromone emission rate, which suggests a repellent effect of the insect if the emission is excessive. The results suggest that the attractant activity was maximized by emitting around 34 µg day^{-1}. The efficacy of the mesoporous dispenser and its possible improvements are discussed.

V.1 Introduction

Striped rice stem borer, *Chilo suppressalis* Walker, is a key pest in the more temperate rice areas in Asia (Japan, Korea and China). It also occurs in Iran and the southern countries of the former USSR; in Europe it is a pest mainly in Spain. The larvae of rice stem borers feed within plant stems causing severe crop losses in many cases (Beevor et al., 1990; Batalla, 1999a). *Chilo* spp is hard to control with foliar contact insecticides because these are only effective within a narrow window during the period between hatching and penetration inside the plant stem (Beevor et al., 1990; Jones, 1998). Nowadays, *C. suppressalis* is being controlled in Spain using aerial applications with insect growth regulators and mating disruption or mass trapping methods in more sensitive areas.

Rice is often grown intensively adjacent to environmentally sensitive coastal areas, estuaries or deltas, which is the case of the two major rice crop areas in Spain. Therefore, it is necessary to replace traditional control techniques by more environmentally friendly methods of integrated pest management such as biological control, mass trapping or mating disruption. The use of these methods has increased since the identification of the rice stem borer sex pheromone, as the three-component blend containing (Z)-11-hexadecenal (Z11-16:Ald), (Z)-13-octadecenal (Z13-18:Ald) and (Z)-9-hexadecenal (Z9-16:Ald), in an approximate ratio of 48:6:5 (Beevor et al., 1990; Tatsuki, 1990).

A biodegradable, low-cost dispenser, with an appropriate pheromone release rate is necessary to reach a good efficiency and to expand the use of these control methods. The ideal dispenser should have a constant release rate during the whole flight period of the pest, independent of weather conditions (Jutsum and Gordon, 1989; Leonhardt et al., 1990; Bradley et al., 1995). Previous work demonstrates that zeolites and other mesoporous materials are suitable matrices for manufacturing dispensers because of their ability for retaining substances depending on their polarities and molecular sizes. Moreover, chemical structure

and properties of these materials can be adapted to release substances at an adequate emission rate over a long period of time (Muñoz-Pallarés et al., 2001). But this optimum rate is not well known in most cases.

We find in the literature some pheromone release threshold values to reach mating disruption depending on the target pest (Ioratti et al., 2004; De Lame et al., 2007; Stelinski et al., 2007). The key to improve control methods based on pheromones as attractants (mass trapping or monitoring) is to know the optimum emission interval, because catches decrease below and above this interval (Jacobson & Beroza, 1964; Zhang & Amalin, 2005). However, there are not many conclusive studies about this subject.

The goal of our study was to obtain the optimum release rate which optimizes the efficacy of an attractant dispenser for the control of the rice stem borer. For this purpose, we compared a standard commercial pheromone dispenser with a new one based on molecular sieves. The efficacy of each dispenser was measured in field trials as number of *C. suppressalis* catches. In order to obtain the optimum pheromone emission rate, we tested four release values: traps baited with one, two or three mesoporous dispensers as well as traps containing one standard dispenser.

V.2 Materials and Methods

V.2.1 Pheromone Dispensers and Traps

V.2.1.1 Standard Dispenser

A closed polyethylene vial FERSEX® CHS C TM, supplied by SEDQ S.L. (Barcelona, Spain) was used as standard dispenser in field and laboratory tests. Vials were 13 mm in diameter and 30 mm high. This type of dispenser is a registered product commonly used in Spain for mass trapping against *C. suppressalis*. The pheromone load for each dispenser was 6 mg.

V.2.1.2 Mesoporous Dispenser

A new pheromone dispenser was elaborated based on a mesoporous material (Corma et al., 1999-2000). It was a cylindrical tablet of 9 mm in diameter and 4 mm high, with a pheromone load of 4 mg (99 % purity). The pheromone was provided by SEDQ S.L. (Barcelona, Spain). For this trial, the mesoporous dispensers were manufactured by means of an industrial process that has around 15% of variability in the initial amount of pheromone.

Initial pheromone loads of all dispensers were determined by extraction and Gas Chromatography analysis in our laboratory. The yield of all extractions was of about 99%.

V.2.1.3 Funnel traps

Lepisan® traps, supplied by SanSan Prodesing S.L (Valencia, Spain) were used in the field test. These traps were made of transparent and green PVC, 19 cm high and 11 cm in diameter. Each trap was baited with the pheromone dispenser and an insecticide strip containing 1.2 g of DDVP (dichlorvos 20% w/w), supplied by Econex S.L. (Murcia, Spain). Lepisan® traps were chosen according to results of a preliminary field study carried out in Amposta (Tarragona, Spain) using different types of moth traps.

V.2.1.4 Light traps

Eight light traps were used in order to monitor the population dynamics of *C. suppressalis* in the rice area where the field test was carried out. These were 15 W UV light traps G8010 model, supplied by Entomopraxis S.L (Barcelona, Spain), powered by a 12V/12A battery. Traps were positioned approximately 1 m above the ground level.

V.2.2 Field trial

The field trial was conducted in a 12 ha mixed-variety rice area located in Amposta (Tarragona, Spain) from May to September 2007. In order to evaluate dispensers catch efficiency, 5 blocks of 4 traps were placed in the field. Intertrap

distance was about 50 m (Hofmeyr and Burger, 1995; Wedding et al., 1995) and distance between blocks was 200 m. Each block contained: trap A, one standard dispenser; trap B, one mesoporous dispenser; trap C, two mesoporous dispensers; and trap D, three mesoporous dispensers. Trap position inside every block was randomized weekly. All traps were placed at 0.5 m above the ground (standard height for mass trapping) hanging from wooden stakes. None of these dispensers were replaced during the whole test. Traps were checked weekly over the entire test period, until the rice crop was harvested.

V.2.3 Pheromone release rates

In parallel with the field trial, all pheromone dispensers were aged in the same field (1 km away from catch traps) at the same time. Dispensers were placed inside Lepisan® traps for up to 124 days. The residual pheromone amount was extracted at different aging times, and then quantified by Gas Chromatography.

Residual pheromone was extracted at 0, 15, 30, 90 and 120 days of aging from standard dispensers, and 0, 7, 18, 29, 47, 64, 90 and 124 days for our new mesoporous dispensers. Two replicates for each aging time were extracted from mesoporous dispensers and three replicates in the case of standard dispensers. The extraction method used for standard dispensers was Pressurized Solvent Extraction using the One PSE® instrument, supplied by Applied Separations Inc. (Allentown, PA, USA). Extraction conditions were 6 cycles of 5 minutes, 100 bars, 60ºC and dichloromethane as solvent. All resulting extracts were concentrated under reduced pressure and analyzed by Gas Chromatography. Previous assays confirmed that no loss of pheromone was produced due to extracts concentration. Mesoporous dispensers were extracted at 40ºC during 2 hours by solvent extraction using tetrahydrofuran. Extracts were then centrifuged at 3000 rpm during 8 minutes. The supernates were collected for their GC analysis.

Z11-16:Ald, the major *C. suppressalis* pheromone component, was quantified by Gas Chromatography with flame ionization detector (GC/FID), with hexadecane as an internal standard. This analysis used a Clarus®500 gas

chromatograph from PerkinElmer Inc. (Wellesley, MA, USA). All injections were made onto a ZB-5MS column (30m x 0.25mm x 0.25µm), that was held at 100ºC for 2 min and then programmed at 15ºC min^{-1} to 170ºC, held at 170ºC for 5 min, and then at 20ºC min^{-1} to 240ºC and held at 240ºC for 1 min. The carrier gas was helium at 1.2 ml min^{-1} with split flow value of 25 ml min^{-1}.

Retention time of Z11-16:Ald was confirmed by GC/FID analysis of commercial pheromone (99% purity), provided by SEDQ S.L. (Barcelona, Spain). The pheromone amount was estimated according to the ratio between Z11-16:Ald and hexadecane responses, by means of a simple regression model.

The minor components of the pheromone (i.e. Z13-18:Ald and Z9-16:Ald) were not determined because previous work using Gas Chromatography coupled with Mass Spectrometry (GC/MS), showed that dispensers ratio of the three components (Z11-16Ald: Z9-16Ald: Z13-18Ald) ranged from 87.5:5.3:7.2 to 87.2:5.3:7.5 from 0 to 120 days of aging. This negligible difference means that the three components of *C. suppressalis* pheromone were released at similar ratio and we can use GC/FID to quantify only the major component of the pheromone to study the release rate. This result was not unexpected given that the chemical structure of the three components is rather similar.

Multiple linear regression was used to study the evolution of residual pheromone load (as mg of Z11-16:Ald) versus time for each type of dispenser. In order to determine if the emission was constant along the time under study, we checked if the quadratic effect was statistically significant.

V.2.4 Statistical analysis

Catch data were collected 23 times during the period 0 to 90 days. Most of these data are zero, and it was decided to group them under a single period for the statistical analysis.

A multifactor ANOVA was carried out to study the effect of three factors on trap catches: (i) day, with 4 levels: 90-102, 102-104, 104-111, 111-116; (ii) block,

and (iii) emission, with 4 levels: SD (standard dispenser), 1MD (one mesoporous dispenser), 2MD (two mesoporous dispensers) and 3MD (three mesoporous dispensers) (LSD test, $P<0.05$). The square root transformation of the number of catches was used to normalize the data.

ANOVA is a useful technique to determine if there are statistical significant differences among the 4 tested levels of pheromone emission. This method treats emission as a qualitative factor. In this case the emission rates are known for each level, and therefore it is of interest to study the linear and quadratic effect of the pheromone emission on the number of captures in order to determine the optimum value. For this purpose we applied a two-factor ANOVA (day and block) using the square root transformation. The residuals from this model were saved and used in a subsequent multiple regression analysis in order to investigate the existence of a relative maximum. Statistical analyses were performed using the Statgraphics plus 5.1 package (StatPoint Technologies, Warrenton, VA, USA).

V.3 Results

V.3.1 Pheromone release rates

Figure V.1 shows the evolution versus time of the remaining load of Z11-16:Ald for the standard and mesoporous dispensers. The residual pheromone load was fitted by simple linear regression for the standard dispenser, resulting R^2=0.893. A similar goodness-of-fit was obtained with the mesoporous dispenser (R^2=0.868). In both cases it was observed that data at day=0 appeared as outliers, which is explained by the higher emission rate during the first days until the dispenser reaches an equilibrium with the environment. Thus, data at day=0 were discarded. Next, we applied a multiple linear regression of the type $y=a+b\,x+c\,x^2$. The quadratic effect was not clearly significant in the mesoporous dispenser (P=0.08) and neither in the standard dispenser (P=0.54). Thus, it was assumed that the residual pheromone load decreased at a constant rate along the period under study. The emission rate is the slope of the linear model. In order to obtain a

better estimation of the coefficients, a multiple linear model was fitted using the type of dispenser as an indicator variable. The resulting slope was 19.2 µg day^{-1} for the mesoporous dispenser and 26.1 µg day^{-1} for the standard dispenser.

This study also showed that the mesoporous dispenser emitted the total of its pheromone load during the trial period, while 30% of the initial pheromone load remained in standard dispenser (see **Figure V.1**).

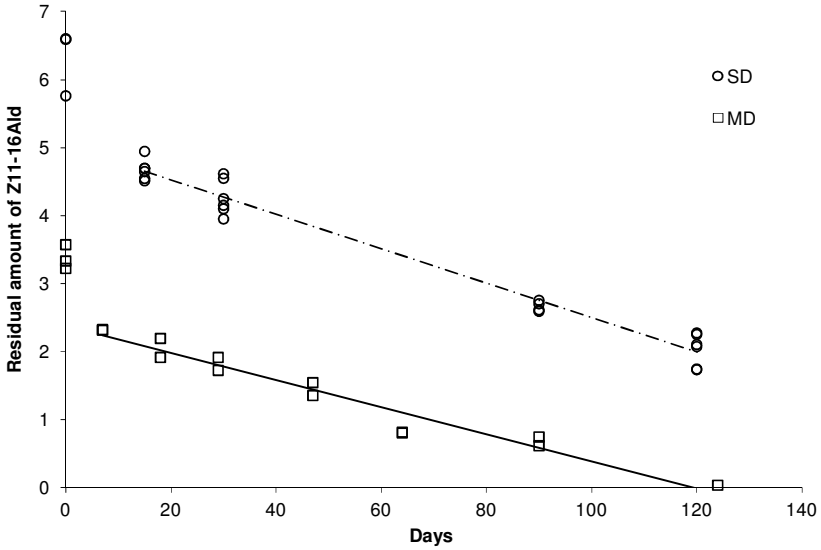

Figure V.1 Release profile of Z11-16:Ald, the major *C. suppressalis* pheromone component, from the two kinds of dispensers tested. Fitted lines show the evolution of remaining mg of pheromone versus time.

V.3.2 Field trial

V.3.2.1 Population dynamics

Light trap catches shown in **Figure V.2** reflect the population dynamics of *C. suppressalis* in the trial area. The first catches were obtained in the middle of July corresponding to a slight first flight (5 moths per trap maximum). Second flight appeared in August, and the third flight in September, when the highest number of moth catches was recorded. These data are consistent with pheromone catches

(**Figure V.3**), showing the same flight periods for both light and pheromone baited traps.

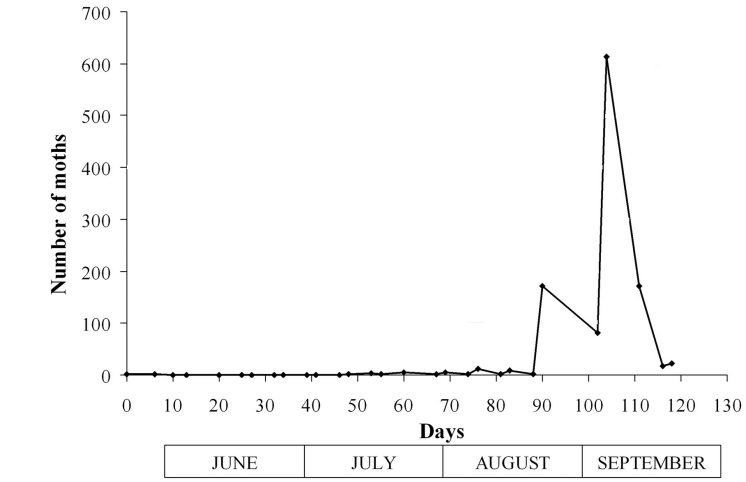

Figure V.2 Population dynamics of *C. suppressalis* in trial area according to the total number of moth catches recorded in 8 light traps.

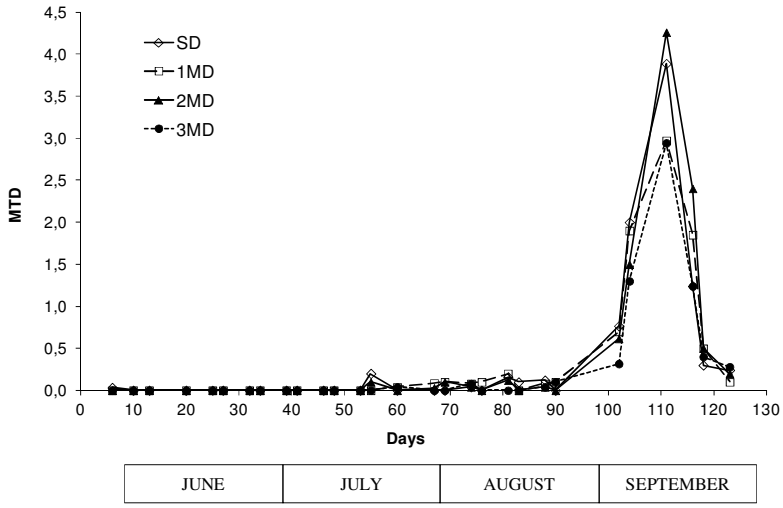

Figure V.3 Moth catches per trap per day (MTD) along the growing season of the rice for each type of baited trap. Catches in Lepisan® traps with DDVP strip, and no dispenser replacement.

Table V.1. **Number of catches of *C. Suppressalis* in traps baited with pheromone dispensers**[a]

Day	Block	Emission levels			
		SD	1MD	2MD	3MD
0-90	A	2	4	5	4
	B	1	1	1	0
	C	5	3	1	0
	D	1	1	1	0
	E	3	7	0	1
90-102	A	5	7	3	4
	B	6	5	9	5
	C	27	12	15	7
	D	2	5	8	2
	E	6	13	2	1
102-104	A	2	3	1	1
	B	2	3	4	2
	C	10	8	5	2
	D	4	3	1	7
	E	2	2	4	1
104-111	A	17	6	21	13
	B	28	17	24	27
	C	51	32	34	23
	D	29	18	47	35
	E	19	31	23	5
111-116	A	0	2	7	3
	B	9	-	10	4
	C	8	16	21	9
	D	10	8	12	11
	E	4	11	10	4
116-123	A	2	0	1	2
	B	5	-	1	5
	C	2	3	5	1
	D	0	1	1	2
	E	0	2	2	1

[a]Data correspond to the number of moth catches collected under the periods of days indicated. Four pheromone emission levels were tested: one standard dispenser (SD), one mesoporous dispenser (1MD), two mesoporous dispensers (2MD) and three mesoporous dispensers (3MD).

V.3.2.2 Trap catches

Figure V.3 shows the average number of catches obtained in traps baited with standard dispenser and one, two or three mesoporous dispensers along the crop season. During the first 90 days, the number of catches was null in most cases. Although these initial data can be grouped into a single period, as indicated in Table 1, they were not considered in the statistical analysis because such low values do not contain reliable information to study the effects of block or emission. Table 1 also shows that the number of catches was rather low at the final period (days 116-123). Around day 120, the residual load of the mesoporous dispenser was very small (**Figure V.1**), and hence it cannot be ensured that the emission rate was constant. Thus, only the catches belonging to the main flight 90 to 116 were considered for the statistical analysis.

Sqrt-transformed catches were analyzed by multifactor ANOVA considering the 3 described factors. Factor day resulted statistically significant (P<0.0001): captures at days 104 to 111 were significantly higher than the rest, because this period corresponds to the maximum pest population (**Figure V.2**).

On the other hand, the effect of factor block was also statistically significant (P<0.0001): the average number of catches at block C was significantly higher than the others, while block A collected the lowest number of catches. This result could be explained by the natural dispersion of the rice stem borer (Alfaro, 2006). In fact, this pest may cause severe damages in a specific area of the plot whilst the rest of the plot may be pest free.

Considering a significance level α=0.05, factor emission also resulted statistically significant (P=0.032). The means plot and LSD intervals for this factor (**Figure V.4**) shows that the highest release rate, emitted from traps baited with three mesoporous dispensers (3MD), captured significantly less than the others. This result suggests that the attractant power decreases above a particular emission value.

Taken into account that the estimated emission rate of the standard and mesoporous dispensers was 26.1 µg day^{-1} and 19.2 µg day^{-1} respectively, the

emission factor was considered as a quantitative variable according to this correspondence: SD=26.1, 1MD=19.2, 2MD=2x19.2=38.5 and 3MD=3x19.2=57.7 µg day^{-1}.

Figure 4 shows the quadratic curve that best fits the four mean values of captures according to the emission rate. This equation was obtained with an Excel® spreadsheet, resulting a relative maximum at about 34 µg day^{-1}.

In order to obtain the optimum pheromone release value which maximizes the attractant activity, a two-factor ANOVA was fitted first with factors day and block. We checked that the interaction was not statistically significant (P=0.46). The residuals of this model do not account for the variability of catches due to emission, since this factor was not included in the ANOVA. Thus, after saving the residuals of this model, they were used to perform a multiple regression analysis in order to study the linear and quadratic effect of emission (eq. 1). In order to obtain the value of emission that maximizes attractant activity, the equation 1 was derived and equalled to zero, resulting an optimum of 34.2 µg day^{-1}. It can be easily checked that equation 2 is mathematically equivalent to equation 1.

$$Residuals = -1.16 + 0.0831 \cdot emission - 0.001214 \cdot emission^2 \quad \text{[eq. 1]}$$

$$Residuals = 0.26 - 0.001214 \cdot (emission - 34.2)^2 \quad \text{[eq. 2]}$$

The coefficient of the quadratic term in equation 1 is statistically significant at α=0.05 (P=0.024) but, in equation 2, it becomes statistically significant at α=0.01 (P=0.0026). This result further confirms the existence of an optimum value of emission that maximizes attractant activity.

Same results were obtained using multiple linear regression with indicator variables for the blocks and time periods. But such analysis is slightly more difficult to interpret for those unfamiliar with the use of indicator variables and we preferred the combined use of ANOVA and multiple linear regression.

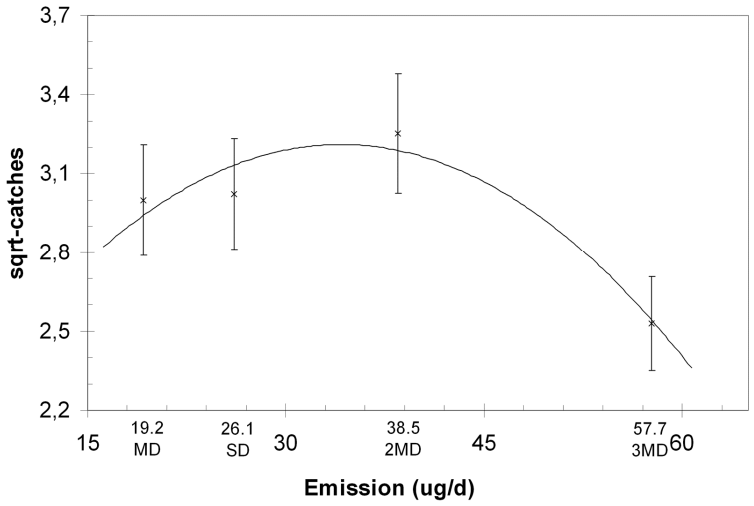

Figure V.4. Means and 95% LSD intervals corresponding to factor emission, from the ANOVA carried out with data in Table 1[a]
[a]The square root transformation was applied to normalize the data. Periods 0-90 and 116-123 were not considered. The curve represents the quadratic model that best fits the mean values.

V.4 Discussion

Control methods based on pheromones are playing an important role in the management of the key pests around the world. In order to ensure the efficacy of these methods, a broad spectrum of controlled release devices have been proposed: hollow fibers, plastic laminates, impregnated ropes, twist-ties, wax formulations, polyethylene vials and rubber septa (Dix et al., 1979; Jutsum & Gordon, 1989; Atterholt et al., 1999; Thorpe et al., 1999; Stipanovic et al., 2004; Trimble, 2007). There are a number of commercial pheromone dispensers available for monitoring and mass trapping of *C. suppressalis* made from polymers. Although polymeric dispensers are effective in many cases (Leonhardt et al., 1993; Shailaja et al., 1997), their main disadvantage is usually that they can accumulate in the environment. In this work, we have studied a new low-cost biodegradable

dispenser based on mesoporous materials for mass trapping and monitoring of *C. suppressalis*. The main advantage of this mesoporous dispenser compared to a standard dispenser also tested, is that the latter retained 30% of the initial pheromone load at the end of the crop season, while the former had a residual load close to zero. Given that the pheromone accounts for the major cost of the dispenser, a competitive one should avoid wasting pheromone at the end of its lifetime.

The study of release kinetics and dispenser field performance plays a crucial role in the development of efficient formulations for dispensers (Shailaja et al., 1997). Both factors should be taken into account in order to establish the connection between attractant activity and pheromone emission.

