UNIVERSIDAD D CÓRDOBA

TESIS DOCTORAL

Control biológico de estados edáficos de tefrítidos (Diptera: Tephritidae) mediante tratamientos de suelo con ascomicetos mitospóricos entomopatógenos (Ascomycota: Hypocreales)

Doctoranda: Dª. Inmaculada Garrido Jurado
Director: Prof. Dr. Enrique Quesada Moraga

Córdoba, 2012

TÍTULO: Control biológico de estados edáficos de tefrítidos (Diptera: Tephritidae) mediante tratamientos de suelo con ascomicetos mitospóricos entomopatógenos (Ascomycota: Hypocreales)

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DOCTORANDO/A: Inmaculada Garrido Jurado

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

La Doctoranda, Dña. Inmaculada Garrido Jurado, se incorporó a nuestro Grupo de Investigación AGR 163 "Entomología Agrícola" en el año 2005 para la realización del Trabajo Profesional Fin de Carrera que da opción al título de Ingeniero Agrónomo. Inmediatamente tras su obtención fue contratada con cargo al Proyecto de Excelencia de la Junta de Andalucía P07-AGR-02933 "Los Hongos Entomopatógenos en la desinfestación biológica del suelo", del que fuimos Coordinador e Investigador Principal junto con el Prof. Dr. D. Cándido Santiago Álvarez.

Los distintos objetivos planteados en esta tesis doctoral se abordan en capítulos como trabajos independientes con formato artículo, ya que la mayor parte de ellos han sido publicados como "full lenght papers" o están en proceso de publicación, como se indica a continuación:

Garrido-Jurado, I., Márquez, M., Ortiz-Urquiza, A., Santiago-Álvarez, C., Iturriaga, E.A., Quesada-Moraga, E., Monte, E., Hermosa, R., 2011. Genetic analyses place most Spanish isolates of *Beauveria bassiana* in a molecular group with word-wide distribution. BMC Microbiology 11, 84.

Garrido-Jurado, I., Torrent, J., Barrón, V., Corpas, A., Quesada-Moraga, E., 2011. Soil properties affect the availability, movement, and virulence of entomopathogenic fungi conidia against puparia of *Ceratitis capitata* (Diptera: Tephritidae). Biological Control 58**,** 277-285.

Garrido-Jurado, I., Valverde-García, P., Quesada-Moraga, E., 2011. Use of a multiple logistic regression model to determine the effects of soil moisture and temperature on the virulence of entomopathogenic fungi against pre-imaginal Mediterranean fruit fly *Ceratitis capitata*. Biological Control 59**,** 366-372.

Garrido-Jurado, I., Ruano, F., Campos, M., Quesada-Moraga, E., 2011. Effects of soil treatments with entomopathogenic fungi on soil dwelling non-target arthropods at a commercial olive orchard. Biological Control 59**,** 239-244.

Quesada-Moraga, E., Valverde-García, P., Garrido-Jurado, I., 2012. The effect of temperature and soil moisture on the development of the pre-imaginal Mediterranean fruit fly *Ceratitis capitata* (Diptera: Tephritidae). Environmental Entomology (Enviado).

Por tanto, se han publicado tres trabajos en la revista *Biological control*, una de las revistas de mayor impacto Q1 del Área de Entomología del Journal of Citation Reports (11 de 83; IP: 2.164; JCR 2010); se trata de una de las revistas de mayor índice de impacto donde se puede publicar un trabajo de este tipo de Control de Plagas en el área de Entomología, pues las que la preceden se refieren a aspectos de investigación básica en fisiología de insectos. Además, se ha publicado otro trabajo en la *revista BMC Microbiology*, situada en Q2 en el área de Microbiología del JCR (38 de 107; IP: 2.960; JCR 2010), y otro se ha enviado a la revista Environmental Entomology, situada en el Q2, en primera posición, en el área de Entomología del JCR (21 de 83; IP: 1.534; JCR 2010), y que es una de las mejores revistas de la Sociedad Americana de Entomología.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 10 de febrero de 2012

Firma del/de los director/es

Fdo.: Dr. D. Enrique Quesada Moraga

I. Los hongos entomopatógenos en el control de tefrítidos

IV. Genetic analyses place most Spanish isolates of Beauveria bassiana in a molecular group with world-wide distribution

V. Soil properties affect the availability, movement, and virulence of entomopathogenic fungi conidia against puparia of Ceratitis capitata (Diptera: Tephritidae)

VI. Use of a multiple logistic regression model to determine the effects of soil moisture and temperature on the virulence of entomopathogenic fungi against pre-imaginal Mediterranean fruit fly Ceratitis capitata

VII. The effect of temperature and soil moisture on the development of the pre-imaginal Mediterranean fruit fly Ceratitis capitata (Diptera: Tephritidae)

VIII. Effects of soil treatments with entomopathogenic fungi on soil dwelling non-target arthropods at a commercial olive orchard

I. Los hongos entomopatógenos en el control de tefrítidos

II. Las moscas de la fruta: biología, comportamiento y control

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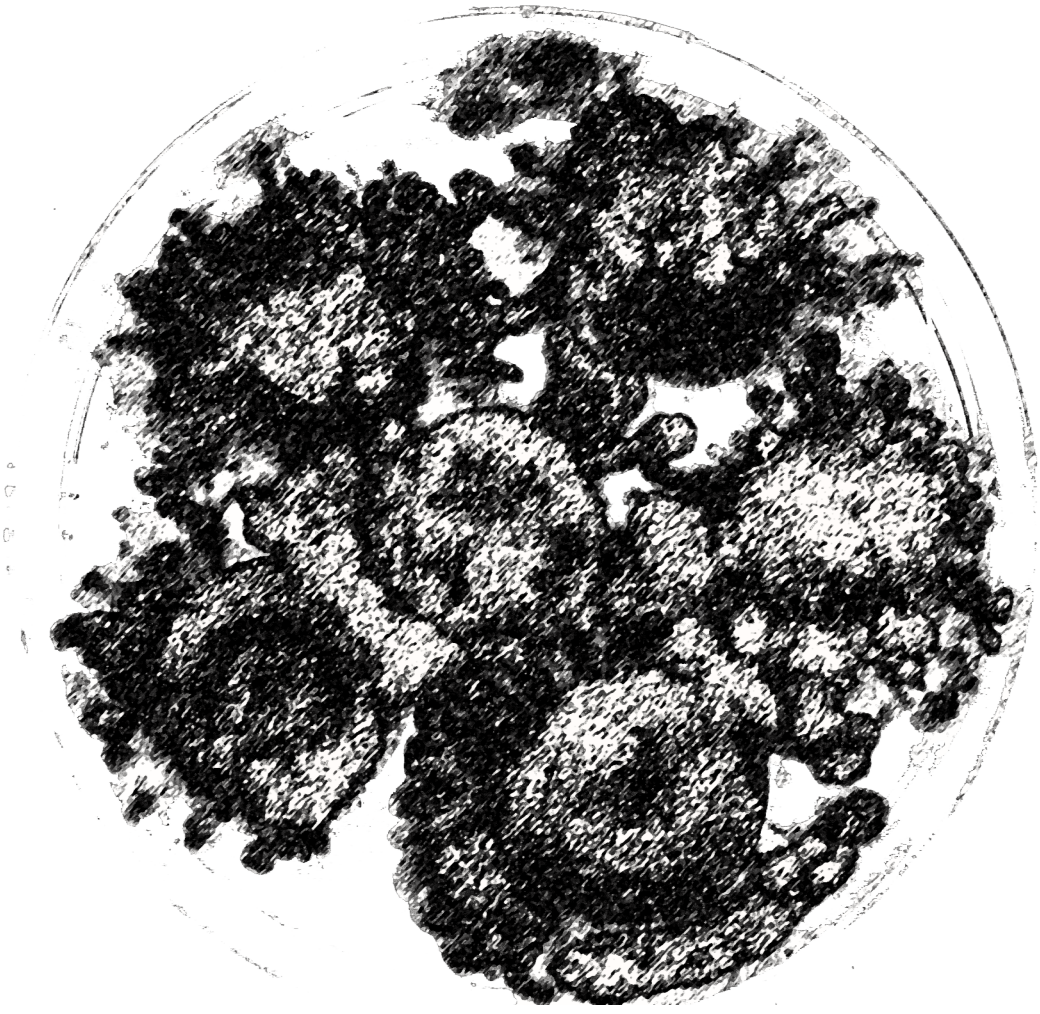
CAPÍTULO I

UNIVERSIDAD DE CÓRDOBA

REVISIÓN

Los hongos entomopatógenos en el control de tefrítidos



Los hongos entomopatógenos en el control de tefrítidos

1. Introducción

La domesticación de plantas y animales fue probablemente el acontecimiento más significativo en la historia de la humanidad, un punto de inflexión, donde la agricultura y ganadería sustituyen a la caza y la recolección de alimentos dentro del ámbito de la actividad humana (Maroto, 1998). Existe apoyo documental de la preocupación del hombre por los trastornos causados a las plantas cultivadas por factores bióticos y abióticos a lo largo de estas 600 generaciones (Jones, 1973; Vázquez-Lesmes y Santiago-Álvarez, 1993; Maroto, 1998), si bien, ha sido en las dos o tres últimas, cuando se han producido los cambios más profundos asociados a la intensificación de la agricultura (Pretty, 2009).

En efecto, el cultivo de las plantas para su aprovechamiento por el hombre genera una modificación del paisaje, que en muchas ocasiones es radical. Los agroecosistemas más recientes y modificados se caracterizan porque son especialmente sencillos en comparación con los sistemas naturales; están formados por pocas especies vegetales mejoradas, muy nutritivas, que ocupan grandes superficies, donde se rompe el equilibrio entre los distintos elementos de la cadena trófica, los productores primarios, la especie cultivada y la flora arvense, y los secundarios, fitófagos, entomófagos y agentes fitopatógenos (Sadras et al., 2009), con resultados muy negativos para los intereses del hombre; se exalta la competencia entre la flora arvense y el cultivo por los recursos, mientras que se favorece la acción de los agentes causantes de daño y enfermedad, que en su conjunto pueden ocasionar una pérdida de un 30-35% de la producción potencial total, sin despreciar las perdidas en poscosecha, que pueden acercarnos a la mitad de la producción agrícola mundial (Oerke, 2006).

La aparición de los insecticidas químicos auguraba malos tiempos a los insectos fitófagos, pero tanto la respuesta de estos, con la aparición de razas resistentes (Rossi y Rainaldi, 2000; Vontas et al., 2011), como los efectos negativos de aquellos sobre el medio ambiente, la fauna útil y los seres humanos, no se hicieron esperar (Peck, 2009). La concienciación medioambiental reinante, y la gran prevalencia de la Agricultura Sostenible como principio rector de las políticas agrarias comunitarias han quedado plasmadas en la Directiva 2009/128/CE del Parlamento Europeo y del Consejo de 21 de octubre de 2009 por la que se establece el marco de la actuación comunitaria para conseguir un uso sostenible de los plaguicidas, que bajo el abrigo del concepto de Gestión Integrada de Plagas, prioriza los métodos no químicos de control de plagas, esto es, agronómicos, físicos, mecánicos o biológicos. La Directiva adquiere el carácter de elemento vertebrador en los Planes de Acción Nacionales, que deben emanar de los distintos estados miembros antes de final de 2011, y establece la obligatoriedad del control integrado de plagas con la llegada de 2014.

Este escenario agudiza la necesidad de nuevas estrategias de control de plagas que se ajusten a los principios de la Agricultura Sostenible, entre las que destaca el control biológico por medio de enemigos naturales entomófagos y entomopatógenos. Más allá, el interés por los microorganismos entomopatógenos como agentes de control ha recibido un gran impulso, gracias a la motivación que prevalece en la realización de investigaciones encaminadas a establecer la efectividad de éstos como agentes bioinsecticidas, y herramientas seguras para el medio ambiente y la salud

pública. En particular los hongos entomopatógenos (HE), únicos agentes de control microbiano que actúan por contacto, por vía tegumentaria, han recibido en los últimos años una gran atención por su presencia natural, diversidad, eficacia insecticida, y adaptación para afrontar el biocontrol de especies de insectos donde la vía digestiva es imposible o está limitada (Quesada-Moraga y Santiago-Álvarez, 2008; Jaronski, 2010).

2. Los Hongos Entomopatógenos

Los HE son enemigos naturales de los artrópodos por lo que tienen un papel fundamental en la regulación de sus poblaciones (Goettel et al., 2005). Estos hongos, presentes de forma natural en el suelo, en el filoplano de las plantas e incluso en el aire (Meyling y Eilenberg, 2006; Quesada-Moraga et al., 2007; Quesada-Moraga y Santiago-Álvarez, 2008), están unidos de manera intrínseca a sus hospedantes, en un proceso de continua coevolución que hace que estén adaptados a vivir a expensas de éstos, de los que obtienen la energía necesaria para su desarrollo (Roy et al., 2006). De esta forma, en función de la asociación trófica de los HE con sus hospedantes, se pueden distinguir tres grupos: (1) hongos biotrofos que se alimentan únicamente de células vivas y tal proceso cesa con la muerte de las mismas, (2) necrotrofos que matan al hospedante y después crecen a expensas de los tejidos muertos, y (3) hemibiotrofos que son inicialmente biotrofos y una vez que el hospedante muere se vuelven necrotrofos (Quesada-Moraga y Santiago-Álvarez, 2008; Vega et al., 2009).

2.1. Posición sistemática de los Hongos Entomopatógenos

La clasificación de los dos grupos principales de hongos entomopatógenos, los Entomophthorales y los Hypocreales ha experimentado cambios recientes en la nueva propuesta de Hibbett et al. (2007) para el Reino de los Hongos (Mycota) (Fig. 1). En lo que respecta a los Entomophthorales, y en concreto los miembros de la familia Entomophthoraceae, originan epizootias naturales, pero al tratarse de biotrofos obligados, existe gran dificultad para producirlos en medio artificial, lo que limita por ahora sus posibilidades de empleo práctico. No obstante, en algunos casos se han obtenido buenos resultados, a nivel experimental,

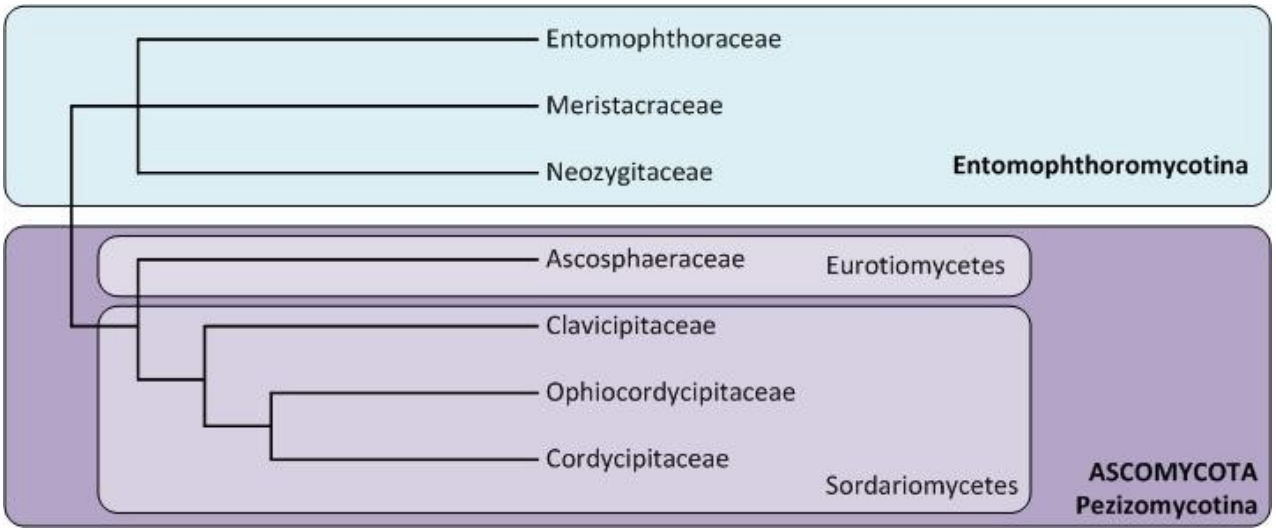


Fig. 1. Relaciones entre las familias más importantes de hongos entomopatógenos dentro de la nueva clasificación del Reino Mycota basado en las propuestas de Hibbett et al., 2007, Keller, 2007 y Sung et al., 2007

sin desarrollo comercial (Pell et al., 2001). Sin embargo, los segundos, los ascomicetos mitospóricos, unen a su amplia distribución y eficacia insecticida, su facilidad de manejo y producción en masa, lo que les ha proporcionado un papel creciente y destacado en el mercado mundial de bioinsecticidas (de Faria y Wraight, 2007).

3. Empleo de ascomicetos mitospóricos entomopatógenos en el control de plagas

En los ascomicetos mitospóricos entomopatógenos (AME), el proceso patogénico comienza generalmente con la adhesión de los conidios a la cutícula del hospedante, donde germinan y emiten un tubo germinativo para atravesarla gracias a una combinación de acciones mecánicas y bioquímicos que facilitan la invasión del hemocele (Hajek y St. leger, 1994; Quesada -Moraga y Santiago-Álvarez, 2008). Una vez dentro, el hongo crece en la cavidad general del insecto en forma de cuerpos hifales si logra vencer la respuesta defensiva del hospedante (Vey et al., 2001), al que provoca la muerte por varias acciones, utilización de nutrientes, invasión de tejidos y órganos o producción de toxinas, cada una por separado o en combinación. La muerte del insecto acarrea el crecimiento necrotrofo del hongo, y en condiciones favorables, las hifas emergen del cadáver, se produce la esporulación y la liberación de los conidios que inician un nuevo ciclo de infección (Inglis et al., 2001; Goettel et al., 2005; Charnley y Collins, 2007).

Este modo de actuación les confiere ventaja en el control de insectos con aparato bucal picador-chupador (Tisanópteros y Hemípteros) (Quesada-Moraga et al., 2006b), en aquellos con aparato bucal masticador entre los que no se conocen enfermedades de etiología viral o bacteriana (algunos Lepidópteros y Coleópteros) (Marannino et al., 2006; 2008), o éstas no son eficaces como ocurre con langostas, saltamontes o cucarachas (Hernández-Crespo y Santiago-Álvarez, 1997; Quesada-Moraga et al., 2004), en insectos del suelo (Quesada-Moraga et al., 2006b), e incluso para algunos parásitos animales de gran importancia como *Varroa destructor* (Acari: Mesostigmata), parásito de la abeja melífera *Apis mellifera* (García-Fernández et al., 2008).

Las estrategias para el empleo de AME en el control de insectos son dos, **inoculativa** mediante la aplicación puntual del inóculo para iniciar ciclos de enfermedad, establecer el hongo en la población del insecto y mantener el control a largo plazo, o **inundativa** con la aplicación del insecticida microbiano, para iniciar una epizootia conducente al declive de la población en un tiempo relativamente corto.

3.1. Papel de los AME en el control integrado de tefrítidos

3.1.1. Tratamientos dirigidos a los adultos

Al igual que los tratamientos químicos convencionales, las estrategias de control de adultos de tefrítidos con AME se basan en tratamientos de pulverización del árbol (Daniel y Wyss, 2010), o mediante la estrategia de atracción e infección que se fundamenta en el empleo de un cebo compatible con los AME (Ekesi et al., 2007; Quesada-Moraga y Santiago-Álvarez, 2008).

3.1.1.1. Aplicación aérea. El AME junto con el cebo se pueden pulverizar en una parte del árbol o dispensarse mediante un dispositivo colocado en una parte de éste, por tanto un aspecto fundamental en este tipo de apli-

caciones es la compatibilidad de los AME con el cebo alimenticio o los atrayentes utilizados. Esta técnica se fundamenta en que el adulto debe ingerir el cebo que contiene al agente de control, o bien éste al posarse fortuitamente sobre la vegetación o los frutos del árbol entra en contacto con las esporas del hongo y se infecta (Ekesi et al., 2007). Además, puesto que los AME pueden transmitirse horizontalmente, es decir de un individuo a otro, los insectos infestados pueden diseminar los conidios adheridos a su cutícula a otros individuos (Quesada-Moraga y Santiago-Álvarez, 2008), lo que sin duda aumenta el potencial de biocontrol de los AME frente a tefrítidos. Por otro lado, las hembras tratadas con AME son menos fecundas que sus congéneres no tratadas (Castillo et al., 2000; Quesada-Moraga et al., 2006b) por lo que el tratamiento con estos incide en el número de frutos picados y en el número de individuos de la generación siguiente. No obstante, para que la aplicación de AME en combinación con cebos o no, sea un método de control de tefrítidos robusto es necesario mejorar la producción y formulación de los conidios para aportar suficiente inóculo en los tratamientos, evitar la inactivación de los conidios por la radiación ultravioleta y favorecer la adhesión de los mismos a los insectos (Ekesi et al., 2007).

3.1.1.2. Atracción e infección. En los últimos años se difunde la estrategia de autodiseminación del inóculo fúngico para el control de fitófagos que responden a estímulos visuales y olfativos, ya que esta estrategia de aplicación reduce la cantidad de inóculo y el área a tratar, lo que minimiza los posibles efectos adversos de los AME sobre los insectos no diana (Ekesi et al., 2007; Quesada-Moraga y Santiago-Álvarez, 2008), a la vez que se reducen los costes de la aplicación. Además, la autodiseminación de AME proporciona una ventaja adicional a la aplicación anterior pues el impacto en la población del fitófago se incrementa más allá del contacto directo (Scholte et al., 2004). Esta técnica se basa en la atracción del adulto mediante señales de distinta naturaleza (visuales, químicas o alimenticias) hasta el lugar en el que se encuentra el AME, donde el insecto se impregna del inóculo y lo disemina entre el resto de la población (Ekesi et al., 2007). Las trampas más extendidas en su empleo son aquellas que contienen feromonas específicas para machos (IAEA, 2003), y las que emplean proteínas y azúcares fermentados que atraen tanto a machos como a hembras (Ekesi et al., 2007). En laboratorio se han realizado diversos estudios de transmisión horizontal de AME mediante el empleo de dispositivos autoinoculadores con atrayentes alimenticios en su interior (Dimbi et al., 2003; Quesada-Moraga y Santiago-Álvarez, 2008). En general, la transmisión es más eficaz cuando se realiza de macho a hembra, ya sea durante los distintos intentos de cópula o durante la misma (Quesada-Moraga y Santiago-Álvarez, 2008), por lo que puede emplearse en programas de Control Integrado para aumentar el éxito del control mediante la suelta de machos estériles (Ekesi et al., 2007).

3.1.2. Tratamientos de suelo dirigidos a estados preimaginales

Los AME han demostrado un gran potencial para el control de adultos de tefrítidos (Quesada-Moraga et al., 2006b), pero en los últimos años, se promulga también su potencial para el control de larvas de tercera edad próximas a pupación y puparios, en tratamientos de suelo en la base del árbol (Ekesi et al., 2007), pues el hongo se encuentra en su hábitat natural, protegido de cualquier factor adverso, donde ejer-

ce su máximo potencial de biocontrol. Para que el empleo de AME sea satisfactorio se deben de cumplir dos objetivos básicos: fácil aplicación en el medio natural del fitófago y aumento de su vida útil y persistencia en aquél tras la aplicación (Ekesi et al., 2007). Es conocido que los AME pueden permanecer e incluso reciclarse en el suelo (St Leger, 2008), por lo que este método de aplicación posibilita reducir progresivamente el número de tratamientos tanto en el espacio, por su localización, como en el tiempo, por la propia ecología de estos agentes de biocontrol. En general, los compuestos seleccionados para la formulación de este tipo de agentes de control no interfieren con el proceso de infección, incluso en el mejor de los casos mejoran la viabilidad, virulencia, transmisión de la enfermedad y persistencia en campo del inóculo (Ekesi et al., 2007). A este respecto, para la toma de decisiones sobre el número de tratamientos a realizar, resulta importante evaluar la evolución del inóculo fúngico en el suelo (Scheepmaker y Butt, 2010).

Más allá, antes de llevar a cabo el registro comercial de alguno de los aislados fúngicos seleccionados por su actividad insecticida frente a tefrítidos, resulta fundamental evaluar su posible impacto sobre otros insectos y artrópodos no diana (Goettel y Jaronski, 1997; Hajek y Goettel, 2000). Sin embargo, los AME generalmente presentan un rango de hospedantes bastante estrecho y su actividad se ve influenciada por factores abióticos y bióticos del medio (Brownbridge y Glare, 2007). Además, los niveles de inóculo en el suelo tras una aplicación descienden a lo largo del tiempo hasta 10^2-10^5 propágulos/g de suelo, y puesto que estos niveles de inóculo de manera natural en el mismo oscilan en torno de 10^3 propágulos/g de suelo, se deduce que los artrópodos no diana ya se encuentran de manera natural en contacto con valores similares de inóculo a los residuales tras una aplicación (Brownbridge y Glare, 2007; Scheepmaker y Butt, 2010). También habría que tener en cuenta los efectos beneficiosos de los AME sobre la fauna microbiana del suelo, ya que su influencia en la densidad y diversidad microbiana edáfica puede ser más importante que los efectos directos sobre la fauna auxiliar.

4. Diversidad y evolución de los ascomicetos mitospóricos entomopatógenos

La investigación sobre los AME se ha limitado a la entomología aplicada y a las consideraciones ecológicas relacionadas con el manejo integrado de plagas. Sin embargo, debido a los últimos descubrimientos de sus diversas e insólitas funciones ecológicas como endófitos de plantas, antagonistas de hongos fitopatógenos, colonizadores de la rizosfera y promotores del crecimiento de las planta (Vega et al., 2009), se ha incrementado el empleo de herramientas moleculares para estudiar su genética y eco-fisiología (Rehner, 2009). La identificación de AME empleando métodos moleculares se basa en polimorfismos que se presentan con elevada frecuencia en el ADN. La mayoría de estos polimorfismos se localizan en regiones no codificantes del genoma como intrones, espaciadores y secuencias repetidas (Castrillo y Humber, 2009), y están basados en el cambio de una sola base (Moore y Frazer, 2002). El genoma fúngico está compuesto casi en su totalidad por ADN nuclear y mitocondrial, aunque también puede albergar algunos elementos extracromosómicos como plásmidos o ARN de doble cadena que pueden contribuir al polimorfismo intraespecies (Castrillo y Humber, 2009). El nivel taxonómico al que se desee llegar determinará la técnica molecular a emplear (Tabla 1), así como el número de muestras

Tabla 1. Técnicas moleculares disponibles en ecología de ascomicetos mitospóricos entomopatógenos y nivel de resolución taxonómica[a]

Tipo de secuencia	Técnica molecular	Nivel taxonómico					Tipo de estudio ecológico			
		Aislado	Población	Subespecie	Especie	Género	Estructura poblacional	Variación genética	Asociación hongo-hospedante	Origen
Conocida	[b]PCR-LP (ITS, microsatélites) PCR-RFLP									
	[c]PCR-SSCP, PCR-DGGE, PCR-TGGE									
Anónima	RAPD									
	Oligonucleótidos universales AFLP									

Los cuadros sombreados en gris indican los niveles taxonómicos que se alcanzan con cada técnica molecular, así como los tipos de estudios ecológicos que se pueden realizar con cada una de ellas.
[a]Basado en Kouvelis et al., 2008; Castrillo y Humber, 2009; Enkerli y Widmer, 2010; Ghikas et al., 2010 ; [b]PCR-LP: PCR-Polimorfismo en la longitud; [c]PCR-SSCP: PCR-Polimorfismo de conformación de las cadenas simples; [d]PCR-DGGE: PCR-electroforesis en gel con gradiente de desnaturalización; [e]PCR-TGGE: PCR-electroforesis en gel con gradiente de temperatura

necesarias para dicha técnica.

4.1. Sistemática molecular de los ascomicetos mitospóricos entomopatógenos

La llegada de los análisis filogenéticos ha revolucionado los estudios de ecología y evolución, pues estos muestran que muchas especies de AME tienen características similares a un ancestro común (Freckleton, 2000). Como muestra la clasificación propuesta por Hibbett et al. (2007), los hongos entomopatógenos se encuentran en los filos Entomophthoromycotina y Ascomycota, aunque este último cuenta con mayor número de representantes (Rehner, 2009). En este filo (clase Sordariomycetes, orden Hypocreales) se encuentra el género *Cordyceps*, que reúne el mayor número de especies entomopatógenas, unas 400, y mayor número de hospedantes (Mains, 1957; 1958; Kobayasi, 1982). Distintos estudios hechos principalmente con ADN ribosomal (Artjariyasripong et al., 2001; Sung et al., 2001; Stensrud et al., 2005) reclasificaron las diferentes especies del género *Cordyceps* ubicándolas por completo en las familias Cordycipitaceae y Ophiocordycipitaceae (Tabla 2) (Spatafora et al., 2007; Sung et al., 2007). El género *Cordyceps* está restringido a los teleomorfos, mientras que sus anamorfos se clasifican en los géneros *Beauveria, Lecanicillium*, *Isaria*,... (Rehner, 2009). Sin embargo, el teleomorfo de *Metarhizium* en principio clasificado en el género *Cordyceps* ha sido transferido al nuevo género *Metacordyceps* (Sung et al., 2007).