There are many studies aimed at obtaining the optimum pheromone release value for mating disruption depending on the target pest (Ioratti et al., 2004; De Lame et al., 2007; Stelinski et al., 2007). However, a pheromone critical release value for mass trapping has been obtained for a few species (Hallett et al., 1999; Zada et al., 2002; Zada et al., 2004; Cross et al., 2006). Different authors have reported that high levels of pheromone tend to confuse insects rather than attract them (Jacobson & Beroza, 1964; Zhang & Amalin, 2005). The present work supports this hypothesis because it was observed that the highest emission rate resulted in lower catches, while emissions around 34 µg day^{-1} maximized the attractant activity. The results are useful for the improvement of monitoring and mass trapping techniques based on pheromones.

The knowledge about the optimum pheromone release rate and the field performance of dispensers based on mesoporous matrices are of relevant interest for further studies. Actually, the optimum emission rate and the pheromone release profile are key parameters for designing the lifetime of each type of dispenser. In addition, these values can be used for different purposes: (i) to adjust the initial pheromone load for new dispensers, (ii) to determine the moment when dispensers need to be replaced, and (iii) to compare between different types of dispensers without the need of a field trial. Studies aimed at increasing the efficacy and

reducing the cost of the treatment are important to optimize and promote the implementation of methods based on pheromones.

Acknowledgements

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Capítulo VI

"Effect of sex pheromone emission on the attraction of *Lobesia botrana*"

Version of the article published in

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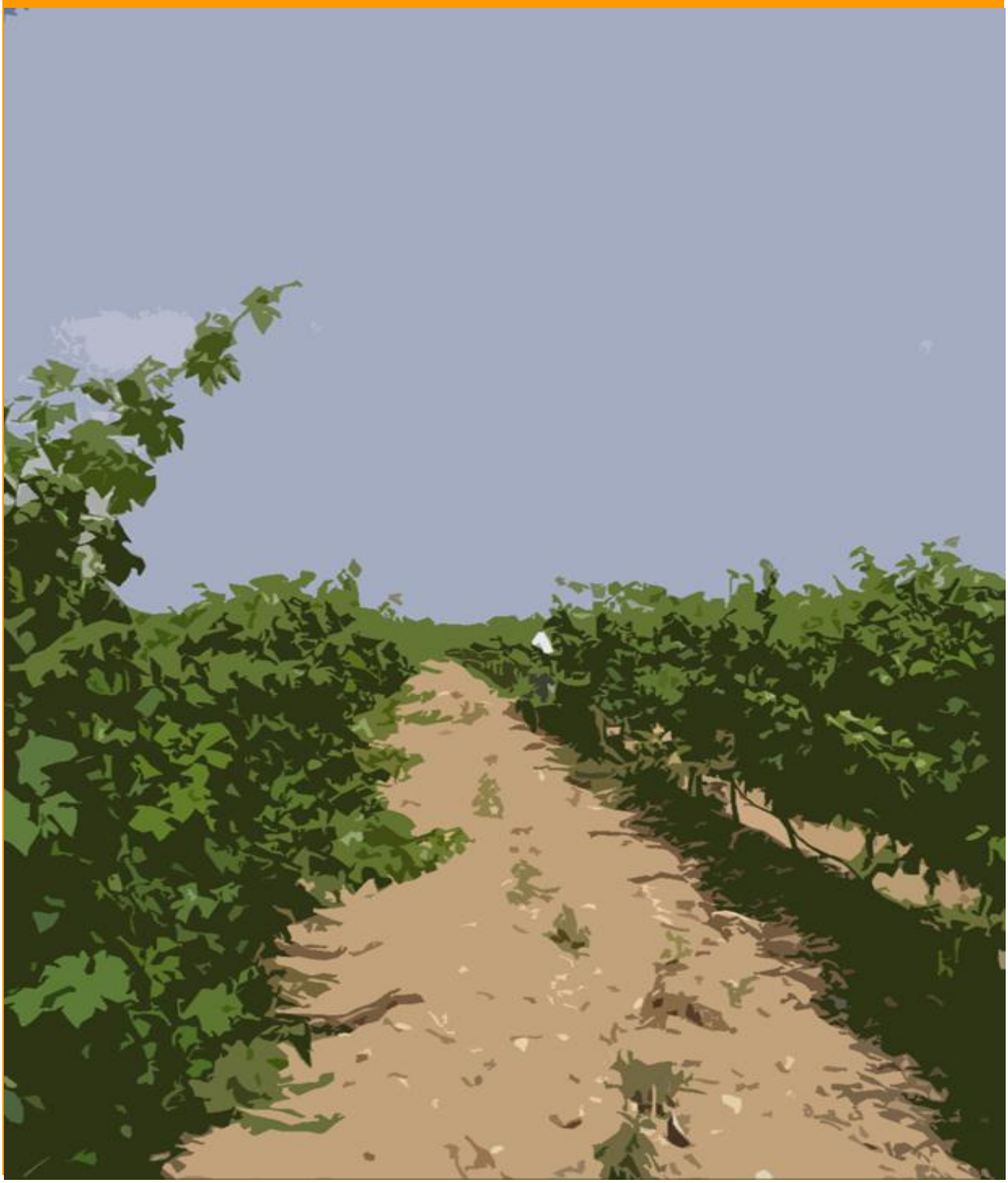

Effect of sex pheromone emission on the attraction of *Lobesia botrana*

Published in Entomologia Experimentalis et Applicata (Vacas S., C. Alfaro, M. Zarzo, V. Navarro-Llopis and J. Primo. 2011)

Abstract. Since the discovery of *Lobesia botrana* Denis & Schiffermüller (Lepidoptera: Tortricidae) sex pheromone, it has played an important role in the control and detection of this pest, for example, through the use of pheromone-baited traps and mating disruption techniques. Rubber septa are the most common pheromone dispensers used in monitoring traps, but often dispenser performance is not optimized. The key to improve methods based on pheromones as attractants (monitoring, mass trapping, or 'attract and kill') is to know the optimum emission interval, because release rates can strongly affect the attraction. In this work, five levels of pheromone load with different release rates were compared in traps using mesoporous pheromone dispensers to investigate the optimum release rate maximizing *L. botrana* catches. Residual pheromone loads of the dispensers were extracted and quantified by gas chromatography, in order to study release profiles and to estimate the various emission levels. The efficacy of pheromone emission was measured in field trials as number of moths caught. A quadratic model was fitted to relate the numbers caught vs. the daily emission rates. The resulting quadratic term was statistically significant, confirming the existence of a relative maximum for *L. botrana* catches. Taking into account that the trial was carried out only in one location, an optimum emission value of ca. 400 µg per day could be considered to enhance the attraction of *L. botrana* under West-Mediterranean weather conditions.

VI.1 Introduction

The European grapevine moth, *Lobesia botrana* Denis & Schiffermüller (Lepidoptera: Tortricidae), is a key pest of grapes in Central Europe and most Mediterranean countries (Anshelevich et al., 1994). Pest damage is mainly caused by larvae feeding on grapes, which leads to fungal colonization of wounds and fruit rot. Traditional chemical control was the main tool to fight *L. botrana*, but since the identification of its sex pheromone in the 1970s (Roelofs et al., 1973), it has been widely used for almost 2 decades against this pest in Germany, Switzerland, and Northern Italy. In other European regions, however, the introduction of pheromone-based methods has been slower (Witzgall et al., 2010). Roelofs et al. (1973) described the main compound of *L. botrana* sex pheromone as (*E,Z*)-7,9-dodecadienyl acetate. Two related compounds were identified later, (*E,Z*)-7,9-dodecadien-1-ol and (Z)-9-dodecenyl acetate, having a synergistic effect on male catches (Arn et al., 1988; El-Sayed et al., 1999). These findings were crucial for the application of pheromone-based control and monitoring techniques. In fact, mating disruption is nowadays the most successful and widespread technique for controlling the moth in Europe. In addition, sex pheromone-baited traps were developed for monitoring *L. botrana* populations, playing an important role in pest detection and treatment timing. Rubber septa are the most common pheromone dispensers used in monitoring traps, but in most cases their performance is not optimized. A dispenser with an appropriate pheromone release rate is necessary to reach a good efficiency and to expand the use of pheromones in pest control systems.

The ideal dispenser should have a constant release rate during the whole flight period of the pest, independent of weather conditions (Jutsum & Gordon, 1989; Leonhardt et al., 1989; Bradley et al., 1995). In order to improve control methods based on pheromones as attractants (monitoring, mass trapping, or 'attract and kill'), the key factor is to know the optimum emission interval, because release rates will strongly affect the attractiveness of the lure, and catches could decrease below and above this interval (Jacobson & Beroza, 1964; Anshelevich et

al., 1994; Zhang & Amalin, 2005). There are some reports of responses of *L. botrana* to different pheromone loads of dispensers (Roehrich et al., 1983; Anshelevich et al., 1994). However, emission rates were not assessed, so trap catches were not correlated with emission values and optimal release rates were not proposed.

The goal of our study was to correlate field trap catches with different pheromone emission values in order to study the optimum emission rate that maximizes the efficiency of the attractant for the control of *L. botrana*. For this purpose, five levels of pheromone load with different release rates of (E,Z)-7,9-dodecadienyl acetate (major active compound) were compared in traps using mesoporous pheromone dispensers. The efficiency of each emission level was measured in field trials as number of moths caught.

VI.2 Material and Methods

VI.2.1 Pheromone dispensers and traps

Three kinds of pheromone dispensers were employed for this trial. All of them were based on a mesoporous material (Corma et al., 1999, 2000), but they differed in size and pheromone load. Dispenser PD1 contained a pheromone load of 1 mg, and it was a cylindrical tablet, 9 mm in diameter and 3.5 mm high. The second (PD10) was loaded with 10 mg of pheromone, and the tablet was 13 mm in diameter and 7.5 mm high. A third dispenser (PD30) was loaded with 30 mg of pheromone; it was 13 mm in diameter and 20 mm high. (*E,Z*)-7,9-dodecadienyl acetate was used as the sex pheromone at 86% isomeric purity. The remaining 13% was the isomer (*E,E*)-7,9-dodecadienyl acetate, according to NMR analysis in our laboratory (data not shown). Previous work on *L. botrana* pheromone synthesis showed that the presence of the (*E,E*)-isomer in the blend did not interfere with the biological activity of the pheromone (Ideses et al., 1982). Pheromone was provided by Ecología y Protección Agrícola SL (Valencia, Spain) and dispensers were loaded with dichloromethane as solvent. For this trial, the mesoporous dispensers

were manufactured by means of an industrial process that has around 15% of variability in the initial amount of pheromone (Ecología y Protección Agrícola SL).

Delta traps and sticky bases used in the field test were supplied by Biagro (Valencia, Spain). Each trap was baited with the corresponding pheromone dispensers, as described below.

VI.2.2 Field trial

The field experiment was carried out from June to August 2009. The trial was designed as follows: four blocks of four traps were placed in a 4-ha Merlot vineyard, cultivated in trellis training. The orchard was in the center of a 16-ha vineyard area located in Fontanars dels Alforins (Valencia, Spain); (Coordinates 38º 45'N, 0º 50' E). Separation was 3 m between rows and 2 m between plants within each row. Distance between blocks was around 45 m and inter-trap distance was 50 m. Traps at each block were baited with a different pheromone dose and will be referred to hereafter as PD1 (one PD1 dispenser), 3PD1 (three PD1dispensers), PD10 (one PD10 dispenser), and 3PD10 (three PD10 dispensers). Thus, their initial pheromone load was 1, 3, 10, and 30 mg, respectively. All traps were hung at 1 m above the ground and their position inside each block was rotated weekly. None of these dispensers were replaced during the test period. The traps were placed on 2 June 2009 and the moths caught were counted weekly during 2 months. According to the results of the first weeks, it was decided to include a higher additional emission level, referred to as 3PD30, so four replicates of the trap baited with three PD30 dispensers (i.e., initial pheromone load 90 mg) were placed in the field 1 month later (24 June). Weather parameters were obtained from the nearest meteorological station located in Montesa (Valencia, Spain), at 20 km from the orchards.

VI.2.3 Pheromone emission rates

During the trial, the three types of dispensers were aged in a vineyard located more than 2 km from the catch traps. Dispensers were placed on 2 June

inside delta traps for 96 days. At different aging intervals a set of nine dispensers, three of each type, was taken to the laboratory to be analyzed.

In order to determine daily emission rates, initial pheromone loads, and the residual pheromone content of aged mesoporous dispensers were extracted in our laboratory by solvent extraction at 40 ºC for 2 h, using dichloromethane/methanol (2:3). The yield of all extractions was around 99%.

Extracts were centrifuged at 3 024 g for 8 min. The supernates were quantified by gas chromatography (GC) with flame ionization detector (GC/FID), using 1-dodecanol as internal standard. For these analyses, a Clarus 500 gas chromatograph from Perkin Elmer (Wellesley, MA, USA) was employed. All injections were made onto a ZB-5MS column (30m × 0.25mm × 0.25µm) that was held at 150 ºC for 3 min and programmed at 20 ºC min^{-1} to 170 ºC, held at 170 ºC for 4 min, and then at 35 ºC min^{-1} to 260 ºC for 2 min. Helium was used as carrier gas at 1.2 ml min^{-1} with a split flow value of 30 ml min^{-1}.

Retention time of the pheromone component was confirmed by GC/FID analysis of commercial pheromone (86% isomeric purity; >99% chemical purity), provided by Shin-Etsu Chemical (Tokyo, Japan). The pheromone amount was calculated based on the ratio between the peak areas of the pheromone component and 1-dodecanol, by means of a simple regression model.

VI.2.4 Statistical analysis

Our main goal was to study the pheromone emission effect on moth attraction and to determine the optimum emission value. First, a multiple linear regression analysis was carried out to model the evolution of residual pheromone load vs. time for each type of dispenser. The first derivative of the resulting equation provides an estimation of the daily emission rate.

Catch data were collected six times for 3PD30 traps and nine times for the others, once every week, during the trial period. The √x-transformation of the numbers caught was used to normalize the data. Following the methodology applied in a previous study (Vacas et al., 2009b), multiple linear regression (MLR)

was used to relate catch data to the emission rate, and to determine the relative maximum. The average number caught was highly variable from week to week. Therefore, polynomial terms of time were introduced as independent variables. Indicator variables were also considered in order to take into account the effect of block. This approach resulted in a rather complicated regression model. In order to obtain a simpler polynomial equation, the effect of time was removed prior to applying MLR by subtracting from each catch datum the average number of moths caught recorded in all traps at a given day. Statistical analyses were performed using the Statgraphics plus 5.1 package (StatPoint Technologies, Warrenton, VA, USA).

VI.3 Results

VI.3.1 Pheromone emission rates

The release profiles of (*E,Z*)-7,9-dodecadienyl acetate for the three types of dispensers employed in this study are shown in **Figure VI.1**. The residual pheromone load [P (µg)] was fitted by polynomial regression in the case of PD1 and PD10 dispensers. The independent variable was the number of days since dispensers were installed in the orchard [t (time)]. For PD1 dispensers, a cubic equation was obtained (equation 1), resulting in a coefficient of determination R^2 = 0.951. No outliers were identified.

$$P_{PD1} = 946.8 - 24.284 \cdot t + 0.311 \cdot t^2 - 0.001488 \cdot t^3 \quad (1)$$

A cubic equation was also obtained for PD10 dispensers. Data at t = 0 did not fit properly and they were disregarded, as well as three outliers, resulting in R^2 = 0.983 (equation 2).

$$P_{PD10} = 11605 - 281.25 \cdot t + 3.40 \cdot t^2 - 0.01511 \cdot t^3 \quad (2)$$

In the case of PD30 dispensers, the residual pheromone load follows an asymptotic trend (**Figure VI.1**) and it was fitted by means of a non-linear exponential model (equation 3; $R^2 = 0.891$).

$P_{PD30} = 19333 + 11148 \cdot \exp(-0.06367 \cdot t)$ (3)

The constant in equation 1 (946.8) coincides with the nominal load of PD1, which was close to 1000 µg. Similarly, when t = 0 in equation 3, P becomes 30481, which is consistent with the initial load of PD30 dispensers. In the case of PD10, **Figure VI.1** shows that the initial pheromone content was 10.8 mg, which is also close to the nominal value. The observed small differences are due to variability of the industrial manufacturing process.

The slope of the lines based on equations 1-3 is not constant (**Figure VI.1**), which implies that the daily emission rate of these pheromone dispensers decreases over time. This rate was estimated at day ti as the first derivative of the fitted equations, i.e., dP/dt (t = ti). Equations 4, 5, and 6 correspond to the first derivative of equations 1, 2, and 3, respectively. For example, 3PD1 traps inspected on 17 June correspond to traps collecting moths in the period of days 8-15 (i.e., t = 8 to t = 15). This trap contains three PD1 dispensers. Thus, the pheromone emission rate was estimated by applying equation 4 at t = 11.5 (i.e., the midpoint of the 8-15 period), and the resulting value was multiplied by 3. The release rate was assumed to be constant along the time interval. All estimated emission values are indicated in the **Appendix**.

$$\frac{dP_{PD1}}{dt} = -24.284 + 0.622 \cdot t - 0.004464 \cdot t^2 \quad (4),$$

$$\frac{dP_{PD10}}{dt} = -281.25 + 6.8 \cdot t - 0.04533 \cdot t^2 \quad (5)$$

$$\frac{dP_{PD30}}{dt} = -709.8 \cdot \exp(-0.06367 \cdot t) \quad (6)$$

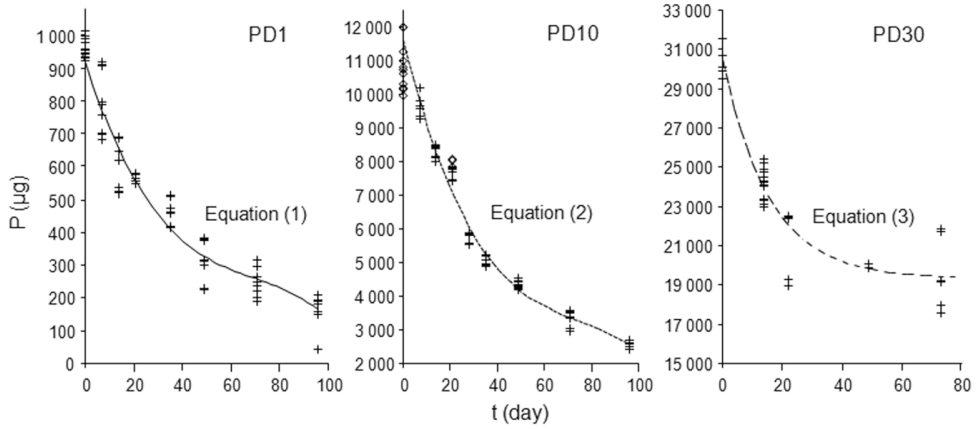

Figure VI.1 Release profiles of (E,Z)-7,9-dodecadienyl acetate, the major *Lobesia botrana* pheromone component, from the three kinds of dispensers tested. Fitted curves describe the pheromone content of the dispenser [P (µg)] vs. time (t = number of days in orchard). For equation 2, points indicated as diamonds (◊) were not taken into account to obtain the regression equation.

VI.3.2 Field trial: Trap catches

The period under study was characterized by the following average weather conditions (from June to August 2009): daily mean T = 25.8 ºC, 59% r.h., and 0.8 m/s wind speed. All traps showed population fluctuations of the pest though at different levels (**Figure VI.2**). First flight began around day 8 (10 June 2009), and the largest catches were recorded on day 22 (24 June). Second and third flights appeared on day 43 (15 July) and day 64 (5 August), respectively. These days correspond to the three flights of the moth cycle.

Most catch data recorded on 10 June and all data recorded on 18 August were null. Therefore, they were not further considered. Data of periods 43-52 and 52-57 were also rather low, 63% being zero. In order to overcome this lack of data variability, which is a problem if studying the effect of emission, both consecutive periods were merged as a single 43-57 interval (see the **Appendix**).

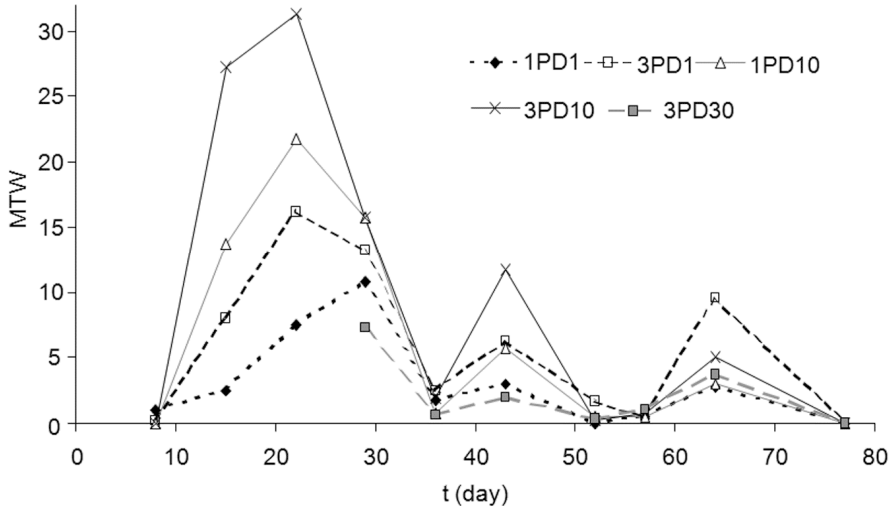

Figure VI.2 Average number of moths caught per trap and week (MTW) for each of five types of baited trap, with t the day of inspection (day 0 corresponds to 2 June 2009 at which most traps were installed). Baited traps were delta traps and dispensers were not replaced.

It was observed that the numbers caught in blocks B and D tend to be higher than in blocks A and C. Actually, by means of one-way ANOVA it was found that the square root of the numbers caught is significantly different between blocks A and C vs. B and D ($F= 8.60$; $df= 1,124$; $P= 0.004$). This result could be explained by the clumped natural distribution of grapevine moth populations (Coscollá et al., 1997; Ifoulis & Savopoulou-Soultani, 2006).

In order to properly fit the square root of the numbers caught ($\sqrt{\mathrm{Nc}}$) to time, block, and emission, it would be necessary to use indicator variables for blocks and polynomial terms of variable t, resulting in a rather complex equation. Instead, it seems preferable in this case to eliminate the effect of block and time prior to applying MLR. For data collected at blocks A and C, we calculated the difference between $\sqrt{\mathrm{Nc}}$ and $\mathrm{ASB_{AC}}$ (average square root of all catch data recorded at blocks A and C). Similarly, for data collected at blocks B and D, $\sqrt{\mathrm{Nc}} - \mathrm{ASB_{BD}}$ was calculated ($\mathrm{ASB_{BD}}$ as average square root of all catch data recorded at blocks B and D). The resulting variable $\sqrt{\mathrm{Nc}} - \mathrm{ASB}$ accounts for the

variability not explained by time or block that could be attributed to emission. Finally, a quadratic model was fitted to relate $\sqrt{\mathrm{Nc}} - \mathrm{ASB}$ to the estimated emission rates (values available in the supplementary material). Taking into account that emission values follow a positive skewed distribution, SRE (square root of emission) was regarded as the independent variable (equation 7).

$$\sqrt{\mathrm{Nc}} - \mathrm{ASB} = -0.784 + 0.129 \cdot \mathrm{SRE} - 0.00322 \cdot \mathrm{SRE}^2 \qquad (7)$$

The goodness-of-fit of equation 7 was low (R^2 = 0.142) but the regression coefficients were statistically significant (P≤0.0001). This result confirms the existence of a relative maximum of catches (**Figure IV.3**). Equation 7 was derived and equaled to zero, resulting in a square root of the optimum emission (SRE) of 19.9. Thus, the pheromone emission rate that maximizes attractant activity is: 19.9^2 = 396 µg day^{-1}.

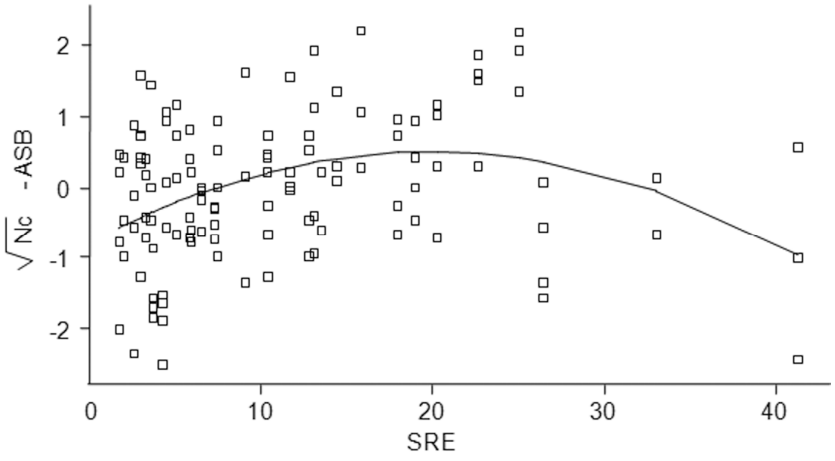

Figure VI.3 Scatter plot and fitted regression model (equation 7) of $\sqrt{\mathrm{Nc}} - \mathrm{ASB}$ vs. SRE (square root of emission). The dependent variable is the square root of numbers caught minus the average square root of catches collected at blocks A and C, or B and D (ASB).

By means of a normal probability plot, it was checked that residuals of equation 7 (i.e., observed minus predicted values) followed approximately a normal distribution and no outliers were identified. It was also found that two of the three highest data of emission act as influential points. Nonetheless, results are very similar if both data are discarded, and the quadratic term is still clearly significant (P = 0.0011). In order to study whether the effects of block and time were properly eliminated with the procedure applied prior to MLR, residuals of equation 7 were used as a dependent variable in a two-way ANOVA with factors block and time. The effect of both factors was not statistically significant (F= 0.05; df= 1,117; *P*=0.83 for block; F= 0.20; df= 6,117; *P*= 0.98 for time).

VI.4 Discussion

Although it is demonstrated that the presence of minor compounds in *L. botrana* pheromone formulations increases biological activity (Arn et al., 1988; El-Sayed et al., 1999), this work employed (*E,Z*)-7,9-dodecadienyl acetate to determine the existence of an optimum sex pheromone release rate, as it is the major pheromone component and the main compound responsible for the attraction (Roelofs et al., 1973; Ideses et al., 1982; Witzgall et al., 2005). The key factor to improve control methods based on pheromones as attractants (monitoring, mass trapping, or 'attract and kill') is to know the optimum emission rate, because insect response to the attractant could decrease below and above this optimal value (Jacobson & Beroza, 1964; Roelofs et al., 1977; Howse, 1998; Zhang & Amalin, 2005). The inhibitory effect of high pheromone doses has been reported for a number of lepidopterans (Roelofs & Cardé, 1974; Wyman, 1979; Millar et al., 1996). However, most of these works discuss insect responses based on initial pheromone loads of the dispensers, which does not give a conclusive idea about the actual release of pheromone, given that daily emission rates, and therefore the amount of airborne pheromone, will depend on dispenser type and weather conditions. The effect of pheromone dispenser type has been studied on

maize stalkborer catches: polyethylene vials loaded with 1 mg pheromone caught significantly more moths than rubber septa loaded with the same amount of ingredient (Critchley et al., 1997). Release kinetics and dispenser field performance are key factors to develop efficient formulations for dispensers, and must be known to establish the relationships between attractant power and pheromone emission.

Some studies compare catches and pheromone doses for lepidopteran pests, resulting in a variety of relationships. Leonhardt et al. (1990) tested cotton wick dispensers for gypsy moth (*Lymantria dispar* (L.)) and proposed an optimal reference release rate of 11.3 µg per day, but plastic laminate dispensers could remain highly attractive by emitting at least 0.72 µg per day. Kehat and coworkers (1994) found increasing catches of codling moth (*Cydia pomonella* (L.)) males with increasing pheromone doses, within the range of 0.1 to 100 µg, but rubber septa loaded with 5 000 µg were significantly less attractive than 100 or 1 000 µg dispensers. Similar behavior was observed for rice leaffolder moth, *Cnaphalocrocis medicinalis* (Guenée) (Kawazu et al., 2004). Vacas et al. (2009b) found decreasing catches of *Chilo suppressalis* (Walker) below and above an optimal release rate of 34 µg per day. And Jactel and coworkers (2006) found an asymptotic increase response of catches of pine processionary moth (*Thaumetopoea pytiocampa* Denis & Schiffermüller) according to increasing doses of its pheromone from 0.5 to 20 mg, with 95% of maximum catch obtained with the 10-mg dosage. This asymptotic pattern has also been observed in other Lepidoptera species (Evenden et al., 1995; Knutson et al., 1998; Rao & Subbaratnam, 1998).

Many papers have studied the effect of dispenser type and pheromone load for a variety of insect families (Mason et al., 1990; Cork et al., 2001; Franklin & Gregoir, 2001; Branco et al., 2004; Kovanci et al., 2006). However, only few studies determined the optimal release rate of attractants (de Groot & DeBarr, 1998; Cross et al., 2006; Vacas et al., 2009b). As mentioned above, catches do not always increase with increasing pheromone doses. Usually, catches increase up to an optimal dose. For higher values, trap catches could remain constant or decrease due to a repellent effect. An optimum pheromone load for *L. botrana* monitoring dispensers has been suggested by Roehrich et al. (1983), who found

that pheromone loads between 1 µg and 10 mg allowed the detection of moths. Anshelevich et al. (1994) reported that *L. botrana* males responded positively to sticky traps baited with rubber septa loaded with increasing doses from 0.1 µg to 0.1 mg pheromone, but loads of 1-10 mg caught significantly fewer moths. However, emission rates were only measured for 1-mg septa, so trap catches were not correlated with emission values and optimal release rates were not proposed. These studies only reported optimum pheromone loads, but the values cannot be adopted as a reference, because it has been demonstrated that similar initial loads in different dispenser types may result in different release rates (Leonhard et al., 1990; Domínguez-Ruiz et al., 2008). Instead, the most suitable reference value to optimize the dispenser performance would be the optimum daily release rate, as this is the actual variable responsible for the airborne pheromone acting in insect attraction. Determination of this value could be of interest to develop more effective dispensers, so that they are able to emit pheromone at the optimum level.

This trial employed different mesoporous dispensers, with pheromone loads ranging from 1 to 30 mg, to obtain the optimum daily emission rate. Release profiles of PD1 and PD10 were fitted to cubic equations, implying that their emission rates were not constant. However, their life span was at least 100 days (**Figure VI.1**) and their residual pheromone loads, at the end of the period under study, were 15% of the initial load for PD1 (equation 1, t = 100) and 22% for PD10 (equation 2, t = 100). On the other hand, the release profile of PD30 was fitted to a model (equation 3) with an asymptote at 19333 µg, which means that about 63% of its initial load was not released, and more than half of the pheromone load was wasted. This is not a suitable feature for an ideal dispenser, as pheromone accounts for 95% of the cost of the dispensers and the use of pheromone must be optimized. Thus, PD30 would need changes in its formulation or design to gain efficiency. However, the life span of PD30 dispensers was enough for the purpose of this work, which was to monitor the main flights of *L. botrana* in the study area.

This study concludes that releasing (*E,Z*)-7,9-dodecadienyl acetate, the major pheromone component of the European grapevine moth, at a rate of about 400 µg per day would maximize moth attraction under West-Mediterranean

weather conditions. Although significant, the scope of the statistical relationship found between catches and emission could be somewhat limited. It should be stressed that the field trial was carried out only in one location and the optimum release rate could be affected by environmental conditions, specially the wind, in so far as pheromone plume is modified (Murlis et al., 1992). Nevertheless, this value could be generalized to catches of *L. botrana* under the average climatic conditions required for its development in temperate Mediterranean areas. An optimum release value is, in any case, a key datum for dispenser manufacturers, as well as a tool to improve *L. botrana* management methods based on pheromones.

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We want to thank Bodegas J. Belda (Fontanars dels Alforins, Valencia, Spain) for providing test orchards and C. Colás for his invaluable field assistance.

Appendix VI Pheromone emission rates and numbers caught of *Lobesia botrana* in traps baited with pheromone dispensers

Day		Trap	Catches at each block[5]				ASB[6]		Emission	
period[1]	Date[2]	code[3]	A	C	B	D	A-C	B-D	(µg day^{-1})	proced.[7]
0-8	10 June	PD1	2	0	-	1	0.18	0.29		
		3PD1	0	0	1	0	0.18	0.29		
		PD10	0	0	0	0	0.18	0.29		
		3PD10	0	0	0	0	0.18	0.29		
8-15	17 June	PD1	1	0	5	4	2.54	3.90	18	$(4)_{t=11.5}$
		3PD1	4	5	10	13	2.54	3.90	53	$3\cdot(4)_{t=11.5}$
		PD10	15	8	16	16	2.54	3.90	209	$(5)_{t=11.5}$
		3PD10	15	20	37	37	2.54	3.90	627	$3\cdot(5)_{t=11.5}$
15-22	24 June	PD1	1	4	12	13	2.87	5.20	14	$(4)_{t=18.5}$
		3PD1	8	5	25	27	2.87	5.20	43	$3\cdot(4)_{t=18.5}$
		PD10	23	6	40	18	2.87	5.20	171	$(5)_{t=18.5}$
		3PD10	10	20	50	45	2.87	5.20	513	$3\cdot(5)_{t=18.5}$
22-29	1 July	PD1	8	4	19	12	2.44	4.18	11	$(4)_{t=25.5}$
		3PD1	3	4	21	25	2.44	4.18	34	$3\cdot(4)_{t=25.5}$
		PD10	7	6	33	17	2.44	4.18	137	$(5)_{t=25.5}$
		3PD10	13	3	20	27	2.44	4.18	412	$3\cdot(5)_{t=25.5}$
		3PD30[4]	-	9	10	3	2.44	4.18	1704	$3\cdot(6)_{t=3.5}$
29-36	8 July	PD1	1	2	0	4	0.67	1.28	9	$(4)_{t=32.5}$
		3PD1	0	2	6	2	0.67	1.28	26	$3\cdot(4)_{t=32.5}$
		PD10	0	2	0	1	0.67	1.28	108	$(5)_{t=32.5}$

		3PD10	2	0	5	1	0.67	1.28	324	$3\cdot(5)_{t=32.5}$
		3PD30	0	0	-	2	0.67	1.28	1091	$3\cdot(6)_{t=10.5}$
36-43	15 July	PD1	0	5	1	6	2.37	1.58	7	$(4)_{t=39.5}$
		3PD1	6	11	7	1	2.37	1.58	20	$3\cdot(4)_{t=39.5}$
		PD10	1	16	3	3	2.37	1.58	83	$(5)_{t=39.5}$
		3PD10	7	21	-	7	2.37	1.58	250	$3\cdot(5)_{t=39.5}$
		3PD30	6	1	1	0	2.37	1.58	699	$3\cdot(6)_{t=17.5}$
43-57	29 July	PD1	0	0	2	0	0.48	1.00	4	$(4)_{t=50}$
		3PD1	0	0	6	1	0.48	1.00	13	$3\cdot(4)_{t=50}$
		PD10	2	1	0	1	0.48	1.00	55	$(5)_{t=50}$
		3PD10	0	1	0	3	0.48	1.00	164	$3\cdot(5)_{t=50}$
		3PD30	0	2	2	1	0.48	1.00	358	$3\cdot(6)_{t=28}$
57-64	5 Aug	PD1	1	5	5	0	1.78	2.03	3	$(4)_{t=60.5}$
		3PD1	-	-	13	6	1.78	2.03	9	$3\cdot(4)_{t=60.5}$
		PD10	4	1	5	2	1.78	2.03	36	$(5)_{t=60.5}$
		3PD10	5	4	5	6	1.78	2.03	107	$3\cdot(5)_{t=60.5}$
		3PD30	-	4	5	2	1.78	2.03	183	$3\cdot(6)_{t=38.5}$
64-77	18 Aug	(all traps)	0	0	0	0	0	0		

[1]Day 0 corresponds to 2 June 2009, when all traps (except 3PD30) were installed.
[2]Date at which traps were inspected for counting.
[3]Initial pheromone load: 1 mg (PD1), 3 mg (3PD1), 10 mg (PD10), 30 mg (3PD10), and 90 mg (3PD30).
[4]Traps 3PD30 were set up on 24 June.
[5]No. moths caught. Missing data are marked as '-'.
[6]Average of the square root of catches recorded at blocks A and C, or B and D.
[7]Procedure used to calculate emission values (see text for a detailed explanation). The equation used is indicated within parentheses, and t is the median number of days in orchard.

Capítulo VII

"Response of two tephritid species, *Bactrocera oleae* and *Ceratitis capitata*, to different emission levels of pheromone and parapheromone"

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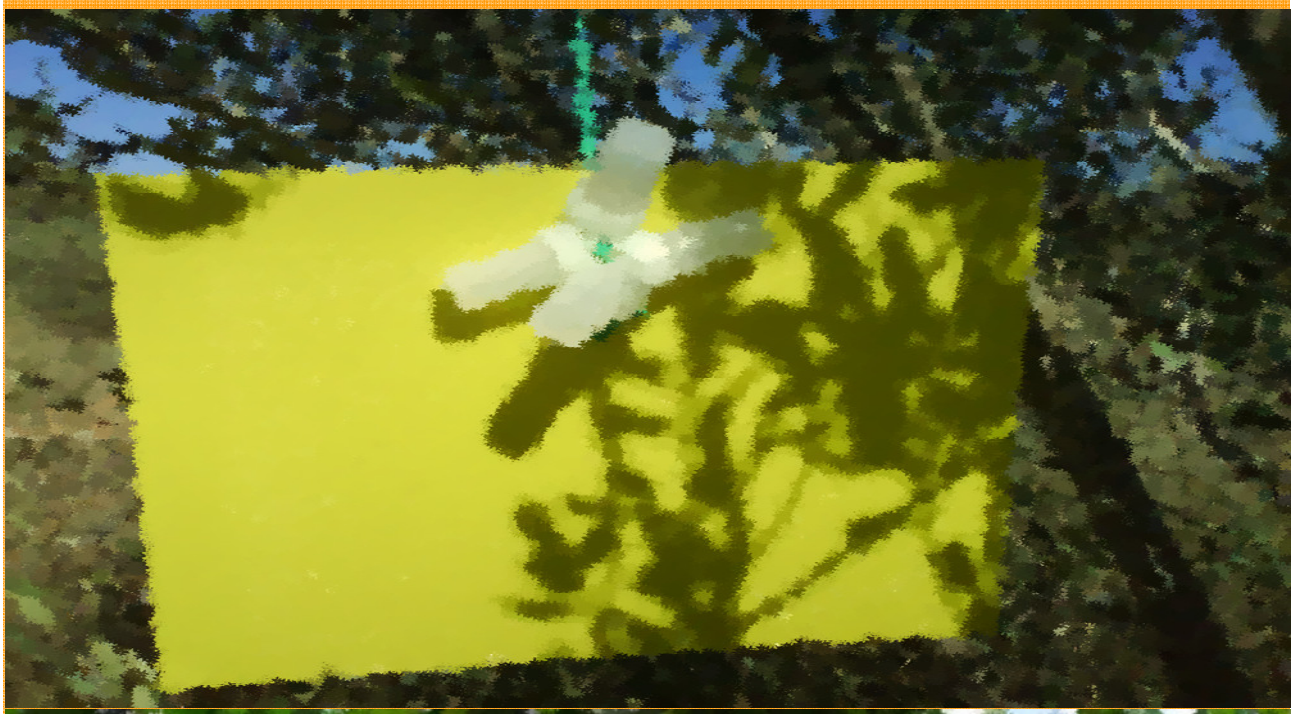

Response of two tephritid species, *Bactrocera oleae* and *Ceratitis capitata*, to different emission levels of pheromone and parapheromone

Published in Crop Protection (Navarro-Llopis V., C. Alfaro, J. Primo and S. Vacas. 2011)*

Abstract. Attractants and pheromones are commonly used in integrated pest management programs in crop systems. However, pheromone dispensers employed in monitoring traps and lure and kill devices are not usually well studied and attractants are released at uncontrolled rates leading to low treatment efficacies and misleading monitoring estimations. Fruit flies are pests of economic importance and monitoring is essential in order to program insecticidal treatments. Moreover, lure and kill techniques are being increasingly used, but the cost of these techniques depends on the number of required traps and, therefore, on the efficacy of the attractants. *Ceratitis capitata* and *Bactrocera oleae* are the two main fruit flies in Mediterranean countries, and the effect of different doses of trimedlure and spiroacetal on fly attraction has been studied. Results showed that a release rate over 1.28 mg/day of spiroacetal reduces *B. oleae* attraction and emission values over 2.4 mg of trimedlure per day did not increase *C. capitata* catches. Under the environmental conditions of our study, an optimum release rate for pheromone attraction in *B. oleae* was determined. Emission values over this optimum level reduced *B. oleae* attraction. However, when a parapheromone was used with *C. capitata*, a fruit fly of the same family, the optimum emission value was not found and higher quantities of parapheromone attracted the same number of flies. The saturation effect of high concentrations of pheromone and parapheromone is discussed.

VII.1 Introduction

Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) and Olive fruit fly, *Bactrocera oleae* (Rossi) are two of the major pests affecting Mediterranean agriculture. *B. oleae* is a monophagous fruit fly which appears in several countries where *Olea europaea* L. is grown. It is endemic in the Mediterranean area but in recent decades it has invaded the olive growing areas in California and Mexico. It is also found in South and Central Africa, Pakistan and the Middle East (Daane and Johnson, 2010; Nardi et al., 2005). Females of the olive fruit fly are the only tephritid females known to produce a sex pheromone, with 1,7-dioxaspiro[5.5]undecane (hereafter spiroacetal) as its major component (Baker et al., 1980; Jones et al., 1983; Haniotakis et al., 1994). Male olive flies also produce this compound, attracting other males, but females are not attracted to spiroacetal emitted from either sex (Haniotakis et al., 1994). In order to follow olive fly populations, two types of traps baited with two kinds of substances are used. McPhail or Olipe traps baited with a mixture of ammonium bicarbonate or ammonium diphosphate are employed for female monitoring. Meanwhile, sticky boards baited with spiroacetal are used for male flight monitoring. In general, males are monitored in the Mediterranean area between May and December when mating activity takes place (Jones et al., 1983).

C. capitata is a polyphagous pest in temperate climates all around the world, with the exception of central and East Asia, developing 4 to 10 generations per year depending on the latitude. The *C. capitata* female pheromone to attract males is not known. Males are responsible for female attraction (Landolt et al., 1992) and much research aimed at finding new substances released by medfly males to attract females has been undertaken. (Jacobson et al., 1973; Ohinata et al., 1977; Baker et al, 1985; Jang et al., 1989). A study relating the sexual behavior of *C. capitata* described how the male raises the tip of his abdomen and emits a long-distance attractant pheromone before mounting the female (Briceño &

Eberhard, 2002). The composition of male pheromone emissions is still under study, and recent works have provided lists which contain the composition of aeration samples of calling males (Gonçalves et al., 2006; Alfaro et al., 2011). In spite of these efforts, full pheromonal attractiveness is not achieved in field tests with blends using major constituents of these pheromone emissions (Light et al., 1999). This would mean that minor components could be having a key effect, and that, therefore, the high complexity of this pheromonal blend makes it very difficult to find an effective mixture.

Trimedlure (TML) was described in 1964 as an attractant for Mediterranean fruit fly (Beroza et al., 1964). TML is a sex-specific attractant widely used in detection and monitoring programs around the world. Ceralure, an iodinated analog of TML was developed by Mcgovern et al. (1988) and later, studies demonstrated that the (-) enantiomer of ceralure, B1, was more effective than trimedlure as a male attractant (Jang et al., 2003). Both trimedlure and ceralure are parapheromones, and no disruption effects have been described although they have been extensively used as male attractants.

Aside from mating disruption, attractants and pheromones have been used in “attract and kill”, chemosterilant (Navarro-Llopis et al., 2007, 2010) or infective devices, but mass trapping is the most widespread technique (El-Sayed et al., 2009). For both fruit flies, the efficacy of this technique depends on the type of trap and the attractant. There are many experiences about the design and color of traps for tephritids (Epsky et al., 1995; Cornelius et al., 1999; Navarro-Llopis et al., 2008) but there are only a few studies comparing pheromone release rate and insect catches (Landolt & Heath, 1990; Domínguez-Ruiz et al., 2008; Suckling et al., 2008). Many studies have compared catches among several types or loads of dispensers for other insect families (Cork et al., 2000; Franklin & Gregoire, 2001; Kovanci et al., 2006) but only a few determined the optimal release rate of attractants in field trials (de Groot & DeBarr, 1998; Cross et al., 2006; Vacas et al., 2009b).

The optimal pheromone release rate is not well known in most cases. Some pheromone release threshold values to achieve mating disruption depending

on the target pest can be found in the literature (Ioratti et al., 2004; de Lame & Gut, 2006; Stelinski et al., 2007). The key to improve control methods based on pheromones as attractants (mass trapping or monitoring) is to know the optimal emission rate, because catches could decrease below and above this optimal value (Jacobson & Beroza, 1964; Roelofs & Cardé, 1977; Zhang & Amalin, 2005). However, there are not many conclusive studies on this subject. The aim of our study was to obtain the optimal release rate which maximizes the efficacy of an attractant for the control of fruit flies. For this purpose, four levels of pheromone emission were compared using traps baited with a different number of standard commercial pheromone dispensers. The efficacy of each trap was measured in field trials as the number of fruit fly catches. These four emission levels have been correlated with field captures to evaluate the existence of an optimal emission rate for both spiroacetal and TML.

VII.2 Material and methods

VII.2.1 Olive fruit fly

VII.2.1.1 Traps and pheromones

Olive fruit flies were captured using yellow sticky traps made of a polyvinyl chloride (PVC) yellow plastic sheet measuring 220 x 175 mm, and coated on both sides with non-drying glue (provided by Biagro SL ,Valencia, Spain).

For the pheromone release study, polyethylene vials (PD) were selected, with a specification of 80 mg (a.i.) for spiroacetal, and were provided by Aragro SA (Madrid, Spain). These dispensers were chosen due to their release pattern, which is constant over time. This allows the study of fly catches over a period of several weeks with a minimum variation of release rate. Pheromone dispensers were inserted in the center of the sticky trap.

VII.2.1.2 Field trial

A field trial was carried out in a 5 ha 10 year old *O. europaea* orchard with trees spaced at 8 by 10 m (125 trees ha^{-1}), located in Betera (Valencia, Spain). The

orchard was divided into four plots to study the effect of four different emission levels in four blocks, placing four traps in a row inside each block. Therefore, each block contained four traps baited with 1, 2, 3 or 4 spiroacetal dispensers. The separation distance between traps was 32 m within each block and their position was randomized in the first plot. The position of traps in the rest of the plots was designed to avoid having traps with the same number of dispensers in the same position. The separation between plots was at least 40 m. Catches were recorded and traps were rotated within each plot every week from October to December 2009.