El género *Beauveria* es uno de los que mejor caracterizado está morfológica y molecularmente. Se trata de un género monofilético (Sung et al., 2007), de amplia distribución geográfica que infecta a más de 700 especies de hospedantes (Meyling y Eilenberg,

Tabla 2. Nueva clasificación de los ascomicetos mitospóricos patógenos de artrópodos

Familia	Estado	Género
Clavicipitaceae	Teleomorfo	*Conoideocrella, Hypocrella, Metacordyceps, Moelleriella, Orbiocrella, Regiocrella, Samuelsia, Shimizuomyces, Villosiclava*
	Anamorfo	*Metarhizium, Paecilomyces s.l., Pochonia*
Cordycipitaceae	Teleomorfo	*Ascopolyporus, Cordyceps, Hyperdermium, Torrubiella*
	Anamorfo	*Akanthomyces, Beauveria, Isaria, Lecanicillium, Simplici-*
Ophiocordycipitaceae	Teleomorfo	*Cordyceps s.l., Elaphocordyceps, Ophiocordyceps*
	Anamorfo	*Haptocillium, Hirsutella, Hymenostilbe*

Esta clasificación se basa en las propuestas de Sung et al., (2007) y Spatafora et al., (2007).

2007). La identificación de aislados en el nivel de especie mediante técnicas moleculares ha mostrado doce linajes internos que incluyen a todas las especies descritas morfológicamente (Rehner et al., 2011). En una primera aproximación empleando el factor de elongación EF1-α aparecen siete linajes de especie, *B. amorpha*, *B. bassiana*, *B. brongniartii*, *B. caledonica*, *B. vermicornia* y dos nuevos linajes, clade C indistinguible morfológicamente de *B. bassiana s.s*. y clade E asociado con el teleomorfo *Cordyceps* (Rehner y Buckley, 2005). Al emplear las regiones intergénicas nucleares Bloc y EFutr (muestran mayor polimorfismo que la anterior) se obtiene que *B. bassiana* s.s. es un complejo críptico de especies con un amplio rango de hospedantes (Rehner et al., 2006; Meyling et al., 2009). Como resultado de la combinación de secuencias parciales de RPB1 (subunidad mayor de la RNA polimerasa II), RPB2 (segunda subunidad mayor de la ARN polimerasa II), EF1-α y Bloc, se obtienen seis linajes ya conocidos (*B. amorpha*, *B. bassiana*, *B. brongniartii*, *B. caledonica*, *B. malawiensis* y *B. vermicornia*), junto con otros seis nuevos (*B. asiatica*, *B. australis*, *B. kipukae*, *B. pseudobassiana*, *B. sungii* y *B. varroae*) (Rehner et al., 2011).

El género *Metarhizium* también es monofilético y está formado por un complejo críptico de nueve especies (Bischoff et al., 2009). Dos de ellas se agrupan en el denominado clade 1 (*M. majus* y *M. guizhouense*) y están separadas del resto (*M. brunneum*, *M. pingshaense*, *M. robertsii*, *M. anisopliae, M. lepidiotae*, *M. acridum* y *M. globosum*) por oligonucleótidos específicos de clade (Schneider et al., 2011).

Otro género importante de AME es *Paecilomyces*. Estudios realizados con ADN ribosomal de distintas especies del género con orígenes diversos, muestran que se trata de un grupo polifilético. Se han dividido cuatro subgrupos en los que en cada uno de ellos se encuentran por ejemplo, *Isaria fumosorosea*, *Paecilomyces marquandii*, *P. lilacinus* y *Paecilomyces* sp. (Freed et al., 2011). El primer subgrupo muestra claras diferencias con el resto y está formado por once especies de *Paecilomyces* sect. *Isarioidea* reclasificadas al género *Isaria* (p. ej. *I. fumosorosea* Wize, *I. javanica* (Frieder. y Bally) Samson y Hywel-Jones y *I. tenuipes* Peck) (Luangsa-Ard et al., 2005).

Por último, *Lecanicillium* es el anamorfo de los géneros *Cordyceps* y *Torrubiella* (Zare y Gams, 2001). Se trata de un género parafilético, intercalado entre especies bien definidas como *Beauveria* e *Isaria* (Sung et al., 2007).

5. Ecología de los ascomicetos mitospóricos entomopatógenos en hábitats epigeos e hipogeos

Conocer el origen y diversidad de los AME resulta primordial para poder entender el proceso de evolución de estos hongos en sus distintas funciones ecológicas. Como se ha visto antes, la ecología molecular profundiza en el estudio de la genética de poblaciones, biodiversidad, conservación, comportamiento e interacción de estos AME con el hábitat (Beebee y Rowe, 2008). Sin embargo, no se debe olvidar su principal función, clave para promover el desarrollo de epizootias como consecuencia de su acción sobre los insectos. Para lo que también es importante la adquisición de un profundo conocimiento de su ambiente, distribución y abundancia en el medio (Fisher et al., 2011).

5.1. Los ascomicetos mitospóricos entomopatógenos en hábitats epígeos

Las epizootias ocurren con mayor frecuencia en hábitats epígeos, en los que la infección se dispersa rápidamente a través de insectos enfermos, de substrato vegetal con conidios en su superficie o de conidios transportados por el viento (Goettel et al., 2005). En este ambiente las principales limitaciones climáticas son la radiación solar, la temperatura y la humedad, que proporcionan una corta persistencia de los propágulos.

La radiación solar, en particular la **radiación ultravioleta** (UV) afecta a la persistencia del inóculo en hábitats epígeos (Inglis et al., 2001). De ella, la UVB ($295 < \lambda < 320$ nm) es la de mayor importancia para los procesos biológicos (Smits et al., 1996). En general, la vida media de los conidios en condiciones naturales de luz solar es de 3-4 h (Roberts y Campbell, 1977), aunque se ha llegado a medir su eficacia sobre insectos hasta 8-14 días después del tratamiento (van der Valk, 2007). Sin embargo, el efecto de la radiación solar puede verse alterado por la formulación y la especie fúngica o aislado (Jaronski, 2010). Además, los estudios recientes muestran la presencia de AME en el filoplano de las plantas, lugar en el que cobra gran importancia el efecto de la radiación UV. La superficie de las hojas rara vez se encuentra en un plano horizontal, lo habitual es tener hojas en distintas orientaciones, que reciben de manera diferente dicha radiación. La parte menos expuesta de la hoja, es decir, el envés, parece albergar más actividad biológica, debido quizás a una menor incidencia de la radiación UV en su superficie (Jaronski, 2010). Parecen pues necesarios estudios que determinen la persistencia del patógeno en cultivos con diferentes tipologías frente a la UVB (Vidal y Fargues, 2007).

La **temperatura** puede afectar a la eficacia insecticida y la persistencia en campo de los AME, por afectar a la germinación de los conidios y a su crecimiento miceliar (Hajek et al., 1990). La respuesta de hongos entomopatógenos a la temperatura sigue una forma de campana desplazada hacia temperaturas más altas (Quesada-Moraga et al., 2006a) con óptimos de crecimiento entre 23 y 28ºC dependiendo del aislado, y cuyo crecimiento disminuye rápidamente en torno a 30ºC. Sin embargo, los valores de temperatura observados en la superficie de la planta pueden superarlos, con incidencia sobre la germinación del inóculo (Noma y Strickler, 1999).

La **humedad** no sólo es esencial para la germinación de los propágulos, sino que también ejerce una fuerte influencia en la conidiogénesis tras la muerte del hospedante (Inglis et al., 2001). El éxito de la in-

fección depende de la relación del hongo con la cutícula del fitófago o con el filoplano de las plantas y sus microclimas (Jaronski, 2010), y no tanto de la humedad general del ambiente (Fargues et al., 2003). Un factor crítico en la influencia de la humedad es la capa límite de la hoja, se trata de una delgada capa de aire inmóvil alrededor del filoplano que se ve afectada por la tipología de la hoja, la temperatura originada por la radiación y el movimiento de aire (Vesala, 1998). En general, la humedad presente en la capa límite de la hoja es superior a la del ambiente (Willmer, 1986). Sin embargo no hay información sobre la capa límite de la cutícula del insecto, aunque numerosos autores especulan con la idea de que los apéndices del aparato bucal, membranas intersegmentales y espiráculos deben tener mayor humedad que el resto de la cutícula por ser a través de ellos donde se hace visible la infección (Ferron, 1977; Charnley, 1989).

5.2. Los ascomicetos mitospóricos entomopatógenos en hábitats hipogeos

El suelo, además de ser reservorio de estos hongos, es el lugar donde se desarrollan complejas interacciones hongo-fitófago, y en las zonas cercanas a la rizosfera también aquellas que relacionan hongo-fitófago-planta (Hu y St Leger, 2002). Las características del suelo (textura, pH, materia orgánica), condiciones ambientales del hábitat (temperatura, humedad), y tamaño y comportamiento de los insectos que habitan en el mismo, hacen que la distribución de conidios en el suelo sea extremadamente heterogénea (Jaronski, 2010). Al aplicarlos por inundación al suelo, estos hongos cambian su proporción en el mismo, favorecidos así en la lucha contra el fitófago. Sin embargo, para un control eficaz, los conidios se ven obligados a permanecer en el medio un periodo más o menos largo de tiempo hasta la llegada del insecto. Esta persistencia en el suelo puede variar entre pocas semanas y más de cuarenta según la especie fúngica y las condiciones del medio que la rodea (Storey et al., 1989; Kabaluk et al., 2007). Además, mediante el análisis con marcadores moleculares se puede constatar la presencia a lo largo del tiempo del aislado con el que se ha realizado el tratamiento (Enkerli et al., 2002).

5.2.1. Factores edáficos de naturaleza biótica pueden intervenir en la eficacia insecticida y la persistencia del inóculo

De todos los factores edáficos de naturaleza biótica, probablemente la **interacción con otros microorganismos** del suelo sea el de mayor relevancia. Muchos suelos de manera natural no permiten el crecimiento en ellos de AME (Stotzky, 1972; Pereira et al., 1993), sin embargo, esta fungistasis desaparece al esterilizar dichos suelos. También es relevante la observación que en ocasiones, dicha fungistasis no se lleva a cabo cuando el suelo es rico en nutrientes, pues los conidios reciben suficiente estímulo nutritivo para su germinación (Groden y Lockwood, 1991). Además, en los últimos años se ha observado el antagonismo en cultivos duales entre estos hongos y los fitopatógenos, pero sólo en medios de cultivo ricos en nutrientes, sin embargo las distintas técnicas empleadas hacen difícil obtener conclusiones al respecto (Jaronski, 2007).

En el suelo se encuentra una zona rica en nutrientes próxima a la **rizosfera** de las plantas, entorno en el que existe una gran actividad de microorganismos, en particular bacterias y hongos (Bowen y Rovira, 1999). Las raíces producen dos tipos de exudados: (1) com-

puestos de bajo peso molecular como aminoácidos, azúcares, ácidos orgánicos y otros metabolitos secundarios, y (2) compuestos de alto peso molecular como proteínas y polisacáridos (Marschner, 1995), que proporcionan un medio favorable para la persistencia de AME (Hu y St Leger, 2002; Bruck, 2005). La presencia de estos hongos en torno a las raíces proporciona además una "barrera repelente" que disuade al fitófago (St Leger, 2008).

Por último, cabe destacar el efecto de la **variabilidad genética** de los AME en la respuesta a las condiciones del medio que puede influir en su virulencia o en su supervivencia en el suelo. Así, Wang y St. Leger (2007) observaron que *M. anisopliae* produce las proteínas MAD1 y MAD2 que se combinan de manera diferente según la adhesión del conidio se produzca en la cutícula del insecto o en la superficie de la planta. Además se han observado diferencias en la persistencia del inóculo en el suelo al emplear una cepa transformada de *M. anisopliae* (Hu y St Leger, 2002).

5.2.2. Factores edáficos de naturaleza abiótica pueden intervenir en la eficacia insecticida y la persistencia del inóculo

Los principales factores abióticos que influyen en los AME son la textura del suelo (tamaño de poro, distribución de partículas), temperatura y humedad, aunque otros como el pH, capacidad de intercambio catiónico o materia orgánica también pueden influir sobre la eficacia insecticida del inóculo y persistencia del mismo (Jaronski, 2010).

El suelo está constituido por partículas que oscilan entre 0.002 y 2 mm de diámetro para la fracción arcilla y arena, respectivamente (Fig. 2). La proporción de las diferentes clases separadas por tamaño determina la **clase textural** de un suelo (arcilloso, limoso, arenoso). Esta superficie mineral del suelo contiene entre un 1 y un 10% de materia orgánica que se supone como un importante reservorio de nutrientes para la biomasa microbiana del suelo (Voroney, 2007).

Esta estructura física define las redes de poros y a su vez el hábitat para la biota del suelo (Fig. 2). La abundancia de AME no está distribuida de manera homogénea a lo largo del perfil del suelo debida a la situación de las distintas partículas en el mismo y los poros originados como consecuencia de ello (Klingen y Haukeland, 2006). La mayoría de los AME se encuentran en los primeros 100 mm del suelo, y su número disminuye a medida que aumenta la profundidad (Ignoffo et al., 1977; Jaronski, 2007). La fracción arcilla muestra mayor influencia sobre los AME debido quizás a poseer mayor superficie específica, mientras que la fracción arena lo hace sobre el movimiento de agua y la aireación (Jaronski, 2007).

La **humedad** del suelo está determinada por el contenido de agua del mismo, que influye en la germinación, infección y esporulación de los AME en el suelo (Inglis et al., 2001). El efecto de la humedad del suelo en los AME es una situación compleja y se considera el factor determinante para el desarrollo de epizootias (Fuxa y Richter, 2004). La interacción entre ambos se crea en función de la textura del suelo vía porosidad. La humedad del espacio poroso del suelo afecta de manera directa a la estabilidad y supervivencia de los conidios (Inglis et al., 2001; Ekesi et al., 2007). A medida que aumenta la humedad disminuye la persistencia del inóculo en el suelo.

Aunque el suelo, en general, sea un hábitat con **temperatura** estable, es cierto que no mantiene uniformidad de la misma en capas superficiales, con variaciones estacionales o diurnas. Sin embargo, a más de 5 cm de profundidad se encuentra mayor estabili-

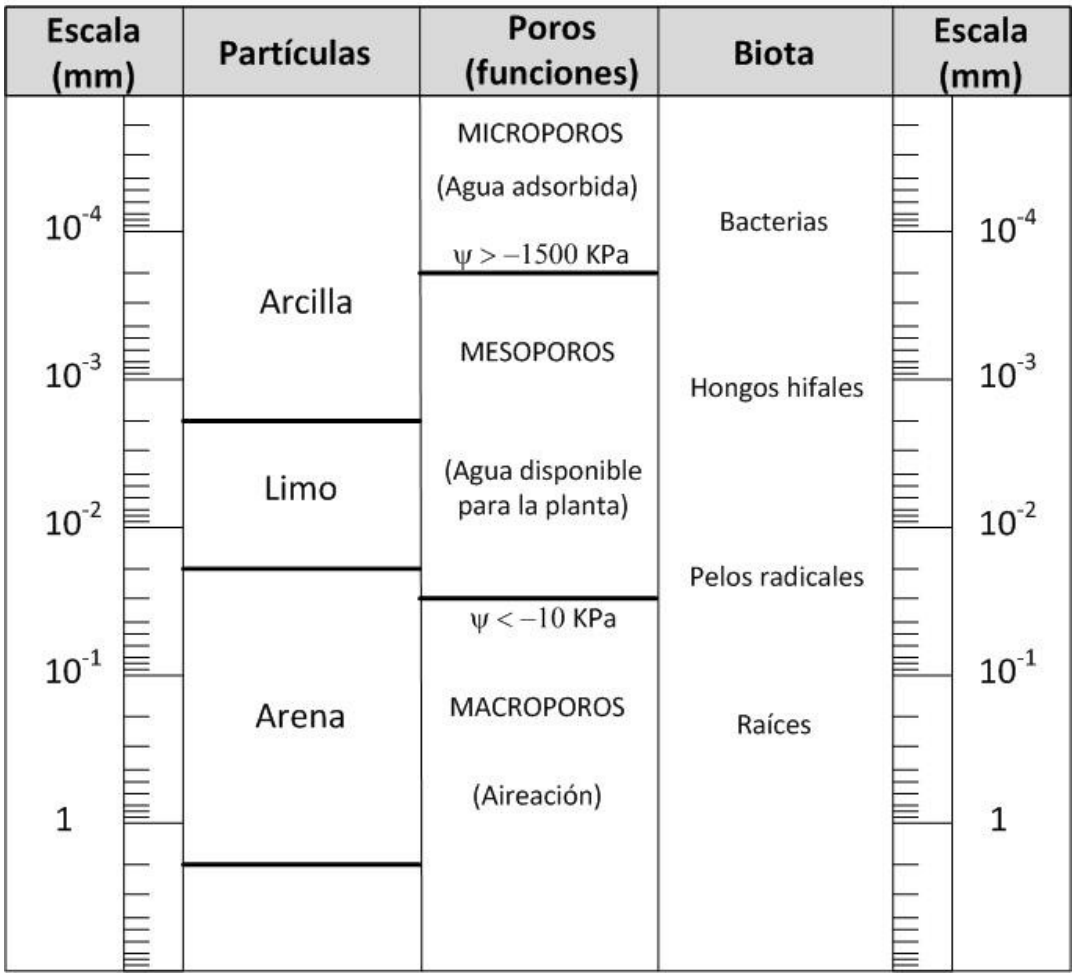


Fig. 2. Dimensiones espaciales de las principales características del suelo (adaptada de Voroney, 2007)

dad con tendencia a ser más frío que en superficie (Jaronski, 2010). La influencia de la temperatura del suelo sobre los AME está íntimamente relacionada con la humedad del suelo. De manera que, debido al calor específico del agua, suelos húmedos están menos sometidos a fluctuaciones de temperatura que suelos secos. Sin embargo, aunque el efecto de la temperatura sobre la persistencia de los AME es variable, éste se encuentra relacionado de manera positiva con la tasa óptima de crecimiento de estos aislados (Quesada-Moraga et al., 2006b; Ekesi et al., 2007).

Aunque como se ha visto hay diversos factores bióticos que inciden sobre los AME, son de menor importancia que los factores abióticos, cuyo conocimiento resulta clave para el éxito de los tratamientos fúngicos al suelo. Este ha sido objetivo fundamental de la presente tesis, aclarar los efectos producidos por los factores abióticos del suelo sobre la presencia, diversidad y persistencia de AME, así como sobre su actividad frente estados preimaginales de *C. capitata*.

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CAPÍTULO II

UNIVERSIDAD DE CÓRDOBA

REVISIÓN

Las moscas de la fruta: biología, comportamiento y control

Las moscas de la fruta: biología, comportamiento y control

1. La familia Tephritidae

En esta familia se encuadran las llamadas "moscas de la fruta", es una de las mayores del orden Diptera, con aproximadamente 4500 especies descritas, en 500 géneros, difundidas en las áreas tropical, subtropical y templada (Maddison y Bartlett, 1989). La entrada en la consideración agronómica se debe al modo de vida de las larvas, unas se desarrollan a expensas de los frutos, carpófagas, las que revisten mayor importancia económica, otras en las inflorescencias de compuestas y labiadas, algunas como minadoras de hojas sin que falten rizófagas ni gallígenas (Tremblay, 1994). La división en subfamilias ha sido objeto de mucha controversia (Tremblay, 1994), la más clásica comprende Dacinae, Tephritinae y Trypetinae (Drew, 1989) pero ahora se completa con Blepharoneurinae, Phytalmiinae y Tachiniscinae (Norrbom et al., 2004). Los géneros *Bactrocera* (520 especies), *Dacus* (243 especies) y *Ceratitis* (78 especies) de Dacinae, *Anastrepha* (198 especies) y *Rhagoletis* (69 especies) de Trypetinae encierran algo más de mil especies (Norrbom et al., 2004), de las que aproximadamente unas 70 alcanzan la consideración de plagas de gran importancia, con los frutales como cultivos más propensos a su ataque. En lo que respecta a la hortofruticultura española, sólo tres especies originan plagas de consideración (Alfaro-Moreno, 2005), *Bactrocera oleae* (Gmelin) o "mosca del olivo", *Ceratitis capitata* (Wiedemann) o "mosca mediterránea de la fruta", y *Rhagoletis cerasi* (Linneo) o "mosca de la cereza", aunque destacan las dos primeras por la importancia económica y extensión de los cultivos a los que atacan.

2. Morfología del adulto

Los adultos son de tamaño pequeño a medio (2.5-10 mm de longitud), si bien éstos pueden oscilar entre 1 y 20 mm, aunque tanto *B. oleae* como *C. capitata* miden entre 4 y 6 mm de longitud (Alvarado et al., 2008). Normalmente son coloreados, con cabeza en rojo en el caso de *B. oleae* o amarilla con una banda parda en el de *C. capitata*. Arista desnuda o ligeramente pubescente; ocelos presentes con sedas ocelares bien desarrolladas, en el caso de *C. capitata* los machos presentan un par anterior modificado con forma de paleta. Sus alas a menudo presentan unas marcas características y la vena subcostal gira bruscamente 90º hacia el margen exterior (Oosterbroek, 2006); las de *C. capitata*, presentan tres bandas anaranjadas además de pequeñas manchas negras en el tercio basal, las de *B. oleae,* son iridiscentes con una mancha oscura cerca del ápice en Fig. 1 (Tzanakakis, 2006). El tórax en *B. oleae* en general es oscuro recubierto de una fina pubescencia blanquecina, con tres líneas longitudinales más oscuras (Alvarado et al., 2008), mientras que en *C. capitata* es gris plateado con manchas negras de formas variadas y largas sedas laterales y posteriores (Fig. 2). Las hembras de ambas especies terminan en un fino y puntiagudo oviscapto no retráctil (Fig. 3) (Oosterbroek, 2006).

3. Morfología de estados pre-imaginales

Los huevos son blancos, menores de 2 mm de longitud, alargados con forma elipsoidal y con un solo eje primario (Fig. 4). En uno de sus extremos presentan un pedicelo, mientras que el otro es liso y redondeado

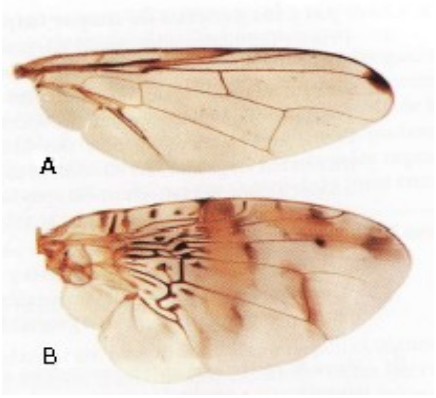

Fig. 1. Patrones de coloración alar en A) *B. oleae* y B) *C. capitata* (de Montoya et al., 2010)

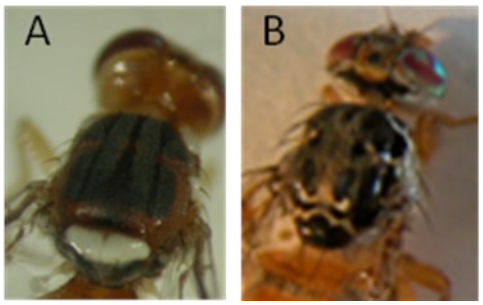

Fig. 2. Tórax en vista dorsal en A) *B. oleae* y B) *C. capitata*

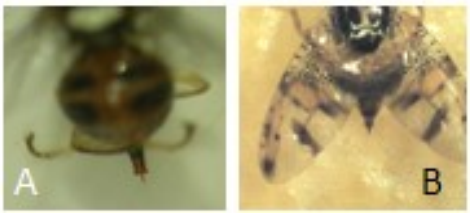

Fig. 3. Oviscapto en la hembra A) *B. oleae* y B) *C. capitata*

sin ninguna abertura o estructura externa (Tzanakakis, 2006). En el ápice del extremo pedicelado se encuentra el micropilo, con la presencia de una o múltiples aberturas, mientras que en el margen lateral los poros aeróspilos circunscriben el pedicelo. La superficie de los huevos es poligonal, típicamente hexagonal, reticulada o con crestas bajorrelieve (Headrick y Goeden, 1998).

La larva es de color blanco o amarillo pálido y su longitud varía de 3 a 15 mm (de 7 a 8 mm en *B. oleae* y *C. capitata*), ápoda, formada por 11 segmentos, ensanchada en su parte posterior y estrechada gradualmente hacia la parte anterior (Headrick y Goeden, 1998). La cabeza es pequeña, retráctil y en forma de cono. Las larvas poseen movilidad y tienen la capacidad de saltar al arquear su cuerpo y distenderlo bruscamente.

La pupa se encuentra confinada en el interior del pupario, formado a partir del último tegumento larvario endurecido. La pupa se desarrolla independientemente del pupario y posee unos espiráculos torácicos bilobulados para la respiración (Headrick y Goeden, 1998). El pupario es cilíndrico, liso con 11 segmentos distinguibles (Santiago-Alvarez y Quesada-Moraga, 2007). El color varía en tonalidad, desde pardo oscuro (*C. capitata*) hasta amarillo, sin olvidar el rojo y pardo claro (*B. oleae*). Su longitud varía entre 3 y 10 mm, si bien ambas especies de interés agrícola en España muestran un tamaño intermedio (5 mm).

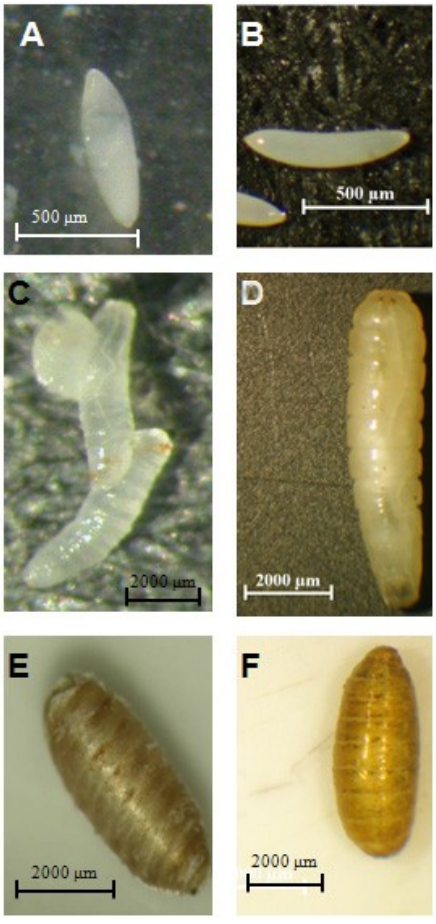

Fig. 4. A) y B) Huevos; C) y D) larvas; E) y F) pupas

4. Distribución geográfica

La familia Tephritidae se encuentra ampliamente distribuida en regiones tropicales y templadas del mundo, sólo está ausente en las zonas polares (Fig. 5).

Los nuevos avances en biología molecular sitúan el origen geográfico de *B. oleae* en África, y una posterior expansión por la cuenca mediterránea, India y parte occidental de Asia (Nardi et al., 2005). En los últimos 10 años ha invadido la costa oeste de Estados Unidos y noroeste de Méjico (Tzanakakis, 2006). En España se establecen tres zonas de ataque: una costera que se extiende desde la provincia de Barcelona hasta Huelva donde la plaga es endémica (Fig. 6); otra inmediata a la anterior, con ataques inconstantes; y una tercera en olivares situados en la región central que presentan daños de menor importancia (Alfaro-Moreno, 2005).

Ceratitis capitata al igual que la anterior es originaria de África (Gasperi et al., 1991), presente en el área subsahariana, la cuenca mediterránea, Oriente Próximo, Arabia, América del Norte, Centro y Sur, Australia Occidental y Hawái (Thomas et al., 2001). En España, se distribuye por toda la zona sur, regiones mediterráneas (incluidas las Islas Baleares), algunas áreas del interior que presentan temperaturas óptimas para su desarrollo y las Islas Canarias (Gómez-Clemente, 1931).