VII.2.2 Mediterranean fruit fly

VII.2.2.1 Traps and pheromones

Mosquisan® traps were used for trapping and were provided by Sansan Prodesing SL (Valencia, Spain). This trap was evaluated and described by Navarro-Llopis et al. (2008). Pheromone dispensers Zentinel Ceca® were provided by Ecología y Protección Agrícola SL (Valencia, Spain). The dispensers were mesoporous (MD) cylindrical tablets containing 1.8 g of TML, which were described in Domínguez-Ruiz et al (2008). In that study, the quantification of isomers was included. Each trap contained the corresponding number of TML dispensers and a 500 mg dichlorvos strip from Agrisense BCS Ltd. (Pontypridd, UK).

VII.2.2.2 Field trial

The field trial was carried out between July and October 2009, when both *C. capitata* population was high enough to obtain a representative number of catches, and citrus fruits begin to ripen. Traps were placed in a 6 ha, 20 year old citrus grove (*Citrus reticulata* Blanco, variety Marisol), with trees spaced at 6 by 4 m (420 trees ha^{-1}), located in Sagunto, near Valencia on the east coast of Spain. The field trial included four plots to study the effect of different TML emission levels. Plots were arranged following the same experimental design as for *B. oleae* trial. Traps with 1, 2, 4 or 6 TML dispensers were placed in each plot. Traps were at separations of 30 m both to avoid direct interaction between traps and natural

spatial population variation. Separation between plots was at least 100 m. *C. capitata* catches were recorded every week, and traps were rotated within each block.

VII.2.3 Release rates

For release rate measurement, 40 dispensers of each type were aged in the same areas, 500 m away from the trial orchards. TML dispensers were aged inside the same Mosquisan® traps and spiroacetal dispensers were attached to sticky traps as in the monitoring traps. Both traps were hung from trees and four aged dispensers were transferred weekly to the laboratory. Residual pheromone was extracted at 0, 5, 7, 10, 14, 21, 28, 35, 42, 49, 55 and 62 days of aging from spiroacetal dispensers and 0, 15, 23, 38, 52, 67 and 82 days for TML dispensers. Three dispensers of spiroacetal and TML for each aging time were extracted. The extraction method used for spiroacetal dispensers was solvent extraction using the Extraction Unit Soxtec® 2043 System (Rose Scientific Ltd. Alberta, Canada). Extraction conditions consisted of 2 hours of extracting followed by 2 hours of washing at 50 ºC with dichloromethane. The TML content from dispensers was obtained by regular solvent extraction. Each dispenser was placed inside a 100 ml tube with a magnet and 50 ml of dichloromethane. The extraction procedure used magnetic agitation at 500 r.p.m. during 2 hours, after which extracts were centrifuged at 3000 r.p.m for 8 min.

Spiroacetal and TML were quantified by gas chromatography with flame ionization detector (GC/FID), using 1-dodecanol and 3-octanol as internal standards respectively. All injections were made in a Clarus500 gas chromatograph from PerkinElmer Inc. (Wellesley, MA) using a ZB-5 column (30 m × 0.25 mm i.d. × 0.25 µm; Phenomenex Inc., Torrance, CA). The carrier gas was helium at 1.2 ml min^{-1}. Retention times of TML and spiroacetal were confirmed with commercial standards provided by Ecología y Protección Agrícola (Valencia, Spain).

VII.2.4 Statistical analysis

Multiple linear regressions were applied to study the evolution of residual pheromone and parapheromone loads versus time for each type of dispenser. To determine whether the emission was constant during the time under study, the significance of the quadratic effect was checked.

The main aim of this work was to study the correlation of the factor emission level with trap catches. For this purpose, variability of catch data due to other factors was first studied by applying a multifactor analysis of variance (ANOVA) to evaluate the effect of date and block factors on the data. The square-root transformation of the number of catches was used to normalize the data. The part of the variability not explained by these two factors will remain on the residuals of the ANOVA. These residuals were used in a subsequent multiple regression analysis to study the effect of factor emission, with four levels for *B. oleae* (1 PD, 2 PD, 3 PD and 4 PD) and *C. capitata* (1 MD, 2, MD, 4 MD and 6 MD). Then, the existence of a relative maximum was investigated.

This methodology had already been applied in Vacas et al (2009b). Statistical analyses were performed using the Statgraphics plus 5.1 package (StatPoint Technologies, Warrenton, VA, USA).

VII.3. Results

VII.3.1 Release Rates

Figures VII.1 and **VII.2** show the evolution of the remaining load of spiroacetal and TML, respectively, versus time for the commercial dispensers. The residual spiroacetal and TML loads were fitted by simple linear regression, resulting in $R^2 = 0.999$ for spiroacetal and $R^2 = 0.918$ for TML. In the case of TML, it was observed that data at day = 0 appeared as outliers, which is explained by the

higher emission rate during the first days until the MD dispenser reaches equilibrium with the environment within one or two weeks. This condition did not exist in the spiroacetal PD dispensers, as they were of a different type of dispenser. Probably, the TML contained in the surface of the MD was released faster. However, the pheromone is inside the polyethylene tube in the PD, not impregnated on the surface, so spiroacetal must pass through the polyethylene walls to be released. Release rate of TML dispensers was not constant during the first two weeks, thus data for the first 15 days were discarded for TML dispensers. Quadratic effect was not significant for spiroacetal and TML release rates (P = 0.07 and P = 0.09, respectively), so it was assumed that the residual pheromone load decreased at a constant rate over the studied period. Therefore, the emission rates for each dispenser were the slopes of their respective linear model. To obtain a better estimation of the coefficients, a multiple linear model was fitted using the type of dispenser as an indicator variable. The resulting slopes were 2.36 mg day^{-1} for the TML dispenser and 0.46 mg day^{-1} for the spiroacetal dispenser.

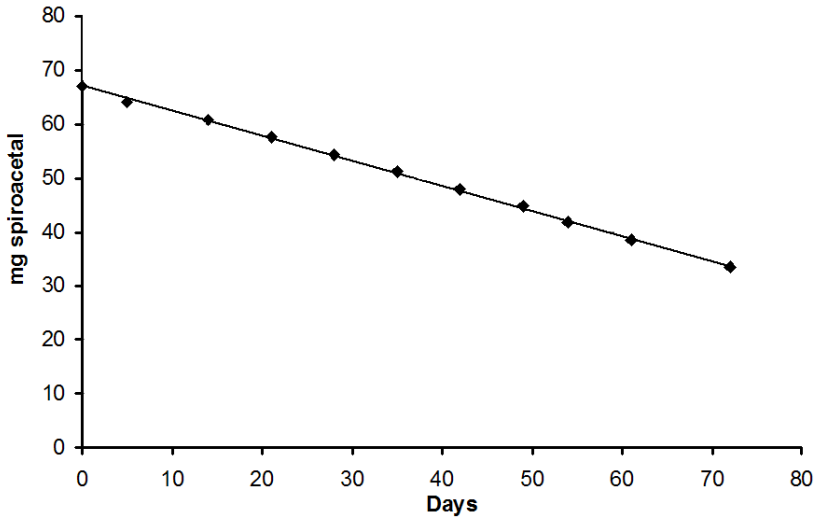

Figure VII.1 Release dynamics of spiroacetal from commercial polyethylene dispensers. Significance of the simple linear regression model was R^2=0.999.

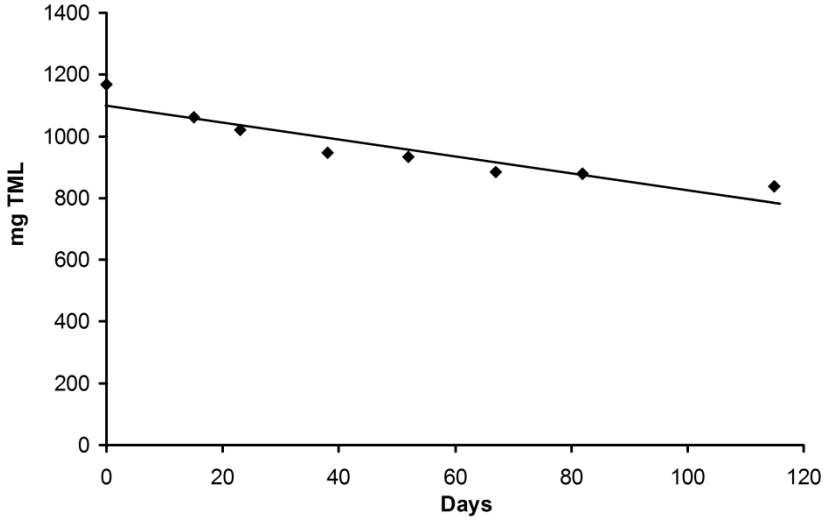

Figure VII.2 Release dynamics of trimedlure from commercial mesoporous dispensers. Significance of the simple linear regression model was R^2=0.918.

VII.3.2 Field Trial

VII.3.2.1 Trap Catches

Figures VII.3 and **VII.4** show the average number of catches obtained in traps baited with spiroacetal and TML commercial dispensers respectively. For spiroacetal dispensers, the olive fly catches went down significantly from the 4th week to the end of the trial (**Figure VII.3**). Therefore, only data from the first three weeks were considered for statistical analysis because such low values do not give sufficiently reliable information to study the effects of block or emission.

Only the catches retrieved from to 3rd to 12th week (**Figure VII.4**) were considered to simplify *C. capitata* statistical analysis because, as mentioned above, TML emission was not constant during the first two weeks of the trial.

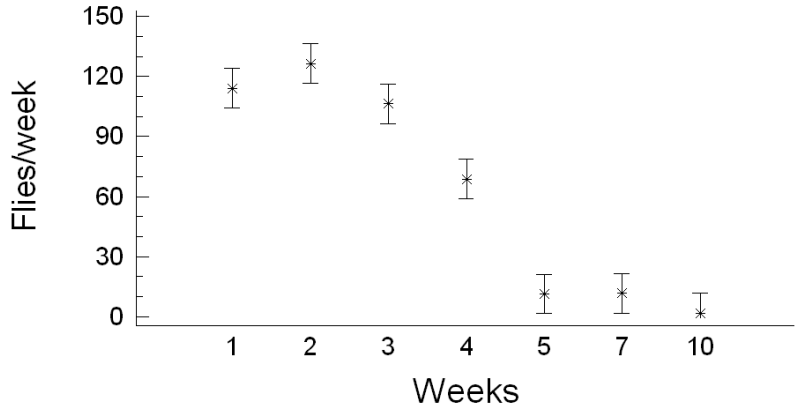

Figure VII.3 Average Olive fruit fly catches per trap per week obtained in yellow PVC sticky boards baited with commercial spiroacetal dispensers.

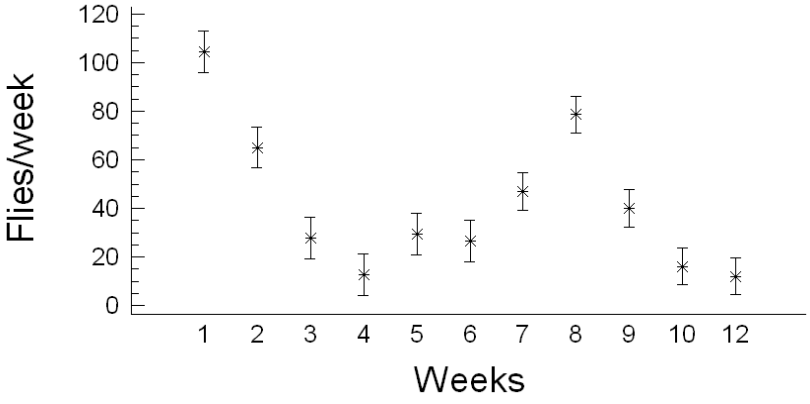

Figure VII.4 Average Mediterranean fruit fly catches per trap per week obtained in Moskisan® traps baited with mesoporous TML dispensers.

VII.3.2.2 Olive fruit fly.

Day factor showed statistical significance (P<0.001): captures of weeks 1st to 3rd were significantly higher than the rest because this period corresponds to the maximum pest population (**Figure VII.3**). The block effect was also statistically significant (P<0.001): the average number of catches in the two central blocks was significantly lower than in the two external blocks. This result could be explained by the barrier effect of the traps described for other tephritids (Cohen & Yuval, 2000).

Emission also showed statistical significance *(*P=0.036). The means plot and LSD intervals for this factor (**Figure VII.5**) show that the highest release rate, emitted from traps baited with 4 PD, captured significantly less than traps baited with 3 PD. This result suggested that attractant efficacy decreases above a particular emission value. Taking into account that the estimated emission rate of the PD dispensers was 0.46 mg day^{-1}, the emission factor was considered as a quantitative variable according to this correspondence: 1 PD = 0.46 mg day^{-1}, 2 PD = 0.92 mg day^{-1}, 3 PD = 1.38 mg day^{-1} and 4 PD = 1.84 mg day^{-1}.

The residuals of the previous ANOVA analysis were used to perform a multiple regression analysis to study the linear and quadratic effect of emission, obtaining the equation 1:

$$Residuals = -2.13\ x^2 + 5.48\ x - 2.91 \qquad (1)$$

The coefficient of the quadratic term in this equation was statistically significant (P=0.03), which reflects a curvature in the model and confirms the existence of an optimum value of emission that maximizes attractant activity. To obtain this value, equation 1 was derived and equaled to zero, resulting in an optimum of 1.28 mg day^{-1}. **Figure VII.5** shows the curvature that best fits the 4 mean values of captures according to the emission rate.

VII.3.2.3 Mediterranean fruit fly

Day factor was statistically significant (P<0.001), probably due to natural population dynamics and to the natural dispersion of *C. capitata*. Captures during the first two weeks were significantly higher than the rest because this period corresponds to the maximum pest population (**Figure VII.4**). The effect of block factor was also statistically significant (P<0.001) and, as in the *B. oleae* study, the average number of medfly catches in the two central blocks was significantly lower than in the two external blocks.

Emission was not statistically significant (P=0.53). Figure 6 shows that the trap baited with 1 MD captured more flies than the others, but these differences were not significant. This result suggests the lack of a maximum attraction value, so the attractant power does not decrease above a particular release value. Taking into account that the estimated emission rate of the MD dispensers was 2.36 mg day^{-1}, the emission factor was considered as a quantitative variable according to this correspondence: 1 MD = 2.36 mg day^{-1}, 2 MD = 4.72 mg day^{-1}, 4 MD = 9.44 mg day^{-1} and 6 MD = 14.16 mg day^{-1}. **Figure VII.6** was obtained using the same methodology as for *B. oleae*. In this figure, no significant reduction of *C. capitata* catches could be observed by increasing the number of TML dispensers (F=1.26; df=140,3; P=0.29). In addition, polynomial regression shows that lineal and quadratic effects are not significant (P= 0.24 and P= 0.35 respectively).

In the case of medfly, the attractant power did not decrease above a particular emission value. The minimum tested concentration was 2.36 mg day^{-1} because it was established as an optimum concentration for *C. capitata* attraction (Domínguez-Ruiz et al., 2008). Although this release rate was multiplied by 6, significant differences among emission levels were not detected.

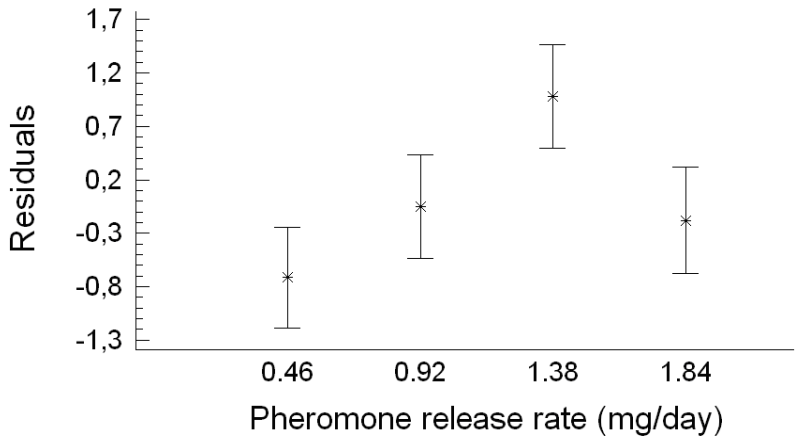

Figure VII.5 Captures of *B. oleae* and 95% LSD intervals corresponding to factor emission for spiroacetal release rates. Curve represents the quadratic model that best fits the mean values of captures according to emission rates. Significance of quadratic term was P=0.03.

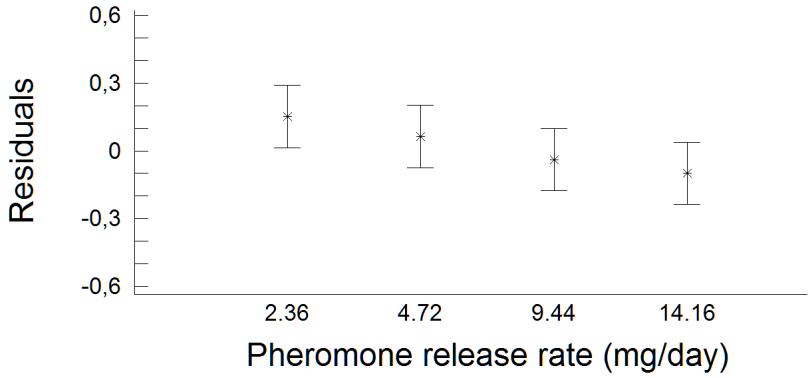

Figure VII.6 Captures of *C. capitata* and 95% LSD intervals corresponding to factor emission for trimedlure release rates. Interval overlapping indicates the lack of a maximum attraction value. Polynomial regression shows that lineal and quadratic effect are not significant (P=0.24 and P=0.35 respectively).

VII.4 Discussion

Different correlations of field trap catches with pheromone release rates have been obtained in this work. *C. capitata* response reached a maximum with a release rate near 2 mg day^{-1} of its parapheromone TML and increasing emission levels did not increase or reduce fly catches. By contrast, *B. oleae* attraction to traps baited with spiroacetal increased up to a limit, with decreasing responses to the highest emission level.

Haniotakis & Pittara (1994) found in laboratory tests that *B. oleae* male responses changed according to different pheromone loads. In this study, the maximum male response was obtained with an intermediate dose of filter paper dispensers releasing 0.59 mg h^{-1} of 1,7-dioxaspiro [5.5] undecane, which probed the existence of an optimal release level. In addition, it was observed that response to the optimum pheromone concentration decreased significantly after 1 min of continuous exposure to the pheromone which means a saturation effect. However, actual release rates and the responses of flies in the field were not measured in this work. There are some studies showing a maximum attraction at a particular pheromone release rate, in Lepidoptera (Vacas et al., 2009b), Coleoptera (Obengofori, 1990; Franklin & Gregoire, 2001; Cross et al., 2006) or other Diptera (Michaelakis et al., 2007). The importance of the determination of optimum pheromone release rates has already been shown in research by Landolt & Heath (1988, 1990), testing behavioral responses of the tephritid female Papaya fruit fly, *Toxotrypana curvicaudata* Gerstaecker. In laboratory bioassays, they found increasing responses to increasing pheromone release rates up to a determined emission value. Later, they confirmed this result in field trials and provided an optimum rate of 960 ng h^{-1} (Landolt & Heath, 1990). The present study supports the existence of these optimum release values in some tephritid species. Specifically, an optimum pheromone release value of 1.28 mg day^{-1} was determined for the attraction of *B. oleae* in the West-Mediterranean conditions in the two months before harvesting. This value may vary under different

environmental conditions, so more replications in different locations should be carried out in order to study variations in this optimum.

Currently, monitoring of olive fruit fly males is carried out in Spain with a pheromone dispenser in the top or in the middle of yellow sticky boards. These dispensers are usually polyethylene vials or rubber septa with loads of pheromone ranging from 1 to 80 mg, having lifespans from 40 to 120 days. These different loads indicate variability in release rates and therefore a variable estimation of the fruit fly population. This would mean that olive fly populations could be over or underestimated depending on the dispenser used. Thus, the type of pheromone dispenser must be taken into account to calculate treatment thresholds. In addition, if manufacturers change dispenser formulations, the calculation of treatment threshold would result in vain. The optimum pheromone release rate, maximizing *B. oleae* captures, has been established by correlating pheromone release rates and field fly catches. This information improves trapping efficiency, so that monitoring results can be optimized and so that fly populations can be studied with maximum sensibility. Moreover, this optimum value will be useful in studies correlating fruit damage with trap catches without dependence on the type of dispenser.

The use of optimum release values is very important to test the actual potential or efficacy of mass trapping programs. Up to now, many mass trapping field trials have been carried out using commercial monitoring dispensers, without information about the attractant release rate (Haniotakis et al., 1991; Broumas et al., 2002). On the other hand, it must be mentioned that release rates are influenced by the type of trap used in the tests (Jones et al., 1983). Therefore, the optimum emission value obtained in this work is only useful for sticky boards and should be recalculated for McPhail, delta or Jackson traps, as aeration of the attractant might be different.

Another factor affecting fly catches is the physiological state of the insects. The field trials which formed part of this research were conducted over the main part of the season for these pests; that is two months before citrus harvesting in *C. capitata* trial and one month before olive harvesting in *B. oleae*. During these

periods of the year, fruit fly populations are usually monitored to determine whether population thresholds are exceeded and if insecticidal treatments should be applied. Moreover, the maturation of eggs and oviposition of *B. oleae* females is taking place during this period when also maximum male catches are detected. (Torres-Vila et al, 2006).

As stated in introduction, *B. oleae* females are the only tephritid known with a well-defined pheromone. In this way, a mating disruption technique could be applied, in principle. This study supports this idea because a decrease of fly catches was detected with high pheromone concentrations. This means that olive fruit flies cannot find the source of odor when pheromone concentration is very high and this is the basis of mating disruption. However, mating disruption experiences carried out against *B.oleae* have obtained inconclusive results in Spain and Greece (Montiel et al, 1982; Montiel and Jones, 2002).

Regarding *C. capitata*, the disruption effect was not observed when different emission levels were tested for the parapheromone TML. The highest catches were obtained with dispensers releasing TML around 2 mg day^{-1}. But fly catches did not significantly decrease even increasing TML release rate more than 6 times. This lack of saturation in response to higher pheromone concentrations could explain why TML do not produce flight disruption in *C. capitata*.

The effect of parapheromones on target insects is very similar to the effect of pheromones, but the specificity of parapheromones is lower. In many cases, substances such as cuelure or methyl eugenol attract many organisms of the same genus, unlike pheromones which are species-specific. Moreover, parapheromones cause a stimulus in the sensilia of the insect which could be as powerful as pheromones, but in most cases this stimulus stops after a few seconds. In the case of pheromones, this effect is very strong in the sensilia and the stimulus could remain much longer giving a high EAD signal. This means that pheromone specificity is very high and the joint place of the pheromone to the receptor can be saturated, whereas parapheromone receptors do not become saturated (Quero et al, 2004). It could explain the existence of an optimum dose for pheromones, as

higher doses saturate the receptors, whereas with parapheromones the emission rate could be increased without saturating the receptors.

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Capítulo VIII

"Solid phase microextraction of volatile emissions of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae): influence of fly sex, age and mating status"

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Solid phase microextraction of volatile emissions of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae): Influence of fly sex, age and mating status

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Abstract. Considerable efforts have been devoted to understanding the courtship behavior and pheromone communication of medflies, however, the sex pheromone composition is still a controversial subject. The discovery of new components affecting medfly behavior would be of interest for medfly control methods based on semiochemicals. This work describes volatile compounds emitted by *Ceratitis capitata* collected using solid phase microextraction. The volatile study was conducted according to an experimental design with three factors (sex, age and mating status) assumed to be relevant for better understanding the chemical communication. Emission data were treated by means of Principal Components Analysis, a statistical methodology not previously applied to the study of volatiles emitted by fruit flies. The characterization of emission patterns could be useful for the selection of compounds to be further investigated in biological assays in order to improve knowledge of the key semiochemicals involved in medfly behavior.

VIII.1 Introduction

The pheromone communication system of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) is complex and not well established. The existence of compounds released by males attracting females of *C. capitata* was reported by Feron (1962) several decades ago and since then, a large amount of research has been conducted on the sexual behavior of *C. capitata* (for review, see Eberhard, 2000). In order to allow the encounter between sexually active males and females, the male raises the tip of its abdomen and emits a long-distance attractant pheromone as a first stage before mounting the female (Briceño, 1996).

One decade after Feron's discovery, Jacobson et al. (1973) described the sex pheromone of *C. capitata* as a mixture of 15 substances, including some carboxylic acids and other compounds including methyl-(*E*)-6-nonenoate and (*E*)-6-nonen-1-ol. Ohinata et al. (1977) reported that these mixtures were attractive to both male and female medflies in laboratory tests, but only attracted males in field experiments. In a subsequent study, these authors developed several formulations of methyl-(*E*)-6-nonenoate which appeared to be as attractive as Trimedlure in outdoor tests (Ohinata et al., 1979). Later, Baker et al. (1985) reported 9 components in the mixture of volatile substances released by male *C. capitata,* including the identification of the cyclic imine 3,4-dihydro-2*H*-pyrrole (1-pyrroline) as a key compound involved in the sexual attraction. Jang et al. (1989) detected 69 compounds from male headspace analysis. In the same work, female headspace samples showed very little material, mainly short chain aldehydes, only at trace levels. Later, Flath et al. revised, in 1993, the composition of virgin male medfly volatiles, and the differences in abundance of the major compounds according to two factors: age and time of day. These reported works (Jang et al., 1989; Flath et al., 1993) agree with the three major components present in male samples: ethyl-(*E*)-3-octenoate, geranyl acetate and (*E,E*)-α-farnesene, and many efforts have been conducted to formulate these key compounds for field testing (Heath et al., 1991; Flath et al., 1993;.Jang et al., 1994; Jang et al., 1996; Light et al, 1999).

However, full pheromonal attractiveness is not achieved with mixtures of the major constituents, probably because of the role of minor components (Light et al., 1999). More recently, Gonçalves et al. (2006) published another list containing the composition of aeration samples of calling males collected in Tenax tubes. It confirmed several compounds of Jang's work and added some others. The reported evidence reveals that the formulation of attractants is a complex problem.

The main aim of this work was to provide the qualitative description of medfly volatile profiles at different stages and to characterize groups of compounds according to their emission pattern. This research presents three novel contributions with respect to similar studies targeted at better understanding volatile compounds emitted by medflies. Firstly, volatiles were collected by solid phase micro-extraction (SPME), a solvent-free sample preparation technique that integrates sampling, isolation and concentration (Ouyang & Pawliszyn, 2008). Since the pioneering work of Pawliszyn who introduced SPME in 1990 (Pawliszyn, 1990), this technique has found application in food (Jelen, 2006; Pontes et al., 2007; Ouyang & Pawliszyn, 2008; Ferreira et al., 2009; Pontes et al, 2009), pesticides (Menezes Filho et al., 2010) and environmental fields (Ouyang & Pawliszyn, 2006, 2007a, 2007b). SPME-gas chromatography (GC) was first applied in 1995 as an alternative method for the study of airborne insect pheromones (Malosse et al., 1995), and since then, SPME has been employed in sampling of insect volatiles, particularly in beetles (Said et al., 2003; Cai et al., 2007; Yasui et al., 2008; Arakaki et al., 2009). SPME has also been recently employed in the identification of chemicals emitted by *Anastrepha serpentine* (Robacker et al., 2009), but its use in medfly volatile collection has not yet been reported, as far as we know. Secondly, the volatile study reported here was conducted according to an experimental design with three key factors (sex, age and mating status) that are assumed to be relevant for better understanding the chemical communication between insects belonging to the same species. This is a novel contribution because three-factorial experimental designs are rarely used in similar studies. Finally, the emission data were analyzed by means of Principal Components Analysis (PCA), which is a statistical methodology rather

unconventional in fruit flies research. This multivariate method has been extensively employed for studies on food volatiles (Jabalpurwala et al., 2009; Elaissi et al., 2010; Giri et al., 2010; Mildner-Szkudlarz & Jelen; 2010; Zhang et al., 2010). But regarding to insects, PCA has been employed only to study the volatiles of Hymenopteran species (Steiner et al., 2007; Coppee et al., 2008). Thus, the present work is the first reported statistical analysis of tephritid volatile profiles using PCA. This study contributes with new substances to the list of volatiles emitted by *C. capitata* and characterizes the emission patterns of medfly volatile constituents according to sex, age and mating status.

VIII.2 Material and methods

VIII.2.1 Insects

Flies were obtained from the mass-rearing colony of the Centro de Ecología Química Agrícola, located at the Universidad Politécnica de Valencia (Valencia, Spain). Larvae were reared on a standard wheat, sugar and yeast diet. Recently emerged flies were separated by sex in one group of 300 males and another group of 300 females in different cages, in order to keep virgin flies for volatile sampling. Flies were held at 24ºC, 60-80% relative humidity, and 16L:8D photoperiod (same as conventional rearing conditions), with ample food and water, until use. Each group of 300 medflies was split into 6 sets of 50. Three sets were taken out from the cage when flies were 3 days old, and the remaining 3 sets consisted of 9-d old flies. Each set was placed inside independent glass chambers for the volatile sampling as described below. The same experiment was repeated with mated males and females, which were obtained from the main mixed cage.

Thus, the experimental design to study volatile compounds emitted by medflies consisted of three factors at two levels: sex (male or female), mating status (virgin or mated) and age (3 or 9 days old). The resulting 8 experimental cases will be referred to hereafter as: virgin 3d-old male (vm3d), virgin 9d-old male (vm9d), mated 3d-old male (mm3d), mated 9d-old male (mm9d), virgin 3d-old

female (vf3d), virgin 9d-old female (vf9d), mated 3d-old female (mf3d), and mated 9d-old female (mf9d). Three replicates of each case were performed, resulting 24 experimental trials (i.e., 24 sets of 50 medflies).

VIII.2.2 Collection of volatiles

The three replicates of each experimental case were simultaneously collected by SPME using three SupelcoTM SPME holders equipped with a polydimethylsiloxane/divinyl benzene fiber (PDMS/DVB; film thickness 100 µm; Supelco Inc., Bellefonte, PA, USA). Each set of 50 medflies was placed inside a 1.3 l glass chamber with a screw-top polytetrafluorethylene (PTFE)-silicone septum cap (Supelco Inc., Bellefonte, PA, USA) in the middle. The density of flies was determined according to desired sensitivity and previous experiences testing 25, 50 and 90 flies per chamber (data not shown). This density is consistent with works using SPME to collect volatiles from other fruit fly (Robacker et al., 2009) and it is in the same order of magnitude than other works with medflies (Jang et al., 1989).

Each chamber was connected to the outlet of an air compressor (Jun-air Intl. A/S, Norresundby, Denmark) coupled with an air purifier system AZ 2020 (Claind Srl, Lenno, Italy) which guarantees the use of ultra-pure air, with a total hydrocarbons amount lower than 0.1 ppm. Between the air compressor and each chamber, a digital flowmeter ELL-FLOW® (Bronkhorst High-Tech BV, Ruurlo, Holland) was installed, according to the method described in a previous study (Alfaro et al., 2008). Each SPME needle was inserted through the septum, and the fibers were exposed to each sample headspace by running 25 ml min^{-1} (the flow rate was measured using flow meters at the inlet and outlet ends) purified air flow rate for 24 h (16L:8D) at 25 ± 1ºC. Each fiber was then removed from the chamber and inserted into the GC injection port to desorb volatiles. Usually, volatiles were collected from 2:00 (p.m.) to 2:00 (p.m.) of the next day. Three “blank” controls were sampled under the same conditions in order to rule out compounds which were not emitted by flies. The presence of other external contaminants from the running air is unlikely due to the use of an ultra-purified air flow through the chamber.

SPME fibers were conditioned before medfly sampling in a GC injector at 250ºC for 15 min with a N_2 flow rate of 30 ml min^{-1}. In order to allow the comparison among samples, the same three fibers were used during all the analyses so that one fiber was associated to each replicate.

VIII.2.3 Detection and identification of volatiles

SPME fibers were thermally desorbed into the GC injector for one min. The injector temperature was set at 250ºC, and it was operated in splitless mode. The extracted medfly volatiles were analyzed by gas chromatography coupled with mass spectrometry (GC-MS) using a Clarus 500 GC-MS from Perkin Elmer Inc. (Wellesley, PA, USA), equipped with a ZB-5-fused silica capillary column (30 m x 0.25 mm i.d. x 0.25 µm; Phenomenex Inc., Torrance, CA, USA). Oven was held at 40ºC for 2 min, then programmed at 5ºC min^{-1} up to 180ºC, and when reached, it was raised to 210ºC at 15ºC min^{-1} and maintained at 210ºC for one min. Helium was used as the carrier gas with a flow of 1.2 ml min^{-1}.

The detection was performed in EI mode (ionization energy 70 eV; source temperature, 180ºC). The spectra acquisition was performed in scanning mode (mass range m/z 35-300 amu). Chromatograms and spectra were recorded by means of GC-MS Turbomass software version 5.1 (Perkin Elmer Inc., Wellesley, PA, USA).

Compounds were identified by comparing their retention times and mass spectra with those of pure standards (confirmed identification), which were purchased from Sigma-Aldrich Química SA (Madrid, Spain), except for ethyl (*E*)-3-octenoate, which was provided by Givaudan Suisse SA (Vernier, Switzerland). The purity of these compounds was >98% by GC-MS, except for farnesene and ocimene which were mixtures of their respective isomers. Nonetheless, it was not possible to obtain the commercial standards of some compounds, so their identification was based on high probability matches (>80%) according to NIST mass spectral library, version 2.0 (Thermo Electron Corporation, Waltham, MA, USA).

To confirm the identification of 1-pyrroline, this compound was prepared by impregnation of sepiolite (Tolsa SA, Madrid, Spain) with its precursor 4-aminobutyraldehyde diethyl acetal (Sigma-Aldrich Química SA, Madrid, Spain) in dichloromethane solution. Sepiolite is a clay mineral with slight acid pH, which leads to the formation of 1-pyrroline as volatile product after impregnation with 4-aminobutyraldehyde diethyl acetal (Yoshikawa et al., 1965; Struve & Christophersen, 2003). Impregnated sepiolite was introduced in 22 ml screw-capped vials (Perkin Elmer Inc., Wellesley, USA) sealed with a PTFE-silicone septum (Supelco Inc., Bellefonte, PA, USA) and a headspace-SPME sample of the volatile products was collected with a PDMS/DVB fiber. Then, it was injected onto GC-MS to obtain retention time and spectral data of 1-pyrroline.

VIII.2.4 Statistical analysis

In total, 70 compounds were reported. For each one of the 24 experimental trials, the peak areas of all compounds were integrated, including both identified and unidentified compounds. The resulting data were arranged as a matrix containing 24 rows, each one corresponding to one trial, by 70 variables (i.e. chemical compounds) in columns. In this dataset, the zero value was assigned to those compounds not detected in a given trial. It was checked that area values followed approximately a log-normal distribution. The minimum value of peak area was around 10^3, the median was ~10^6 and the maximum was ~10^{10}. Therefore, for compounds detected at trace levels, below the integration threshold, we used 100 as area value which is one log-unit below the minimum integrated area.

Attempting to normalize the data distribution, area values were transformed by applying the quadratic root transformation (i.e., $x^{0.25}$), resulting a dataset of emission profiles that will be referred to hereafter as emission matrix. This transformation has advantages for ANOVA and for the multivariate analysis conducted in this study. Actually, ANOVA assumes a normal distribution of the residuals. We checked that this hypothesis was better satisfied by applying the quadratic root transformation prior to the analysis.

The emission matrix was analyzed using PCA in order to understand the similarities and dissimilarities among variables. Principal components (PCs) are directions of maximum data variance obtained as linear combinations of the original variables. The projections of observations (i.e. experimental trials in this case) over the direction determined by the first principal component (PC1) are called t[1] scores; t[2] are the projections over PC2, and so on. The contributions of variables (compounds) in the formation of a given component are called loadings, being p[1] the loadings in the formation of PC1; p[2], the loadings of PC2, etc. (Wold et al., 1987). A scatter plot of the scores corresponding to two different PCs is referred to as score plot. Observations appearing close to each other in the score plot will correspond to experimental cases with a similar volatile profile. A scatterplot of the loadings corresponding to two different components is referred to as loading plot. PCA is sometimes considered a bilinear model because score plots and loading plots provide complementary information.

Conducting a 3-way ANOVA with each compound becomes complex due to the high number of volatiles. Thus, it was decided to use PCA as a preliminary method in order to obtain clusters of compounds with a similar emission pattern. Three different PCA models were obtained: one with the emission matrix, the second selecting data from male flies, and a third using female's data. Variables were autoscaled (i.e. mean-centered and scaled to unit variance) prior to the PCA, which is the common pretreatment when the variance is rather different among variables, as it is the case here. The analysis was carried out using the software SIMCA-P 10.0 (Umetrics AB, Malmö, Sweden). Once identified the basic clusters by means of PCA, one compound was then selected from each cluster and, next, a 3-way ANOVA was applied using the software Statgrapics plus 5.1 (StatPoint Technologies Inc., Warrenton, VA, USA) in order to study in detail the emission pattern of these representative compounds according to sex, age and mating status.

VIII.3 Results and Discussion

VIII.3.1 Overview of identified compounds

Table VII.1 lists the 70 detected compounds emitted by *C. capitata* and not detected in blank experiments, showing their retention times. Thirty five of them were confirmed with commercial standard, and 17 were tentatively identified. The rest unknown volatiles were minor components, being less than 1% of the total released compounds, except c22 which represents about 3% of the total compounds emitted by vm3d, being also abundant in young females. All compounds were detected in males whereas only 37 were found in female volatile profile.

Twenty five of the 70 compounds detected in our study have also been reported in similar works aimed at identifying volatiles from male medflies (literature references indicated in **Table VIII.1**). Mavraganis et al. (2008) made extracts of the whole body of male medflies and identified some already reported constituents, as well as cis-geraniol and decanal, apart from other compounds never described before. These previous works employed analytical techniques based on headspace, thermal desorption and solvent extraction, what could explain the differences in medfly volatile profiles. Volatile collection techniques have been compared by some authors (Cavalli et al., 2003; González-Mas et al., 2009), demonstrating that the technique employed has influence on the kind of compounds which were collected and identified.

New medfly volatiles, not previously reported in the literature, were identified in the present study, which confirms that SPME is adequate to investigate insect volatile compounds as reported for other species (Cai et al., 2007; Arakaki et al., 2009; Robacker et al., 2009).

The percentage of each compound with respect to the total integrated peaks was calculated to compare the emission among compounds in the same sample, in order to identify the major compounds. The main constituents of male volatile sampling were geranyl acetate, (*E,E*)-α-farnesene and ethyl (3*E*)-3-

octenoate, which is consistent with the results of similar studies (Baker et al., 1985; Jang et al., 1989; Flath et al., 1993; Light et al., 1999), but our analysis added 2-ethylhexanoic acid to these main components. Jang et al. (1989) reported that the only compounds attributable to the presence of females were some short-chain aldehydes at trace levels. By contrast, we have identified acetophenone, decanal, trimethylamine and 2-ethylhexanoic acid as the most abundant compounds released by female medflies.

Some compounds identified in the present study have not been previously described as being part of the volatile constituents of *C. capitata*. These include acetophenone, geranic acid, methyl-eugenol and 2-piperidone. Specifically, acetophenone has been described as a volatile component emitted by females of the *Dendroctonus* spp. (Pureswaran & Borden, 2004), as active component of the oviposition pheromone of *Schistocerca gregaria* (Rai et al., 1997) and even as an attractant to the *C. capitata* parasitoid *Diachasmimorpha longicaudata* (Rohrig et al., 2008). On the other hand, it was unexpected to find methyl-eugenol on medfly volatile profiles, as it is known as highly attractive kairomone for many *Bactrocera* species, and it is widely used in male annihilation to eradicate *Bactrocera dorsalis* pest (Vargas et al., 2000; OIEA, 2005). In addition, methyl-eugenol also occurs naturally in numerous essential oils (Poucher, 1974). These factors might suggest a matter of contamination. However, this was not likely to occur, as this compound was not previously employed in our laboratory and it was not detected in the blank samples. If methyl-eugenol had been a contaminant, it would have appeared randomly. However, methyl-eugenol was detected in all samples from males.

The present study did not detect some compounds reported by other authors, but most of them are minor compounds. Only one major and one intermediate compounds, ethyl acetate and ethyl (*E*)-3-hexenoate, respectively, described by Jang et al. (1989) have not been detected in this work. This fact could be explained by the different technique applied for volatile collection, as mentioned above, or other experimental factors (e.g. sampling duration or air-flow) as pointed out by other authors (Flath et al., 1993). Actually, taking into account that this kind of experiences were carried out with *C. capitata* mass-rearing colonies, the rearing

conditions could also have influence on the detected compounds. Volatiles were collected in 24 hours period, thus, some detected compounds could not be directly related to chemical communication processes and different results might have been obtained using a shorter time. Other authors have exactly determined the time of day when virgin males call (Jang et al., 1989; Wong et al., 1978), but the aim of this study was to describe the maximum number of compounds and to check differences in the emission regarding to the three studied factors. Therefore, the 24 hours period was chosen attempting to detect minor components and to achieve the maximum sensitivity. If sampling periods are limited to the calling males, compounds possibly correlated with other behavioral processes would be missed, such as oviposition, repellency, etc.

Table VIII.1 Compounds detected in the experiments of medfly emissions according to sex, age and mating status

Clu.[a]	code[b]	RT[c]	Id.[d]	Compound	Lite.[e]
A	c17	13.78	C	nonanal	*8,9,41*
A	c24	16.83	C	decanal	*41*
A	c43	22.44	T	longifolene	
B1	c6	7.46	C	2,5-dimethylpyrazine	*14*
B1	c12	12.44	C	acetophenone	
B1	c18	13.95	C	phenyl ethyl alcohol	
B2	c15	13.12	C	α,α-dimethylbenzyl alcohol	
B2	c19	14.33	C	2-ethylhexanoic acid	
C	c9	10.22	C	β-myrcene	*8,9*
C	c46	24.04	C	1-dodecanol	
C	c61	27.76	T	6-phenylundecane	
D1	c4	5.59	C	3-methylbutanoic acid	*5,6,41*
D1	c5	5.97	C	2-methylbutanoic acid	*41*
D2	c10	11.73	C	(*E*)-β-ocimene	*8,9,14*
D2	c11	12.06	C	(*Z*)-β-ocimene	*8,9*
E1	c14	12.93	C	3-ethyl-2,5-dimethylpyrazine	*7,8,10*
E1	c31	19.68		Unknown	
E2	c8	10.02	C	hexanoic acid	*4,5,9*
E2	c16	13.68	C	linalool	*7-10,14,41*
E2	c23	16.68	C	ethyl (*E*)-3-octenoate	*7-10,14*
F	c20	14.98		Unknown	
F	c22	16.35		Unknown	
F	c35	20.85	T	2-methyl-2,2-dimethyl-1-(2-hydroxy-1-methylethyl) propyl ester of 2-methylpropanoic acid	
F	c38	21.44	T	3-Hydroxy-2,2,4-trimethylpentyl ester of 2-methylpropanoic acid	
F	c54	25.77	T	4-phenyldecane	
F	c62	27.85	T	5-phenylundecane	
F	c64	28.07	T	4-phenylundecane	
G	c1	0.20	C	trimethylamine	*8*
G	c7	8.52	C	α-methyl-ɣ-butyrolactone	*7-10,41*
H	c2	1.55	C	1-pyrroline	*7-9*
H	c13	12.69	C	2-hexenoic acid	*7,9,10,41*
H	c33	20.10	C	methyl geranate	*8,9*
H	c39	21.65	C	geranyl acetate	*7-10 ,14*
I1	c27	18.35		Unknown	
I1	c28	18.60	C	α-citral	
I1	c40	21.93	T	7-methylindole	
I1	c44	23.60	C	β-farnesene	*8*
I1	c45	23.98	C	geranyl propionate	*8,14*
I1	c47	24.30		Unknown	

I1	c53	25.68		Unknown	
I1	c55	25.89		Unknown	
I1	c56	25.96		Unknown	
I1	c58	27.37		Unknown	
I1	c59	27.48	T	lanceol	
I1	c60	27.63		Unknown	
I1	c42	22.44	T	p-mentha-2,8-dien-1-yl acetate	
I1	c48	24.38	T	2,6,6-trimethyl bicyclo [3.1.1]hept-2-en-4-yl acetate	
I1	c66	28.36		Unknown	
I1	c67	28.65		Unknown	
I1	c68	29.01	T	(*Z*)-α-bergamotene	*14*
I1	c69	29.25		Unknown	
I2	c49	24.54	C	(Z,E)-α-farnesene	9
I2	c52	25.54		Unknown	
I3	c30	19.12	C	indole	*8,9,14,41*
I3	c32	19.85		Unknown	
I3	c50	24.88	C	(*E,E*)-α-farnesene	*7-10,14,41*
J	c21	15.92	C	2-piperidone	
J	c36	21.02	C	geranic acid	
J	c37	21.17	T	hydroxylinalool	*41*
K	c3	4.01	C	4-methyl-3-penten-2-one	
K	c41	22.12	C	methyleugenol	
K	c57	27.03	T	α-patchoulene	
K	c70	29.91	T	6-phenyldodecane	
-	c25	17.10	C	2-phenoxyethanol	
-	c26	18.20	C	cis-geraniol	*41*
-	c29	18.86	T	Dicyclopentenyl alcohol	
-	c34	20.63		Unknown	
-	c51	25.20	T	α-panasinsen	
-	c63	27,87		Unknown	
-	c65	28.20		Unknown	

[a]Cluster assigned to the compound according to Figure 2 and Figure 4. Seven compounds were not included in any cluster.

[b]Compound code as it appears in Figures 2, 3, 4, and 5, which was assigned by increasing values of retention time (code 1 for the compound with least retention time and 70 for the highest value).

[c]Retention time, in min.

[d]Identification: confirmed by means of a commercial standard (C) or tentative (T).

[e]Literature cites where the compound was described in medfly (numbers according to reference list).

VI.3.2 Data pretreatment

PCA was applied to the emission matrix that contains the integrated peak areas from GC after applying the quadratic root transformation, which allows the comparison of a given compound among samples given that constant sampling conditions were used for all experimental trials.

The purpose of this work was not the quantitative determination, as SPME is a non-quantitative analytical technique (Serrano et al., 2009; Alonso et al., 2009), unless a proper calibration is conducted for each compound. Actually, the amount of each compound trapped in the fiber is not directly proportional to its concentration in the experimental glass chamber given that it also depends on the affinity of the compound to be adsorbed on the fiber. The purpose was to study the emission pattern for a given compound according to the three experimental factors. Therefore, compounds clustered together mean that their emission pattern is similar among insect stages, regardless of the concentration.

VI.3.4 PCA: Score plots

The score plot for PC1 and PC2 corresponding to the emission matrix (**Figure VIII.1a**) highlights the relationships among samples. The three replicates of each case are located close to each other. Taking into account that each replicate corresponds to a different fiber, this result suggests that the performance of the three employed PDMS/DVB fibers was similar. This means that the same type of fiber, from the same manufacturer, gives similar results, which justifies the direct data comparison of replicates.