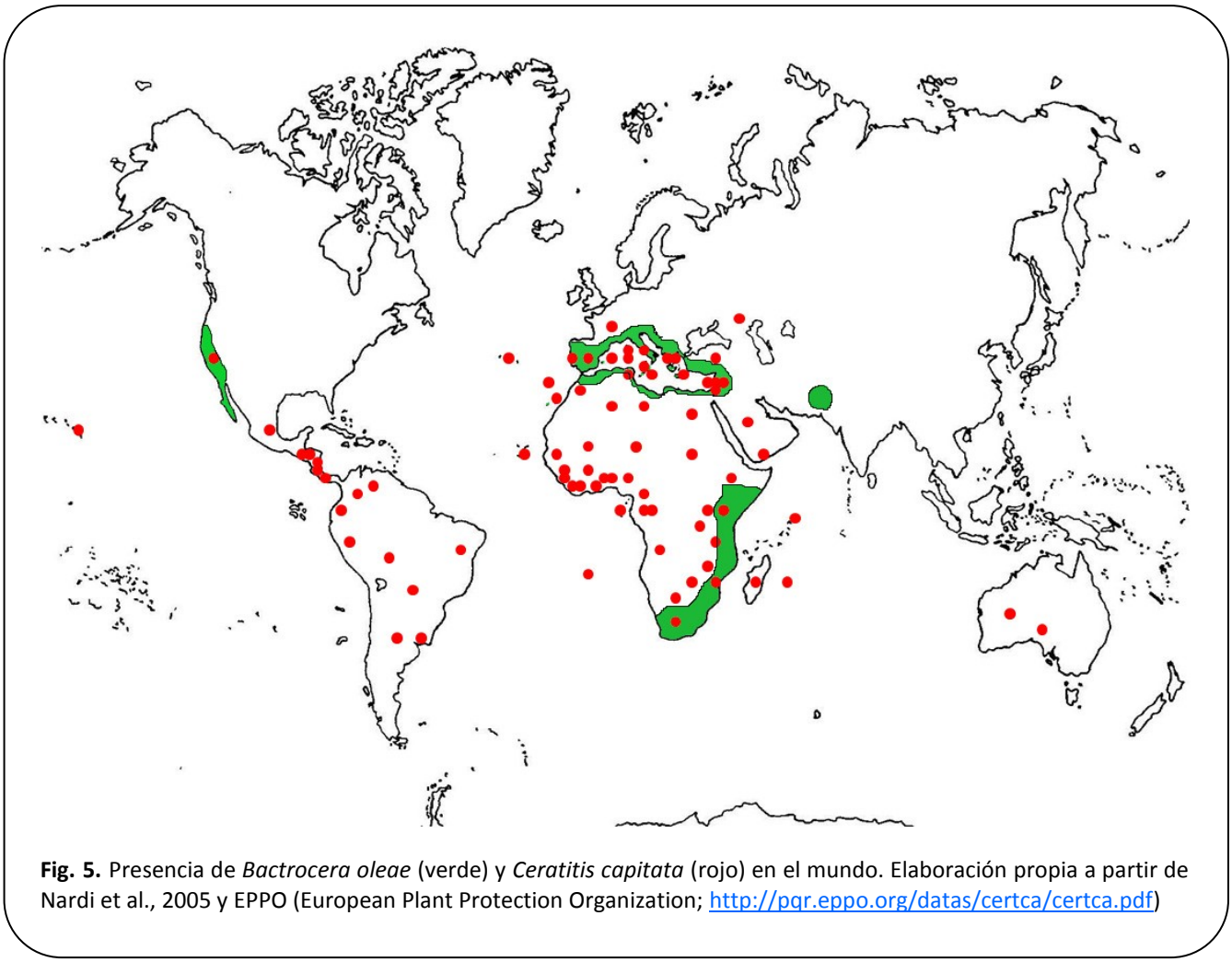

Fig. 5. Presencia de *Bactrocera oleae* (verde) y *Ceratitis capitata* (rojo) en el mundo. Elaboración propia a partir de Nardi et al., 2005 y EPPO (European Plant Protection Organization; http://pqr.eppo.org/datas/certca/certca.pdf)

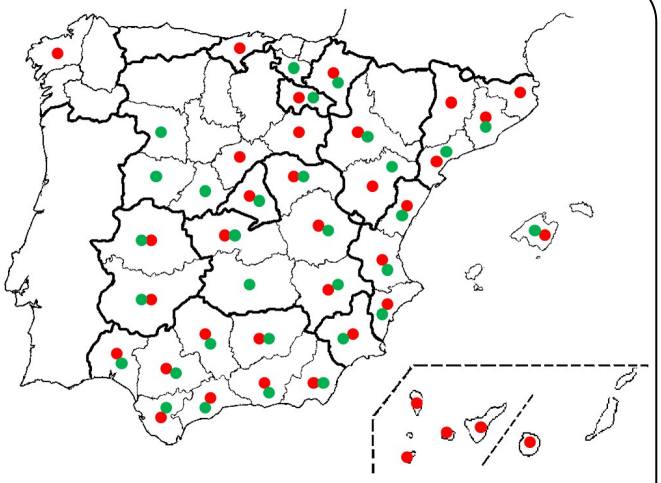

Fig. 6. Presencia de *Bactrocera oleae* (verde) y *Ceratitis capitata* (rojo) en España

5. Biología y comportamiento

Los tefrítidos tienen un ciclo de vida completo u holometábolo, con la existencia de los estados de huevo, larva, pupa y adulto. Tras el apareamiento, la hembra realiza la puesta bajo la epidermis del fruto (en la pulpa o en la cáscara) (Oosterbroek, 2006). Dependiendo de la especie, los huevos son depositados individualmente o en grupos de 2 a 10 huevos. El tiempo de desarrollo de estos huevos es variable y dependiente de la especie, temperatura y naturaleza del fruto, de manera que este puede durar entre 1 y 5 días. Las larvas se alimentan de la pulpa o de las semillas hasta completar los tres estadios larvarios (Oosterbroek, 2006), en lo que invierten de 4 a 25 días. En las especies univoltinas la duración del estado de pupa en condiciones controladas de laboratorio suele ser de un año, mientras que en las multivoltinas puede variar entre 7 y 25 días (Headrick y Goeden, 1998). Los adultos recién emergidos no poseen la coloración típica de la especie, además necesitan tiempo para alcanzar la madurez sexual, periodo que puede oscilar entre 5 y 20 días según especie y sexo. El apareamiento en los tefrítidos sigue dos estrategias diferentes, la defensa del recurso y el "lek" o estrategia compleja. La primera estrategia suele ser llevada a cabo por especies univoltinas y monófagas, donde el macho escoge un fruto como su territorio y lo protege del resto de los machos enfrentándose a ellos batiendo sus alas o sus patas ("pateando" o "boxeando"), cuando una hembra lo invade en busca de un sitio para la oviposición éste fuerza la cópula. No suele producir feromonas de atracción sexual o en caso contrario son de corto alcance (Sivinski y Burk, 1989). En la estrategia compleja los machos se agrupan formando un lek, es decir, se reúnen para atraer hembras con el único propósito de copular (Pie, 1998). Estos machos danzan en forma rítmica y liberan feromona sexual que atrae a nuevos machos para formar el lek y así captar hembras receptivas para la cópula (Sivinski y Calkins, 1986). En el caso de *C. capitata*, el tamaño del lek influye en que los machos tengan mayor éxito individual en la cópula (Shelly, 2001). La hembra escoge al macho y lo aparta del grupo para realizar el cortejo y la cópula. En este momento, las hembras tienen la necesidad de ingerir sustancias ricas en proteína, buscan alimento y un lugar donde depositar los huevos.

5.1. Ceratitis capitata

Especie hemodinámica, desarrollo continuo, sin diapausa, que en las zonas con inviernos suaves pasa la estación en estado de adulto o en estado de larva en el interior de los frutos (Tremblay, 1994), mientras que en las zonas más frías inverna en estado de pupa enterrada en el suelo a una profundidad que no suele exceder los 80 mm (Delrio, 1986; Alfaro-Moreno, 2005). Tras el apareamiento, la hembra realiza la puesta, en grupos de 2 a 10 huevos aproximadamente, para ello atraviesa con su oviscapto el epicarpo

que, dependiendo del espesor del mismo quedan situados en el mesocarpo, como ocurre con los melocotones, o retenidos en aquél, como en los cítricos. Cada hembra es capaz de poner entre 300 y 400 huevos durante su vida adulta (Thomas et al., 2001), aunque no toda ella es fértil, porque la maduración sexual, en clara dependencia con la estación del año, consume 4-7 días (verano) ó 10-12 días (otoño-invierno) de edad. La fase de huevo dura 2-5 días dependiendo de la temperatura y naturaleza del fruto, tras la eclosión, las larvas enseguida barrenan en aquél, a cuyas expensas completan su desarrollo, en lo que invierten de 9-15 días, para finalmente volver a su superficie y dejarse caer al suelo para pupar. Los adultos emergen en 10-11 días, se alimentan libando sustancias de origen animal o vegetal que encuentra sobre los árboles frutales, lo que permite la maduración sexual de las hembras, antesala del apareamiento y puesta (Alfaro-Moreno, 2005). Así se suceden varias generaciones en el curso del año, en número estrechamente dependiente de la temperatura, cuando ésta es favorable y las moscas encuentran frutos adecuados para hacer la puesta y sostener su desarrollo, pueden llegar a tener 6-7 generaciones anuales. En las zonas del interior de España, donde las temperaturas son más bajas, no existen las generaciones de otoño e invierno. La Fig. 7 resume como se suceden las distintas generaciones y sobre qué cultivos se desarrollan las mismas.

5.2. Bactrocera oleae

Se trata de una especie multivoltina que presenta entre 2 y 5 generaciones por año en función de las condiciones de humedad y temperatura locales y regionales (Fig. 8) (Ruiz Castro, 1948). Las moscas adultas muestran una alta actividad durante el día, en busca de alimento para su supervivencia y reproducción. Estas se alimentan de diversas sustancias orgánicas, líquidas o sólidas, melaza, néctar u otros exudados de plantas, polen, etc… La puesta se inicia cuando las aceitunas se acercan a su tamaño final y se vuelven lo suficientemente blandas como para ser atravesadas por el oviscapto de la hembra, hecho que generalmente se produce a partir de primeros-mediados de verano y continua hasta principios de invierno (Santiago-Alvarez y Quesada-Moraga, 2007).

La hembra pone de 10 a 12 huevos diariamente, como norma uno por fruto, y entre 200 y 250 a lo largo de toda su vida (De Andrés-Cantero, 2001; Weems y Nation, 2009). Ésta es atraída a aceitunas receptivas guiada por estímulos visuales y químicos (Girolami et al., 1975), perfora el fruto con el oviscapto e introduce por lo general un sólo huevo en el mesocarpio del mismo. Sin embargo este número de huevos por fruto puede variar por reducida disponibilidad de aceitunas, como consecuencia de baja floración o fallo en el cuajado, por inusitada densidad de población de mos-

Fig. 7. Especies vegetales sobre las que se desarrolla *Ceratitis capitata* a lo largo del año

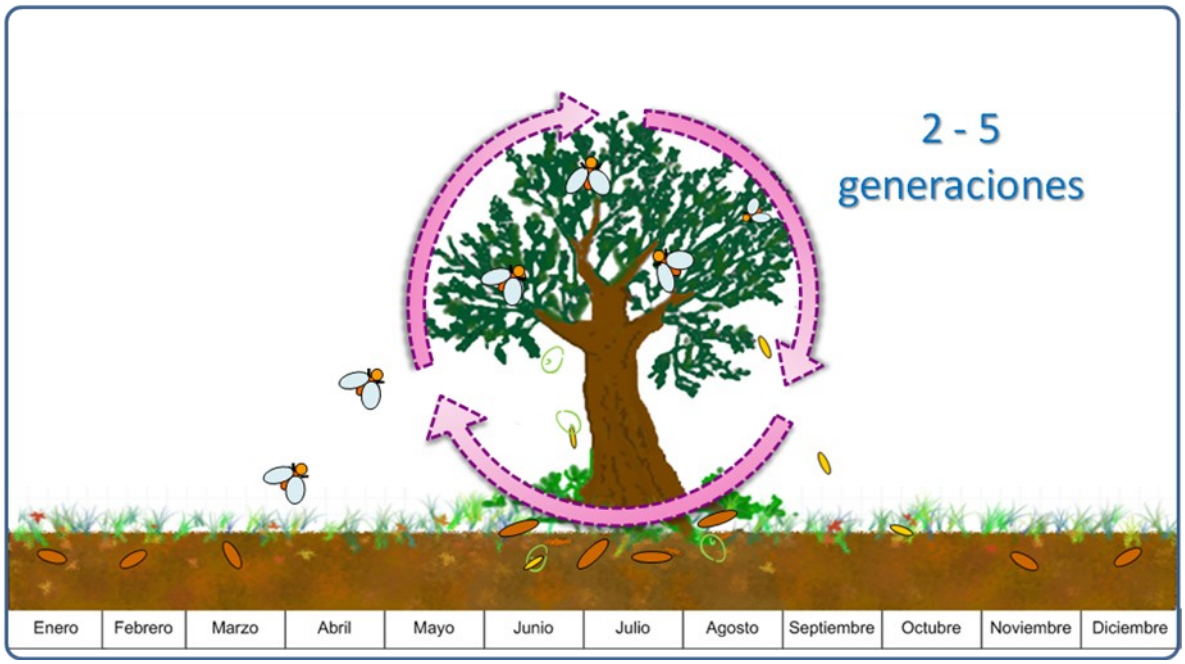

Fig. 8. Ciclo biológico anual de *Bactrocera oleae*

ca o por ambas causas conjuntamente (Santiago-Alvarez y Quesada-Moraga, 2007). Tras la eclosión las larvas excavan una galería en la que crecen y terminan su desarrollo larvario. Bajo condiciones favorables nutricionales y de temperatura, las que se suelen dar en cría de laboratorio, este ciclo de vida se completa en aproximadamente un mes (Tzanakakis, 2006). En condiciones de campo, las larvas de tercer estadio salen a la superficie de la aceituna y caen al suelo para la pupación (Ruiz Castro, 1948; Tremblay, 1994; Alfaro -Moreno, 2005) (Figura 8); el 80% la realizan en los 3 primeros cm del suelo, situadas de preferencia entre el S y el O de la superficie que interesa la copa del árbol, con una duración del estado pupal entre los 10 días y varios meses (hasta 4) según la estación del año. La mayor parte de la población de mosca del olivo pasa el invierno como pupa varios cm por debajo de la tierra, aunque también pueden pasar el invierno en estado adultos refugiados en el olivar o en el exterior del mismo (Santiago-Alvarez y Quesada-Moraga, 2007) (Fig. 8). Este es el periodo en el que se consideran más vulnerables y en el que experimentan mayores disminuciones de su potencial biótico. Las moscas adultas aparecen con el inicio de la primavera (De Andrés-Cantero, 2001), y la maduración sexual es altamente sincronizada con la disponibilidad de frutos adecuados Normalmente, la supervivencia de los adultos alcanza hasta final del mes de abril y alguno puede alcanzar el mes de julio (Arambourg y Pralavorio, 1972). La actividad es retomada cuando la temperatura se encuentra entre 6-7º C, dando algún vuelo a 13-14º C y comenzando a ovipositar a 16-17º C y, en presencia de olivas del año precedente, a 18-19º C están en plena actividad (Girolami, 1979).

6. Daños e importancia económica

Los tefrítidos son responsables de importantes pérdidas en la producción de numerosos frutales. La presencia de picaduras como consecuencia de la oviposición origina daños directos al depreciar los frutos (Rice, 2000), el pequeño orificio producido en la superficie alrededor del cual aparece una mancha oscura a la vez que marca un punto de entrada de microor-

ganismos fitopatógenos. Los daños producidos por estos se ven acrecentados con la actividad de alimentación de las larvas, que permite la entrada de oxígeno lo que provoca procesos de oxidación, maduración prematura y pudrición de la fruta, inservible ya para el mercado (Rice, 2000). A estos daños directos sobre los frutos se une la pérdida de mercados internacionales para la exportación de fruta como consecuencia de las estrictas normas de cuarentena asociadas a la presencia de *C. capitata* en el listado A2 de la European and Mediterranean Plant Protection Organization (EPPO). En el caso de *B. oleae*, las aceitunas picadas destinadas a la producción de aceite afectan sustancialmente a la cantidad y calidad del mismo. Las larvas se alimentan del 10-30 % de la pulpa de la aceituna con una reducción cuantitativa de la cantidad de aceite producida, además este es de mayor acidez y nada deseable por el consumidor. Se estima que la mosca del olivo es causante del 5% de las pérdidas en la producción mundial de aceituna con un valor de 1000 millones de euros al año (Nardi et al., 2005).

7. Control de tefrítidos

Las medidas de control de tefrítidos pretenden evitar, o reducir de manera significativa, que las hembras realicen la puesta, causa inicial de la depreciación del fruto, el aspecto cosmético, a la que sigue el barrenado de larvas, que disminuye su calidad organoléptica. La solución en plena producción se puede conseguir por tanto, bien con tratamientos adulticidas, para evitar la puesta, larvicidas, dirigidos a las larvas dentro de fruto con productos con capacidad de penetración o sistémicos, y finalmente, mediante tratamientos de suelo dirigidos a las larvas de tercer estadio que se dirigen al mismo para pupar. Estos insectos amplían su distribución utilizando sus mecanismos naturales de dispersión, sin embargo, el comercio internacional ha facilitado dicha dispersión. Por ello, dentro de las ocho regiones fitosanitarias de la FAO, los 45 países que integran la European Plant Protection Organisation (EPPO), tratan de protegerse frente a la entrada de nuevas especies de esta familia mediante sus mecanismos de cuarentena. Esto obliga a la aplicación de tratamientos cuarentenarios de desinfestación de frutos provenientes de zonas infestadas. Hasta la fecha estos tratamientos están basados en métodos químicos, aplicación de sustancias volátiles (la mayoría de uso restringido) para producir la muerte del insecto en cámaras, y físicos como la inmersión en agua caliente, aire caliente húmedo forzado, vapor caliente, bajas temperaturas, irradiación, impulso eléctrico y uso de radio frecuencias y microondas (Hernández et al., 2010). Esto es válido para *C. capitata*, incluida en la lista A2 de la EPPO (http://www.eppo.org/QUARANTINE/list.html).

En el ámbito nacional, la lucha contra *C. capitata* está regulada por ley (Real Decreto 461/2004 de 18 de Marzo BOE 079 de 01/04/2004), mientras que en el caso de *B. oleae*, es cada Comunidad Autónoma la que decide establecer las medidas fitosanitarias pertinentes dentro del marco normativo relacionado con la Producción Integrada. Así nos encontramos la Orden de 15 de abril de 2008 (BOJA núm. 83, del 25), por la que se aprueba el Reglamento Específico de Producción Integrada en Olivar en Andalucía, en el que se combinan todos los métodos de control disponibles.

7.1. Control de adultos

7.1.1. Medidas basadas en prácticas agronómicas

La aplicación de distintas prácticas agronómicas puede tener repercusión en la incidencia de tefrítidos.

Entran en esta consideración la elección de cultivares resistentes o menos susceptibles o bien que la cosecha escape al ataque de mosca (Alfaro-Moreno, 2005; Quesada-Moraga et al., 2009b; Santiago-Álvarez et al., 2011); condiciones de protección como invernadero o embolsado de frutos (Domínguez, 1989) ambas con un coste elevado para la explotación, manejo de la flora arvense, cubiertas vegetales, riego etc., por su influencia sobre el estado vegetativo del árbol, y sobre la diversidad de enemigos naturales, tipo y momento de la recolección, etc (Quesada-Moraga et al., 2009a).

7.1.2. Capturas de adultos

Tienen el objetivo de reducir las poblaciones de tefrítidos mediante estímulos físicos, químicos, etc. de atracción o rechazo. El **estímulo visual** se suele emplear en las capturas de adultos, donde colores como el amarillo (*B. oleae, C. capitata*), el verde o el rojo ejercen gran atracción cromotrópica según la especie (Katsoyannos, 1987). Además, existen trampas que simulan frutos como la trampa Ladd, que consiste en un panel rectangular amarillo entre dos hemisferios rojos, de manera que atrae a moscas adultas que no han alcanzado su madurez sexual con el primero y a moscas maduras con los segundos (Montoya et al., 2010).

El empleo de dispositivos de captura (mosqueros) de diferentes tipos o diseños (tipo trip, Mcphail, olipe, etc.) cebados con **atrayentes** es una práctica que se realiza desde hace tiempo (Gómez-Clemente, 1929; 1939). Los atrayentes son compuestos que afectan de una u otra forma el comportamiento de los insectos y constituyen un grupo importante en número. Los primeros atrayentes que se usaron fueron el vinagre, agua de maceración de salvado, agua y salvado, agua, salvado y vinagre, y concentrado de naranja y otros jugos de frutas (Gómez-Clemente, 1929; 1939). Están constituidos por soluciones de azúcar fermentada y proteínas hidrolizadas líquidas, a las que es necesario adicionar conservadores (bórax), a fin de reducir la contaminación microbiana y alargar el tiempo de efectividad de la mezcla. En la actualidad, entre los atrayentes alimenticios de mayor uso se encuentran las proteínas hidrolizadas que liberan distintas sales de amonio y otros compuestos atrayentes durante su fermentación. En los últimos años la investigación se ha enfocado en el desarrollo de atrayentes alimenticios sintéticos, más específicos, efectivos y con mayor estabilidad en el medio (Ros, 2001). Un ejemplo es el Biolure, producto que combina los atrayentes putrescina (1,4-diamino butano), trimetilamina y acetato de amonio, y es muy eficaz para atraer a las hembras con la consiguiente reducción de la puesta. Este atrayente es efectivo para varias especies de los géneros *Ceratitis* y *Bactrocera* (Montoya et al., 2010). Otros atrayentes sintéticos evaluados recientemente para *C. capitata* han sido una mezcla de amonio y trimetilamina; la combinación de acetato de amonio, trimetilamina y cadaverina; y acetato de amonio y n-metil pirrolidina, ambas con capturas similares al Biolure (Navarro-Llopis et al., 2008). En el caso de la mosca del olivo, el bicarbonato de amonio ha resultado ser el atrayente más efectivo. Por lo general, los atrayentes son compuestos altamente volátiles, que se presentan en formulaciones con liberación prolongada bajo condiciones de campo (hasta 10 semanas). El primer atrayente específico para machos del género *Bactrocera* que se registró fue el metileugenol (ME) (FAO/IAEA 2005). El trimedlure (TML) fue desarrollado en 1961 como un atrayente específico para machos de *C. capitata* y el Cuelure (CUE) se descubrió como un compuesto sumamente efectivo para diferentes especies del género

Bactrocera. En los últimos años se ha estudiado el cerelure B1 que es un atrayente sintético para machos de *C. capitata* mostrando una mayor atracción que el trimedlure, pero el alto costo de su síntesis limita su aplicación (Jang et al., 2003).

Otro grupo de atrayentes son las feromonas sexuales, señales químicas volátiles producidas por las hembras vírgenes o los machos que estimulan a los individuos del sexo contrario a volar hacia ellos. Según Jacobson et al. (1973) el macho de *C. capitata* produce dos feromonas identificadas como metil-(E)-6-nonenoate (1) y (E)-6-nonen1-ol, sin embargo dada la complejidad de su composición molecular quedan excluidas de su uso comercial. No es el caso de la producida por *B. oleae* denominada "spiroketal (1-7)-dioxaspiro-[5,5]-undecano" (OFP) incluida en el registro FAO/IAEA 2005.

7.1.3. Lucha autocida por medio de machos estériles

Consiste en la liberación en forma sistemática de un gran número de insectos esterilizados en laboratorio para reducir la posibilidad de reproducción entre insectos de una población natural de la misma especie (Mitchell y Saul, 1990). Esta técnica se basa en la producción de un gran número de insectos en plantas de cría masiva, que son esterilizados mediante radiaciones ionizantes. Inicialmente se utilizaron rayos-X, posteriormente radiaciones gamma a partir de Cobalto 60 o Cesio 137 por presentar éstas un menor riesgo en la operación y mayor efectividad en la esterilización. El éxito de esta técnica depende de que los machos estériles copulen con las hembras silvestre, para lo que es necesario que estos machos sean capaces de sobrevivir en campo lo suficiente, con comportamiento similar a los silvestres, para garantizar su acoplamiento con las hembras; resulta pues limitante que las operaciones de cría en masa en laboratorio, la radiación, o el manejo de los insectos puedan alterar su comportamiento (Bush et al., 1976; McInnis et al., 1996; Briceno y Ederhard, 1998; Yuval et al., 1998), con la paralela reducción en su capacidad de apareamiento (Shelly y Dewire, 1994; Lance et al., 2000). Algunas de las especies de dípteros tefrítidos en las que se ha aplicado esta técnica son *B. oleae, C. capitata*, *Anastrepha ludens* (Loew), *A. obliqua*, *B. cucurbitae*, *B. dorsalis* y *B. tryonii* (Economopoulos et al., 1977; Liedo et al., 2010).

La eficiencia de esta técnica se incrementa cuando se integra un sistema de sexado genético en el proceso de esterilización para practicar la esterilización a los machos de manera exclusiva (Gazit et al., 2004; Rendon et al., 2004). Actualmente se han construido varias cepas unisexuales o sexadas genéticamente en dípteros, pero en tefrítidos, sólo *C. capitata* ha sido posible llevarla a los niveles de cría masiva requeridos por este método de control, su programa de sexado es uno de los más sobresalientes. En ella se han utilizado como marcadores genéticos dos mutaciones: sensibilidad letal a la temperatura (tsl) para eliminar a las hembras y pupa blanca (wp) la cual se localiza en el mismo cromosoma y muy cerca de tsl, funcionando como un control visible (Franz, 2005). El sexado de individuos se realiza en estado de huevo por razones estrictamente económicas, los huevos se incuban en agua a 34º durante una hora, de esta forma los embriones hembra sensibles a la temperatura mueren por lo que sólo sobreviven los embriones macho y si alguna hembra consigue sobrevivir, queda delatada por el color blanco de la pupa (Franz, 2005).

En la actualidad es posible evaluar la eficiencia de acoplamiento de los machos estériles en campo mediante técnicas moleculares basadas en la PCR (San Andres et al., 2007), aspecto importante para evaluar

la eficacia de esta técnica.

7.1.4. Control químico

En el control de las moscas de la fruta se han empleado tradicionalmente insecticidas de amplio espectro (organofosforados y carbamatos) en tratamientos generalizados de todo el árbol, vía aérea mediante la técnica de ultra-bajo volumen, donde la toxicidad por contacto o ingestión de pequeñas gotas puede causar la mortalidad de las moscas (Mohammad y Aliniazee, 1989). Para este tipo de tratamientos, el Reglamento (CE) Nº 848/2008 de la Comisión de 28 de agosto de 2008 por el que se modifica el Reglamento (CE) Nº 2076/2002 y la Decisión 2003/565/CE, en lo relativo al período previsto en el artículo 8, apartado 2, de la Directiva 91/414/CEE del Consejo, prohíbe la utilización de insecticidas organofosforados como el malatión y el diclorvos, así como los tratamientos aéreos, lo que reduce las opciones a los tratamientos terrestres, para los que sólo se dispone de los organofosforados fosmet y metil clorpirifos, los piretroides deltametrin y lambda cihalotrin, y los nuevos neurotóxicos imidacloprid y etofenprox para *C. capitata*, o fosmet, dimetoato, e imidacloprid en el caso de *B. oleae* (Registro de productos fitosanitarios MARM 2011, http://www.marm.es/es/agricultura/temas/medios-de-produccion/productos-fitosanitarios/registro/menu.asp).

Los tratamientos en forma de cebo se aplican mediante pulverización foliar en la parte más soleada del árbol o en un dispositivo (mosquero), lo que minimiza los residuos del insecticida en el ambiente y en los frutos. Estos tratamientos consisten en la mezcla de un insecticida con un atrayente alimenticio, generalmente proteína hidrolizada, para dirigir a los insectos al cebo donde mueren como consecuencia de ingerir o entrar en contacto con el insecticida. Las hembras se sienten especialmente atraídas por estos tipos de cebos, ya que necesitan un aporte de proteínas para la maduración de los ovarios (Ros et al., 1979; Ros, 1988).

La tendencia actual en el control químico es el desarrollo de insecticidas selectivos que generen mezclas más efectivas y con menor impacto al medio ambiente. Un producto que ha resultado seguro para el medio y ha mostrado potencial contra *C. capitata* y *B. oleae* es el caolín (polvo humectable de silicato de aluminio) que además de controlar los adultos reduce la infección de frutos (Saour y Makee, 2004) pues los hace menos atractivos para las hembras. Otra opción la presentan los insecticidas del grupo de las difenilureas, como el diflubenzurón y luferulón, que son inhibidores de la síntesis de quitina que ingeridos por las hembras causan reducción en la fecundidad de las mismas y en la viabilidad de los huevos "esterilidad" en hembras lo que puede dar origen a una significativa reducción de las poblaciones de campo (Albajes y Santiago-Álvarez, 1979; Sarasua y Santiago-Álvarez, 1983; Navarro-Llopis et al., 2007).

A este respecto, la búsqueda de nuevos insecticidas respetuosos con el medio ambiente eleva al mayor protagonismo a productos **derivados de plantas superiores o de microorganismos**. La azadiractina procede de *Azadirachta indica* o árbol del neem. Se trata de un inhibidor del crecimiento que bloquea la síntesis de los ecdisteroides pues evita la liberación de la hormona protoracicotrópica producida por las células neurosecretoras, por lo que las mudas no se realizan con normalidad. Este producto se vende para el control de *C. capitata* bajo diferentes formulaciones (Di Ilio et al., 1999; Viñuela et al., 2000). Otros derivados de plantas empleados en el control de *B. oleae*

son las piretrinas naturales extraídas de *Chrysanthemum cinerariaefolium* (Trev.) Bocc, rotenona extraída de *Derris* spp, *Lonchocarpus* spp y *Terphrosia* spp y preparados de *Quassia amara* L. (Reglamento (CEE) 2092/91 del Consejo sobre Producción Ecológica). Derivados de bacterias se encuentran el spinosad y la abamectina, metabolitos secundarios obtenidos a partir de la fermentación de los actinomicetos *Saccharopolyspora spinosa* (Mertz y Yao) y *Streptomyces avermitilis* (Burg) Kim y Goodfellow, respectivamente. La eficiencia del spinosad en el control de tefrítidos en general y de *C. capitata* (Adan et al., 1996; Burns et al., 2001; McQuate et al., 2005; Urbaneja et al., 2009) y *B. oleae* (Collier y Van Steenwyk, 2003) en particular está probada por diversos estudios. La abamectina se ha evaluado frente varios tefrítidos incluido *C. capitata* mostrando buenos resultados en condiciones experimentales (Albrecht y Sherman, 1987; Hu et al., 2000; Heath et al., 2009). Por último, también los hongos producen una gran variedad de compuestos que pueden ser empleados en el control de tefrítidos. Así podemos encontrar metabolitos secundarios producidos por el hongo fitopatógeno *Mucor hiemalis* Wehmer y los HE *Metarhizium anisopliae* (Metsch.) Sorok. y *Paecilomyces chrysogenum* con actividad insecticida cuando se administran por ingestión a *C. capitata* (Castillo et al., 2000; Konstantopoulou et al., 2006); y proteínas fúngicas con gran acción insecticida por ingestión (Ortiz-Urquiza et al., 2009; Ortiz-Urquiza et al., 2010a). Estas últimas, como factores de virulencia, son consideradas un gran blanco para la selección y mejora de hongos entomopatógenos con la reducción de los tiempos letales (Ortiz-Urquiza et al., 2010b).

7.1.5. Control biológico

El control biológico por medio de enemigos naturales se presenta como una alternativa respetuosa con el medio, tanto en la faceta de control macrobiano que implica a organismos entomófagos: vertebrados (pequeños mamíferos, aves) e invertebrados (parasitoides y depredadores, artrópodos: arácnidos o insectos), como en la de control microbiano basada en microorganismos entomopatógenos: virus, bacterias, hongos, nemátodos, protozoos, o sus productos.

7.1.5.1. Control macrobiano. Existen distintas estrategias de control biológico en la lucha contra tefrítidos. El control biológico **clásico** se define como la liberación de enemigos naturales exóticos para su establecimiento en el medio. La búsqueda de agentes naturales de control de la mosca mediterránea se inicia a finales del siglo XIX comienzos del XX como ponen en evidencian las famosas expediciones de Compere (Alfaro-Moreno, 2005) y de Silvestri en 1913 por África (Cánovas, 1940). A principios de los años 30 la Estación de Fitopatología Agrícola de Levante (Valencia) importó dos Braconidae, *Psyttalia humilis* (Silvestri) y *Diachasmimorpha tryoni* (Cameron), para el control de *C. capitata* que no llegaron a establecerse. Posteriormente, en los años 60-70, se intentó con *Tetrastichus giffardianus* (Silvestri) y *P. concolor* (Szèpligeti) pero los resultados tampoco fueron satisfactorios. En el olivar, las especies *P. concolor* y *Pnigalio mediterraneus* (Ferriere y Delucchi) han sido incluidas en el Reglamento Específico de Producción Integrada en Olivar en Andalucía. Sin embargo, este tipo de control tiene la limitación que representa el bajo porcentaje de parasitismo natural observado en campo (Wang y Messing, 2003; Argov y Gazit, 2008).

El control biológico por **conservación** consiste en el mantenimiento o modificación del medio a través de las prácticas agrícolas, con el fin de favorecer la

acción de los enemigos naturales. La aplicación de este enfoque contra tefrítidos ha sido planteada por algunos autores que destacan la importancia que pueden jugar ciertos arbustos y árboles nativos, como reservorios para mantenimiento de los parasitoides en periodos de escasez del fitófago. La idea de permitir el crecimiento y floración de ciertas plantas cerca del olivar como reservorio de enemigos naturales de la mosca del olivo ya fue apuntada por Ruiz-Castro en 1951 (Quesada-Moraga et al., 2009a). Las inflorescencias de muchas especies de umbelíferas sirven como alimento a larvas y adultos del neuróptero *Chrysoperla carnea* (Stephens), uno de los depredadores más importantes del olivar.

La liberación **inoculativa** consiste en la suelta de enemigos naturales con el fin de que se establezcan y controlen al fitófago durante un periodo de tiempo determinado. El desarrollo de las técnicas de cría masiva de parasitoides y los estrictos umbrales económicos establecidos para las exportaciones de fruta, presentan a este tipo de control el más viable con parasitoides. En la actualidad existe en la Comunidad Valenciana un Plan Integral de Actuación contra la mosca, por lo que en 2002 el Instituto Valenciano de Investigaciones Agrarias (IVIA) importó desde Hawái las especies *D. tryoni* y *Fopius arisanus* (Sonan) para contemplar las posibilidades de su uso in situ (Santiago et al., 2006).

La liberación **inundativa**, consiste en sueltas periódicas con entomófagos para el control a corto plazo de la población. Este tipo de control con depredadores no se contempla todavía debido a la escasez de estudios que evalúen la acción de los mismos en poblaciones de tefrítidos y a la dificultad de su cría masiva en laboratorio.

7.1.5.2. Control microbiano. Los parasitoides y depredadores han protagonizado algunos éxitos de gran resonancia en el control de tefrítidos, pero no han satisfecho todas las expectativas puestas en ellos cuando se trata de aplicaciones por inundación dada la dificultad para la cría en masa (Cancino y Ruíz, 2010). Sin embargo, los microorganismos entomopatógenos unen a su seguridad de empleo la facilidad de manejo por lo que ofrecen un gran potencial para programas de control integrado.

Los **virus** han sido poco explorados, a pesar de haber mostrado gran potencial con reovirus probado frente a adultos de *B. oleae* (Manousis et al., 1987; Anagnou-Veroniki et al., 1997) y *C. capitata* (Plus, 1989). Además, el Picornavirus V y el Reovirus I son capaces de replicarse de forma natural y matar adultos de *C. capitata*, *B. oleae* y otros tefrítidos (Plus y Cavalloro, 1983; Plus, 1989). Sin embargo, no se ha descubierto alguno que produzca una elevada mortalidad en campo sin causar perjuicio sobre la salud humana y el medio ambiente (Anagnou-Veroniki et al., 1997).

De todas las **bacterias**, *Bacillus thuringiensis* (Berliner) (Bt) ha sido con la que más se ha experimentado hasta el momento. Ésta produce la δ-endotoxina que debe ser ingerida en forma de cristal para causar la muerte, por lo que puede ser diluida y mezclada con proteína hidrolizada. La **bacteria** *B. thuringiensis* (Bt) ha mostrado cierta eficacia en experimentos de laboratorio frente a adultos de *C. capitata* y *B. oleae* (Alberola et al., 1999; Sivropoulou et al., 2000), aunque éstos no invitan a su aplicación en campo. En consecuencia, hasta la fecha hay pocas referencias sobre su empleo práctico en campo.

Los microorganismos entomopatógenos más idóneos para un control efectivo son los que invaden al hospedante susceptible por la vía tegumentaria tal

como lo hacen los hongos y **nemátodos** entomopatógenos. Sin embargo, estos últimos producen una baja infección en adultos de tefrítidos.

Los **hongos** entomopatógenos (HE) son los microorganismos que presentan mayor potencial para el control microbiano ya que actúan por contacto, es decir, alcanzan la cavidad general del insecto por la vía tegumentaria sin necesidad de ser ingeridos, mecanismo de acción que les hace idóneos para el control de tefrítidos, lo que se ha revelado en numerosos trabajos para *C. capitata* (Ekesi et al., 2003; Quesada-Moraga et al., 2006; Quesada-Moraga et al., 2008), *B. oleae* (Anagnou-Veroniki et al., 2005; Konstantopoulou y Mazomenos, 2005), *A. ludens* (Lezama-Gutierrez et al., 2000; De la Rosa et al., 2002), *A. fraterculus* Schiner (Carneiro y Salles, 1994; Destefano et al., 2005), *B. tryoni* (Carswell et al., 1998) o *R. indifferens* (Yee y Lacey, 2005).

Estos agentes, están presentes de forma natural en el suelo y en las poblaciones de insectos, por lo que respecta a nuestras condiciones edafoclimáticas los aislados que se obtienen con mayor frecuencia corresponden a especies de los géneros *Beauveria* y *Metarhizium* (Quesada-Moraga y Santiago-Álvarez, 2008). En cualquier caso se trata de poner los conidios del hongo en contacto con el tegumento del insecto, una vez que aquellos germinan, las hifas de penetración colonizan el hemocele, con consecuencia de muerte para el insecto: por utilización de los nutrientes, invasión de sus tejidos y órganos, asfixia al desarrollarse en el sistema respiratorio, y en ocasiones por secretar metabolitos tóxicos.

El control de adultos de tefrítidos puede hacerse por distintas vías: 1) tratamientos de pulverización del árbol al igual que los tratamientos químicos (Daniel y Wyss, 2010); 2) mediante la técnica de atracción infección utilizando cebos compatibles con los HE (atrayente + conidios) mediante tratamientos localizados en una parte del árbol o colocados en un dispositivo en el mismo (Ekesi et al., 2007; Quesada-Moraga et al., 2008); 3) liberando machos estériles como agentes de dispersión, previamente infestados con conidios (Montoya y Toledo, 2010), o con tratamientos al suelo antes de la salida de los adultos, que se impregnan con los conidios disponibles en el suelo.

No obstante, para que la aplicación de HE sea un método de control robusto es necesario mejorar la producción y formulación. Ésta última puede aumentar la eficacia de los conidios influyendo en su virulencia, tolerancia a la desecación, tolerancia térmica y a UV, velocidad de germinación e infección.

7.2. Control de estados pre-imaginales

7.2.1. Medidas basadas en prácticas agronómicas

El principal objetivo es reducir los focos de infestación, y consisten en la destrucción de frutos atacados tanto los que se encuentren en el árbol como los caídos al suelo, así como las labores de este para la destrucción de pupas. El riego juega un papel importante pues modifica el microclima del suelo con influencia sobre las pupas de tefrítidos (Tsitsipis y Papanicolaou, 1979; Orsini et al., 2007).

7.2.2. Control químico

Tradicionalmente se han utilizado insecticidas organofosforados como el dimetoato para obtener una acción sistémica frente a huevos y larvas de tefrítidos que se desarrollan en el interior del fruto picado (Conti y Pusino, 1994; Alvarado et al., 2008). Estos tratamientos larvicidas generan ciertos problemas de residuos además de presentar plazos de seguridad relativamen-

te largos, de hasta 60 días (http://www.marm.es/es/agricultura/temas/medios-de-produccion/productos-fitosanitarios/registro/menu.asp).

Es posible la aplicación del insecticida al suelo para controlar los estados inmaduros de tefrítidos. Se recomienda aplicar el producto en el área bajo árbol, preferentemente donde se encuentra la fruta infestada. En este tipo de control se han utilizado los piretroides teflutrin y lambda-cihalotrin (Stark y Vargas, 2009), con el tratamiento de 1-2 m^2 de la falda del árbol para control de larvas de tercer estadio próximas a pupación y pupas de *C. capitata*.

7.2.3. Control biológico

Se ha podido constatar la infección de larvas en varias poblaciones de *B. oleae* en laboratorio y en poblaciones de campo en Grecia por un **picornavirus** (Manousis et al., 1987; Knowles, 1998). Además Anagnou-Veroniki et al. (1997) observaron grandes cantidades de viriones en las heces de moscas del olivo adultas que contribuían a la dispersión del virus tanto horizontal como vertical por contaminación de la dieta larvaria y los huevos durante la oviposición.

Los **nemátodos** entomopatógenos se emplean en el control de larvas de tercer estadio. Los estados juveniles infectivos penetran en el interior del insecto a través de las aberturas naturales. Una vez dentro, liberan una bacteria simbiótica, que es la responsable de originar la muerte del insecto hospedante. Los juveniles infectivos se alimentan de tejido utilizado por la bacteria para su multiplicación hasta alcanzar la fase adulta y reproducirse. Estos se han probado frente a diferentes tefrítidos, así en larvas de *C. capitata* los nematodos de los géneros *Steinernema* y *Heterorhabditis* han ocasionado altas mortalidades en suelos con diferencias texturales y condiciones ambientales (Gazit et al., 2000; Rohde et al., 2010). La facilidad de multiplicación a gran escala en fermentadores ha propiciado la elaboración de preparados comerciales, sin embargo aún no se ha conseguido desarrollar formulaciones para su empleo a gran escala.

Los **hongos** entomopatógenos pueden ser aplicados al suelo bajo la proyección de la copa del árbol. El suelo es uno de los principales hábitat de los HE, éste les confiere protección frente a la radiación ultravioleta y son menores las fluctuaciones de temperatura y humedad (Quesada-Moraga y Santiago-Álvarez, 2008). En el suelo los conidios de estos hongos entomopatógenos pueden permanecer durante un largo periodo de tiempo o incluso reciclarse en la rizosfera o sobre insectos que habitan permanente o temporalmente en él. El carácter que muestran de patógenos facultativos facilita su multiplicación a gran escala sobre una gran variedad de sustratos y los propágulos infectivos obtenidos, los conidios, son susceptibles de manipulación para elaborar formulaciones insecticidas que se pueden aplicar por los métodos convencionales. La selección de un aislado adecuado para el desarrollo de un micoinsecticida es un proceso complejo, además de mostrar alta virulencia, actividad insecticida, tiene que estar adaptada a los factores ambientales que resultan críticos para el crecimiento del hongo y la virulencia, como la temperatura y la humedad. Los conidios del hongo, una vez que alcanzan la cutícula del insecto y se adhieren a la misma para invadir el cuerpo de su hospedante en las primeras 24h. Durante el proceso inicial de la infección (germinación y penetración de la cutícula) el hongo es muy susceptible a factores ambientales, tales como la temperatura, humedad, radiación UV, lluvia.... Además, antes de llevar a cabo la aplicación comercial de algún aislado fúngico seleccionado por su actividad insecticida, re-

sulta fundamental evaluar su posible impacto sobre otros insectos y artrópodos no diana. En España todavía no existen formulados para el control de tefrítidos en el suelo aunque se dispone de experiencias en laboratorio enfocadas al control en el mismo de puparios de *B. oleae* y *C. capitata* (Quesada-Moraga et al., 2006; Quesada-Moraga et al., 2008).

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CAPÍTULO III

UNIVERSIDAD D CÓRDOBA

JUSTIFICACIÓN Y OBJETIVOS

Justificación y objetivos

Existe una necesidad urgente de nuevas medidas de control de plagas de tefrítidos que se adapten a los principios de la Agricultura Sostenible, recogidos por la Directiva 2009/128/CE del Parlamento Europeo y del Consejo de 21 de octubre de 2009 por la que se establece el marco de actuación comunitaria para conseguir un uso sostenible de los plaguicidas, cuya transposición y regulación en España tienen lugar en estos días, y que desembocará en la obligatoriedad del Control Integrado de Plagas desde 1 de enero de 2014. Por su presencia natural en plantaciones frutales y de olivo en España, así como por su modo de acción por contacto, vía tegumentaria, los ascomicetos mitospóricos entomopatógenos (AME) constituyen posiblemente la mejor alternativa para el control microbiano de tefrítidos (Quesada-Moraga et al., 2007; Quesada-Moraga y Santiago-Álvarez, 2008). Los trabajos realizados en el seno de nuestro Grupo de Investigación PAIDI AGR 163 "Entomología Agrícola" antes de iniciar esta Tesis Doctoral, pusieron de manifiesto la eficacia de aislados autóctonos de *B. bassiana* y *M. anisopliae* frente a adultos de tefrítidos en tratamientos aéreos, bien mediante pulverización o mediante "atracción e infección" (Quesada-Moraga et al., 2006; Quesada-Moraga et al., 2008). No obstante, también se disponía de evidencia experimental sobre la eficacia de algún aislado frente a larvas de tercera edad próximas a pupación y puparios, en tratamientos de suelo en la base del árbol (Quesada-Moraga et al., 2006; Eldesouki-Arafat, 2007), así como frente a adultos que se infestan con los conidios en el suelo al abandonar los puparios (Eldesouki-Arafat, 2007).

Sin embargo, el éxito de esta estrategia de aplicación al suelo depende del conocimiento de aquellos factores, tanto genéticos como ambientales, que pueden influir sobre la dinámica del inóculo en el suelo y sobre la eficacia del mismo. Por ello, esta Tesis Doctoral aborda, posiblemente por primera vez, la interacción hongo-suelo-insecto, desde los acontecimientos más íntimamente ligados al microbiota, hasta los que conectan con el macrobiota. Por un lado, se intenta conocer el origen y diversidad de los AME que resulta primordial para poder entender el proceso de evolución de estos hongos en sus distintas funciones ecológicas. Pero sin olvidar su principal función como agentes de control, para lo que estudia la posible interacción de los factores físico-químicos y ambientales del suelo con su eficacia insecticida frente a estados pre-imaginales de tefrítidos en el suelo, además de los posibles efectos de estos tratamientos sobre la artropofauna no diana de este medio.

Por tanto, atendiendo a todo lo anteriormente expuesto, los objetivos concretos de esta Tesis Doctoral son:

1. Determinar la diversidad genética de aislados autóctonos del género *Beauveria* procedentes de suelo e insectos, y su relación con el origen geográfico y hábitat de aislamiento
2. Evaluar el efecto de la textura y pH del suelo sobre la persistencia en el mismo y la eficacia de aislados de los hongos *Beauveria bassiana* y *Metarhizium anisopliae* que en trabajos previos del Grupo de Investigación mostraron actividad insecticida frente a estados pre-imaginales y adultos de *Ceratitis capitata*
3. Evaluar el efecto de la temperatura y humedad del suelo sobre la actividad insecticida de aislados de *B. bassiana* y *M. anisopliae* candidatos para su aplicación al suelo para el control de estados pre-

imaginales de tefrítidos

4. Evaluar el efecto de la temperatura y humedad del suelo sobre estados pre-imaginales de *C. capitata* para la sincronización de los calendarios de aplicación de insecticidas en los programas de control integrado de plagas
5. Determinar el impacto sobre la artropofauna edáfica del olivar del tratamiento realizado al suelo con el aislado EAMa 01/58-Su de *M. anisopliae* dirigido al control de estados pre-imaginales de la mosca del olivo *Bactrocera oleae*, con énfasis en las comunidades de hormigas de la especie *Tapinoma nigerrimum*

Los resultados relativos al primer objetivo se recogen en el capítulo 4, que comprende el manuscrito **"Genetic analyses place most Spanish isolates of *Beauveria bassiana* in a molecular group with wordwide distribution"** publicado en BMC Microbiology 11 (2011) 84. Los resultados del segundo objetivo se recogen en el capítulo 5 en el manuscrito **"Soil properties affect the availability, movement, and virulence of entomopathogenic fungi conidia against puparia of *Ceratitis capitata* (Diptera: Tephritidae)"** publicado en Biological Control 58 (2011) 277–285. El tercer objetivo queda recogido en el capítulo 6 dentro del manuscrito **"Use of a multiple logistic regression model to determine the effects of soil moisture and temperature on the virulence of entomopathogenic fungi against pre-imaginal Mediterranean fruit fly *Ceratitis capitata*"** publicado en Biological Control 59 (2011) 366–372. El cuarto objetivo corresponde al capítulo 7 de esta Tesis Doctoral "**The effect of temperature and soil moisture on the development of the pre-imaginal Mediterranean fruit fly *Ceratitis capitata* (Diptera: Tephritidae)"**, artículo enviado recientemente a Environmental Entomology. Finalmente, el último objetivo de esta Tesis Doctoral se recoge en el capítulo 8, que comprende el manuscrito **"Effects of soil treatments with entomopathogenic fungi on soil dwelling non-target arthropods at a commercial olive orchard"** publicado en Biological Control 59 (2011) 239–244.

CAPÍTULO IV

Este capítulo es una versión adaptada del artículo

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BMC Microbiology

RESEARCH ARTICLE Open Access

Genetic analyses place most Spanish isolates of *Beauveria bassiana* in a molecular group with word-wide distribution

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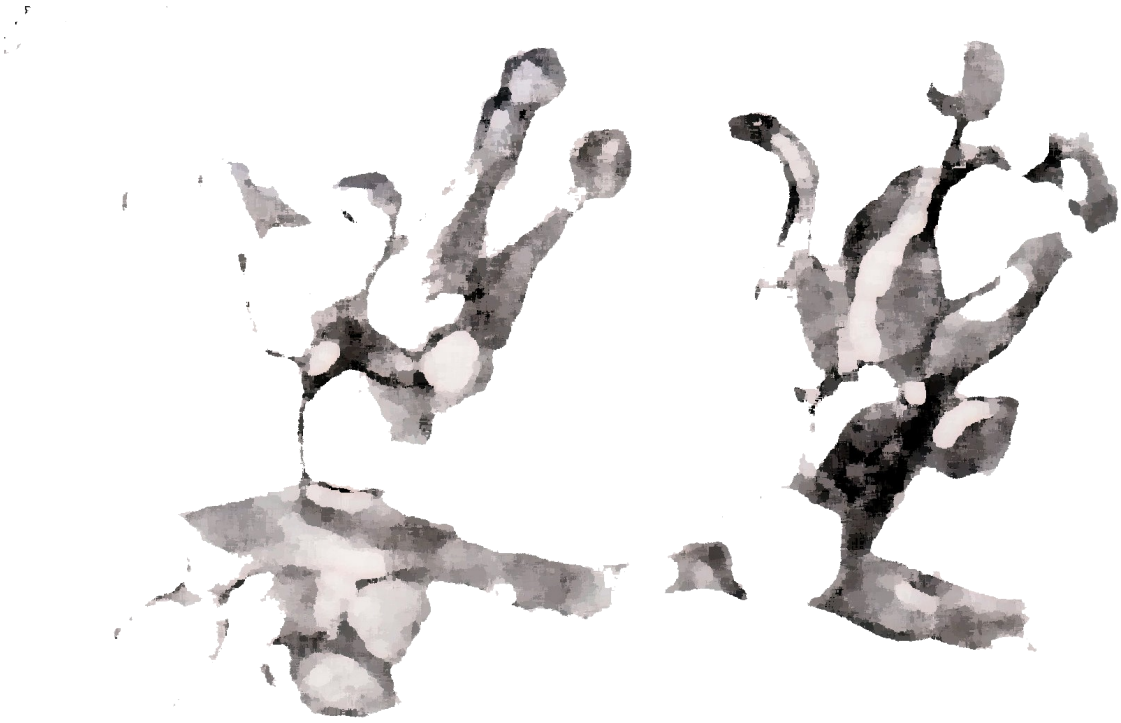

Genetic analyses place most Spanish isolates of *Beauveria bassiana* in a molecular group with word-wide distribution

ABSTRACT

Background: The entomopathogenic anamorphic fungus *Beauveria bassiana* is currently used as a biocontrol agent (BCA) of insects. Fifty-seven *Beauveria bassiana* isolates -53 from Spain- were characterized, integrating group I intron insertion patterns at the 3'-end of the nuclear large subunit ribosomal gene (LSU rDNA) and elongation factor 1-alpha (EF1-α) phylogenetic information, in order to assess the genetic structure and diversity of this Spanish collection of *B. bassiana*.

Results: Group I intron genotype analysis was based on the four highly conserved insertion sites of the LSU (Ec2653, Ec2449, Ec2066, Ec1921). Of the 16 possible combinations/genotypes, only four were detected, two of which were predominant, containing 44 and 9 members out of 57 isolates, respectively. Interestingly, the members of the latter two genotypes showed unique differences in their growth temperatures. In follow, EF1-α phylogeny served to classify most of the strains in the *B. bassiana* s.s. (*sensu stricto*) group and separate them into 5 molecular subgroups, all of which contained a group I intron belonging to the IC1 subtype at the Ec1921 position. A number of parameters such as thermal growth or origin (host, geographic location and climatic conditions) were also examined but in general no association could be found.

Conclusion: Most Spanish *B. bassiana* isolates (77.2%) are grouped into a major phylogenetic subgroup with wordwide distribution. However, high phylogenetic diversity was also detected among Spanish isolates from close geographic zones with low climatic variation. In general, no correlation was observed between the molecular distribution and geographic origin or climatic characteristics where the Spanish *B. bassiana* isolates were sampled.

1. Background

The anamorphic fungus *Beauveria bassiana* (Bals.) Vuill. (teleomorph: *Cordyceps bassiana*) is the most widely used mycopesticide for the biological control of insect pests (Wraight et al., 2000; Quesada-Moraga et al., 2006), formulations based on this fungus being available for commercial use (Wraight et al., 2001). However, there are still many unresolved questions in our understanding of the life of fungal entomopathogens, including their population characteristics and relationships between genotypes and habitats or host -pathogen interactions (Enkerli and Widmer, 2010). For predictable and successful application of biological control agents (BCAs) to control diseases and pests in natural environments, their biology and ecology must be well understood (Meyling and Eilenberg, 2007; Kouvelis et al., 2008; Meyling et al., 2009).

The morphological features of conidia are common tools for identification in *Beauveria*. Morphological and molecular studies have shown that the broad patterns of diversity in *Beauveria* have been accurately predicted in previous morphological studies. However, they have also shown that these approaches are insufficient to investigate species such as *B. bassiana* (Rehner and Buckley, 2005). Molecular data applied to taxonomic investigations have demonstrated that *B. bassiana* is a species complex with several cryptic species and have corroborated their link to *Cordyceps* teleomorphs (Gaitan et al., 2002; Aquino de Muro et al., 2005; Rehner and Buckley, 2005; Devi et al., 2006; Rehner et al., 2006). In this sense, phylogenetic studies based on nuclear ITS and elongation factor 1-alpha (EF1-α) sequences have demonstrated the monophyly of *Beauveria* and the existence of at least two lineages within *B. bassiana s.l.* (*sensu lato*), and also that EF1-α sequences provide adequate information for the inference of relationships in this genus (Rehner and Buckley, 2005). Studies on the genetic variability of BCAs such as *B. bassiana* are crucial for the development of molecular tools for their monitoring in the natural environment (Kouvelis et al., 2008).

Minisatellite loci (Coates et al., 2002), random amplified polymorphism DNA (RAPD) (Castrillo et al.,

2003), universally primed (UP) PCR (Meyling and Eilenberg, 2006), amplified fragment length polymorphism (AFLP) (Aquino de Muro et al., 2003), isoenzyme analyses (St Leger et al., 1992), or combinations of these methods (Fernandes et al., 2009) have provided useful polymorphisms to access genetic diversity among *B. bassiana* isolates.

Although some molecular studies have correlated *B. bassiana* genetic groups and host affiliation (Berreta et al., 1998; Gaitan et al., 2002), more recent evidence indicates that this is not the case since *B. bassiana* contains generalist enthomopathogens with no particular phylogenetic association with their insect host (Fernandes et al., 2009; Meyling et al., 2009), environmental factors being the prime selective forces for genotypic evolution in *B. bassiana* (Meyling et al., 2009). In this sense, several studies have demonstrated the association between *B. bassiana* genetic groups and Canadian (Bidochka et al., 2002), Brazilian (Fernandes et al., 2009) and worldwide (Ghikas et al., 2010) climatic zones.

Entomopathogenic species displayed a high degree of variability-mainly attributed to the presence of group I introns- at specific sites of the coding regions of small and large subunits of nuclear ribosomal RNA genes (SSU rDNA and LSU rDNA). Group I introns in entomopathogenic fungi were initially reported in *Beauveria brongniartii* LSU genes (Neuvéglise and Brygoo, 1994). Work addressing the presence and usefulness of these non-coding elements has been reported for *Beauveria*. For example, Neuvéglise et al. (1997) found 14 form variants of introns, differing in size and restriction patterns, at four different LSU positions from among a panel of 47 isolates of *B. brongniartii*, two of *B. bassiana*, and one of *Metarhizium anisopliae* from several geographic origins. Coates et al. (2002) found 12 intron forms in the SSU from 35 *Beauveria* isolates. Wang et al. (2003) analyzed the presence of group I introns in the four LSU insertion positions, designated Bb1 (also known as Ec2563), Bb2 (Ec2449), Bb3 (Ec2066) and Bb4 (Ec1921), and distributed a collection of 125 *B. bassiana* isolates in 13 different genotypes. In that study, their sequence analyses confirmed that the introns were invariably inserted in specific target sequences, and a strong correlation between specific insertion sites and intron subgroups was also observed. In addition, the features and behaviour of these group I intron were also detected in related genera such as *Cordyceps* (Nikoh and Fukatsu, 2001) and *Metarhizium* (Pantou et al., 2003).

The present study was undertaken to investigate the genetic variability existing in a collection of 53 Spanish isolates of *B. bassiana*, obtained from different substrates or insect hosts, and 4 isolates from other European countries. The insertion patterns of group I introns at the 3'- end of the LSU rDNA genes and EF1-a phylogenetic distribution were integrated in order to explore any possible correlation between genetic groups and geographical/climate origin, and habitat or insect host.

2. Results

2.1. Analysis of group I introns in 3' LSU rDNA

The 3'-end of the nuclear LSU rDNA genes of the 57 *B. bassiana* isolates (Table 1) was amplified with primers I29 and M1 and four different sizes of PCR products were observed on agarose gels, ranging from 0.79 to 1.77 kb. The sizes were as follows: about 1650 bp for 44 isolates; 1770 bp for one isolate; 1280 bp for 9 isolates, and 790 bp for 3 isolates. All amplicons were purified and sequenced in order to determine whether the insertion of multiple sequences, a feature described for members of this and other entomopathogenic genera, was responsible for the diversity

Table 1: Information concerning the *Beauveria bassiana* isolates analyzed in this study.