PC1 explains 74.5% of the total data variability, while PC2 accounts for 13.1% (**Table VIII.2**). PC3 is also relevant according to the cross-validation criterion (Q^2>Q^2 limit). These results were consistent with the fact that data were obtained from a 3-factorial design of experiments. **Figure VIII.1a** shows that PC1 discriminates the 24 observations according to sex, which indicates the existence of substantial differences in the volatile profiles of males versus females. However, vm3d and mf9d appear next to each other in the plot, somewhat intermediate of

males and females, and determine an independent direction of data variability which is PC2. This result indicates that the emission profile of virgin males at the age of 3 days is similar to the profile of mated females at 9-d old, which seems rather surprising because their sex, age and mating status are completely different.

Taking into account that PC1 discriminates male's versus female's data, it was decided to conduct a new PCA model for each sex. Regarding to males, although vm9d is very close to mm3d and mm9d in **Figure VIII.1a**, **Figure VIII.1b** suggests that the volatile profile of vm9d is somewhat different to that of mated males. These differences are subtle because PC2 of male's PCA is determined by vm9d, and this PC satisfies the eigenvalue criterion (λ>1) but not clearly the cross-validation criterion given that Q^2=0.0935 ~ 0.104 (**Table VIII.2**).

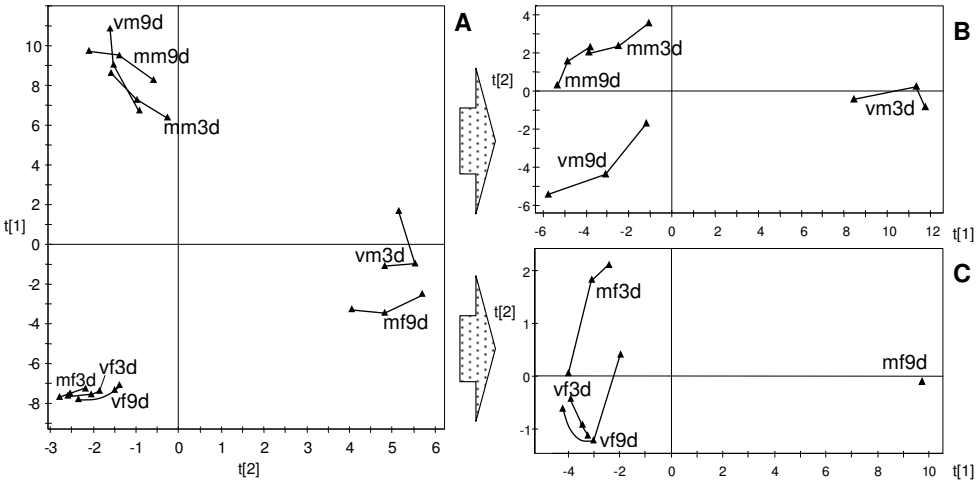

Figure VIII.1. Score plot (t[1] vs. t[2]) for the first and second principal components obtained from the emission matrix (**a**). The score plot in **b** (t[2] vs. t[1]) corresponds to the PCA using male's observations. The score plot in **c** (t[2] vs. t[1]) was obtained with female's data. Prior to the analyses, data (i.e. peak area values) were transformed using the quadratic root and autoscaled. Observation codes: first letter, male (m) or female (f); second letter, virgin (v) or mated (m); number, 3-d old (3d) or 9-d old (9d). The three replicates of each experimental case are joined with solid lines.

In the case of females, the emission profile changes considerably for mated females at 9 days of age. The differences among mf3d, vf3d and vf9d were more relevant because PC2 of female's data (Q^2=0.354 > 0.115) significantly discriminates mf3d versus virgin females (**Figure VIII.1c**). All the 37 compounds released by females were detected in mf9d whereas only 15 were detected in vf3d, 18 in vf9d and 26 in mf3d.

Table VIII.2. Summary overview of the four principal components (PC) obtained from the emission matrix[a]

Observations	PC	R^2_x	λ	Q^2	Q^2 limit
All	1	0.745	17.9	0.729	0.055
All	2	0.131	3.15	0.443	0.057
All	3	0.038	0.90	0.067	0.059
All	4	0.018	0.43	-0.218	0.061
Male's	1	0.621	7.46	0.517	0.096
Male's	2	0.109	1.31	0.094	0.104
Male's	3	0.064	0.77	-0.075	0.113
Female's	1	0.940	11.3	0.933	0.107
Female's	2	0.030	0.36	0.354	0.115
Female's	3	0.013	0.15	-0.006	0.125

[a]Variance explained (R^2_x), eigenvalue (λ), variance explained by cross-validation (Q^2) and threshold value (Q^2 limit). Results are also indicated for the PCA carried out using male's observations, and for the PCA using female's data. Values were autoscaled prior to PCA.

VI.3.5 PCA: Loading plots

Many variables in **Figure VIII.2** have positive loadings in PC1 (i.e., p[1]>0), which indicates that the amount of volatile compounds emitted by male medflies was considerably higher than females, as it was described by Jang et al. (1994). Compounds below the dashed line were emitted only by males while those above the line were emitted by both sexes, with only 4 exceptions (c9, c26, c29, and c65). Taking into account the correspondence between **Figure VIII.1a** and **Figure VIII.2**, compounds with high p[2] values will be those preferentially emitted by vm3d and mf9d. Regarding female's PCA, the loading plot (**Figure VIII.3**) suggests that most compounds emitted by females followed a similar pattern, so PC1 accounts for 94% of the total variance. Taking into account **Figure VIII.1c**, this result implies that the highest values of emission corresponded to mf9d. The variability of p[2] loadings corresponds to the slight different profile between mf3d and virgin females.

By contrast, the loading plot obtained using male's observations (**Figure VIII.4**) results far more scattered, which indicates differences in the emission profile according to age and mating status. The comparison of **Figure VIII.1b** and **Figure VIII.4** suggests that the emission of compounds below the lower dashed line in **Figure VIII.4** is significantly higher in virgin than in mated males ($P<0.05$) and the opposite applies to compounds above the upper dashed line (one-way ANOVA considering mating status as factor and using the quadratic root transformation).

Given that PC1 and PC2 of the three PCA models summarize the relevant information of the emission matrix, compounds close to each other in **Figures VIII.2**, **VIII.3** and **VIII.4** will have a similar emission profile. However, it does not imply similar area values, as discussed above, because the scale was corrected in the data treatment applied prior to PCA.

The task of clustering was simplified by finding sets of compounds close to each other in **Figure VIII.2** and **Figure VIII.4**, which makes sense assuming that the pattern of all compounds emitted by females was rather similar. Using this criterion, 5 clusters were established for compounds emitted only by males (C, J,

K, I_1 and I_2) and 11 clusters for volatiles emitted by both sexes (A, B, D, E, F, G, H and I_3). Cluster B was split in two subsets, B_1 and B_2, which are next to each other in **Figure VIII.2** but not so much in **Figure VIII.4**. Clusters D and E were also divided in two subgroups. Cluster I in **Figure VIII.4** contains 23 compounds and, together with c27 but excluding c26, it was subdivided as I_1, I_2 and I_3 in **Figure VIII.2**. The different emission pattern of each cluster was characterized by means of ANOVA as described next

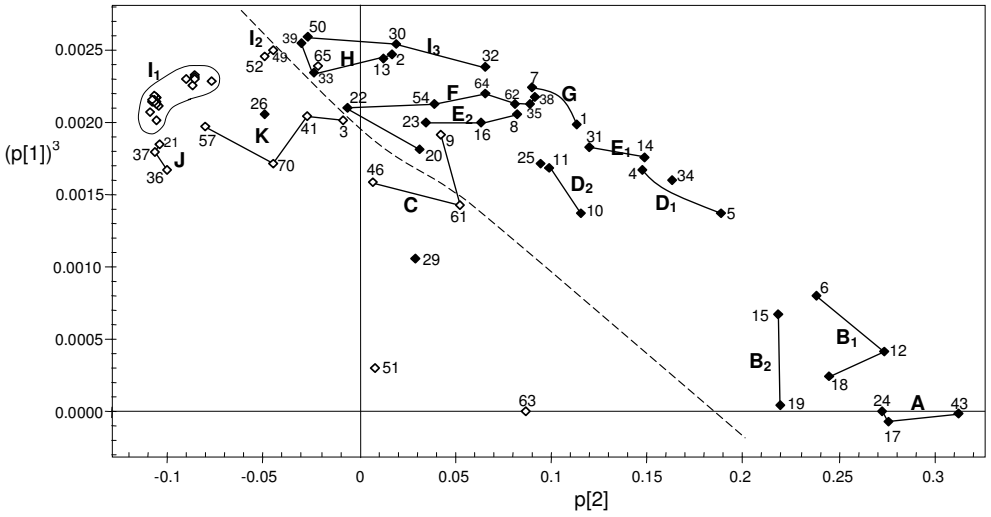

Figure VIII.2 Loading plot ($p[1]^3$ vs. p[2]) for the PCA carried out with the emission matrix, corresponding to the score plot in **Figure VIII.1A**. Filled diamonds are compounds emitted by male and female medflies (codes as indicated in **Table VIII.1**). White diamonds are compounds emitted only by males, most of which appear below the dashed line. Cluster I_1 contains 18 compounds (codes: 27, 28, 40, 42, 44, 45, 47, 48, 53, 55, 56, 58, 59, 60, 66, 67, 68 and 69). Sets of compounds appearing close to each other in **Figure VIII.2** and **Figure VIII.4** are joined with solid lines and labeled with boldface letters.

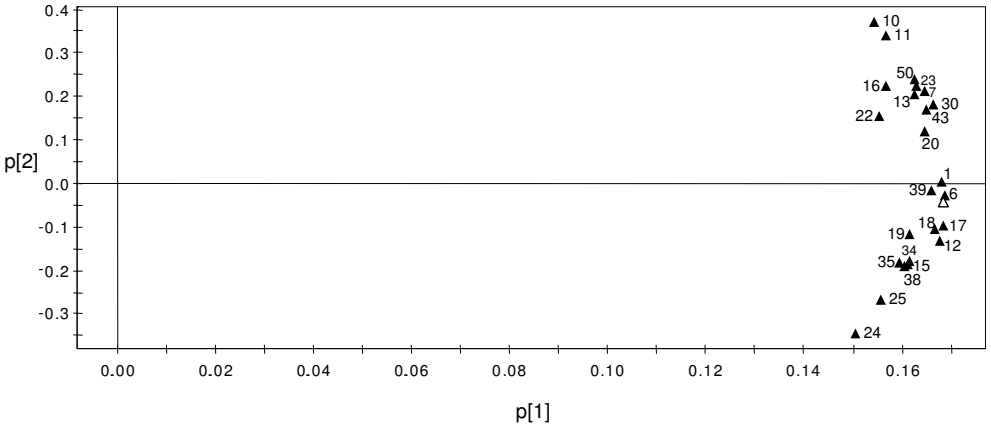

Figure VIII.3 Loading plot (p[2] vs. p[1]) for the PCA carried out using female's observations, corresponding to the score plot in **Figure VIII.1C**. The white triangle next to c6 accounts for 13 compounds (codes 2, 4, 5, 8, 14, 26, 29, 31, 32, 33, 54, 62, 64) which were only emitted by mated females at 9 days of age.

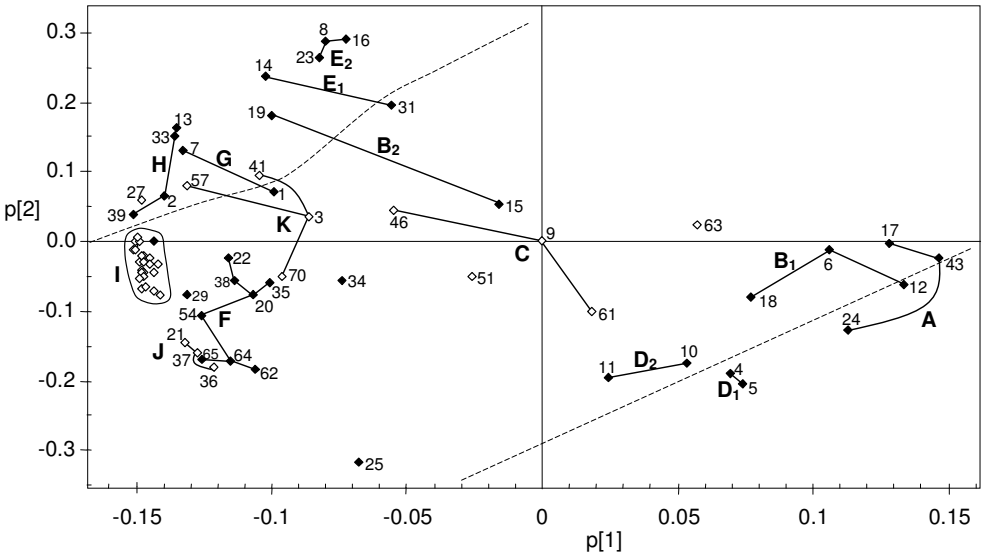

Figure VIII.4. Loading plot (p[2] vs. p[1]) for the PCA carried out using male's observations, corresponding to the score plot in **Figure VIII.1B**. Filled diamonds are compounds emitted by male and female medflies. White diamonds are compounds emitted only by males. Cluster I contains 23 volatiles (codes 26, 28, 30, 32, 40, 42, 44, 45, 47, 48, 49, 50, 52, 53, 55, 56, 58, 59, 60, 66, 67, 68, 69). Clusters highlighted by solid lines and labeled with boldface letters are the same as in **Figure VIII.2** except I (I ∪ c27 in Fig. 4 ≡ $I_1 \cup I_2 \cup I_3 \cup$ c26 in **Figure VIII.2**). The emission of compounds below the lower dashed line was significantly higher in virgin than in mated male medflies (P<0.05), while the opposite applies to those above the upper dashed line.

VI.3.6 ANOVA results

Data from a 3-factorial design of experiments are usually analyzed by means of a 3-way ANOVA in order to study the simple effect of each factor as well as the interactions. In this case, the triple interaction resulted statistically significant ($P<0.01$) in 47 of the 70 compounds, which becomes complex due to the high number of variables. In order to ease the interpretation of interactions, a new factor called "age×status" was created with 4 variants: virgin 3-d old (v3), virgin 9-d old (v9), mated 3-d old (m3), and mated 9-d old (m9). Next, a two-way ANOVA was conducted with age×status and sex. The interaction plot of both factors was checked for each compound because it characterizes the emission pattern. Following this procedure, it was observed that compounds from the same cluster present a similar pattern, and one of them was selected as a representative of the cluster. Thus, 16 interaction plots were obtained for the 16 clusters, 10 of which are depicted in **Figure VIII.5**. The remaining 6 plots (B_2, C, D_2, E_1, G and I_2) are not shown because their emission pattern can be deduced from the others taking into account the relative position of clusters in **Figure VIII.2** and **Figure VIII.4**.

In the lower part of **Figure VIII.5** (from left to right: I_3, F, D_1, B_1, A), mf9d was the case with highest emission among female medflies, which was comparable to values emitted by one of the four male cases. By contrast, the emission pattern of males follows a changing trend from cluster A to I_3 (**Figure VIII.5**). The highest emission in A and B_1 corresponded to vm3d. Cluster A comprises nonanal, decanal and longifolene, basically emitted by vm3d and mf9d. Cluster B was established with 5 compounds appearing close to each other in **Figure VIII.2** (B_1: c6, c12, c18; B_2: c15, c19), which suggests that they share a common emission pattern. Nonetheless, c15 and c19 were regarded as a different subset (B_2) because are characterized by a higher emission of mated males, while the opposite applies to B_1.

Cluster D is characterized by an emission of virgin males higher than in mated males. These differences are statistically significant ($P<0.05$) only in methyl butanoic acids (D_1). For this reason, compounds from D_1 were separated from ocimenes (D_2) as different subsets.

The trend changes in cluster F because the emission of vm3d was the lowest, in average, among the male cases. The same occurs for indole and (*E,E*)-α-farnesene in cluster I_3, which present the highest differences between males and females among compounds emitted by both sexes.

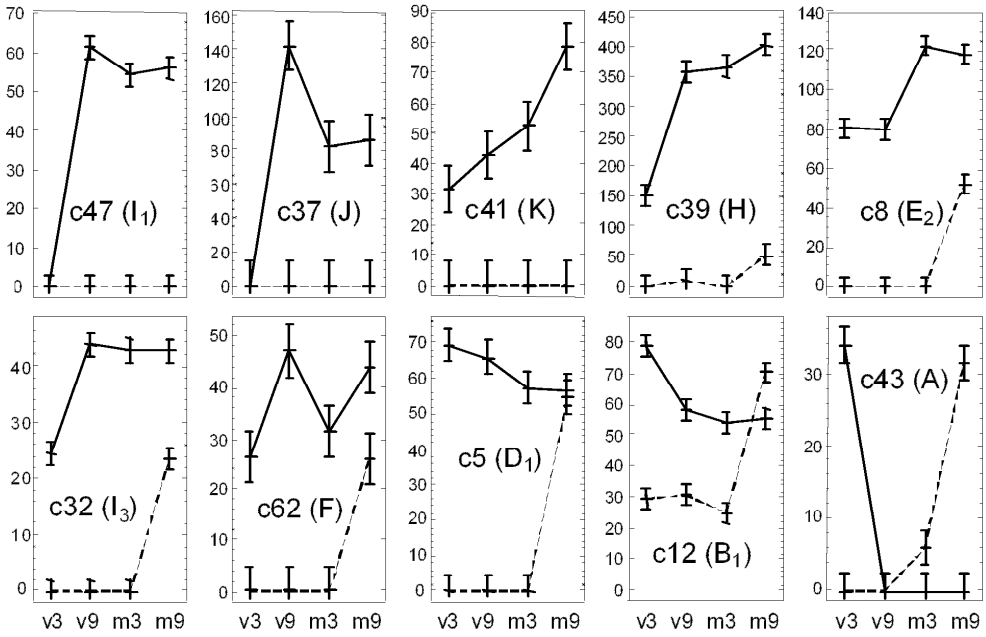

Figure VIII.5. Interaction plot and 95% LSD intervals of 10 ANOVAs conducted with factor sex (males: thicker solid lines; females: thinner dashed lines) and factor age×status with 4 variants (v3: virgin 3-d old; v9: virgin 9-d old; m3: mated 3-d old; m9: mated 9-d old). Each ANOVA was carried out using the peak area values for a different compound that was selected as the best representative of the basic clusters identified in **Figure 2** and **Figure 4** (compound code and cluster, indicated inside each plot). The quadratic root transformation was applied prior to the analyses.

Regarding to the upper part of **Figure VIII.5**, the emission of vm3d was the lowest among males, or at least similar to other male cases. Moreover, females' emission was the lowest and even null for clusters I_1, J and K. In hexanoic acid (cluster E_2), the emission among virgin males was very similar, but not in the case of compounds in cluster H, like geranyl acetate and 1-pyrroline. The pattern of

geranyl acetate (c39) and methyl-eugenol (c41) is somewhat similar, but vm3d differs significantly from vm9d in geranyl acetate given that the LSD intervals do not overlap, whereas in methyl eugenol the emission from virgin males did not show significant differences according to age. Finally, hydroxylinalool (c37) and c47 (unknown) display a similar pattern: no emission was observed for vm3d, whereas the highest values corresponded to vm9d. Thus, age had an influence on virgin males but not on the mated for these compounds.

Generally speaking, the emission of vm3d with respect to the other male cases highly determines the differences among clusters. This result is consistent with **Figure VIII.1a**, which highlights vm3d as the most different case among males.

A set of seven compounds were not assigned to any cluster (**Table VIII.1**) because their relative position in **Figure VIII.2** and **Figure VIII.4** was somewhat different. The emission pattern of these compounds can be deduced approximately taking into account their relative position with respect to the reference compounds in **Figure VIII.5**.

Regarding to relationships between chemical structures and emission patterns, the similar position of nonanal (c17) and decanal (c24) in **Figure VIII.2** and **Figure VIII.4** might be related with the similar chemical structure of both compounds. In fact, these aldehydes were attributed to the presence of females *(8)*. The same applies to 3-methylbutanoic acid and 2-methylbutanoic acid, both grouped as cluster D_1, which only differ in the position of the methyl group. Cluster F also comprises 3 molecules very similar: 4-phenyldecane, 4-phenylundecane and 5-phenylundecane. However, in the case of 6-phenyldodecane (cluster K), the position is clearly different. Another similar molecule is 6-phenylundecane (cluster C), but this compound was detected at trace levels and then its emission pattern could not be properly characterized. The presence of these phenyl-alkanes could be regarded as contamination, but their absence in blank experiments and the use of ultra-pure air suggested that they were emitted from the sample. The two isomers (*E*)-β-ocimene and (*Z*)-β-ocimene were grouped as cluster D_2. Another pair of isomers, (*E,E*)-α-farnesene and (*Z,E*)-α-farnesene, also presents a similar

position in **Figure VIII.2** and **Figure VIII.4**. Their emission pattern was nearly identical in males, but they were not grouped in the same cluster because (*E,E*)-α-farnesene was emitted by females, but not the (*Z,E*) isomer. Hexanoic acid (cluster E2) and 2-hexenoic acid (cluster H) are also related molecules, but their emission profile is different. The same applies to indole (cluster I3) versus 7-methylindole (cluster I_1), as well as in the case of geranyl acetate (cluster H) vs. geranyl propionate (cluster I_1), which also differ in one carbon atom. The results suggest that compounds with a very similar chemical structure are likely to show a similar emission pattern, maybe because they share common metabolic routes.

VI.3.7 Relationship between emission pattern and reported blends

Mixtures of 3 or 5 major constituents have been tested for medfly attraction by some authors. Laboratory results were encouraging, but field experiences were not successful *(9-13)*. This may suggest that other minor compounds could be responsible for long range attraction and these compounds should be added to the mixtures. The three major compounds described and tested by Heath et al., (*10*) and Jang et al *(11)*, correspond to different clusters: ethyl (*E*)-3-octenoate (cluster E), geranyl acetate (cluster H) and (*E,E*)-α-farnesene (cluster I_3). In addition, 1-pyrroline was added by Jang et al., *(8)* and it is also located in cluster H. However, according to **Figure VIII.5**, the emission pattern of males in these three clusters is not so different, having the lowest emission values for vm3d. Light et al. *(13)* included some intermediate and minor compounds in the tested blends, such as linalool and methyl geranate, which are also included in clusters E_2 and H. Many of the compounds previously described and tested as potential semiochemicals appear in similar clusters (E, H, I), which suggests the suitability of this methodology for analyzing medfly volatile compounds. These three clusters, as well as cluster J (including the new identified compounds geranic acid and 2-piperidone), appear characterized by higher emission in adult males (**Figure VIII.5)**, what would suggest female attraction bioassays with these compounds. On the other hand, the emission pattern of compounds belonging to clusters with higher emissions in adult mated females would suggest oviposition deterrent

bioassays. This would be the case of the new identified compound, acetophenone, included in cluster B_1.

VI.4 Conclusions

New compounds emitted by *C. capitata* were detected and identified, such as acetophenone, geranic acid, methyl-eugenol and 2-piperidone, among others. The first application of SPME to collect medfly volatiles allowed the detection of compounds not previously described up to now. The application of an experimental design with three factors (sex, age and mating status) is reported here for the first time in the study of *C. capitata* volatiles. The results showed that these factors have a key influence, with complex interactions, highlighting the difficult task of finding blends affecting medfly behavior.

The PCA application and the characterization of the emission pattern for each detected compound according to these factors have allowed their classification into different clusters. The methodology applied in this work would require further experimental testing in the search for semiochemicals, but allows the selection of compounds to be tested in the different biological assays (attraction, repellency, oviposition…) to improve the existing tools for the control of *C. capitata*.