Code	Isolate	Location	Climate	Habitat/Host (Order)
Bb1	EABb 01/145-Su	Sevilla (Spain)	M	olive
Bb2	EABb 01/160-Su	Huelva (Spain)	M	oak
Bb3	EABb 01/164-Su	Huelva (Spain)	M	pine
Bb4	EABb 01/168-Su	Huelva (Spain)	M	scrubland
Bb5	EABb 01/171-Su	Huelva (Spain)	M	cotton
Bb6	EABb 01/15-Su	Almería (Spain)	M	dessert
Bb7	EABb 01/126-Su	Cádiz (Spain)	M	olive
Bb8	EABb 01/75-Su	Almería (Spain)	M	seaside
Bb9	EABb 01/116-Su	Sevilla (Spain)	M	olive
Bb10	EABb 01/112-Su	Sevilla (Spain)	M	wheat
Bb11	EABb 01/125-Su	Cádiz (Spain)	M	fallow land
Bb12	EABb 00/10-Su	Jaén (Spain)	M	olive
Bb13	EABb 00/11-Su	Jaén (Spain)	M	scrubland
Bb14	EABb 00/13-Su	Jaén (Spain)	M	woodland
Bb15	EABb 00/16-Su	Almería (Spain)	M	scrubland
Bb16	EABb 00/17-Su	Almería (Spain)	M	dessert
Bb17	EABb 01/07-Su	Córdoba (Spain)	M	meadow
Bb18	EABb 01/19-Su	Granada (Spain)	M	wheat
Bb19	EABb 01/22-Su	Córdoba (Spain)	M	scrubland
Bb20	EABb 01/25-Su	Córdoba (Spain)	M	olive
Bb21	EABb 01/27-Su	Córdoba (Spain)	M	wheat
Bb22	EABb 01/33-Su	Cádiz (Spain)	M	olive
Bb23	EABb 01/34-Su	Málaga (Spain)	M	olive
Bb24	EABb 01/35-Su	Málaga (Spain)	M	scrubland
Bb25	EABb 01/36-Su	Málaga (Spain)	M	meadow
Bb26	EABb 01/37-Su	Málaga (Spain)	M	olive
Bb27	EABb 01/43-Su	Jaén (Spain)	M	olive
Bb28	EABb 01/45-Su	Jaén (Spain)	M	scrubland
Bb29	EABb 01/64-Su	Granada (Spain)	M	woodland
Bb30	EABb 01/73-Su	Granada (Spain)	M	scrubland
Bb31	EABb 01/76-Su	Granada (Spain)	M	scrubland
Bb32	EABb 01/100-Su	Sevilla (Spain)	M	olive
Bb33	EABb 01/103-Su	Sevilla (Spain)	M	woodland
Bb34	EABb 01/105-Su	Sevilla (Spain)	M	cotton
Bb35	EABb 01/130-Su	Cádiz (Spain)	M	pine
Bb36	EABb 01/132-Su	Cádiz (Spain)	M	cotton
Bb37	EABb 90/2-Dm	Badajoz (Spain)	M	*Dociostaurus maroccanus* (Orthoptera)
Bb38	EABb 90/4-Cb	Badajoz (Spain)	M	*Chortipus bicolor* (Orthoptera)
Bb39	EABb 91/6-Ci	Badajoz (Spain)	M	*Calliptamus italicus* (Orthoptera)
Bb40	EABb 91/7-Dm	Badajoz (Spain)	M	*D. maroccanus* (Orthoptera)
Bb41	EaBb 92/10-Dm	Badajoz (Spain)	M	*D. maroccanus* (Orthoptera)
Bb42	EABb 92/11Dm	Badajoz (Spain)	M	*D. maroccanus* (Orthoptera)
Bb43	EABb 93/14-Tp	Córdoba (Spain)	M	*Thaumetopea pytiocampa* (Lepidoptera)
Bb44	EABb 04/01-Tip	Sevilla (Spain)	M	*Timaspis papaveris* (Hymenoptera)
Bb45	EABb 01/88-Su	South Portugal	M	sunflower
Bb46	EABb 01/39-Su	Málaga (Spain)	M	almond
Bb47	EABb 01/110-Su	Sevilla (Spain)	M	holm oak
Bb48	EABb 04/06-Su	Córdoba (Spain)	M	cork oak
Bb49	EABb 04/08-Su	Córdoba (Spain)	M	hazel
Bb50	EABb 04/02-Su	Santander (Spain)	HO	Ebro river
Bb51	EABb 04/03-Su	Santander (Spain)	HO	grassland
Bb52	EABb 04/05-Su	Álava (Spain)	C	leek
Bb53	EABb 04/09-Su	Madrid (Spain)	C	grassland
Bb54	EABb 04/10-Su	Gerona (Spain)	M	olive
Bb55	EABb 04/12-Su	Georgia	C	inculto
Bb56	B.bassiana 1333	Greece	M	*Bactrocera oleae* (Diptera)
Bb57	B.bassiana 3395	Poland	C	No data available

Code: reference as each isolate is cited in the text.

Source: reference as received from the Collection from the Department of Ciencias y Recursos Agrícolas y Forestales (CRAF) of the University of Córdoba, Spain.

Climatic: zones where isolates were collected (M: subtropical Mediterranean, C: continental, HO: humid oceanic).

of their lengths.

After sequencing analysis (Table 2), we observed that the smallest PCR products were detected in 3 out of the 57 isolates studied - coded Bb19, Bb50 and Bb57- indicating that these isolates had no introns, and the intronless sequence size was 790 bp; identical in composition to a homologous fragment of *B. bassiana s.l.* (Wang et al., 2003) described previously. The other 54 isolates exhibited introns inserted at one or more of the four possible conserved positions. Among these 54 intron-containing isolates, the insertion was as follows: 44 showed inserted sequences at positions 1 (Ec2563) and 4 (Ec1921); one isolate, Bb51, with a sequence size of 1770 bp, contained two introns at positions 2 (Ec2449) and 4 (Ec1921), and nine isolates contained only one intron at position 4.

The presence/absence of introns at the 3'-end of the nuclear LSU rDNA of the 57 isolates analyzed allowed their distribution in the following genotypes: A1B2B3A4, B1A2B3A4, B1B2B3A4 and B1B2B3B4 (A = presence, B = absence; according to Wang et al. 2003). Insertion sites are numbered from 1 to 4, also following Wang's terminology (Wang et al., 2003): Ec2563 (position 1), Ec2449 (position 2), Ec2066 (position 3) and Ec1921 (position 4). These genotypes and their distribution frequencies are shown in Table 2. Three out of the 57 isolates had no introns; nine contained one, and forty-five had two introns. Fifty-four of 57 isolates showed an inserted intron at position 4, and 44 isolates at position 1, whereas only one isolate had an inserted intron at position 2. None of the 57 isolates had introns at the 3 insertion site.

There was a significant correlation between belonging to an intron genotype and the mean of the optimal ($F_{1,84}$: 57.20°C; $P < 0.001$) and highest ($F_{1,84}$: 27.39°C; $P < 0.001$) growth temperatures, which were significantly lower in the genotype B1B2B3A4, with T_{opt} and T_{max} values of 24.3 and 33.9°C, respectively, than those obtained for A1B2B3A4, with T_{opt} of 26.7 and T_{max} 35.6°C (data not shown).

Two different intron sequence sizes, 427 or 443 bp in length, were detected at position 4 within the 54 *Beauveria* isolates that bore an insertion at this site, allowing the distribution of the isolates into two subgenotypes (Table 2). Three of these 54 isolates had a sequence of 427 bp, showing 100% identity with the 4 -position intron sequence reported for *B. bassiana* Bb232 (Wang et al., 2003).In 51 of the *B. bassiana* isolates, the inserted sequence length at this position was 443 bp, and four variants with few nucleotide differences were observed after alignment of these sequences, showing identity values of 98 to 100% with

Table 2: Genotypes derived from the presence/absence of introns in LSU rDNA genes for 57 *Beauveria bassiana* isolates and types of intron sequences

Genotype* (%)	Isolate code	No. isolates	GenBank position 1 (Ec2563)	GenBank position 2 (Ec2449)	GenBank position 4** (Ec1921)
A1B2B3A4	Bb2-5, Bb32-33, Bb35, Bb45, Bb48-49	10	EF115312		EF115308 (433a)
	Bb1, Bb6-12, Bb14-17, Bb20-21, Bb23-31, Bb34, Bb36, Bb41-42, Bb44, Bb46-47,				
	Bb52-54, Bb56	34	EF115312		EF115307 (443b)
B1A2B3A4	Bb51	1		EF115313	EF115309 (427)
B1B2B3A4	Bb13, Bb18,	2			EF115310 (443c)
	Bb22, Bb37, Bb39-40, Bb43,	5			EF115311 (443d)
	Bb38, Bb55	2			EF115309 (427)
B1B2B3B4	Bb19, Bb50, Bb57	3			

*A, presence, and B, absence of a given intron at the 3'-end of the nuclear LSU rDNA genes. Numbers 1-4 represent insertion sites Ec2563, Ec2449, Ec2066 and Ec1921, respectively, as previously described (Wang et al., 2003)

**Sequence types inserted at position 4 (Ec1921) are indicated by their sizes in bp, followed by a letter for identical sizes (i.e., 443a, b, c,d) to indicate small differences in their composition.

another sequence detected at the same position in *B. bassiana* Bb726 (Coates et al., 2002).

The intron sequence inserted at position 2 was only detected for Bb51, an isolate obtained in Santander (North Spain), and was 502 bp long. This intron shared 99 and 98% identity with two sequences previously detected at the same position in the LSU of *B. bassiana* isolates 178 and 1121 (Coates et al., 2002; Wang et al., 2003). A 387-bp intron was identified in 44 isolates at position 1. Alignment of these sequences revealed that the 387-bp sequence was conserved in the 44 *B. bassiana* isolates, where this intron was observed, and this sequence had identity values of 98% with the previously described sequence of *B. bassiana* ECBL16 (Coates et al., 2002).

The seven different *B. bassiana* intron sequences exhibited the typical characteristics of group I and no ORFs were detected. These intron sequences from *B. bassiana* were compared with other fungal intron sequences available in databases for their placement in previously reported subgroups (Michel and Westhof, 1990). The introns inserted at positions 2 and 4 were placed in the IC1 subgroup (one of the 15 subgroups, based on their secondary structure, described within the group I introns), and that inserted at position 1 was placed in the IE subgroup. As previously observed in group I introns (Nikoh and Fukatsu, 2001; Pantou et al., 2003; Wang et al., 2005), those inserted at the same site all belonged to the same subgroup. The intron sequences obtained in this work were compared with other *B. bassiana* intron sequences representing different subgroups to examine their polymorphisms (data not shown). Intron size and nucleotide identity differences were observed but P, Q, R and S motif elements, which are needed for the formation of the secondary structure of group I introns (Cech, 1988), were highly conserved among the introns inserted at the same site, particularly for position 1. The highest polymorphism was observed in introns inserted at 2, the P1-P3 helices being the source of this variation, and at 4, in the P5, P6 and P8 helices.

The MP tree obtained after an alignment of the 7 different intron sequence types identified from 57 *B. bassiana* isolates and another 24 GenBank-deposited sequences, which represent intron sequences from *M. anisopliae*, *B. bassiana* and *Cordyceps profilica*, together with the subsequent phylogenetic analysis are shown in Fig. 1. The tree reveals the separation of four independent groups, supported by high bootstrap values, corresponding to the four positions reported previously (Wang et al., 2003): Ec1921 (position 4), Ec2066 (position 3), Ec2449 (position 2) and Ec2563 (position 1), where intron insertions occurred. The tree shows that the sequence group located at position 4 is closer to those at position 2 and both contain IC1 subgroup introns. Similarly, position 3 sequences are closer to position 1 sequences, and both groups have IE subgroup introns. Within position 4, *Cordyceps* and *Metarhizium* were separated from *Beauveria* sequences and formed an independent group, supported by a bootstrap value of 100%. In addition, the five different *Beauveria* sequences obtained here were separated into two of the four observed groups at this position, supported by bootstrap values of 94% and 60%. This separation was in accordance with the two sequence sizes detected: 443 and 427-bp in length. However, the four different sequence types detected for 443-bp-sized introns were not separated after phylogenetic analysis.

2.2. EF1-α gene analysis

With the exception of isolate Bb49, where no amplification was observed, all isolates afforded PCR products of 1.1 kb for the EF1-α gene with the primers tef1fw and 1750-R. Eleven different EF1-α gene se-

quences were identified among the 56 isolates. The alignment and comparison of these 11 sequences and another 18 GenBank-deposited sequences, representing different lineages from *B. bassiana s.s.* (*sensu stricto*), *B. brongniartii* and *B. bassiana* clade C (Rehner and Buckley, 2005; Rehner et al., 2006; Meyling et al., 2009), produced 1757 aligned positions, with 1542 constant characters and 114 parsimony-informative characters. The MP tree is shown in Figure 2. Of the 56 isolates analyzed, 94.6% (53 isolates) were located in the *B. bassiana s.s*. clade, and 5.4% (3 isolates) in clade C, which includes *B. cf.* (uncertain taxonomy) *bassiana* isolates. Within *B. bassiana s.s*., the 53 isolates analyzed in this study were separated in five subgroups (Eu-7, Eu-8 and Eu-9 with isolates from Spain and Portugal; Eu-3 from Spain, France and Denmark; and Wd-2 with world-wide distribution), supported by bootstrap values higher than 50%.

2.3. Integration of intron insertion patterns and EF1-α phylogenetic distribution

In order to assess the phylogenic distribution of the different intron configuration types, they were mapped on the EF1-α tree (Fig. 2). All 53 *B. bassiana* s.s. isolates showed an intron IC1 inserted at position 4. However, the IE intron inserted at position 1 was only present in the 10 isolates from subgroup Eu-7 and 33 out of 39 isolates from subgroup Wd-2. In particular, this subgroup included most of the Spanish isolates of *B. bassiana* forming an EF1-α phylogenetic group with isolates 681 from Romania and 792 from the USA (Rehner and Buckley, 2005) but displaying two different intron insertion models. Bb51 showed a unique intron insertion pattern, with an IC1 intron at position 2, and located separately in the Eu-9 subgroup. No introns were detected at any position in the three *B. cf. bassiana* isolates from clade C.

No correlation between EF1-α phylogenetic groups and insect host was observed. Although Eu-7 subgroup did not included isolates of insect origin, the Wd-2 subgroup grouped isolates collected from Diptera, Hymenoptera, Lepidoptera and Orthoptera. Moreover, Wd-2 isolates from Orthoptera displayed different intron insertion models (i.e., Bb37, Bb39 and Bb40, and Bb42).

Forty-nine Spanish and one Portuguese isolates of *B. bassiana s.s*. were collected from subtropical Mediterranean climate zones and were distributed in the Eu-7, Eu-3, Wd-2 and Eu-8 subgroups. Two Spanish isolates, Bb52 and Bb53, were collected from continental climate locations and were placed within subgroups Eu-7 and Wd-2, respectively. The only *B. bassiana s.s*. isolate from a humid oceanic climate included in this work, Bb51 from Santander, displayed a characteristic intron insertion model and formed the EF1-α subgroup Eu-9. In addition, Bb51 produced smaller conidia than the rest of *B. bassiana* isolates, this morphological feature being statistically significant (data not shown). Nevertheless, other isolate from the same climatic zone, Bb50, was grouped with other European isolates in *B. cf. bassiana* clade C.

3. Discussion

In the present study, we have identified different *B. bassiana* genotypes and phylogenetic subgroups in a collection of 57 isolates of this fungus, based on intron insertion patterns and EF1-α phylogenies, respectively.

The variability in group I introns from rDNA genes has been used as a molecular tool for the identification of polymorphisms in entomopathogenic fungi (Neuvéglise et al., 1997; Mavridou et al., 2000; Márquez et al., 2006). Our study of *B. bassiana* LSU rDNA identified 99 introns among the 57 isolates ana-

lyzed. Four specific sites of intron insertion have been described previously in *Beauveria* species (Neuvéglise et al., 1997; Wang et al., 2003), but in our collection introns were only detected at positions 1, 2 or 4.

Particularly, our study shows that 100% of *B. bassiana s.s.* isolates had an intron inserted at position 4. This position was also preferential for intron insertion (84.4%) in a population of 125 *B. bassiana* isolates (Wang et al., 2003). The number of introns found in the 57 isolates was in agreement with the 199 introns detected in 125 *B. bassiana* isolates by Wang et al. (2003); the 44 introns detected in 26 *M. anisopliae* isolates by Márquez et al. (2006), and the 69 introns found in 28 representative members of the genus *Cordyceps* by Nikoh and Fukatsu (2001). However, only four intron insertion patterns were present in our *B. bassiana* collection while greater variability was found in other studies: 13, 7 and 9 insertion patterns within 125 *B. bassiana* (Wang et al. 2003), 26 *M. anisopliae (*Márquez et al. 2006) and 47 *B. brongniartii* (Neuvéglise et al., 1997) isolates, respectively.

The MP tree based on intron sequences shows that they were distributed in four large groups, with bootstrap values of 100%, corresponding to four insertion positions (Fig. 1). As could be expected (Wang et al. 2003; Michel and Westhof, 1990), the introns inserted at the same site always belonged to the same subgroup: IC1 at positions 2 and 4, and IE at position 1.

Although the origin and transmission mechanisms of group I introns have generated controversy (Nikoh and Fukatsu, 2001), this distribution of sequences is in agreement with previously reported observations (Wang et al. 2003) and means that introns inserted at the same position have a monophyletic origin and are transmitted vertically. In subsequent events intron speciation and diversification take place as occurs at position 4, where *B. bassiana* introns are separated from *Metarhizium* and *Cordyceps* introns, and two *B. bassiana* IC1 sequence sizes were located in two different sub-clades, supported by high bootstrap values.

Rehner and Buckley's study (2005) based on EF1-α and ITS phylogenies has revealed that i) six clades can be resolved within *Beauveria* (A-F) and, excepting those corresponding to *B. bassiana* (A and C), they are closely to species previously described on the basis of their morphology, and ii) *B. bassiana s.s*. (A) was determined almost entirely from nucleotide variation at EF1-α. Further phylogenetic studies carried out with nuclear and/or mitochondrial DNA regions of *B. bassiana* from all continents have served to resolve lineage diversity within this species (Rehner et al., 2006; Fernandes et al., 2009; Meyling et al., 2009; Ghikaset al., 2010). Since phylogenetic species by continent and in the order of their discovery have been designated previously (Meyling et al., 2009), we followed this nomenclature to refer the new phylogenetic subgroups identified among the Spanish *B. bassiana s.s*. isolates as Eu-7, Eu-8 and Eu-9. The results obtained from MP analyses (Fig. 2), using a 1.1 kb fragment of the EF1–α gene from 56 isolates from our collection, confirmed that 53 isolates were *B. bassiana s.s.* (A), and three isolates grouped in three different phylogenetic subgroups within *B. cf. bassiana* (C). As in a previous study Meyling et al., 2009), the collection of Spanish isolates of *B. bassiana s.s*. was separated in five phylogenetic subgroups. However, only isolate Bb38, sampled from insects, was grouped with one (Eu-3) of the five phylogenetic species proposed by those authors working with a Danish collection of *B. bassiana s.s.* (Meyling et al., 2009), including insect isolates only. Interestingly, three phylogenetic subgroups (Eu-7, Eu -8 and Eu- 9) were only formed by isolates from Spanish and Portuguese isolates. However, most of

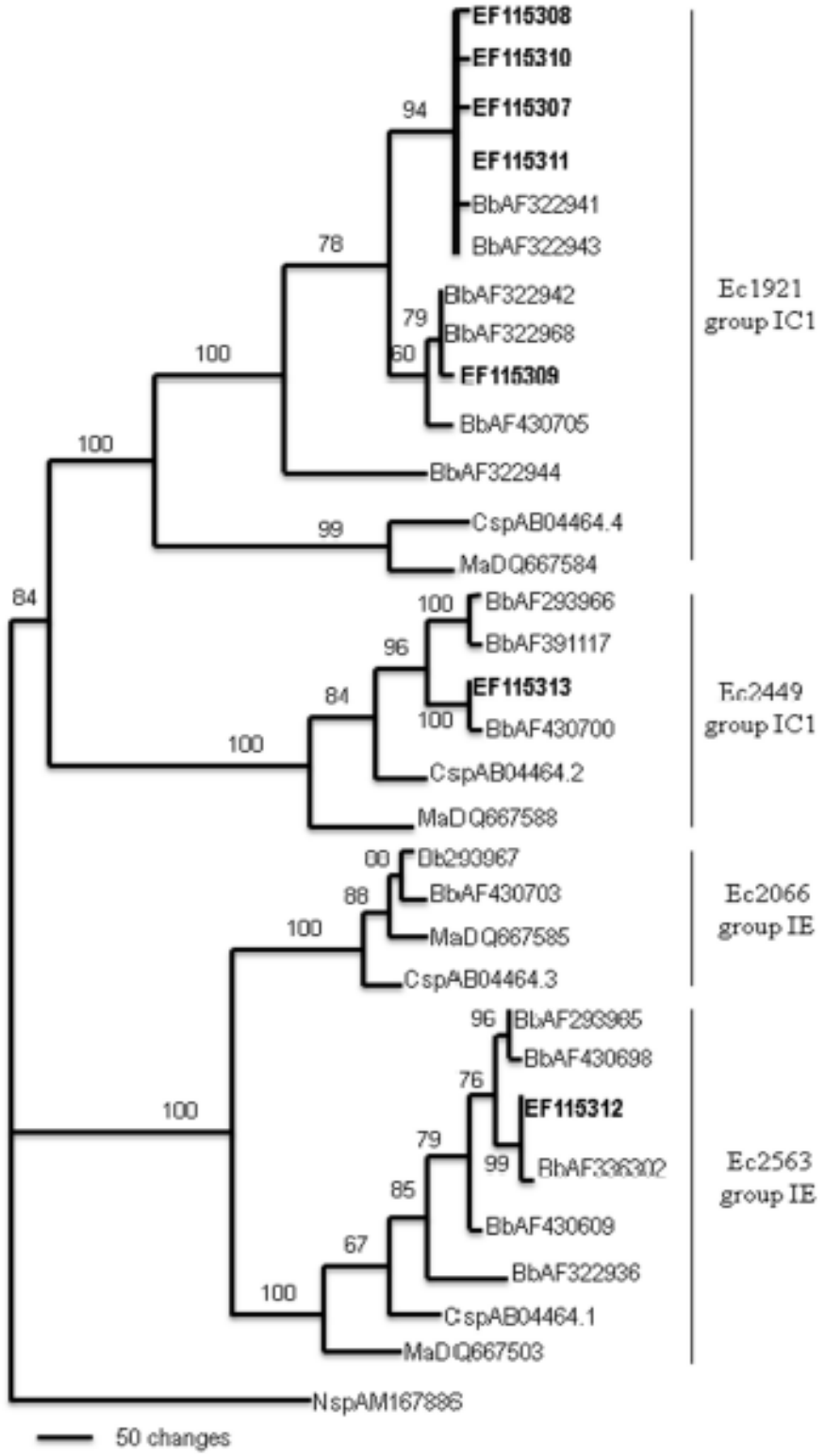

Fig. 1. Phylogenetic analysis of group I introns inserted in the LSU rDNA genes of entomopathogenic fungi. The MP tree was generated by parsimony analysis after heuristic searches (TBR option). A bootstrap full heuristic analysis, with bootstrap intervals from 1000 replications and nodes supported in >50% of bootstrap replicates, was generated using the PAUP* 4.0 program. Branch lengths are proportional to the number of changes. Seven different intron sequence types (bolded) identified from 57 *B. bassiana* isolates were aligned with 24 representative intron sequences from *Metarhizium anisopliae* (Ma), *Beauveria bassiana* (Bb) and *Cordyceps profilica* (Csp), and an intron sequence from *Naegleria* sp. (Nsp) was used as outgroup. The four group I intron insertion positions are shown as Ec1921 (position 4), Ec2066 (position 3), Ec2449 (position 2) and Ec2563 (position 1)

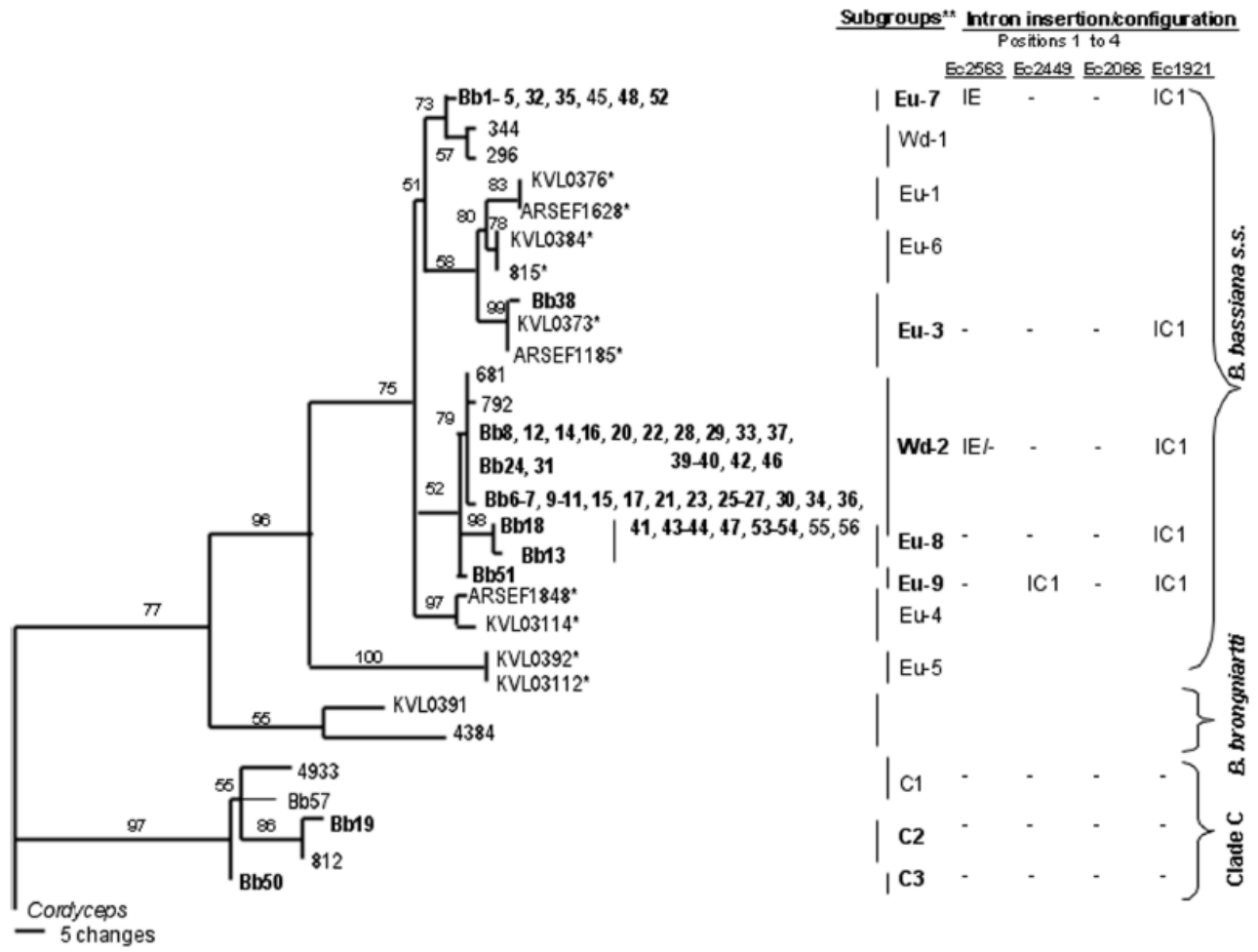

Fig. 2. Phylogenetic analysis based on EF1-α sequences from *Beauveria bassiana*. The MP tree was generated by parsimony analysis after heuristic searches (TBR option). A bootstrap full heuristic analysis, with bootstrap intervals from 1000 replications and nodes supported in >50% of bootstrap replicates, was generated using the PAUP* 4.0 program. Branch lengths are proportional to the number of changes. Eleven sequence types identified from 56 *B. bassiana* isolates, of which 52 were sampled in Spain (bolded), were aligned with 18 GenBank *B. bassiana s.s*., *B. brongniartii* and *B. cf. bassiana* (clade C) sequences, indicated by accession numbers as in previous works (Rehner and Buckley, 2005; Meyling et al., 2009). *B. bassiana s.s*. EF1-α sequences representing European subgroups (Meyling et al., 2009) are marked with an asterisk. Reference isolates from countries different to Spain, are referred to as: Eu-1 (KVL0376 from Denmark and ARSEF1628 from Hungary), Eu-3 (KVL0373 from Denmark and ARSEF1185 from France), Eu-4 (KVL03114 from Denmark and ARSEF1848 from Belgium), Eu-5 (KVL0392 and KVL03112 from Denmark), Eu-6 (KVL0384 from Denmark and 815 from France), Eu- 7 (Bb45 from Portugal), Wd-1 (296 and 344 from USA), Wd-2 (681 from Romania, 792 from USA, Bb55 from Georgia and Bb56 from Greece), C1 (4933 from France and Bb57 from Poland), C2 (812 from France) and *B. brogniartii* (KVL0392 from Denmark and 4384 from China). *Cordyceps cf. scarabaeicola* (EFCC 2533) was the outgroup. Identifiers of EF1-α subgroups and intron configuration patterns are indicated

the isolates in our collection (39 out of 56) were grouped with isolates from Romania and the USA in the world-wide phylogenetic subgroup Wd-2, which includes isolates from Europe, Africa and North America (Rehner and Buckley, 2005).