Acknowledgements

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DISCUSIÓN GENERAL

DISCUSIÓN GENERAL

Los emisores mesoporosos

Para el buen funcionamiento de las técnicas de control de plagas basadas en el uso de semioquímicos, es necesario disponer de un emisor que reúna las características ya mencionadas en la introducción (Jones, 1998; Suckling, 2000; Muñoz-Pallarés et al., 2001). Además de ser biodegradable y de bajo coste, debe proporcionar una emisión controlada de la sustancia o sustancias en cuestión, al nivel que requiera la técnica. Existen numerosos tipos de emisores comerciales basados en polímeros plásticos, pero también existen otras formulaciones de semioquímicos más ecológicas como las de cera (p.e. *soywax*) (Behle et al., 2008) o las emulsiones de parafina (Atterholt et al., 1998; Atterholt et al., 1999; Jenkins y Isaacs, 2008; Stelinski et al., 2010). En el Centro de Ecología Química Agrícola (CEQA-IAM) se ha trabajado extensamente sobre el desarrollo de nuevos emisores para semioquímicos, basados en materiales inorgánicos naturales porosos, como son las zeolitas, atapulgita, sepiolita y otros. El uso de estos soportes mesoporosos se describe en dos patentes del Consejo Superior de Investigaciones Científicas (CSIC) y de la Universidad Politécnica de Valencia (UPV) (Corma et al., 1999; Corma et al., 2000). Siguiendo esta tecnología, el CEQA-IAM ha desarrollo con éxito emisores para atrayentes de machos (paraferomona trimedlure) y hembras (acetato amónico y N-metilpirrolidina) de la mosca de la fruta, *Ceratitis capitata* (Domínguez-Ruiz, 2007; Domínguez-Ruiz et al., 2008); y también emisores para tratamientos de confusión sexual contra *Lobesia botrana* y *Cydia pomonella* (Femenia, 2011).

En el trabajo descrito por Domínguez-Ruiz (2007), se comprobó cómo los emisores de tipo polimérico eran sensibles a los incrementos de temperatura, lo que impedía que tuviesen una emisión constante y una vida útil suficiente. Los emisores con soporte mesoporoso consiguen tener una vida útil mayor debido a que la liberación de los ingredientes activos se debe a procesos controlados por

difusión y no por evaporación como ocurre en los soportes poliméricos, cuya vida útil y capacidad de atracción se acorta después de periodos con altas temperaturas. Sin embargo, los emisores mesoporosos se caracterizan por tener una emisión controlada, lo cual optimiza el uso de la feromona, y además quedan con bajos niveles residuales de carga. También se confirma en los trabajos descritos por Femenia (2011), que los emisores mesoporosos tienen cinéticas más próximas a las de orden cero, y proporcionan mayores tiempos de vida útil que los emisores poliméricos comerciales.

En cuanto a la composición de nuestros emisores, el porcentaje de feromona respecto al soporte y la proporción de aditivos compactantes son clave para conseguir la velocidad de emisión adecuada. Al aumentar la proporción de aditivos, aumenta la retención de feromona y disminuye la velocidad de emisión (Domínguez-Ruiz, 2007; Femenia, 2011) y al contrario, cuando se aumenta el porcentaje de feromona, aumenta su velocidad de emisión. Así que, con las formulaciones desarrolladas en estos trabajos previos ya se consiguió un diseño equilibrado en el que los aditivos incorporados a la composición aseguraban la estabilidad química de la feromona dispersa en la sepiolita y conferían al emisor consistencia y resistencia durante toda la campaña. Tomando como base las experiencias en formulación de estos trabajos previos, en la presente tesis se han desarrollado nuevos emisores de sepiolita para la confusión sexual del piojo rojo de California (*Aonidiella aurantii*) (Vacas et al., 2009a; Vacas et al., 2010) y de la polilla del tomate (*Tuta absoluta*) (Vacas et al., 2011a), y emisores de atracción para el barrenador del arroz (*Chilo suppressalis*) (Vacas et al., 2009b) y la polilla del racimo (*Lobesia botrana*) (Vacas et al., 2011b).

Experiencias de confusión sexual

Piojo rojo de California

Capítulo I "The first account of the mating disruption technique for the control of California red scale, *Aonidiella aurantii* Maskell (Hemiptera: Diaspididae) using new biodegradable dispensers"

Capítulo II "Mating disruption of California red scale, *Aonidiella aurantii* Maskell (Hemiptera: Diaspididae), using biodegradable mesoporous pheromone dispensers"

Capítulo III "Different strategies to apply mating disruption for the control of *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae)"

Desde el descubrimiento de la primera feromona en 1960, la aplicación de los llamados compuestos semioquímicos se ha extendido a cientos de plagas de diferentes órdenes de insectos. En el caso de los diaspinos, se han identificado las feromonas sexuales de cinco especies de este orden de insectos: *A. aurantii* (Roelofs et al., 1977), *A. citrina* Coquillett (Gieselmann et al., 1979a), *Aspidiotus nerii* Bouché (Einhorn et al., 1998), *Pseudaulacaspis pentagona* Targioni-Tozzeti (Heath et al., 1979) y *Quadraspidiotus perniciosus* Comstock (Gieselmann et al., 1979b). Hasta el momento no había sido descrita ninguna aplicación con éxito de un método de control basado en confusión sexual contra un diaspino. En nuestro caso, consideramos que el piojo rojo de California (PRC) era un candidato ideal para la aplicación de la técnica de confusión sexual, por ser conocida su feromona sexual y ser una especie de escasa movilidad, que vive muy íntimamente ligada a su hospedero. Los únicos estadios de este insecto capaces de desplazarse son las ninfas móviles y los machos adultos (Bodenheimer, 1951). Los machos, por su parte, vuelan algunos metros en busca de la cópula, pero no pueden iniciar nuevas infestaciones por sí mismos, son las ninfas móviles el principal agente de dispersión de la plaga (Greathead, 1990). Estas ninfas se desplazan hasta 50 cm

para fijarse en un órgano de la planta, lo que explica su dispersión entre el ramaje del propio árbol, pero no a largas distancias (Bodenheimer, 1951). Por este motivo, la inmigración natural de la plaga entre parcelas es difícil, y la intrusión por parte de las hembras adultas de PRC es imposible, debido a su condición sésil. De acuerdo con estos aspectos de la biología de *A. aurantii*, los ensayos para confusión sexual se diseñaron en bloques de algo menos de 1 ha, lo que no sería adecuado para ensayar esta técnica con plagas de insectos de mayor movilidad. Por ejemplo, en el caso de *C. pomonella*, las experiencias de confusión sexual recomiendan grandes áreas aisladas (Cardé y Minks, 1995; Witzgall et al., 2008; Witzgall et al., 2010), para evitar la intrusión de hembras grávidas desde parcelas colindantes.

Las experiencias previas de confusión sexual llevadas a cabo en Israel no daban una idea clara de la efectividad de la técnica contra PRC (Barzakay et al., 1986; Hefetz et al., 1988). Sin embargo, demostraban que emisores formulados con sólo uno de los componentes de su feromona sexual conseguían reducir las capturas de machos respecto a una parcela control. Por este motivo, en el CEQA-IAM se decidió abordar la aplicación de esta técnica y formular nuevos emisores mesoporosos para PRC con la mezcla diastereomérica de sólo uno de los componentes de su feromona sexual, (3*S*,6*R* y 3*S*,6*S*) del acetato de 3-metil-6-isopropenil-9-decenilo. La posibilidad de controlar la población con una mezcla feromonal de diastereómeros supone una considerable reducción en los costes del tratamiento de confusión sexual, ya que la feromona supone alrededor del 90% del coste del emisor y la obtención de un solo diastereómero encarece mucho su síntesis. Como mencionan otros autores, no se requiere la mezcla feromonal natural para que la confusión sexual sea eficaz, al contrario que para la captura en trampas (Witzgall et al., 2010); de hecho, existen trabajos en los que se consigue confundir poblaciones de lepidópteros con mezclas feromonales poco atrayentes (Bengtsson et al., 1994; Cork et al., 1996; Stelinski et al., 2008).

Por otra parte, los ensayos de confusión contra PRC de Hefetz y colaboradores presentaron ciertos problemas. Ensayaron dosis de 12 g ha^{-1} de

feromona, liberada desde emisores tipo *rubber*, que se reponían cada 2 meses, consiguiendo índices de inhibición de capturas de entre 60-80%, pero no incluían una valoración del daño final en fruto, ni una descripción de los tratamientos llevados a cabo en su parcela de referencia (Barzakay et al., 1986; Hefetz et al., 1988). Respecto al tipo de emisor utilizado, los emisores *rubber* tienen elevadas velocidades de emisión durante las primeras semanas, pero después pierden eficacia. Además, no son biodegradables, necesitan reposición para proteger completamente el ciclo del cultivo y su emisión es altamente dependiente de la temperatura (McDonough et al., 1989). Los *rubber* empleados en los ensayos de Israel, iban cargados como máximo con 6 mg de la feromona de *A. aurantii*, cantidad claramente insuficiente para conseguir una buena eficacia de confusión, como quedó demostrado durante la campaña de 2006, en la que se ensayaron los nuevos emisores mesoporosos desarrollados en el CEQA-IAM cargados con 8 y 20 mg de feromona (D8 y D20; Capítulo I). Solo en el bloque tratado con emisores de 20 mg se observó un cierto efecto de desorientación en los machos, aunque no se consiguió controlar el daño en fruto (Vacas et al., 2009a). Para que la confusión sexual sea eficaz, debe haber cantidad suficiente de feromona en el ambiente durante todo el periodo en que los insectos son sexualmente activos (Cardé et al., 1975; Howse, 1998), por ello es necesario que los emisores liberen la feromona de PRC en cantidad adecuada durante los siete meses que dura el ciclo del insecto. Los resultados de la campaña de 2006 (Capítulo I) indicaron que la feromona emitida no fue suficiente para confundir a los machos en segunda y tercera generación. La carga residual de los emisores ensayados durante 2006 (D8 y D20) resultó muy elevada, cercana al 40% al final del ensayo, lo que supone una gran cantidad de feromona no liberada y malgastada. Teniendo en cuenta estos dos factores, baja emisión y elevada carga residual, se decidió continuar las experiencias en la campaña de 2007 con dos nuevas formulaciones (Capítulo I). Estos nuevos emisores cargados con 50 y 100 mg de feromona (D50 y D100, respectivamente) consiguieron bajos porcentajes de feromona residual y mayores velocidades de emisión. Además, el control de las capturas de machos y el conteo de daño en fruto al final de la campaña pusieron de manifiesto que ambos tratamientos afectaron significativamente la comunicación química del PRC (Vacas

et al., 2009a). Comparando con una parcela de referencia sin tratamientos, ambos emisores proporcionaban un control eficaz de la plaga, sin diferencias significativas entre ellos. Debido al coste de la feromona y la necesidad de métodos de control que resulten económicamente viables para el productor, decidimos seleccionar el emisor D50 para los posteriores ensayos de eficacia en la temporada 2008 (Capítulo II). Hay que destacar que el emisor D50 se acerca a las características exigidas a un emisor ideal (Muñoz-Pallarés et al., 2001). Es efectivo durante un periodo prolongado (al menos 6 meses), emite la feromona de forma gradual, no deja feromona residual al final del tratamiento y su base es una matriz biodegradable que puede integrarse en el medio sin contaminar, lo que le proporciona un valor añadido para incluir esta técnica en los programas de control integrado de plagas.

Posteriormente, se demostró la eficacia de la técnica de confusión sexual contra PRC, con los emisores mesoporosos desarrollados, en tres ensayos diferentes llevados a cabo en dos provincias españolas: Huelva y Valencia (Capítulo II) (Vacas et al., 2010). En la provincia de Huelva, el PRC es también una de las principales plagas en cítricos y, dadas las altas temperaturas medias que se suelen alcanzar en esta zona geográfica, puede llegar a desarrollar hasta cuatro generaciones completas. En estos ensayos llevados a cabo en Picasent (Valencia) y Río Tinto (Huelva), se plantearon nuevas cuestiones referentes a la influencia de varios factores sobre la eficacia de la confusión: las variedades de cítricos, el momento de colocación de los emisores de feromona y la vida útil de los mismos. En los tres ensayos se observó efecto de desorientación de machos y reducción de daño, respecto a sus respectivas parcelas de referencia. Sin embargo, en los ensayos de Picasent se registraron capturas de machos más altas de las esperadas durante el tercer vuelo (Agosto-Octubre), en comparación con las obtenidas en Rio Tinto. Este fenómeno se atribuyó a la fecha de colocación de los emisores, que fue dos semanas antes en los ensayos de Picasent (21 Febrero, frente al 5 Marzo en Río Tinto). Por lo tanto, el periodo útil de 6 meses de los emisores se habría agotado al comienzo de Septiembre y la feromona presente en aire podría no ser suficiente para confundir a los machos

completamente durante el tercer vuelo y, especialmente, para controlar el cuarto vuelo que tuvo lugar ese mismo año. La importancia de la duración de los emisores está muy relacionada con la variedad de cítricos en la que se aplica la confusión. El PRC está ampliamente distribuido y aunque la susceptibilidad del hospedero está relacionada con el número de glándulas de aceite en las hojas (McClure, 1990; Asplanato y García-Marí, 1998), todas las variedades de cítricos son susceptibles a su ataque. Para variedades tempranas o medias, como Navelina y Clemenules (ensayos 1 y 2 en Capítulo II), los emisores colocados al inicio de Marzo con una vida útil de 6 meses fueron adecuados para controlar los 3 vuelos que generalmente tienen lugar en España. En variedades tardías, como las Valencia (ensayo 3 en Capítulo II), el final de la tercera y la posible cuarta generación no controlada afectarían gravemente al fruto aún sin recolectar. Esto motivó posteriores estudios para ajustar la fecha de colocación de los emisores de confusión sexual y promover la emisión de feromona hasta completar el ciclo de PRC (Capítulo III). Aunque se pudiese pensar en utilizar mayores dosis de feromona para cubrir periodos más prolongados, esto supondría un aumento notable en el coste de la técnica; por lo tanto, resultaría más conveniente ajustar la fecha de colocación, que elevar la dosis de feromona.

Generalmente, la primera generación de PRC en nuestra área geográfica no suele ser la más abundante, además estos individuos no suelen encontrarse colonizando los frutos. La segunda generación, que tiene lugar al comienzo del verano, es generalmente la principal responsable del daño en fruto (Rodrigo et al., 2004). Por este motivo, en el Capítulo III se proponen dos fechas de colocación para los emisores mesoporosos: antes de la primera generación (Marzo), para empezar a controlar la plaga desde el primer vuelo de machos y antes de la segunda generación (Mayo), para frenar el segundo vuelo que daría lugar a las ninfas más dañinas de la segunda generación. También se incluyó el estudio de una tercera opción: colocación tardía de feromona (Mayo) más un tratamiento convencional con aceite contra las ninfas de primera generación (Vacas et al., 2011c). Los resultados demostraron que aunque se obtuvieron índices de

inhibición de capturas mayores del 80% con ambas estrategias de colocación, el control del daño en fruto fue más eficaz colocando los emisores en el mes de Mayo (6% en parcela de colocación Marzo, frente a 1,5% colocando en Mayo). Esto se explica por la cinética de emisión y la vida útil de los emisores, que en esta ocasión fue de únicamente 5 meses. Estableciendo la confusión sexual en el mes de Marzo, este periodo de 5 meses no protegería el cultivo hasta el final de Agosto, por lo que no se estaría produciendo confusión en el tercer vuelo, permitiendo las cópulas y el desarrollo de una generación que colonizará los frutos. La colocación en Mayo cubriría el tercer vuelo con velocidades de emisión de feromona adecuadas, protegiendo así el cultivo. Por lo tanto, en zonas con niveles medios de población, el control de la primera generación de ninfas del PRC parece no ser crucial y el cultivo quedaría protegido con el establecimiento de la confusión sexual justo antes del segundo vuelo. Este hecho está en contraposición a lo que ocurre con las plagas de lepidópteros. Numerosos autores demuestran la importancia de controlar la emergencia de las primeras polillas y recomiendan la aplicación temprana de feromona para prevenir el desarrollo de las subsiguientes generaciones a lo largo de la campaña (Staten et al., 1987; Kehat et al., 1995; Lykouressis et al., 2005).

Los tratamientos que se hacen habitualmente contra PRC son pulverizaciones con aceites minerales, insecticidas organofosforados como clorpirifos o IGRs como piriproxifen. Las cochinillas son especialmente sensibles a los aceites minerales cuando se encuentran en el estadio de ninfa móvil y la susceptibilidad decrece con la edad de la cochinilla, debido a que la presencia de un escudo más grueso y el desarrollo de la membrana ventral, son características que interfieren en la penetración y dispersión del producto debajo del escudo (Riehl, 1988; Rill et al., 2007). Diversos estudios demuestran la compatibilidad de las pulverizaciones de aceite con el control biológico de PRC (Liang et al., 2010). Sin embargo, los plaguicidas convencionales son los que pueden suponer una amenaza para la conservación de la fauna útil. En el Capítulo III de la presente tesis también se demuestra la compatibilidad de las aplicaciones de aceite con la confusión sexual. Concretamente, para niveles poblacionales medios, el

tratamiento de aceite no mejora el resultado de eficacia del tratamiento combinado con feromona (Vacas et al., 2011c), por lo que se podrían suprimir estas pulverizaciones. Sin embargo, se podría recomendar la aplicación de aceite en ciertas situaciones: (1) tratamiento de aceite dirigido a las ninfas de primera generación (Mayo), cuando se va a instalar por primera vez la confusión sexual y se parte de poblaciones altas conocidas (daño en fruto en la campaña anterior mayor al 20%); (2) tratamiento de aceite cada dos o tres años, para impedir los brotes de cochinillas secundarias. Se podría pensar en suprimir las pulverizaciones cuando la confusión sexual está instaurada, sin embargo esos tratamientos podrían controlar a otros diaspinos de baja incidencia como *Parlatoria pergandii* Comstock o *Cornuaspis beckii* (Newman). Por lo tanto, se desaconseja la eliminación total de los tratamientos de aceite. Por otro lado, es conocido que los aceites minerales pueden causar cierta fitotoxicidad y por ello se han descrito recientemente nuevas formulaciones de aceites vegetales como plaguicidas contra PRC, buscando menor fitotoxicidad (p.e. emulsiones con semillas de brasicáceas (Rongai et al., 2008)).

El papel de los enemigos naturales en el control de *A. aurantii* es importante (Furness et al., 1983; Moreno y Luck, 1992; Bedford, 1996), aunque, generalmente, no proporcionan un control suficiente como para mantener los niveles de población de esta cochinilla por debajo de los umbrales de tratamiento (Ishaaya et al., 1992; Grafton-Cardwell y Reagan, 1995; Jacas y Urbaneja, 2010). Sin embargo, es necesario comprobar que las nuevas técnicas que se introduzcan en programas de control integrado no perjudican a la fauna útil. Por este motivo, se han planteado ensayos, tanto en laboratorio como en campo, para evaluar el efecto de los ambientes de confusión sexual (con altas concentraciones de feromona) en el nivel de parasitismo infligido sobre PRC por parte de su enemigo natural *Aphytis melinus* (Vacas et al., 2011d).

La polilla del tomate

Capítulo IV "Studies on the development of a mating disruption system to control *Tuta absoluta* Povolny (Lepidoptera: Gelechiidae)"

La dificultad de lograr un control químico eficaz sobre la polilla del tomate, reside en la propia biología de la plaga: (1) los hábitos alimenticios de las larvas que dificultan su contacto con los insecticidas, y (2) el elevado número de generaciones que provoca que convivan todos los estadíos del insecto. Esto tiene como consecuencia que los productos no se apliquen siempre en el momento de mayor susceptibilidad, o que las aplicaciones hayan de ser demasiado frecuentes, promoviendo la selección de biotipos resistentes. Este es un problema común a otras especies de polillas de la familia Gelechiidae, como *Keiferia lycopersicella* (Walsingham) (van Steenwyk y Oatman, 1983), *Tecia solanivora* Povolny (Bosa et al., 2006), o lepidópteros de otras familias como el barrenador del arroz (*Chilo suppressalis*) (Ramoneda y Roig, 1989), o el del maíz (*Sesamia nonagrioides* (Lefebvre)) (Albajes et al., 2002), entre otros. En todas estas especies, las larvas se encuentran protegidas en el interior de galerías, por lo que es muy importante determinar el momento de aplicación de los tratamientos, para afectar a las larvas en el exterior, antes de que formen las galerías, o cuando salen de ellas para pupar. Por este motivo, es muy importante disponer de técnicas de control que no se basen en la afectación de las larvas que requieren repetidas aplicaciones de insecticidas. El control basado en feromonas puede proporcionar una solución a este problema, ya que, al ser sustancias implicadas en la comunicación entre insectos, van a actuar directamente sobre los machos y hembras adultos de las polillas. Por lo tanto, las técnicas que utilizan feromonas como atrayentes o para interferir en su comunicación se presentan como una buena alternativa para el control de la polilla del tomate.

Michereff y colaboradores publicaron en el 2000(b), sus estudios iniciales sobre confusión sexual de la polilla del tomate. En su trabajo se describe el potencial del componente principal de la feromona de *T. absoluta*, acetato de

3*E*,8*Z*,11*Z*-tetradecatrienilo (TDTA), para interferir en la comunicación sexual de la plaga. En este trabajo, se describe que la habilidad de los machos de la polilla para llegar a las trampas cebadas con feromona se redujo en los bloques tratados con feromona, por lo que se demostraba cierto efecto de desorientación. Sin embargo, ninguna de las dosis de feromona probadas consiguió reducir el número de galerías en hojas, ni el número de frutos dañados (Michereff et al., 2000b), por lo que la eficacia de la técnica no quedaba confirmada. En el Capítulo IV de esta tesis se describen los experimentos que demuestran la eficacia de la confusión sexual aplicada a *T. absoluta*, empleando la tecnología de emisores mesoporosos de feromona desarrollada en el CEQA-IAM.

Uno de los motivos que expone Michereff para explicar el poco éxito de sus ensayos es un nivel de emisión inapropiado, que crea una concentración de feromona insuficiente para inhibir las cópulas. De hecho, empleaba emisores tipo *rubber* que, como ya se ha mencionado en el capítulo anterior sobre PRC, tienen una emisión de feromona irregular y son muy dependientes de las condiciones climáticas. Además, estos emisores no eran de larga duración y requerían una reposición cada 28 días, lo que resultaba muy poco económico en la práctica. El uso de los emisores mesoporosos, que presentan una velocidad de emisión constante durante al menos 120 días (Capítulo IV), supone un gran avance respecto a los emisores utilizados anteriormente.

Otro aspecto muy importante a tener en cuenta es la pequeña superficie (200 m^2) en la que se desarrollaron las experiencias al aire libre en Brasil (Michereff et al., 2000b). También, como se ha citado anteriormente, es importante aplicar la técnica de confusión sexual contra lepidópteros en grandes áreas (Cardé y Minks, 1995), para asegurar la eficacia de la técnica. Como el mismo Michereff afirma, es muy probable que en su ensayo se produjese la intrusión de individuos de *T. absoluta* desde zonas no tratadas con feromona. En la presente tesis (Capítulo IV) se ha ensayado la eficacia de la confusión sexual con distintas concentraciones de feromona en invernaderos con diferentes grados de aislamiento. Con los datos obtenidos no se puede concluir si se podría aplicar la confusión sexual en el cultivo de tomate llevado a cabo al aire libre, dado que se

requeriría mucha más cantidad de feromona por ha, y el elevado coste de la misma haría que los tratamientos no fuesen económicamente viables.

Lo que confirma la presente investigación es la desorientación de machos y el control de daño en el cultivo de tomate desarrollado en un invernadero con un buen aislamiento. Cuando se trata de invernaderos de malla poco tupida, aumenta la posibilidad de intrusión de polillas. En nuestros ensayos, comprobamos que en estas condiciones no tuvo lugar una inhibición de capturas y, por lo tanto, no se estaba produciendo una confusión sexual que consiguiese reducir el daño con dosis de 10-40 g ha^{-1}, en comparación con un control que recibió cuatro tratamientos químicos. Los resultados cambian notablemente cuando las experiencias se realizan bajo invernaderos de malla tupida, de plástico o vidrio, con buen aislamiento, reduciendo así la posibilidad de intrusión de plaga. En estos casos, una dosis de 30 g ha^{-1} (ensayo Paiporta 2009 en Capítulo IV) consiguió inhibir las capturas de machos y controlar el daño en fruto de igual forma que siete tratamientos durante el ciclo de cultivo, con *Bt* e indoxacarb. En los posteriores ensayos de eficacia llevados a cabo en Alicante y Paiporta, se consiguieron buenos resultados de control de la plaga, pero también se comprobó la necesidad de revisar las formulaciones de los emisores. En el ensayo de Paiporta 2010, los daños fueron mayores de lo esperado hacia el final de la campaña, lo que se justifica con el periodo útil de los emisores, que fue solo de 4 meses, quedando en ellos una elevada carga de feromona residual. Este porcentaje se debe reducir y se debe, asimismo, alargar la vida útil del emisor, de manera que se emitan alrededor de 170 µg $día^{-1}$ durante al menos 6 meses, para cubrir los ciclos de cultivo más largos y proporcionar dosis de feromona de al menos 30 g ha^{-1}. Este valor está de acuerdo con lo que proponían Michereff y colaboradores (2000b), quienes observaron la desorientación en los machos de parcelas tratadas con 35-50 g ha^{-1} de la feromona de *T. absoluta*. Además, en una revisión de Witzgall (2010) sobre el uso de feromonas en el manejo integrado de plagas, se refiere de forma general que dosis de 10 a 100 g ha^{-1} de feromona por campaña son necesarias para conseguir interferir en la comunicación sexual de los insectos.

En el capítulo III se ha descrito la importancia de controlar la emergencia temprana de los primeros individuos cuando se habla de plagas de lepidópteros. En todos los ensayos realizados en esta tesis sobre *T. absoluta*, los emisores se colocaron pocos días después de la plantación del cultivo (plantas de 10 cm de altura como máximo). Los resultados han demostrado que, con la formulación adecuada, la aplicación temprana fue suficiente para proteger el cultivo durante toda la campaña. Michereff (2000b) también apunta el momento de aplicación como un factor decisivo para la eficacia de la confusión y, sin embargo, es uno de los motivos del fracaso de su ensayo, ya que en su caso, por problemas técnicos, los dispositivos se colocaron 30 días después de la plantación, y en ese momento, la plaga ya estaba establecida y los niveles de infestación eran altos.

Por último, Michereff (2000b) menciona que el uso de únicamente el compuesto mayoritario de la feromona pudo ser la causa de la falta de eficacia en sus ensayos. Sin embargo, está ampliamente establecido que para tratamientos de confusión sexual no se requiere la feromona natural, y que se pueden confundir poblaciones de lepidópteros con mezclas feromonales poco atrayentes, o únicamente con componentes mayoritarios, en función del mecanismo de confusión que esté funcionando (Witzgall et al., 2010).

Optimización de emisores de atracción

Capítulo V "Study on the optimum pheromone release rate for attraction of *Chilo suppressalis* Walker (Lepidoptera: Pyralidae)"

Capítulo VI "Effect of sex pheromone emission on the attraction of *Lobesia botrana*"

Capítulo VII "Response of two tephritid species, Bactrocera oleae and *Ceratitis capitata*, to different emission levels of pheromone and parapheromone"

El seguimiento de poblaciones mediante trampas de feromona permite la programación de los métodos de control para realizar las actuaciones en el momento más adecuado. El éxito de las técnicas de seguimiento depende en gran medida de la disponibilidad de trampas adecuadas y de emisores que liberen el compuesto atrayente con la velocidad óptima (Suckling, 2000). La mayoría de los difusores que se emplean para los métodos basados en atracción son formulados en emisores tipo *rubber*, que como ya se ha mencionado, no tienen una emisión adecuada. El problema fundamental de este tipo de emisores es que la orientación de un insecto en vuelo hacia la fuente de feromona se produce o inhibe dentro de un rango estrecho de concentraciones de feromona (Zhang y Amalin, 2005).

Se encuentran en la literatura numerosos estudios sobre umbrales de emisión necesarios para producir confusión sexual en función de la especie (Cardé y Minks, 1995; Ioratti et al., 2004; De Lame et al., 2007; Stelinski et al., 2007; Witzgall et al., 2010). Sin embargo, son pocos los estudios que correlacionan los niveles de emisión de feromona con el nivel de capturas en condiciones de campo, estableciendo un rango de emisión que optimice las capturas. La clave para la mejora de los métodos de control basados en feromonas como atrayente (trampeo masivo, atracción y muerte) reside en el conocimiento de ese nivel óptimo de emisión, ya que la respuesta de los insectos al atrayente en cuestión puede disminuir por encima o por debajo de ese valor óptimo (Jacobson y Beroza, 1964;

Roelofs et al., 1977; Howse, 1998; Zhang y Amalin, 2005). Este fenómeno ha quedado demostrado para los lepidópteros *C. suppressalis* y *L. botrana* (Capítulos V y VI).

El trabajo descrito en el Capítulo V de la presente tesis muestra la respuesta de la plaga del barrenador del arroz, *C. suppressalis*, frente a cuatro niveles de emisión del componente mayoritario de su feromona sexual, el (Z)-11-hexadecenal (Vacas et al., 2009b). Utilizando la tecnología de emisores basados en tamices moleculares, se consiguieron trampas con 3 niveles de emisión, al cargarlas con 1, 2 ó 3 emisores de la feromona de *C. suppressalis*. El cuarto nivel de emisión se consiguió con un emisor comercial de la empresa SEDQ (Barcelona, España), del tipo vial de polietileno. En primer lugar, estudiando la cinética de emisión de los dos tipos de emisores utilizados (comercial y mesoporoso), se obtuvo que ambos emitían la feromona de forma constante durante el periodo de ensayo. Por lo tanto, los cuatro niveles de emisión ensayados fueron: 26,1 µg día^{-1} (dado por el emisor comercial), 19,1 µg día^{-1} (dado por el emisor mesoporoso), 38,5 µg día^{-1} (2 emisores mesoporosos) y 57,7 µg día^{-1} (3 emisores mesoporosos). El análisis de la varianza, con las capturas obtenidas, demostró que el factor nivel de emisión era significativo, y por lo tanto el nivel de capturas estaba influenciado por la velocidad de emisión de feromona. Para abordar esta relación, se aplicó un análisis de regresión polinomial que correlacionaba los valores medios de capturas con los valores de emisión de feromona, obteniendo un modelo cuadrático (Capítulo V) que evidenciaba la existencia de una velocidad de emisión para un máximo valor de capturas. El valor obtenido y propuesto fue de 34 µg día^{-1}; por lo tanto, con valores de emisión por encima y por debajo de este nivel no se consigue un funcionamiento óptimo en un sistema de trampeo para el barrenador del arroz, bajo las condiciones ambientales propias de este cultivo en la zona Mediterránea.

Del mismo modo, en el capítulo VI se comprueba que la atracción de los machos de la polilla del racimo hacia trampas cebadas con su feromona sexual también es dependiente del nivel de emisión, y la relación encontrada fue la misma que para *C. suppressalis*. Anshelevich (1994) ya dio evidencias de esta

dependencia en el comportamiento de *L. botrana*, pero el estudio no proporcionaba valores reales de emisión y por lo tanto, no se establecía la correlación capturas-nivel de emisión (Anshelevich et al., 1994). En el capítulo VI se detalla como las cinéticas de los emisores mesoporosos empleados se ajustaron a modelos polinómicos, que permitieron calcular las velocidades de emisión en cada periodo de capturas y establecer la correlación buscada. La velocidad de emisión óptima obtenida para *L. botrana* fue de unos 400 µg día^{-1}, valor que resultó del orden de 10 veces mayor que para el barrenador del arroz.

El efecto inhibidor que pueden tener las dosis altas de feromona sobre las capturas de insectos, ha sido demostrado para otros lepidópteros (Roelofs y Cardé, 1974; Wyman, 1979; Millar et al., 1996). Sin embargo, muchos de estos trabajos se basan en el dato de carga inicial de los emisores utilizados, lo que no se correlaciona directamente con la emisión real de feromona en un determinado momento ni con la concentración de feromona activa en el ambiente. Por ejemplo, el efecto del tipo de emisor sobre las capturas del barrenador del maíz fue estudiado por Critchley y colaboradores: viales de polietileno cargados con 1 mg de feromona capturaban significativamente más polillas que emisores tipo *rubber* con la misma carga de ingrediente activo (Critchley et al., 1997), lo cual indicaba que el nivel de emisión real no era el mismo. Otro ejemplo es el trabajo de Knutson y colaboradores (1998) que compara ocho soportes diferentes para la emisión de la feromona de otro barrenador del maíz, o trabajos de Leonhardt y colaboradores con diversos tipos de emisores para la feromona de la polilla *Lymantria dispar* (L.) (Leonhardt et al., 1990).

El tipo de respuesta encontrada en el comportamiento de *C. suppressalis* y *L. botrana* para los diferentes niveles de emisión ensayados en nuestros trabajos (Capítulos V y VI), también fue observada para otros lepidópteros: *L. dispar* (Leonhardt et al., 1990), *C. pomonella* (Kehat et al., 1994), *Cnaphalocrocis medicinalis* (Guenée) (Kawazu et al., 2004); y también para otras familias de insectos, como coleópteros (Mason et al., 1990; Franklin y Gregoir, 2001), hemípteros (Branco et al., 2004) y dípteros, como *Culex pipiens* (Michaelakis et al., 2007).

Por lo que respecta a los dípteros tefrítidos, la importancia de la determinación de un nivel óptimo de emisión ya había sido mencionado por Landolt y Heath (1988,1990). En sus ensayos de laboratorio con la mosca de la papaya, *Toxotrypana curvicaudata* Gerstaecker, observaron que la respuesta a la feromona aumentaba con el nivel de emisión hasta cierto límite. Posteriormente, observaron el mismo comportamiento en campo y propusieron una velocidad óptima de 23 µg día^{-1} (Landolt y Heath, 1990). Los ensayos realizados y descritos en el capítulo VII de la presente tesis apoyan la existencia de estos valores óptimos de emisión en tefrítidos. La metodología ya descrita (Capítulos V y VI) se aplicó a la mosca del olivo *Bactrocera oleae* respecto a su feromona spiroacetal y a la mosca del Mediterráneo *Ceratitis capitata* respecto a su paraferomona trimedlure (Navarro-Llopis et al., 2011). Haniotakis y Pittara (1994) demostraron en ensayos de laboratorio que la máxima respuesta de los machos de *B. oleae* a su feromona se obtenía con dosis intermedias de feromona, pero no proporcionaban datos de emisión ni ensayos de campo. Mediante el estudio descrito en el capítulo VII, ensayando diferentes niveles de emisión, se determinó un valor óptimo de 1,28 mg día^{-1} para la atracción de *B. oleae*, en las condiciones ambientales que tienen lugar en los olivares de la zona Oeste del Mediterráneo. En este mismo capítulo se estudió la relación emisión-capturas para *C. capitata* y su paraferomona trimedlure. Sin embargo, la respuesta fue muy distinta a la encontrada en *B. oleae*. Para el trimedlure, no se observó un efecto de saturación con altas dosis, si no que a partir de una emisión de 2,4 mg día^{-1}, cantidades mayores de trimedlure no capturaban significativamente más moscas. Por lo tanto, en este caso se obtiene una respuesta de tipo asintótico, que también ha sido observada en algunas especies de lepidópteros (Evenden et al., 1995; Knutson et al., 1998; Rao y Subbaratnam, 1998; Jactel et al., 2006).

Este estudio de las cinéticas de emisión y su correlación con el poder atrayente son claves para el desarrollo de formulaciones eficaces.

Búsqueda de semioquímicos

Capítulo VIII "Solid phase microextraction of volatile emissions of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae): Influence of fly sex, age and mating status"

La investigación sobre búsqueda, aislamiento e identificación de semioquímicos de insectos es un campo en el que aún queda mucho por explorar. Como ya se ha indicado, las feromonas de lepidópteros son las más estudiadas, ya sea por sus estructuras químicas sencillas y muy similares unas a otras, o por el buen conocimiento de su biología (Primo-Yúfera, 1991). El caso de los dípteros es muy diferente al de los lepidópteros, tanto en lo que se refiere al comportamiento sexual, como en la variedad de las estructuras de sus feromonas. Se han descrito las feromonas sexuales para más de 30 especies de dípteros (El Sayed, 2011). Entre ellos, han sido ampliamente estudiadas las feromonas sexuales cuticulares, con estructura de hidrocarburo, producidas por moscas de las familias Drosophilidae (*Drosophila melanogaster* Meigen), Muscidae (*Musca domestica* L.) y Glossinidae (*Glossina morsitans* Newstead) (Carlson et al., 1971; Langley y Carlson, 1983; Wicker y Jallon, 1995; Tillman et al., 1999). La familia Tephritidae, a la que pertenece la mosca Mediterránea de la fruta (*C. capitata*), presenta un sistema de apareamiento tipo *lek*, descrito ampliamente para especies de los géneros *Bactrocera* (Miyatake y Haraguchi, 1996; Jackson y Long, 1997; Shelly, 2001; Weldon, 2007), *Anastrepha* (Sivinski, 1989; Segura et al., 2007) y *Ceratitis* (Arita y Kaneshiro, 1985; Kaspi y Yuval, 1999), por el que los machos forman grupos y emiten señales para atraer a las hembras. Por esta particularidad en su comportamiento sexual, la mayoría de las feromonas descritas para tefrítidos son emitidas por los machos de la especie en cuestión (Kobayashi et al., 1978; Burk, 1983; Robacker y Hart, 1985; Lu y Teal, 2001). Únicamente en el caso de *B. oleae* se ha descrito una feromona sexual emitida por hembras de tefrítidos (Baker et al., 1980).

Desde principios de los años 70 se viene investigando sobre la composición de la feromona del macho de *C. capitata*. Jacobson y sus colaboradores describieron una mezcla de 15 sustancias, que incluía ácidos carboxílicos y los compuestos metil (E)-6-nonenoato y (E)-6-nonen-1-ol (Jacobson et al., 1973). Esta mezcla resultó ser atractiva para machos y hembras en laboratorio, pero en el campo, lo fue solo para machos (Ohinata et al., 1977; Ohinata et al., 1979). Desde estos hallazgos, se han desarrollado trabajos que estudian las emisiones volátiles de machos de *C. capitata* tomando muestras en materiales adsorbentes diversos, como Tenax y Porapak (Baker et al., 1985; Jang et al., 1989; Flath et al., 1993; Gonçalves et al., 2006). Sin embargo, no existen citas sobre la aplicación de la técnica de microextracción en fase sólida (SPME) para la toma de muestras de las emisiones volátiles de la mosca del Mediterráneo. Esta técnica fue introducida por Pawliszyn en 1990, pero no es hasta 1995 cuando se cita como método alternativo para el estudio de feromonas de insectos (Malosse et al., 1995). La técnica de SPME ha sido empleada principalmente para estudios en coleópteros (Said et al., 2003; Cai et al., 2007; Yasui et al., 2008; Arakaki et al., 2009), y recientemente para la identificación de sustancias emitidas por el tefrítido *Anastrepha serpentina* (Wiedemann) (Robacker et al., 2009).

En la presente tesis (Capítulo VIII) se procedió a la captura e identificación de los volátiles emitidos por los machos y hembras de *C. capitata* mediante SPME, introduciendo como novedad la influencia de tres factores: sexo, edad y estado sexual (Alfaro et al., 2011). Los datos obtenidos se sometieron a un análisis de componentes principales (PCA), una metodología poco convencional en la búsqueda de volátiles de insectos y sin precedentes en el campo de las moscas de la fruta. Este método estadístico multivariante es muy común en otros campos, como el análisis alimentario (Jabalpurwala et al., 2009; Elaissi et al., 2010; Giri et al., 2010; Mildner-Szkudlarz y Jelen, 2010; Zhang et al., 2010), pero por lo que respecta a los insectos, solo se ha aplicado a especies de himenópteros (Steiner et al., 2007; Coppee et al., 2008). La técnica de PCA permite realizar agrupaciones de los compuestos identificados de acuerdo con los factores estudiados. Por lo tanto, el análisis en este caso agrupa aquellos compuestos que

presentan pautas de emisión similares, que según su abundancia relativa en cada uno de los estadios de las moscas, pueden sugerir diferentes mezclas para ensayos biológicos de comportamiento. Por ejemplo, compuestos muy abundantes en machos vírgenes adultos sugerirían ensayos de atracción de hembras; compuestos abundantes en hembras copuladas adultas podrían sugerir ensayos para compuestos inhibidores de la oviposición.

Este trabajo contribuye también con nuevos compuestos, nunca antes descritos en *C. capitata*, y sin embargo no se han detectado otros citados anteriormente en la literatura científica. Esto podría ser debido a la utilización de técnicas diferentes para la toma de volátiles y a otros factores experimentales, como la duración del muestreo o el flujo de aire (Flath et al., 1993). Además, teniendo en cuenta que este tipo de ensayos suelen hacerse con insectos de colonias de laboratorio, las condiciones de la cría también podrían influir en los resultados.

Los constituyentes mayoritarios encontrados en las emisiones de los machos fueron acetato de geranilo, (*E,E*)-α-farneseno y el (*E*)-3-octenoato de etilo, lo que coincide con lo descrito por otros estudios similares (Baker et al., 1985; Jang et al., 1989; Flath et al., 1993; Light et al., 1999), pero nuestro análisis añade, a esta lista, el ácido 2-etilhexanoico. Respecto a las hembras de *C. capitata*, únicamente se les atribuía emisiones de aldehídos a nivel de trazas (Jang et al., 1989); sin embargo, en nuestro trabajo se identificó, además de decanal, otro tipo de compuestos como acetofenona, trimetilamina y acido 2-etilhexanoico, como las emisiones más abundantes en hembras.

Quizá las observaciones más importantes se extraen de los conjuntos en los que el PCA sitúa a los compuestos anteriormente citados en bibliografía y que se relacionan con el comportamiento sexual de *C. capitata*. Por ejemplo, los tres compuestos mayoritarios descritos y ensayados anteriormente en otros trabajos (Heath et al., 1991; Jang et al., 1994), se sitúan en diferentes conjuntos: (*E*)-3-octenoato de etilo (conjunto E), acetato de geranilo (conjunto H), y (*E,E*)-α-farneseno (conjunto I_3). Además, el compuesto 1-pirrolina, citado como compuesto implicado en la atracción sexual (Baker et al., 1985), se sitúa también en el

conjunto H. Sin embargo, el patrón de emisión de estos tres conjuntos (E, H, I_3) es muy similar y se caracteriza por tener muy baja emisión en el caso de machos jóvenes vírgenes. Otros compuestos de abundancia intermedia, como linalol y metil-geranato, también descritos por Light (1999), se incluyen en los conjuntos E_2 y H. Según lo descrito, compuestos previamente citados y probados como potenciales semioquímicos de *C. capitata* se agrupan en tres conjuntos similares: E, H e I, lo que sugiere la conveniencia de la aplicación del PCA para estudiar estos volátiles. Estos tres conjuntos, junto al J (que incluye nuevos compuestos identificados, como ácido geránico y 2-piperidona) se caracterizan por tener elevada emisión cuando los machos son adultos y, por tanto, sería interesante realizar ensayos con las sustancias incluidas en estos conjuntos para probar su efecto atrayente frente a hembras de *C. capitata.* Por otro lado, los compuestos más abundantes en las emisiones de hembras copuladas adultas pueden sugerir ensayos de inhibidores de la oviposición, como sería el caso del nuevo compuesto identificado acetofenona, incluida en el conjunto B_1.

Los resultados de este estudio junto con ensayos complementarios de electroantenografía pueden sentar las bases para el establecimiento de mezclas de componentes naturales eficaces como atrayentes para machos o hembras de *C. capitata* que puedan utilizarse en técnicas de control.

CONCLUSIONES

CONCLUSIONES

Los resultados obtenidos en el conjunto de los ensayos llevados a cabo durante la realización de esta tesis doctoral permiten extraer las siguientes conclusiones:

Estudios sobre confusión sexual (Capítulos I-IV)

1. La confusión sexual ha demostrado ser una técnica eficaz en la lucha contra el piojo rojo de California (A*onidiella. aurantii*), controlando el nivel de daño en fruto de la misma forma que tratamientos de aceites convencionales bien aplicados. La eficacia ha sido demostrada en diferentes localizaciones y durante varios años, con dosis de feromona de ~30 g por ha y colocando un emisor por árbol (o al menos 400 emisores por ha), protegiendo el cultivo durante al menos 6 meses sin necesidad de reposición.
2. La confusión sexual contra *A. aurantii* consigue inhibir las capturas de machos en más de un 90% y reducir el daño en fruto en hasta un 80%, comparado con testigos sin tratamientos.
3. La inhibición del primer vuelo de los machos de *A. aurantii* no es decisiva para el éxito de la técnica, ya que la aplicación de la confusión sexual justo antes del segundo vuelo de la plaga consigue reducir el daño igualmente.
4. La lucha contra la polilla del tomate (*Tuta absoluta*) es posible en invernaderos con buen aislamiento, protegiendo el cultivo durante al menos 4 meses, con dosis de ~30 g por ha.
5. Considerando el precio actual de la feromona, la aplicación de la confusión sexual en el cultivo de tomate al aire libre no es viable económicamente.

Optimización de emisores para sistemas de atracción (Capítulo V-VII)

6. La atracción de los machos del lepidóptero *Chilo suppressalis* hacia trampas cargadas con su feromona sexual, es dependiente del nivel de emisión de la misma. El modelo cuadrático obtenido sugiere la existencia de un efecto repelente de la feromona cuando la dosis es excesiva, y se propone una velocidad óptima de emisión de ~34 µg por día de (Z)-11-hexadecenal, para maximizar la actividad atrayente de la feromona en la zona arrocera Mediterránea.
7. En el caso del lepidóptero *Lobesia botrana*, la respuesta al componente mayoritario de su feromona, (*E,Z*)-7,9-dodecadienil acetato, también es dependiente del nivel de emisión. Se obtuvo un modelo cuadrático, con una velocidad óptima de emisión de 400 µg por día en las condiciones climáticas típicas de la región Mediterránea.
8. Para los machos del díptero *Bactrocera oleae* se obtuvo también un tipo de respuesta cuadrática respecto al nivel de emisión de su feromona spiroacetal. Existe un valor de emisión óptima de ~1,3 mg por día, por encima y por debajo del cual disminuye la eficacia de las trampas cebadas con spiroacetal.
9. Para *Ceratitis capitata*, no se observó un óptimo de emisión cuando el atrayente utilizado es su paraferomona trimedlure, obteniendo un aumento no significativo de las capturas al superar un valor de emisión determinado (~2,4 mg por día).

Estudio de los volátiles emitidos por *C. capitata* (Capítulo VIII)

10. La técnica de SPME permite detectar nuevos compuestos en las emisiones de *C. capitata* como acetofenona, ácido geránico y 2-piperidona, no descritos anteriormente al usar otras técnicas de toma de muestra. En total se han detectado 70 compuestos, todos ellos emitidos por machos y sólo 37 por las hembras.
11. El análisis de componentes principales es adecuado para la agrupación de los compuestos detectados en diferentes conjuntos según su pauta de emisión y ha permitido la identificación de ciertas mezclas de compuestos susceptibles de ser ensayadas biológicamente.

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