When the different intron insertion patterns were mapped on the *B. bassiana* EF1-α phylogeny (Fig. 2), the existence of a same intron genotype in a given phylogenetic subgroup could be indicative of its clonal origin as it is the case of Eu-7 and Eu-8. Previous studies have shown that Eu-3, where Bb38 is located, is a clonal group (Meyling et al., 2009). Isolate Bb51 was the only member of Eu-9 and the separated phylogenetic grouping of this isolate is supported by a charac-

teristic intron insertion pattern and the production of statistically significant smaller conidia than those from any other intron genotype (data not shown). The two different intron genotypes observed among the isolates from the complex phylogenetic subgroup Wd-2, may indicate that homologous recombination is involved in the IE intron loss at position 1. Previous studies have shown frequent intron losses of group I introns in the nuclear rDNAs of *Cordyceps* (Nikoh and Fukatsu, 2001). Recently, a low frequency of sexual reproduction was observed in Eu-1 (Meyling et al., 2009); this could also be the case of Wd-2 where the absence of an IE intron at position 1 was only observed in 6 out of 39 isolates of this phylogenetic subgroup.

The genetic diversity of Spanish *B. bassiana s.s.* isolates was compared in relation to their hosts and geographical provenance and according to the latter view (Ghikas et al., 2010), no general correlation can be observed between the molecular variability among isolates and host and/or geographical origin. Although most of the isolates in our study were collected from soil, 8 out of 9 isolates from insects were grouped together in the subgroup Wd-2 although they derived from different insect orders. Phylogenetic subgroups only indicated a tenuous dependence upon geographic origin (i.e., Bb2-5 located in Eu- 7 or Bb23-26 and Bb29 -31 located in Wd-2). A recent phylogeographic report (Fernandes et al., 2009) has provided evidence that the genetic distance of Brazilian *B. bassiana* isolates correlates with geographical distance, suggesting that according to Rehner's study (2006) allopatry plays an important role in the phylogenetic diversification of *B. bassiana*. The authors of another recent study (Meyling et al., 2009) concluded that multiple phylogenetic species of *B. bassiana s.s*. co-exist in sympatry within the limited natural habitat of a bordering hedgerow. We observed that isolates sampled in close locations were placed in different phylogenetic subgroups (i.e., Bb35 and Bb36, from Cádiz, belong to Eu-7 and Wd-2, respectively; and Bb38, and Bb39-40 and Bb42, from Badajoz, group within Eu-3 and Wd-2, respectively, Bb39-40 and Bb42 having different intron genotypes). According to Meyling's study (2009), the high phylogenetic diversity of the Spanish isolates of *B. bassiana s.s.* could be explained by the untilled habitats where most of them were sampled (i.e., olive, oak, pine, meadow or scrubland).

Previous studies have suggested that the saprophytic phase of entomopathogenic fungi exerts evolutionary pressure on the genotype and that adaptation to a habitat type is associated with their environmental preferences (Bidochka et al., 2002). Recent studies have also pointed out the importance of climatic conditions in the prevalence and distribution of *B. bassiana* genotypes (Ghikas et al., 2010). Our study was carried out on 51 isolates from subtropical Mediterranean climate locations that were distributed within the phylogenetic subgroups Eu-3, Eu-7, Eu-8, Wd-2 and clade C; 4 isolates were from continental climate sites and grouped in Eu-7, Wd-2 and clade C; and 2 isolates came from a humid oceanic climate zone, being located in Eu-9 and clade C. Interestingly, the only *B. bassiana s.s*. from a humid oceanic climate was the singular isolate Bb51. The fact that isolates from Mediterranean or continental climates overlapped in different phylogenetic subgroups, could be due to lower differences among the abiotic conditions existing in Spain, a country covering far smaller geographical surface and with much less variability than that considered in other Canadian, Brazilian or worldwide studies where phylogenetic species showed a better correlation with climate characteristics (Ghikas et al., 2010), biogeographic distribution (Fernandes et al., 2009) and habitat (Bidochka et al., 2002). In a thermal growth study (Bidochka et al., 2002) it was described that *B. bassiana* genetic groups from different

habitats in Canada were associated with temperature preferences. When we explored the thermal preferences within a set of Spanish *B. bassiana s.s.* isolates belonging to the two main intron genotypes (A1B2B3A4 and B1B2B3A4) and four phylogenetic EF1-α subgroups (data not shown), a correlation between intron genotypes and the mean optimal and maximum temperatures for growth was observed, both growth temperatures being significantly lower in the B1B2B3A4 genotype with respect to A1B2B3A4. However, no correlation was observed between thermal preferences and the climatic origin of the Spanish *B. bassiana* isolates.

4. Conclusion

Four intron genotypes, and five and three phylogenetic subgroups within *B. bassiana s.s.* and *B. cf. bassiana* (clade C) have been identified, respectively, in a collection of 57 *B. bassiana* isolates -53 from Spain. The highest polymorphism was observed in introns inserted at positions 2 and 4. All *B. bassiana s.s*. displayed an IC1 intron inserted at position 4. Integration of intron insertion patterns and EF1-α phylogenetic distribution served to demonstrate the monophyletic origin and vertical transmission of introns inserted at the same site. In subsequent events intron speciation and diversification take place as occurs at site 4, where *B. bassiana* introns are separated from *Metarhizium* and *Cordyceps* introns. No general correlation was observed between the molecular data and insect host, but a tenuous correlation was detected with the geographic origins. The high phylogenetic diversity of the Spanish isolates of *B. bassiana s.s.* could be due to the untilled habitats where most of them were sampled.

5. Methods

5.1. Fungal isolates and morphological studies

The 57 isolates of *B. bassiana* used in this study were selected from a Spanish collection of 960 records at the CRAF (Ciencias y Recursos Agrícolas y Forestales) Department of the University of Cordoba (Córdoba, Spain), representing different geographic origins, habitats/hosts and climates. Fifty-three Spanish isolates were studied, 51 of them being collected from subtropical Mediterranean climate zones -characterized by warm to hot, dry summers and mild to cool, wet winters and 2 from a humid oceanic climate. Forty five out of these 53 isolates were from soil, most of them from poorly tilled or untilled fields (i.e., olive, oak, pine or scrubland) and 8 were isolated from insects. Information about these isolates is provided in Table 1. All fungal isolates were derived from single conidial spores grown on Malt Extract Agar plates (MEA, Difco Becton Dickinson, Sparks, MD).

5.2. DNA extraction, PCR amplification, and sequencing

Mycelia for DNA extraction were obtained as previously described (Márquez et al., 2006). Total DNA was extracted using the method previously described (Möller et al., 1992).

Two nuclear gene regions, LSU rDNA and EF1-α, were amplified, sequenced and analyzed. The 3'-end of the nuclear LSU rDNA cluster was also amplified with primers I29 (5'-CTGCCCAGTGCTCTGAATGTC-3') (Wang et al., 2003) and M1 (5'-GGTAAAACTAACCTGTCTCACG-3') (Márquez et al., 2006) for the 57 isolates of *Beauveria* included in the study. The distribution of putative introns was investigated using the following combinations of previously described primers: I29-I38, I31-I32, I21-I22 and E23-M1 (Wang et al., 2003; Márquez et al., 2006). A 1100 bp fragment spanning the 3' 2/3 of the EF1-α gene was

amplified with primers tef1fw (5'- GTGAGCGTGGTAT-CACCA-3') (O'Donnell et al., 1998) and 1750-R (5'-GACGCATGTCACGGACGGC-3') for all isolates, except Bb49. The oligonucleotide 1750-R was designed at the 3'-end of an alignment of *Beauveria* EF1-α genes obtained from databases. PCR was performed in a total volume of 50 μl containing 25 ng of genomic DNA and 0.20 μM concentrations of each of the above primers, using the Taq polymerase system (Biotools B&M Labs, Madrid, Spain) and following the manufacturer's instructions. The amplification program included an initial denaturing cycle of 1 min at 94°C, followed by 35 cycles of 1 min 30 s at 94°C, 2 min (for EF1-α) or 2 min 30 sec for (LSU rDNA) at 55 (for EF1-α) or 57°C (for LSU rDNA) and 3 min at 72°C, and a final extension step of 7 min at 72°C in a PCR System 9700 Genetic Thermal Cycler (Applied Biosystems, Foster City, CA). The PCR products were electrophoresed on 1% agarose gels buffered with 1 × TAE (Sambrook et al., 1989) and stained with ethidium bromide. A 100-bp ladder molecular weight standard (Roche Mannheim, Mannheim, Germany) was also used.

The PCR products were purified from agarose gels using the Geneclean II kit® system (Q-Biogene, Carlsbad, CA), following the manufacturer's protocol. DNA sequences were obtained using an automated ABI 377 Prism Sequencer (Applied Biosystems, Foster City, CA) with fluorescent terminators at the Department of Microbiology and Genetics of the University of Salamanca. All PCR products were sequenced in both directions, using amplification primers and internal primers when necessary.

The intron and EF1-α sequences obtained in this study were deposited in the GenBank database. Intron and EF1-α sequence accession numbers are available in Table 2 and additional file 1 respectively.

5.3. Molecular analyses

The presence or absence of introns at the 3'-end of the nuclear LSU rDNA of each isolate was determined by detecting previously described target sequences (Wang et al. 2003). In order to compare the results obtained in this study with the *B. bassiana* genotypes based on previously reported intron insertion patterns in the LSU rDNA gene, Wang's terminology was used (Wang et al., 2003). The intron sequences detected in each insertion point were aligned with representative *Beauveria* sequences to examine their polymorphisms and to identify conserved motifs. Intron subgroups were determined by comparison with representative secondary structures from previous studies (Mavridou et al., 2000; Nikoh and Fukatsu, 2001; Pantou et al., 2003; Wang et al., 2003).

Intron and EF1-α sequences were analyzed separately. Published sequences for isolates included within the genera *Beauveria*, *Metarhizium* and *Cordyceps* were retrieved from GenBank and included in the alignments. Alignments were generated using the MegAlign (DNASTAR package, 1989-92, London, UK) and the CLUSTALX 1.81 program (Thompson et al., 1997). Phylogenetic analyses were carried out with the PAUP* version 4.0 b10 program. Gaps, encoded as missing data, and uninformative characters were excluded from the analyses. Most-parsimonious (MP) trees were obtained for intron and EF1-α data from heuristic searches using TBR branch-swapping (Felsenstein et al. 1985), and all MP trees were summarized in a single tree in which all branch lengths equal to zero were collapsed by polytomies. An intron sequence of *Naegleria* sp. (AM167886) and the EF1-α gene of *Cordyceps* cf. *scarabaeicola* (AY531967) were used as outgroups in the analysis of intron and EF1-α sequences, respectively. A bootstrap full heuristic

analysis consisting of 1000 replicates was performed, and a 50% majority rule tree was produced.

6. Additional material

Additional file 1 (Table 3).

7. Acknowledgements

This manuscript is in memoriam of Marcela Márquez, deceased in the course of this research. This work has been funded by the Spanish Ministry of Education and Science, projects AGL2004-06322-C02-02/ AGR and AGL2008- 0512/AGR; and Junta de Castilla y León, project GR67.

Table 3. GenBank accession numbers of EF1-*a* sequences obtained in this study from 57 *Beauveria bassiana* isolates and EF1-a subgroups

Code[a]	Isolate[b]	EF1-a	EF1-a subgroup	Code[a]	Isolate[b]	EF1-a	EF1-a subgroup
Bb1	EABb 01/145-Su	FJ545730	Eu-7	Bb30	EABb 01/73-Su	FJ545732	Wd-2
Bb2	EABb 01/160-Su	FJ545730	Eu-7	Bb31	EABb 01/76-Su	FJ545739	Wd-2
Bb3	EABb 01/164-Su	FJ545730	Eu-7	Bb32	EABb 01/100-Su	FJ545730	Eu-7
Bb4	EABb 01/168-Su	FJ545730	Eu-7	Bb33	EABb 01/103-Su	EF545731	Wd-2
Bb5	EABb 01/171-Su	FJ545730	Eu-7	Bb34	EABb 01/105-Su	FJ545732	Wd-2
Bb6	EABb 01/15-Su	FJ545732	Wd-2	Bb35	EABb 01/130-Su	FJ545730	Eu-7
Bb7	EABb 01/126-Su	FJ545732	Wd-2	Bb36	EABb 01/132-Su	FJ545732	Wd-2
Bb8	EABb 01/75-Su	FJ545731	Wd-2	Bb37	EABb 90/2-Dm	FJ545731	Wd-2
Bb9	EABb 01/116-Su	FJ545732	Wd-2	Bb38	EABb 90/4-Cb	FJ545734	Eu-3
Bb10	EABb 01/112-Su	FJ545732	Wd-2	Bb39	EABb 91/6-Ci	FJ545731	Wd-2
Bb11	EABb 01/125-Su	FJ545732	Wd-d	Bb40	EABb 91/7-Dm	FJ545731	Wd-2
Bb12	EABb 00/10-Su	FJ545731	Wd-2	Bb41	EaBb 92/10-Dm	FJ545732	Wd-2
Bb13	EABb 00/11-Su	FJ545740	Eu-8	Bb42	EABb 92/11Dm	FJ545731	Wd-2
Bb14	EABb 00/13-Su	FJ545731	Wd-2	Bb43	EABb 93/14-Tp	FJ545732	Wd-2
Bb15	EABb 00/16-Su	FJ545732	Wd-2	Bb44	EABb 04/01-Tip	FJ545732	Wd-2
Bb16	EABb 00/17-Su	FJ545731	Wd-2	Bb45	EABb 01/88-Su	FJ545730	Eu-7
Bb17	EABb 01/07-Su	FJ545732	Wd-2	Bb46	EABb 01/39-Su	FJ545731	Wd-2
Bb18	EABb 01/19-Su	FJ545733	Eu-8	Bb47	EABb 01/110-Su	FJ545732	Wd-2
Bb19	EABb 01/22-Su	FJ545735	C2	Bb48	EABb 04/06-Su	FJ545730	Eu-7
Bb20	EABb 01/25-Su	FJ545731	Wd-2	Bb49	EABb 04/08-Su	Not available	Unknown
Bb21	EABb 01/27-Su	FJ545732	Wd-2	Bb50	EABb 04/02-Su	FJ545736	C3
Bb22	EABb 01/33-Su	FJ545731	Wd-2	Bb51	EABb 04/03-Su	FJ545737	Eu-9
Bb23	EABb 01/34-Su	FJ545732	Wd-2	Bb52	EABb 04/05-Su	FJ545730	Eu-7
Bb24	EABb 01/35-Su	FJ545739	Wd-2	Bb53	EABb 04/09-Su	FJ545732	Wd-2
Bb25	EABb 01/36-Su	FJ545732	Wd-2	Bb54	EABb 04/10-Su	FJ545732	Wd-2
Bb26	EABb 01/37-Su	FJ545732	Wd-2	Bb55	EABb 04/12-Su	FJ545732	Wd-2
Bb27	EABb 01/43-Su	FJ545732	Wd-2	Bb56	*B. bassiana* 1333	FJ545732	Wd-2
Bb28	EABb 01/45-Su	FJ545731	Wd-2	Bb57	*B. bassiana* 3395	FJ545738	C1
Bb29	EABb 01/64-Su	FJ545731	Wd-2				

[a] Code: reference as each isolate is cited in the text.

[b] Source: reference as received from the Collection from the Department of *Ciencias y Recursos Agrícolas y Forestales (CRAF)* of the University of Córdoba, Spain.

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CAPÍTULO V

Este capítulo es una versión adaptada del artículo

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Soil properties affect the availability, movement, and virulence of entomopathogenic fungi conidia against puparia of *Ceratitis capitata* (Diptera: Tephritidae)

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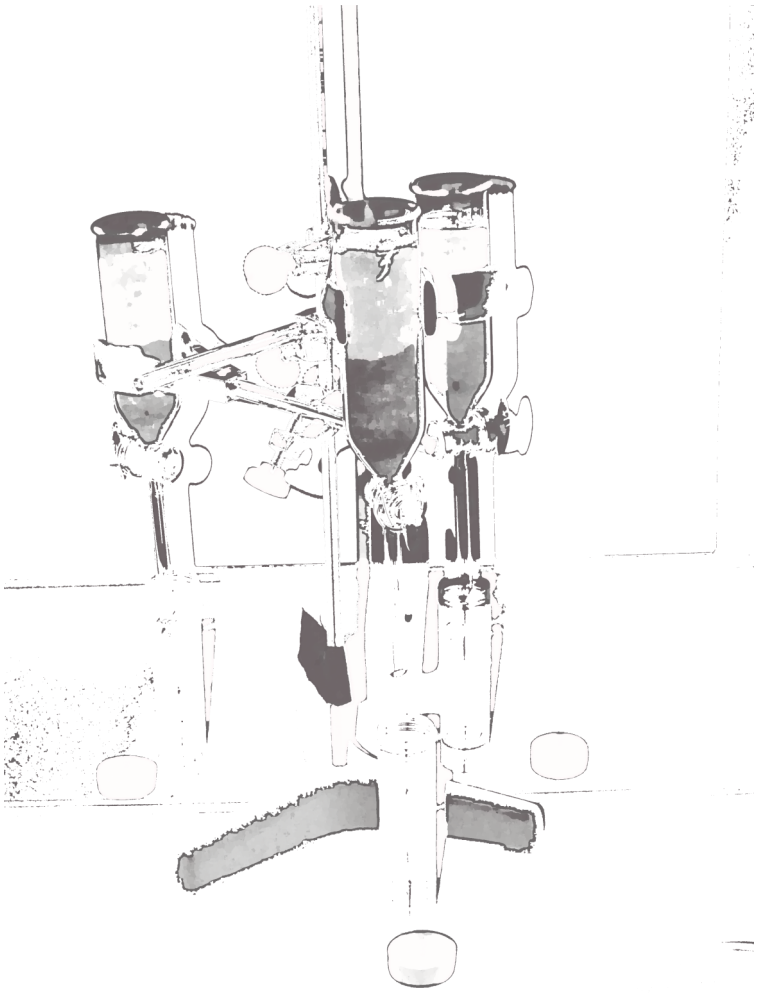

Soil properties affect the availability, movement, and virulence of entomopathogenic fungi conidia against puparia of *Ceratitis capitata* (Diptera: Tephritidae)

ABSTRACT

The Mediterranean fruit fly, *Ceratitis capitata* Wiedemann (Diptera: Tephritidae), is the major tephritid pest in the Mediterranean region. This insect may overwinter as pupae inside fruits or in soil. Therefore, infection with entomopathogenic fungi is a potentially useful control technique during the insect's soil-dwelling stage. Entomopathogenic fungi have an important role in Integrated Pest Management programs as an alternative to conventional chemical control, but they have been usually selected on the basis of laboratory results with little regard to fungal ecology. In this work, we designed several experiments to study the availability and movement of the EF *Beauveria bassiana* (Balsamo) Vuill. and *Metarhizium anisopliae* (Metsch.) conidia in 16 soils differing widely in pH, texture, organic matter, and carbonate contents. Experiments of adsorption and drag of conidia by soil particles suspended in $CaCl_2$ solutions of different ionic strength showed *B. bassiana* conidia to be retained by clay particles, and this effect disappeared with increasing ionic strength. The availability of *M. anisopliae* conidia in the suspension tended to be lower for sandy than for clayey soils and was not influenced by ionic strength. Regardless of soil properties, over 90% of the added fungal propagules were recovered from the surface layer of columns of packed soils representing model combinations of texture (sandy or clayey) and pH values (acid or alkaline). However, retention of *B. bassiana* conidia in the surface layer was higher in clayey than in sandy soils, and the retention of *M. anisopliae* conidia in the surface layer was higher in sandy than in clayey soils. Finally, neither soil texture nor ionic strength affected the infectivity of conidia of both fungal strains to *C. capitata* puparia.

1. Introduction

The Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wied.), is arguably one of the most serious tephritid pests in the Mediterranean region, particularly in Spain (Tremblay, 1994; Alfaro-Moreno, 2005). Medfly eggs are laid directly into the host fruit (such as oranges, apricots, peaches or pears) where larvae feed and develop causing direct damage. Currently, the control of medflies relies mainly on targeting adults with chemical insecticides, which are used as bait (insecticides mixed with an attractant) or cover sprays (Ros et al., 2002) that have ecological and toxicological side effects (Purcell and Schroeder, 1996). Third instar prepupating medfly larvae drop from fruits to the ground and burrow in the soil to locate pupation sites, and they pupate within the first 2–4 cm of the soil (Jackson et al., 1998). Thus, the soil-dwelling stage of tephritid flies offers an excellent opportunity for an effective management, but this opportunity has been poorly explored (Ekesi et al., 2007).

As an alternative to chemical control or as part of the Integrated Pest Management programs, there is resurgence in the use of microbial insecticides for biological control of tephritid flies, particularly entomopathogenic fungi. The strategic options for medfly control with entomopathogenic fungi include aerial applications and autodissemination to target adults and soil inoculation to target pupariating larvae and puparia (Ekesi et al., 2007). In addition, several isolates of the mitosporic ascomycetes including *Metarhizium anisopliae* (Metsch.) Sorok. and *Beauveria bassiana* (Balsamo) Vuill. have potential for medfly adult and puparia control (Quesada-Moraga et al., 2006, 2008).

Soil is an important reservoir for insect pathogenic fungi, and it is generally considered as a favorable environment for fungal microbial control because it provides shelter from environmental extremes, therefore, increasing the persistence of conidia and their ability to thrive (Jackson et al., 2000; Ekesi et al., 2007; Jaronski, 2007). Nonetheless, the effective use of *M. anisopliae* or *B. bassiana* as effective microbial insecticides against medfly puparia necessitates a solid understanding of the key factors controlling the migration and retention

of infective propagules in the soil. Fungal conidia transport and retention can be influenced by many factors including soil physicochemical properties, substratum hydrophobicity, and electrostatic interactions (Banks et al., 2003; Quesada-Moraga et al., 2007; Jaronski, 2010). With the exception of preliminary studies by Ignoffo et al. (1977), Wollum and Cassel (1978) and Storey and Gardner (1987), there are not any published studies on the vertical movement and availability of *B. bassiana* and *M. anisopliae* conidia in soil under microcosm conditions. Thus, the first aim of this study was to evaluate the adsorption, i.e., the action of a soil particle in attracting and holding on its surface, of conidia of one *B. bassiana* and one *M. anisopliae* strain in 16 Mediterranean soils ranging widely in texture and chemical properties. The transport behavior of the conidia was examined using laboratory scale columns packed with four selected soils representing different combinations of texture, pH, and ionic strength. We used this information to elucidate whether conidia accumulated at or near the surface of the soil where puparia may be exposed to the fungus, or if they penetrated deeper in the soil profile.

Finally, we determined the effect of the soil properties on the virulence of *B. bassiana* and *M. anisopliae* against puparia of *C. capitata*.

2. Materials and methods

2.1. Propagation of fungal isolates

The fungal isolates, *B. bassiana* EABb 01/110-Su and *M. anisopliae* EAMa 01/58-Su were selected because previous studies have shown them to be the most virulent of each fungal species against medfly puparia (Quesada-Moraga et al., 2006). These strains belonged to the culture collection at the Department of Agricultural and Forestry Sciences and Resources (AFSR) of the University of Córdoba (Spain). The *M. anisopliae* EAMa 01/58-Su strain was originally isolated from the soil of a wheat crop at Hinojosa del Duque (Province of Córdoba, Spain), and the *B. bassiana* EABb 01/110-Su strain was originally isolated from the soil of a holm oak forest in the Province of Seville (Spain). Fungi were grown on malt agar (MA) (BioCult Laboratorios, Madrid, Spain) at 25 ºC in darkness and were then lyophilized in a LyoQuest laboratory freeze dryer (Telstar, Barcelona, Spain) and stored at -80 ºC.

For inoculum preparation, the isolates were subcultured on MA for 15 days at 25 ºC in darkness. Conidial suspensions were prepared by scraping conidia from well sporulated (15 days) cultures in Petri plates into sterile $CaCl_2$ solutions (varying $CaCl_2$ concentrations of 2×10^{-4}, 2×10^{-3}, 6×10^{-3}, and 2×10^{-2} M). The number of conidia was estimated using a hemocytometer. Viability of conidia was checked before preparation of suspensions by germinating tests in liquid Czapek-Dox broth plus 1% (w/v) yeast extract medium. In all experiments, germination rates were higher than 90%. Sterile distilled water was used as a control suspension. The conidial suspensions used in the experiments were adjusted by diluting conidia with each $CaCl_2$ solution to a final concentration of 1.0×10^7 conidia/ml.

2.2. Soils

Sixteen soils from the collection at the AFSR of the University of Córdoba (Spain) were used in the different experiments. The basic properties of these soils are shown in Table 1. Additional details of the analytical procedures used in this study have been previously

described by Cañasveras et al. (2010).

2.3. Influence of soil type and electrolyte concentration on adsorption and drag of conidia on soil

2.3.1. Influence of soil type

Sixteen soils were selected to determinate the influence of soil type on the conidia adsorption. A soil suspension (10 g) in 50 ml of a 2 x 10^{-3} M $CaCl_2$ conidial suspension was prepared in a 45 mm in diameter and 70 mm in height glass beaker and stirred for 30 min (Fig. 1). A portion (0.5 ml) of the suspension (soil mixed with the fungal suspension) was taken with a pipette at a depth of 20 mm at 0, 15, and 30 min (these times were based on previous experiments with fungi suspensions to determine time of assay and fungal concentration) and transferred to an Eppendorf tube (1.5 ml). A portion (0.1 ml) of each of these three suspensions was spread uniformly over the surface of a Petri plate (90 mm in diameter) containing Sabouraud Dextrose Agar with Chloramphenicol (SDAC) medium. After incubation, the *B. bassiana* and *M. anisopliae* colonies were counted, and the number of conidia per milliliter was determined by multiplying the plate count by the dilution factor. Controls consisted of conidia suspension without soil.

In a parallel experiment about the influence of soil type, the sedimentation rate of the soil was accelerated by increasing the concentration of $CaCl_2$. This promotes clay flocculation, i.e., aggregation of clay particles in so-called flocs, and, consequently, a much faster sedimentation rate than in the case of dispersed, individual clay particles. In concentrated $CaCl_2$ solution, soils particles are thus forced down and changes occur in their binding time

Table 1. Geographical location and properties a of the soil samples used in this work

Name of soil	Geographical location		Soil Order	Soil factors			Textural class	OM (g/kg)	$CaCO_3$ (g/kg)	pH	EC 1:5 µS/cm	CEC ($cmol_c$/kg)	DEI (g/kg)	DC (g/kg)
	Locality	Province		Sand (g/kg)	Silt (g/kg)	Clay (g/kg)								
AG3	Córdoba	Córdoba	Vertisol	175	315	510	Clay	15	245	8.1	301	48	7	100
AG4	Córdoba	Córdoba	Inceptisol	224	318	458	Clay	20	335	8.2	398	41	8	98
AG6	Obejo	Córdoba	Entisol	660	230	110	Sandy loam	18	0	6.8	219	16	19	59
AG15	Castro del Río	Córdoba	Inceptisol	178	255	567	Clay	17	545	8.6	118	32	1	95
AG20	Luque	Córdoba	Inceptisol	405	245	350	Clay loam	12	221	8.8	98	23	10	89
AG35	Pozoblanco	Córdoba	Alfisol	860	90	50	Sandy	14	2	6.0	17	7	3	23
AG39	Fuenteobejuna	Córdoba	Alfisol	630	110	260	Sandy clay loam	11	5	5.5	21	20	21	48
AG41	Azuaga	Badajoz	Alfisol	770	80	150	Sandy loam	11	4	6.1	23	12	18	20
AG51	La Luisiana	Sevilla	Alfisol	400	120	480	Clay	6	0	6.3	402	8	25	153
AG53	Santa Cruz	Córdoba	Vertisol	159	250	592	Clay	5	215	8.5	105	13	5	187
AG54	Santa Cruz	Córdoba	Vertisol	176	257	567	Clay	7	221	8.5	88	10	4	214
AG56	Baena	Córdoba	Alfisol	334	266	400	Clay loam	3	250	8.1	895	23	7	182
INM6	Jerez	Cádiz	Inceptisol	370	330	300	Clay loam	19	597	8.2	271	17	2	150
INM9	Fuente de piedra	Málaga	Alfisol	545	255	200	Sandy clay loam	24	674	8.4	330	9	4	124
INM13	La Rambla	Córdoba	Inceptisol	500	245	255	Sandy clay loam	9	602	8.4	174	21	2	105
INM19	Donadío	Jaén	Inceptisol	187	450	364	Clay loam	1	692	8.6	85	37	1	108

O M: organic matter; EC: electrical conductivity; CEC: cation exchange capacity; DEI: dithionite extractable iron; DC: dispersable clay

with conidia. For that an aliquot (0.2 ml) of the 2 x 10^{-2} M $CaCl_2$ solution was added to the Eppendorf tube containing the 2 x 10^{-3} M $CaCl_2$ suspension. An aliquot (0.1 ml) from the supernatant was then spread on SDAC plates in the same way as before.

2.3.2. Effect of the electrolyte concentration

The AG3 and AG35 soils were selected as representatives of clayey and sandy textural classes, respectively. Soil suspensions (10 g of soil in 50 ml of a $CaCl_2$ conidial suspension) at $CaCl_2$ concentrations of 0, 2 x 10^{-4}, 2 x 10^{-3}, 6 x 10^{-3}, and 2 x 10^{-2} M were prepared in glass beakers (45 mm in diameter and 70 mm in height) and were stirred for 30 min. As before, an aliquot (0.1 ml) of each suspension was placed into separate SDAC plates, which were incubated before counting the number of colony-forming units (CFUs).

2.4. Movement of conidia on soil

Columns of four soils representing model combinations of texture (sandy or clayey) and pH (acid or alkaline) (AG35, AG51, AG53, and INM9) were prepared by uniformly packing 100 g of sterile soil in methacrylate cylinders (140 mm in height and 40 mm in diameter) (Fig. 2). The packed columns were saturated with 40–45 ml of a 1.0 x 10^7 conidia/ml suspension with either 2 x 10^{-2} M $CaCl_2$, 2 x 10^{-3} M $CaCl_2$, or sterile deionized water. After each run, the column and effluent were sampled and analyzed for conidia distribution. Each column was divided into three equal parts from which samples were taken. To assess the conidial density in each sample, the number of CFU per gram of soil was determined on the SDAC in Petri dishes. One gram of the homogenized sample of soil was added to 9 ml of sterile distilled water, and this mixture was shaken for 20–60 min. After homogenization, aliquots (0.1 ml) were spread onto the medium. In some cases, it was necessary to dilute the soil solution before spreading. Four plates per sample were used.

2.5. Infectivity of fruit fly puparia

The infectivity experiment was conducted using each of the two fungal isolates (EABb 01/110-Su and

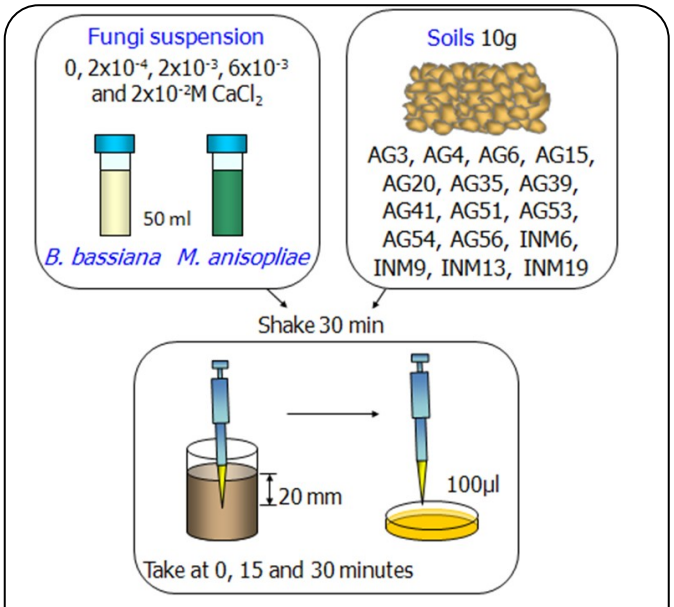

Fig. 1. Illustration of the method used to determine the influence of soil type and electrolyte concentration on adsorption and drag of conidia in soil

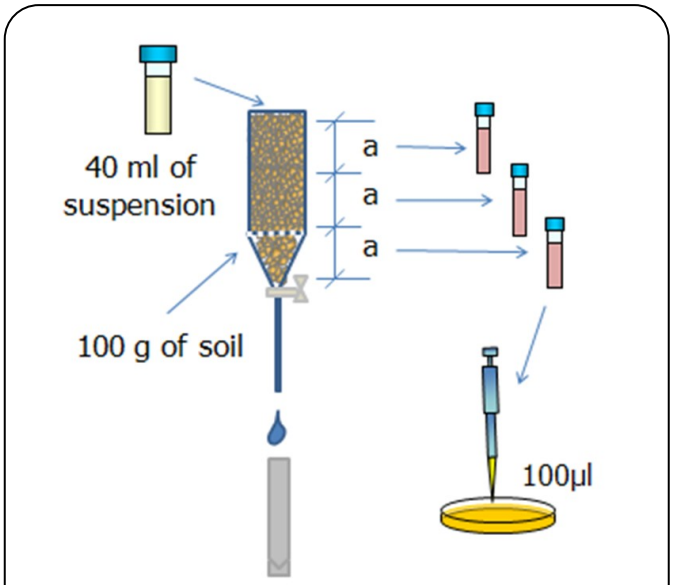

Fig. 2. Illustration of the method used to study the movement of conidia in soil

(AG35, AG51, AG53, and INM9). The experimental units were transparent containers (80 mm x 80 mm x 55 mm) with 30 g of soil in each container. Conidial suspensions prepared in sterile water, 2 x 10^{-3} M $CaCl_2$ or 2 x 10^{-2} M $CaCl_2$ were added to obtain a water potential of -0.5 MPa (5.0%; wt:wt), which was measured using a psychrometer apparatus (WP4 Decagon). Controls consisted of soils treated with the same solutions without conidia. The soil was vigorously shaken for 5 min to achieve a uniform distribution of inoculum. Ten pupariating late third instars of *C. capitata* larvae were released into the soil. The containers were then placed inside a large plastic container (250 mm x 450 mm), which was covered with filter paper that was periodically wetted. The periodic wetting of the filter paper helped to maintain soil moisture within ±3% of its initial content (Ekesi et al., 2003). Each treatment was replicated three times. The containers were incubated at 25 ºC until adult emergence. The puparia that failed to emerge were dipped in 1% sodium hypochlorite for 3 min and rinsed twice with sterilized water. Puparia were placed on a damp, sterilized filter paper contained in a Petri dish, which was then sealed with parafilm, and the puparia were kept at room temperature to be inspected for development of mycelium.

2.6. Statistical analysis

To find associations between variables, principal component analysis (PCA) was used to reduce the dimensionality of the data table. In this analysis, the measured variables (organic matter, clay content, sand content, and pH) were linear functions of non-measured variables, which were called principal components (PCs). The eigenvalues of the PCs were a measure of their associated variance, and the participation of the observable variables in the PCs was given by the loadings. For adsorption and drag of conidia in soil, linear regression analysis was used. The analysis of variance (ANOVA) with a split-split-plot design was performed for the movement of conidia in soil. Mortality data were analyzed using one-way ANOVA, and the means were compared with the LSD test. Before the ANOVA, all mortality and mycosis percentages were transformed using the arcsin transformation (Steel and Torrie, 1980). Statistix 9 (Analytical Software, 2008) was used in all statistical analyses.

3. Results

3.1. Influence of soil type and electrolyte concentration on adsorption and drag of conidia on soil

3.1.1. Influence of soil type

Table 2 summarizes the PCA results for the relationship between the concentration of *B. bassiana* and *M. anisopliae* conidia in suspension and soil variables including loadings (participation of the original variables in the new ones) and eigenvalues for each PC.

For *B. bassiana*, the first three PCs accounted for 81.5%, 81.0%, and 79.7% of the cumulative variance at 0, 15, and 30 min, respectively. Because there was no significant increase in variance with PC4, only the first three PCs were considered in the interpretation of results. PC1 accounted for 48.4%, 49.5%, and 42.3% of the variance at 0, 15, and 30 min, respectively. The respective values for PC2 (20.1%, 19.7%, and 24.0%) and PC3 (13.0%, 11.9%, and 13.4%) were markedly lower. The properties that yielded on average the highest loadings were as follows: (1) sand (positive) and pH (negative) for PC1; (2) dithionite-extractable iron (positive), $CaCO_3$ (negative), and organic matter

(OM) (negative) for PC2; and (3) cation exchange capacity (CEC) (positive at 0 min and negative at 15 and 30 min) for PC3.

For *M. anisopliae* the first three PCs accounted for 76.0%, 79.7%, and 78.4% of the cumulative variance at 0, 15, and 30 min, respectively. PC1 accounted for 41.8%, 46.8%, and 48.3% of the variance at 0, 15, and 30 min, respectively. The respective values for PC2 (23.0%, 19.2%, and 17.8%) and PC3 (11.3%, 13.6%, and 12.3%) were markedly lower. The properties that yielded on average the highest loadings were as follows: (1) sand (negative) and pH (positive) for PC1; (2) $CaCO_3$ and OM (negative) for PC2; and (3) CEC (negative) for PC3.

The slopes of the regression lines representing the time course of conidia concentration in the soil suspension for the 16 soils are shown in Table 3. The slope was seemingly related to the fungal species and the textural class. For *B. bassiana*, the higher slopes corresponded in general to the sandy soils and the lower slopes to the clayey soils. For *M. anisopliae,* however, the higher slopes corresponded to the clayey soils and the lower slopes to the sandy soils. The concentration of *B. bassiana* conidia in the suspension decreased more steeply with time for the model sandy soils than for the model clayey soils (Fig. 3).

The evolution of the conidia concentration in the soil suspension was further evaluated under flocculation conditions, which forces soil particles to sediment rapidly (Table 4). The regression slopes were related to pH even though the relationship varied among fungal species. For *B. bassiana*, the higher slopes corresponded mostly to acidic soils, and the lower slopes corresponded to the alkaline soils. For *M. anisopliae*, no clear relationship was observed (Fig. 4).

3.1.2. Effect of the electrolyte concentration

The effects of electrolyte concentration on the conidia concentration in the soil suspension at different times for the AG3 and AG35 soils (models for the clayey and sandy textural classes, respectively) are shown in Figs. 5 and 6. Conidia concentration decreased with increasing $CaCl_2$ concentration at all time points (0, 15, and 30 min) for the control (without soil) and the two soils. For *B. bassiana*, the highest conidia concentration was detected at 0 M $CaCl_2$, and the lowest conidia concentration was detected at 2×10^{-2} M $CaCl_2$. The conidia concentration decreased with time. For example, the conidia concentrations at 0 M $CaCl_2$ were 8.1×10^6, 3.4×10^6, and 0.3×10^6 conidia/ml at 0, 15, and 30 min, respectively. The conidia concentration was higher in the sandy soil (AG35) than in the clayey soil (AG3) at all time points (Fig. 5). For *M. anisopliae*, the highest conidia concentration was found at 2×10^{-3} M for 0 and 15 min, and the conidia concentration was extremely low at 30 min (Fig. 6). No clear differences in conidia concentration between the two soils were observed throughout the experiment (Fig. 6).

3.2. Movement of conidia in soil

There were significant effects of soil type ($F_{3,107}$ = 14.90 and P = 0.0035) and depth ($F_{2,107}$ = 950.34 and P < 0.001) on *B. bassiana* conidia concentration (CFU) in the soil. Although the majority of the fungal propagules were recovered from the surface layer of the column, differences in conidia movement occurred in response to soil type (Table 5). When sterile deionized water was used as the solution, the surface layer of the AG51 and AG53 soil columns (clayey soils) retained over 93% and 91% of conidia, respectively, which was more than the conidia retention found for the sandy AG35 and INM9 soils with values of only 84% and 76%, respectively.

Table 2. Loadings of nine soil variables in three significant principal components for 16 soil samples in *Beauveria bassiana* or *Metarhizium anisopliae* soil suspensions in 2.0×10^{-3} M $ClCa_2$

Time (min)	Variable	*Beauveria bassiana*			*Metarhizium anisopliae*		
		PC1	PC2	PC3	PC1	PC2	PC3
0	Dispersable clay	-0.38	0.23	-0.40	0.28	0.52	0.24
	Electrical conductivity	-0.22	0.20	-0.63	0.05	0.40	0.32
	Cation exchange capacity	-0.27	-0.03	0.55	0.28	-0.27	-0.39
	$CaCO_3$	-0.36	-0.41	-0.03	0.35	-0.36	-0.35
	Dithionite extractable iron	0.29	0.47	-0.11	-0.37	0.30	-0.40
	Clay	-0.37	0.38	0.13	0.37	0.28	-0.50
	Sand	0.44	-0.21	0.14	-0.48	-0.13	0.31
	Organic matter	0.05	-0.51	-0.32	-0.11	-0.41	-0.19
	pH	-0.43	-0.25	-0.03	0.46	-0.12	-0.15
	Eigenvalue	4.36	1.81	1.17	3.76	2.07	1.01
	% Variance explained	48.4	20.1	13.0	41.8	23.0	11.3
	% Cumulative variance	48.4	68.5	81.5	41.8	64.8	76.0
15	Dispersable clay	-0.37	0.30	0.38	0.41	0.29	0.20
	Electrical conductivity	-0.21	0.28	0.40	0.18	0.01	-0.38
	Cation exchange capacity	-0.25	-0.08	-0.64	0.15	-0.28	-0.75
	$CaCO_3$	-0.33	-0.44	0.16	0.28	-0.52	0.18
	Dithionite extractable iron	0.34	0.39	-0.16	-0.33	0.31	-0.39
	Clay	-0.37	0.29	-0.29	0.40	0.30	-0.16
	Sand	0.44	-0.15	0.23	-0.45	-0.13	0.19
	Organic matter	0.08	-0.57	-0.01	-0.14	-0.58	-0.08
	pH	-0.44	-0.22	0.13	0.45	-0.19	0.11
	Eigenvalue	4.45	1.77	1.07	4.21	1.73	1.23
	% Variance explained	49.5	19.7	11.9	46.8	19.2	13.6
	% Cumulative variance	49.5	69.1	81.0	46.8	66.0	79.7
30	Dispersable clay	-0.38	0.26	0.44	0.38	0.39	-0.26
	Electrical conductivity	-0.17	0.23	0.37	0.18	0.16	0.12
	Cation exchange capacity	-0.21	-0.02	-0.73	0.16	-0.35	0.76
	$CaCO_3$	-0.32	-0.46	0.11	0.33	-0.45	-0.26
	Dithionite extractable iron	0.35	0.40	-0.08	-0.40	0.25	0.31
	Clay	-0.36	0.38	-0.24	0.36	0.30	0.32
	Sand	0.46	-0.21	0.22	-0.44	-0.11	-0.25
	Organic matter	0.05	-0.51	-0.05	-0.06	-0.57	-0.02
	pH	-0.46	-0.23	0.08	0.45	-0.12	-0.14
	Eigenvalue	3.80	2.16	1.21	4.35	1.61	1.11
	% Variance explained	42.3	24.0	13.4	48.3	17.8	12.3
	% Cumulative variance	42.3	66.3	79.7	48.3	66.1	78.4

The differences became smaller when 2×10^{-3} and 2×10^{-2} M $CaCl_2$ solutions were used. Conidia transport was also influenced by pH with the conidia concentration in the surface layer for the 2×10^{-2} M $CaCl_2$ treatment being higher in the columns of alkaline soils (AG53 and INM9). As for *B. bassiana*, there were significant effects of soil type ($F_{3,107}$ = 18.88 and P = 0.0019) and depth ($F_{2,107}$ = 1017.29 and P < 0.001) on

Table 3. Slope of regression lines representing the time course of conidia concentration in the soil suspension (16 soils)

Beauveria bassiana				*Metarhizium anisopliae*			
Soil	**Slope***	**Textural class**	**pH**	**Soil**	**Slope ***	**Textural class**	**pH**
Without soil	45.8	-	-	Without soil	142.7	-	-
AG6	41.2	Sandy loam	6.8	AG56	129.3	Clay loam	8.1
AG41	38.2	Sandy loam	6.1	INM19	126.2	Clay loam	8.6
INM9	37.7	Sandy clay loam	8.4	AG15	111.2	Clay	8.6
INM13	35.7	Sandy clay loam	8.4	AG53	101.2	Clay	8.5
AG35	29.7	Sandy	6.0	AG20	96.3	Clay loam	8.8
AG51	26.2	Clay	6.3	AG3	64.5	Clay	6.1
AG39	26.0	Sandy clay loam	5.5	AG4	64.5	Clay	8.2
AG15	19.5	Clay	8.6	AG51	54.8	Clay	6.3
INM19	18.7	Clay loam	8.6	AG54	47.5	Clay	8.5
INM6	15.8	Clay loam	8.2	INM13	44.3	Sandy clay loam	8.4
AG56	14.3	Clay loam	8.1	INM6	42.5	Clay loam	8.2
AG20	13.7	Clay loam	8.8	INM9	41.5	Sandy clay loam	8.4
AG4	13.7	Clay	8.2	AG35	39.8	Sandy	6.0
AG53	11.5	Clay	8.5	AG39	38.8	Sandy clay loam	5.5
AG3	11.3	Clay	6.1	AG6	31.5	Sandy loam	6.8
AG54	11.0	Clay	8.5	AG41	29.5	Sandy loam	6.1

* Slope × 10^3 conidia ml^{-1} s^{-1}

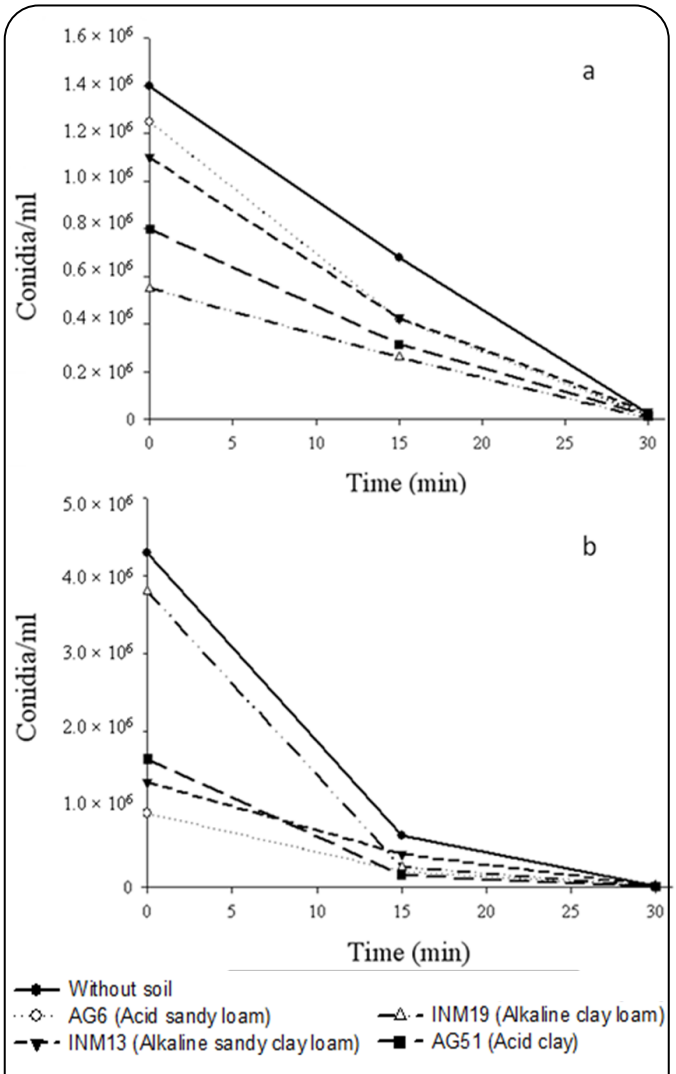

Fig. 3. Time course of (a) *Beauveria bassiana* and (b) *Metarhizium anisopliae* conidia concentration in the suspensions of four soils with different texture and pH in 2 x 10^{-3} M $CaCl_2$

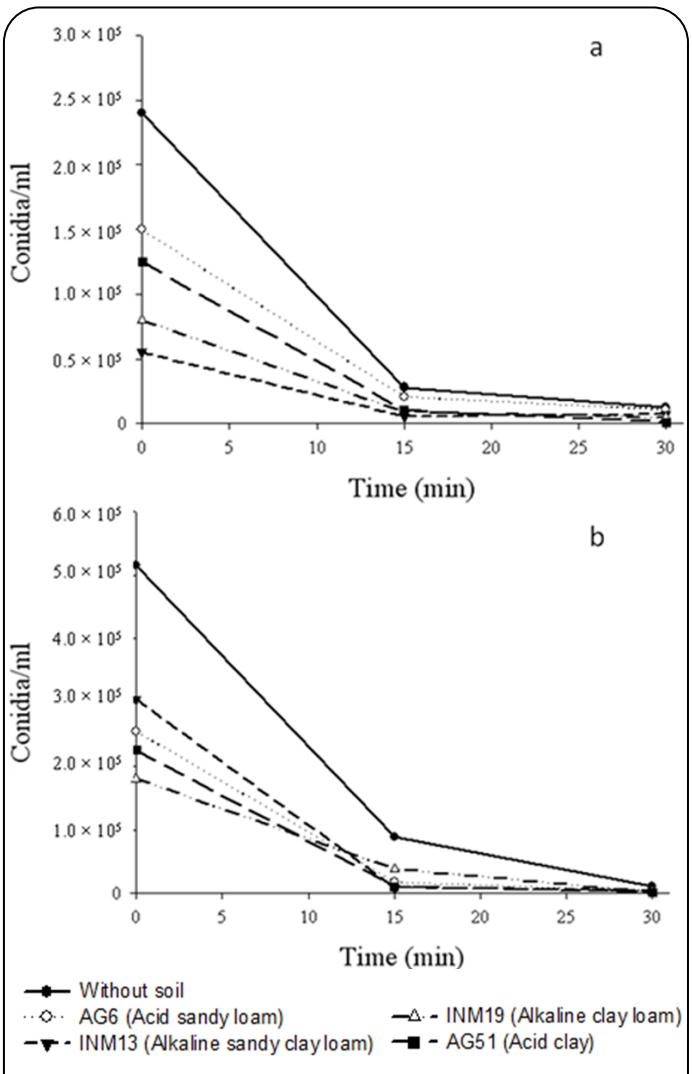

Fig. 4. Time course of (a) *Beauveria bassiana* and (b) *Metarhizium anisopliae* conidia concentration in the suspensions of four soils with different texture and pH in 2 x 10^{-2} M $CaCl_2$

Table 4. Slope of regression equations relating number of colony forming units to time in different soils under flocculation conditions

Beauveria bassiana			
Soil	**Slope***	**Textural class**	**pH**
Without soil	7.6	-	-
AG35	5.2	Sandy	6.0
AG3	4.9	Clay	8.1
AG41	4.9	Sandy loam	6.1
AG6	4.6	Sandy loam	6.8
AG4	4.2	Clay	8.2
AG20	4.1	Clay loam	8.8
AG51	4.1	Clay	6.3
INM9	2.8	Sandy clay loam	8.4
INM19	2.5	Clay loam	8.6
AG54	2.4	Clay	8.5
INM13	1.6	Sandy clay loam	8.4
AG53	1.6	Clay	8.5
INM6	1.5	Clay loam	8.2
AG39	1.4	Sandy clay loam	5.5
AG56	1.2	Clay loam	8.1
AG15	0.5	Clay	8.6

Metarhizium anisopliae			
Soil	**Slope***	**Textural class**	**pH**
Without soil	16.8	-	-
AG53	15.4	Clay	8.5
AG15	13.3	Clay	8.6
INM9	10.6	Sandy clay loam	8.4
INM13	10.0	Sandy clay loam	8.4
AG39	9.8	Sandy clay loam	5.5
AG35	9.4	Sandy	6.0
AG6	8.4	Sandy loam	6.8
AG41	8.0	Sandy loam	6.1
AG51	7.5	Clay	6.3
INM6	7.1	Clay loam	8.2
INM19	5.9	Clay loam	8.6
AG56	5.1	Clay loam	8.1
AG54	4.6	Clay	8.5
AG20	5.1	Clay loam	8.8
AG4	4.9	Clay	8.2
AG3	1.6	Clay	8.1

* Slope × 10^3 conidia ml^{-1} s^{-1}

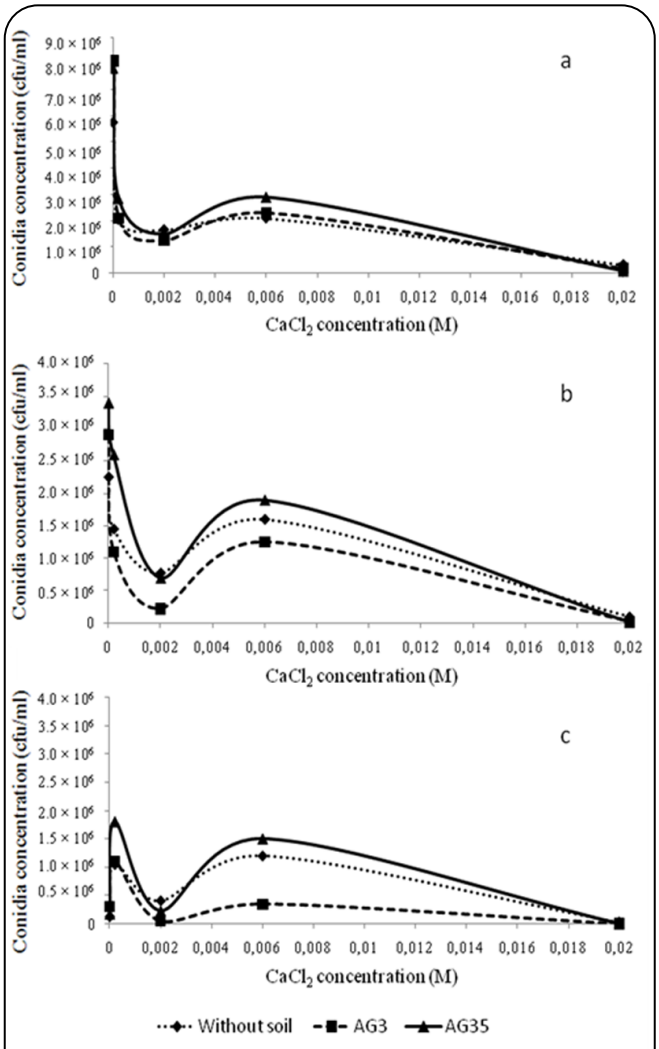

Fig. 5. Concentration of *Beauveria bassiana* conidia in the soil solution at: (a) 0 min, (b) 15 min, and (c) 30 min. Experimental data are represented by diamonds (control), squares (AG3), and triangles (AG35)

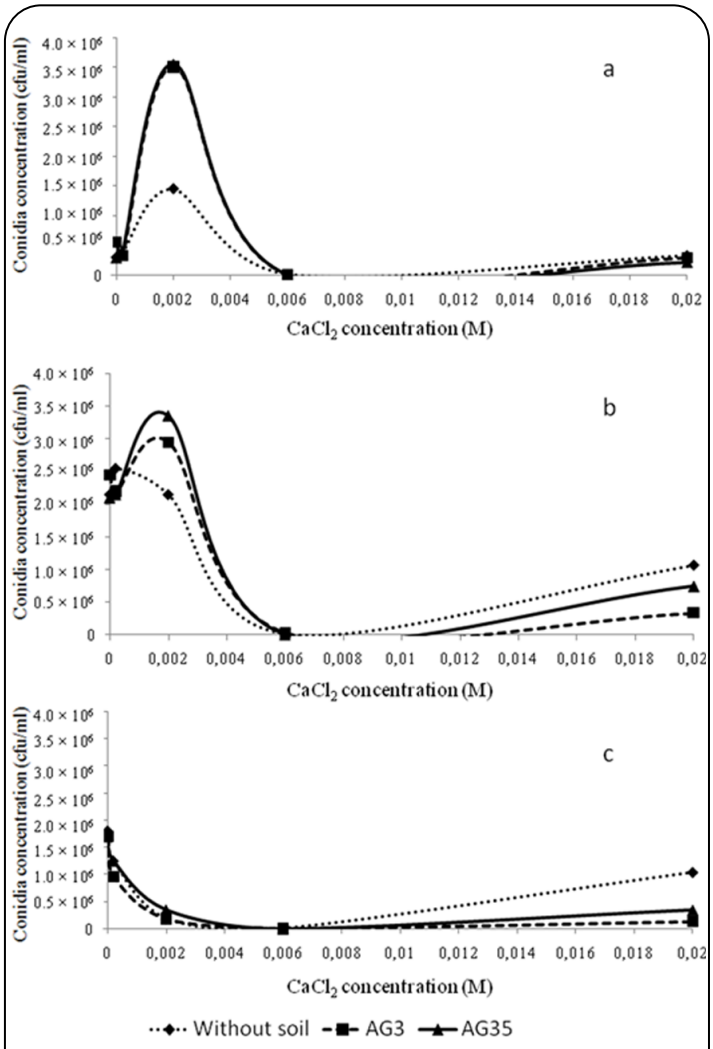

Fig. 6. Concentration of *Metarhizium anisopliae* conidia in the soil solution at (a) 0 min, (b) 15 min, and (c) 30 min. Experimental data are represented by diamonds (control), squares (AG3), and triangles (AG35)

M. anisopliae conidia concentration (CFU) in the soil. Generally, the sandy soils (AG35 and INM9) retained more conidia than the clayey soils (AG51 and AG53), especially in the surface layer (Table 5). Although no significant effect of ionic strength was found, the total number of conidia recovered from the soil decreased with increasing $CaCl_2$ concentration. Thus, only 30% of the total number of conidia was recovered from the acidic soils (AG51 and AG35), and 50% of the total number of conidia was recovered from the alkaline soils (AG53 and INM9) (Table 5).

3.3. Infectivity of fruit fly puparia

The *B. bassiana* treatment had significant impact on total *C. capitata* puparia mortality ($F_{1,71}$ = 76.91 and P = 0.0031) with mortality rates in the control (3.3–6.7%) significantly lower than the mortality rates in the fungal-treated soils (Table 6). Neither soil type nor electrolyte ($CaCl_2$) concentration significantly affected puparia mortality. For the AG51 soil (an acidic clayey soil), no significant differences in total puparia mortalities were observed among the three electrolyte concentrations (Table 6). For the AG53, AG35 and INM9 soils, a slight decrease in total puparia mortality with increasing $CaCl_2$ concentration was observed (Table 6). Similarly, puparia mycosis, which ranged from 6.7% to 16.7%, was not significantly affected by soil type or electrolyte concentration (Table 6). *C. capitata* puparia total mortality was significantly lower in the control soil than in the *M. anisopliae*-treated soil for conidial suspensions at the three $CaCl_2$ concentrations ($F_{1,71}$ = 142.7 and P = 0.0013) with total mortality values ranging from 6.7% to 20.0% for the control soils and total mortality values ranging from 40.0% to 83.3% for the treated soils (Table 6). Although a slight total mortality increase was observed with increasing $CaCl_2$ concentration, significant differences in puparia mortality did not occur between soil types or $CaCl_2$ concentrations. The puparia mycosis caused by *M. anisopliae* was not significantly affected by soil type or $CaCl_2$ concentration. The highest percentage of total mortality and mycosis for all soils and electrolytes was 50.0% (AG51 and AG53 soils in 2 x 10^{-3} M $CaCl_2$), and the lowest percentage of total mortality and mycosis was 26.7% (AG35 soil in 2 x 10^{-2} M $CaCl_2$).

4. Discussion

Although soil moisture and temperature have been reported as important abiotic factors influencing the efficacy of entomopathogenic fungi against soil-dwelling insect pests (McCoy et al., 1992), the present study indicated that soil texture and pH are also influential variables. This study demonstrated clear differences between the EABb 01/110-Su *B. bassiana* strain and EAMa 01/58-Su *M. anisopliae* strain in their interaction with soil. The adsorption, drag, and column experiments revealed that *B. bassiana* conidia tended to be retained by clay particles, but *M. anisopliae* did not, which is a consequence of the larger size (Ignoffo et al., 1977) and hydrophobic nature (Jeffs et al., 1999) of the latter. Also, Salazar et al. (2007) suggested that movement of *M. anisopliae* conidia in soil was favored by the larger macropores and less tortuosity of sandy soils when compared to the clayey soils, thereby enhancing vertical movement of water. Thus, *B. bassiana* has been reported to predominate over *M. anisopliae* in soils with high clay content from natural and cultivated areas in all regions of Spain and Portugal, including the mainland and islands (Quesada-Moraga et al., 2007).

The adsorption and drag experiments supported

The existence of an interaction among *B. bassiana* conidia and soil clay minerals, which was suggested by the fact that the availability of conidia was always lower in the clayey soil than in the sandy suspensions. The cell wall in aerial conidia of *B. bassiana* is composed of a great diversity of carbohydrates that contribute to the formation of hydrogen bonds between the conidia and hydrophobic and hydrophilic surfaces (Holder and Keyhani, 2005; Wanchoo et al., 2009), which may be involved in the interaction of conidia with the clay surfaces for ion exchange as previously reported for *Histoplasma capsulatum* (Ascomycota: Onygenales) (Lavie and Stotzky, 1986). Nonetheless, Storey and Gardner (1987) did not observe adsorption to clay particles with commercially formulated *B. bassiana*, which was attributed to the hydrophilic character of the formulate after being amended with a wetting agent. In this study, the principal component analysis showed that dithionite-extractable iron, which is a measure of the iron oxides associated with the silicate clays of the soil, principally influence *B. bassiana* conidia retention in soil. This influence may be explained by the ability of fungi to produce iron-transporting siderophores to accumulate and transport iron from the environment into the cells (Lavie and Stotzky, 1986) in addition to the adsorption phenomena being greatly promoted on hydroxylated Fe/OH surfaces (Cornejo et al., 2004). Similarly, extracellular siderophores are essential for many fungal/host interactions (Johnson, 2008), and uptake of iron by fungal conidia may be performed by extracellular siderophores that directly transfer iron from the environment into conidia (Horowitz et al., 1976; Harper et al., 1980; Graham and Harper, 1983). In addition, clay particles can be adhered to the fungal surface, thereby, interfering with the iron nutrition of the fungus by sequestering fungal siderophores (Jaronski, 2007). The adsorption of *B. bassiana*, which may have saprophytic growth (Hajek, 1997), onto clay particles

Table 5. Relative percentage of *Beauveria bassiana* and *Metarhizium anisopliae* conidia recovered from three depths in columns of four soils as a function of electrolyte concentration

	Soil	$CaCl_2$ concentration											
		0 M				2×10^{-3} M				2×10^{-2} M			
		Recovery (%)	Relative percentage			Recovery (%)	Relative percentage			Recovery (%)	Relative percentage		
			Surface	Middle	Lower		Surface	Middle	Lower		Surface	Middle	Lower
Beauveria bassiana	AG51	41	93	5	2	31	94	3	3	18	94	6	0
	AG53	35	91	6	3	27	92	4	4	27	92	4	4
	AG35	13	84	8	8	23	96	4	0	17	94	6	0
	INM9	17	76	12	12	18	94	6	0	27	92	4	4
Metarhizium anisopliae	AG51	37	95	5	0	29	93	7	0	9	89	11	0
	AG53	30	97	3	0	13	92	8	0	14	93	7	0
	AG35	92	98	1	1	20	90	5	5	28	89	7	4
	INM9	80	99	1	0	24	92	4	4	42	93	5	2

Table 6. Puparial mortality (% ± SE) in *Ceratitis capitata* after treatment with 10^8 CFU per ml of *B. bassiana* and *M. anisopliae* suspension in four soil types

		$CaCl_2$ concentration								
		0 M			2×10^{-3} M			2×10^{-2} M		
		Total mortality (%)			Total mortality (%)			Total mortality (%)		
	Soil	Control	Treated	Mycosis (%)	Control	Treated	Mycosis (%)	Control	Treated	Mycosis (%)
Beauveria bassiana	AG51	3.3 ± 3.3a	26.7 ± 3.3b	16.7 ± 3.3c	6.7 ± 3.3a	23.3 ± 3.3b	6.7 ± 3.3c	6.7 ± 3.3a	26.7 ± 3.3b	16.7 ± 3.3c
	AG53	3.3 ± 3.3a	23.3 ± 8.8b	16.7 ± 8.8c	3.3 ± 3.3a	20.0 ± 5.8b	13.3 ± 6.7c	3.3 ± 3.3a	13.3 ± 3.3b	13.3 ± 3.3c
	AG35	3.3 ± 3.3a	33.3 ± 8.8b	6.7 ± 3.3c	6.7 ± 3.3a	16.7 ± 3.3b	10.0 ± 5.8c	6.7 ± 3.3a	10.0 ± 3.3b	6.7 ± 3.3c
	INM9	0.0 ± 0.0a	33.3 ± 6.7b	13.3 ± 6.7c	6.7 ± 3.3a	26.7 ± 3.3b	6.7 ± 3.3c	3.3 ± 3.3a	13.3 ± 8.8b	10.0 ± 5.8c
Metarhizium anisopliae	AG51	16.7 ± 3.3a	66.7 ± 8.8 b	46.7 ± 8.8c	20.0 ± 5.8a	70.0 ± 0.0b	50.0 ± 10.0c	10.0 ± 5.7a	83.3 ± 3.3b	33.3 ± 3.3c
	AG53	13.3 ± 6.7a	56.7 ± 3.3b	26.7 ± 3.3c	20.0 ± 0.0a	63.3 ± 13.3b	50.0 ± 20.0c	16.7 ± 3.3a	40.0 ± 5.8b	26.7 ± 3.3c
	AG35	13.3 ± 6.7a	60.0 ± 0.0b	36.7 ± 3.3c	13.3 ± 3.3a	66.7 ± 3.3b	30.0 ± 5.8c	20.0 ± 5.7a	66.7 ± 3.3b	46.7 ± 6.7c
	INM9	6.7 ± 3.3a	43.3 ± 3.3b	33.3 ± 3.3c	16.7 ± 3.3a	63.3 ± 8.8b	43.3 ± 6.7c	16.7 ± 6.7a	60.0 ± 5.8b	33.3 ± 3.3c

For each $CaCl_2$ concentration and mortality percent data followed by the same letter are not significantly different ($\alpha = 0.05$) according to the LSD test. For each $CaCl_2$ concentration and mycosis percent data followed by the same letter are not significantly different ($\alpha = 0.05$) according to the LSD test

can also be attributed to enhanced iron solubility resulting in better nutrition for the fungus caused by acidification of the soil. Moreover, conidia adsorbed onto clays may be available for biocontrol purposes. Clays are used in many biocontrol formulations, particularly as clay/chitosan complex coatings (Cohen and Joseph, 2009). This study supports the biocontrol idea because the observed differences among soils on conidia absorption were not reflected in differences in puparia mortality.

Regarding conidia movement in soil, this study agreed with the report published by Wollum and Cassel (1978), which reported that most of the organisms are trapped within the soil column for a period of time. In our study, active conidia were not recovered from the effluent even though they were present at the surface layer of the soil columns and differences in their movement occurred in response to soil type. Similar results were reported by Ignoffo et al. (1977) who revealed that superficial layers retain greater amounts of conidia when compared to deeper layers in columns of clayey and sandy soils inoculated with *B. bassiana* and *M. anisopliae*, respectively.

$CaCl_2$ affects soil structure through the well known effect of exchangeable Ca^{2+} on the attractive forces between clay particles. The addition of Ca^{2+} also causes a pH decrease because Ca^{2+} ions replace some of the exchangeable H^+ (Ritchey et al., 1982). These Ca^{2+} effects may explain why increasing concentrations of $CaCl_2$ in alkaline soils promoted the presence of resting *B. bassiana* conidia in the superficial layers.

Soil texture and $CaCl_2$ concentration did not affect the conidia infectivity of either fungal strain to medfly puparia. The size and distribution of pore spaces affect the infectivity of *Beauveria* spp. and *Metarhizium* spp. conidia by mediating physical contact (Jaronski, 2010). In addition, saturated soil has water-filled macropores, and

passing through a fungus-treated soil may not encounter conidia (Jaronski, 2007). Our previous studies (Quesada-Moraga et al., 2006) clearly revealed the effect of water potential on the pathogenicity of *B. bassiana* and *M. anisopliae* against medfly puparia. Based on these studies, to determinate the effect of soil texture and pH in the fungal infectivity against puparia, we selected the optimal water potential conditions. Therefore, we conclude that optimal water conditions prevented the interaction between soil texture and pathogenicity of EF against *C. capitata* puparia under changing electrolyte concentrations.

Soil is the natural ecosystem of entomopathogenic fungi where they share a habitat with soil-dwelling insect stages. The environment of entomopathogenic fungi provides protection against UV radiation, optimal temperature conditions, and optimal moisture conditions. In addition, fungi may survive in soil by recycling in insects or roots (St. Leger, 2008) providing a long term strategy for puparia control. The persistence of the entomopathogenic fungi inoculum in soil is a prerequisite for successful control efficacy because this persistence is related to the retention and viability of conidia in the soil. The results of this study indicated that the availability of *B. bassiana* and *M. anisopliae* conidia in the soil is significantly affected by the properties of the soil. Therefore, the movement of fungi throughout the soil profile is quite low, so their availability to infect propagules resides in the superficial soil layer. Finally, this study also suggested that the pathogenicity of *B. bassiana* and *M. anisopliae* against medfly puparia is not significantly affected by soil properties.

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CAPÍTULO VI

Este capítulo es una versión adaptada del artículo

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Use of a multiple logistic regression model to determine the effects of soil moisture and temperature on the virulence of entomopathogenic fungi against pre-imaginal Mediterranean fruit fly *Ceratitis capitata*

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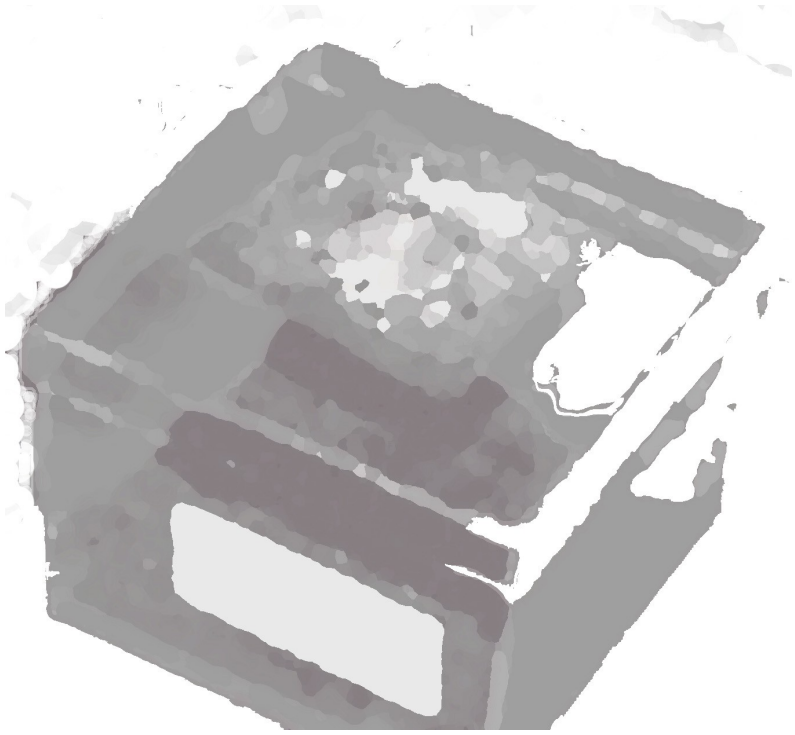

Use of a multiple logistic regression model to determine the effects of soil moisture and temperature on the virulence of entomopathogenic fungi against pre-imaginal Mediterranean fruit fly *Ceratitis capitata*

ABSTRACT

Last-instar larvae of the Mediterranean fruit fly, *Ceratitis capitata*, were exposed to *Beauveria bassiana* and *Metarhizium anisopliae* isolates in sterile soil under controlled conditions. A multiple logistic regression model for mycosis was applied, and different temperature and moisture patterns for each of the four isolates were observed (Bb-1333, EABb 01/33-Su, EAMa 01/58-Su, EAMa 01/158-Su). In general, *B. bassiana* isolates were less virulent than *M. anisopliae* isolates. For all of the fungal isolates, lower mycosis values were observed under extreme moisture conditions (1% and 17% wt.:wt.). For the most virulent isolate, *M. anisopliae* EAMa 01/58-Su, higher mycosis values were observed at intermediate temperatures. Conversely a direct relationship between temperature and puparia mortality was observed for *M. anisopliae* isolate EAMa 01/158-Su. Both *B. bassiana* isolates displayed a parabolic relationship with moisture, and mycosis was observed at low temperatures (15–20.1 ºC). This work provides additional evidence on the fact that for each fungal species, each isolate is an independent biological entity with different responses to environmental conditions; therefore, entomopathogenic fungal isolates for pest control should have a range of temperatures and humidities that can be matched to the desired environment.

1. Introduction

The Mediterranean fruit fly *Ceratitis capitata* or medfly (Wiedemann) (Diptera: Tephritidae) is a major insect pest of fruit trees in the Mediterranean region. Damage to fruit is caused by gravid females that oviposit under fruit skin and larvae that feed on the flesh of the fruit. Damage by fruit flies leads to rotten and inedible fruits that usually drop to the ground prematurely, causing large direct economic losses (Tremblay, 1994; Alfaro-Moreno, 2005). Similarly, indirect economic losses are caused by strict quarantine regulations that are imposed by importing countries to avoid entry and establishment of medflies. During development, third instar *C. capitata* drop from fruits to the ground, burrow into the soil, and form a puparium (Tremblay, 1994; Alfaro-Moreno, 2005). Despite the fact that *C. capitata* control measures may be performed on both adults and prepupating larvae and puparia in the soil, control methods are usually directed at adults with broadspectrum chemical insecticides in bait sprays (Ros et al., 2002), which may have adverse effects on humans, non-target organisms, and the environment.

In recent years, scientists have sought environmentally friendly medfly control methods, such as microbial control with entomopathogenic fungi (Ekesi et al., 2007). Strategic options in the use of entomopathogenic fungi for medfly control include aerial applications and autodissemination, which target adults, and soil inoculation, which targets pupariating larvae and puparia (Ekesi et al., 2007). Moreover, the aforementioned methods can be used in a biocontrol strategy that targets both adults and prepupariating larvae and puparia. Such a strategy will require the identification of a fungal isolate that is active against both insect stages. Although several studies are published on the virulence of *Metarhizium anisopliae* and *Beauveria bassiana* against *C. capitata* adults (Garcia et al., 1989; Castillo et al., 2000; Ekesi et al., 2002; Dimbi et al., 2003; Ekesi et al., 2003, 2005; Konstantopoulou and Mazomenos, 2005), reports on fungal strains that show pathogenicity to both *C. capitata* adults and puparia are scarce (Dimbi et al., 2003). In a previous study, we demonstrated that several *M. anisopliae* and *B. bassiana* indigenous isolates are moderately to highly virulent against both *C. capitata* adults and puparia (Quesada-Moraga et al., 2006b, 2008).

The soil is an important reservoir for insect pathogenic fungi and is generally considered a favorable

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environment for fungal microbial control because the soil provides shelter from environmental extremes, which increases the persistence of conidia and their ability to thrive (Jackson et al., 2000; Ekesi et al., 2007). For the successful application of *M. anisopliae* or *B. bassiana* as a biological insecticide against medfly puparia, environmental soil factors related to the use of these fungi must be evaluated (Jackson et al., 2000). Similarly, the isolation, distribution, and efficacy of entomopathogenic fungi, such as *B. bassiana* and *M. anisopliae* in the soil are subject to a matrix of interacting abiotic and biotic factors (Jaronski, 2007; Quesada-Moraga et al., 2007) that are currently being evaluated within our ongoing medfly control research program.

Soil moisture and temperature are key abiotic factors that affect the persistence and virulence of entomopathogenic fungi in the soil (Jaronski, 2007). Similarly, soil moisture and temperature are among the main factors that affect the susceptibility of soil dwelling-insects to fungal infection (McCoy et al., 1992). Surprisingly, only a few studies on the effects of soil moisture and temperature on the virulence of entomopathogenic fungi to tephritid puparia have been performed (Ekesi et al., 2007). In our previous work on *M. anisopliae* EAMa 01/58-Su, we found that an increase in soil moisture levels at 25 ºC did not result in higher pupal mortalities, which is expected for entomopathogenic fungi (Quesada- Moraga et al., 2006b). Ekesi et al. (2003) also observed a similar effect of soil moisture on the infectivity of African isolates of *M. anisopliae* on *C. capitata* puparia. The authors hypothesized that this effect may be due to the existence of certain physiological factors that limit infectivity of conidia in water-saturated soil. However, whether the effect of soil moisture on fungal virulence is a general phenomenon for *B. bassiana* and *M. anisopliae* is unclear. In the present study, a multiple logistic regression model was constructed to evaluate the effect of soil moisture, temperature and their interaction on the virulence of four indigenous *M. anisopliae* and *B. bassiana* against pre-imaginal stages of *C. capitata*.

2. Materials and methods

2.1. Insects

C. capitata were obtained from mass-rearing stock maintained at our insectary since 2004, which was initially derived from the stock colony of El Encin (INIA, Madrid), and were reared under a photoperiod of 16:8 (L:D) h at 50–60% RH and a temperature of 26 ± 2 ºC. Adult flies were provided with water and an artificial diet based on a mixture of protein-hydrolysate (Yeast Hydrolysate Enzymatic, ICN Biomedicals, Aurora, Ohio, USA) and sucrose (Panreac) (1:4 wt.:wt.). Larvae were reared on a diet of wheat bran, sucrose, brewer's yeast, nipagin, nipasol, benzoic acid, and water (300 g/kg, 75 g/kg, 36 g/kg, 2 g/kg, 2 g/kg, 2.4 g/kg, and 600 ml/kg, respectively).

2.2. Fungal isolates

The isolates were obtained from the culture collection at the Department of Agricultural and Forestry Sciences and Resources (AFSR) of the University of Cordoba (Spain). The fungi were maintained in slant monoconidial cultures on malt agar (MA) at 25 ºC in the dark and were stored at -80 ºC (Table 1). The fungal isolates were selected based on the results of previous studies, which showed that the fungi are highly

virulent against medfly adults and are pathogenic to puparia (Quesada-Moraga et al., 2006b; Eldesouki-Arafat, 2007).

2.3. Inoculum preparation

The isolates were grown on MA slants and were subcultured by conidia transfer to MA petri plates that were grown for 15 days at 25 ºC in the dark. Conidia from 15-day old cultures were used in all of the experiments. Conidial suspensions were prepared by scraping conidia from Petri plates into a sterile aqueous solution of 0.1% (v/v) Tween 80. The concentration of conidia was assessed according to the colony-forming unit (cfu) method (Goettel and Inglis, 1996). The conidial suspension was diluted with 0.1% (v/v) Tween 80 to obtain a final concentration of 1.0 x 10^8 cfu/ml. Finally, the fungal cultures were evaluated for the ability of the spores to germinate in Petri dishes on a water agar substrate at 25 ºC, showing that spores germinated 12 h after the inoculation and the germination rate was always greater than 95%.

2.4. Experiment design

Identical experiments were conducted on each of the four fungal isolates (Bb-1333, EABb 01/33-Su, EAMa 01/58-Su and EAMa 01/158-Su). The soil used in all of the experiments was collected from the field in Córdoba, Spain and was characterized as a sandy-loam (78% sand, 17% silt, 5% clay, and 0.2 organic matter, pH 8.4). The soil was sieved (2 mm mesh), air-dried at 26 ºC for 10 days, and sterilized prior to use. Sterile distilled water volumes of 1.5, 2.7, 3.9 and 5.1 ml containing 1.8 x 10^8 cfu of the isolates were added to transparent containers (80 x 80 x 55 mm) containing 30 g of soil. Soil water potential (matric and osmotic components) was determined by the psychrometric method. According to a WP4 Decagon psychrometer the aforementioned water volumes corresponded to water potentials of -0.5 MPa (mega Pascal) [5.0% (wt.:wt.)], -0.47 MPa [9.0% (wt.:wt.)], -0.28 MPa [13.0% (wt.:wt.)] and -0.23 MPa [17.0% (wt.:wt.)]. To attain a water potential of -2.14 MPa [1% (wt.:wt.)], 0.3 ml of distilled water containing a fungal suspension of 3.0 x 10^8 cfu/ml was added to the container. A control lot of soil was treated with the same quantity of sterile distilled water containing 0.1% Tween 80. The soil was thoroughly mixed by shaking for 5 min to achieve a uniform distribution of the inoculum, and 10 pupariating late third instars of *C. capitata* were released into the soil. The containers were placed inside a large plastic container (250 by 450 mm), and was covered with a damp filter paper, which was moistened periodically to maintain a ±3% loss of the initial soil water content (Ekesi et al., 2003). In total, three replicates were conducted, and 10 insects per replicate were used for each isolate and control. The containers were incubated at 15, 20, 25, 30 and 35 ºC until emergence. Puparia that failed to emerge were immediately surface disinfested with 1% sodium hypochlorite followed by three rinses with sterile distilled water. Then they were placed on sterile wet filter paper in sterile Petri dishes that were sealed with parafilm and kept at 25 ºC to be inspected for development of mycosis on the pupae.

Table 1. Fungal isolates from the culture collection at AFSR that were evaluated against *C. capitata* puparia

Fungal species	Isolate	Host or source	Site and date of origin
B. bassiana	Bb-1333	*Bactrocera oleae* (Gmel.) (Diptera: Tephritidae)	Grece, 1961
	EABb 01/33-Su	Soil (olive tree orchard)	Cádiz (Spain), 2001
M. anisopliae	EAMa 01/58-Su	Soil (wheat crop)	Córdoba (Spain), 2001
	EAMa 01/158-Su	Soil (olive tree orchard)	Sevilla (Spain), 2001

2.5. Statistical analysis

Puparia mortality is binary data (yes or no). Generalized linear models that do not assume a normal distribution of mortality are more appropriate than the continuous/normal regression approach (McCullagh and Nelder, 1989). Indeed, both approaches use mycosis as a percentage, whereas in the former the total number of exposed puparia is considered while in the later it is not.

A multiple logistic regression model was developed for each fungal isolate. The model included the linear and quadratic effects of temperature (T), soil moisture (SM), and the interaction between temperature and soil moisture (T x SM). The proposed equation is a second order surface model.

$$Y = 1 \quad \text{if mycosed puparium}$$
$$\text{logit}[P(Y=1)] = \log\left[\frac{P(Y=1)}{1-P(Y=1)}\right] = \alpha + \beta_1 T^2 + \beta_2 SM^2 + \beta_3 T + \beta_4 SM + \beta_5 (T \times SM)$$

The logistic models (distribution = binomial; link = logit; estimation method = maximum likelihood) were fitted according to JMP 9.0 (generalized linear model module, SAS GENMOD procedure) (SAS-Institute-Inc., 2010). The statistical significance of the global model and the linear, quadratic and interaction effects of temperature and soil moisture were determined by conducting likelihood-ratio χ^2 tests (alpha level = 0.05, prob > χ^2 values less than 0.05 were considered significant). The goodness of fit was determined by Pearson's χ^2 test (alpha level = 0.05), and prob > χ^2 values less than 0.05 indicated that higher-order terms or more covariates should be added to the model (JMP 9.0).

For each fungal isolate, the temperature and soil moisture required to achieve the maximum response (proportion of mycosed puparia) was calculated using the profiler module for generalized linear models (JMP 9.0).

3. Results

The logistic model for the proportion of mycosed puparia revealed differing trends in the effects of temperature and moisture for each of the four isolates (Bb-1333, EABb 01/33-Su, EAMa 01/58-Su and EAMa 01/158-Su). Overall, the two *M. anisopliae* isolates produced greater proportions of mycosis than the two *B. bassiana* isolates under the entire range of temperatures and soil moistures. The mean proportion of mycosed puparia across at all of the studied temperatures and soil moistures was 47.4% for isolate EAMa 01/158-Su, 26.5% for EAMa 01/158-Su, 17.8% for EABb 01/33-Su and 16.4% for isolate Bb-1333.

The logistic model was significant for all of the isolates, and the prob > χ^2 values ranged from <0.0001 to 0.0359 (Table 2). For isolate EAMa 01/158-Su, temperature had a significant positive linear effect on mycosis (the proportion of mycosis increased with an increase in temperature, prob > χ^2 < 0.0001, df = 1). Moreover, soil moisture had a significant positive linear effect (prob > χ^2 = 0.0030, df = 1), and the interaction between temperature and soil moisture was statistically significant (prob > χ^2 = 0.0426, df = 1). Quadratic effects were not significant for temperature or soil moisture. The maximum predicted proportion of mycosed puparia was 64.3% and was observed at 35 ºC and 17.0% wt.:wt. (Table 2).

Temperature had significant negative linear and quadratic effects on mycosis for isolate EAMa 01/58-Su (prob > χ^2 = 0.0002, df = 1 for T; prob > χ^2 = 0.0136, df = 1 for T2), and soil moisture had a significant negative quadratic effect (the rate of mycosis increased to

Table 2. Maximum predicted values for mycosis of the generalized linear model for the studied isolates

	Max. Predicted proportion of mycosis					Model fit test Likelihood-ratio χ2 (df=5)		Goodness of fit test Pearson's χ2 (df=744)	
Isolate	Temperature (ºC)	Moisture (% wt:wt)	Proportion of mycosis	L CI	U CI	χ2	Prob> χ2	χ2	Prob> χ2
Bb-1333	15.0	11.9	0.24	0.16	0.32	11.92	0.0359	747.98	0.4522
EABb 01/33-Su	15.0	9.9	0.38	0.29	0.48	40.86	<0.0001	751.60	0.4154
EAMa 01/58-Su	20.1	10.8	0.63	0.56	0.68	32.92	<0.0001	749.93	0.4322
EAMa 01/158-Su	35.0	17.0	0.64	0.52	0.75	61.81	<0.0001	755.54	0.3764

a maximum and then decreased, prob > χ^2 = 0.0022, df = 1). Alternatively, the interaction between temperature and soil moisture was not significant. The maximum predicted proportion of mycosed puparia was 62.3% and was attained at 20.1 ºC and 10.8% wt.:wt. (Table 2).

For isolate EABb 01/33-Su (Fig. 1), temperature had a significant negative linear effect, (prob > χ^2 = 0.0137, df = 1), and soil moisture had a significant negative quadratic effect (prob > χ^2 < 0.0001, df = 1). The interaction between temperature and soil moisture was not significant. The maximum predicted proportion of mycosed puparia was 37.6% and was attained at 15.0 ºC and 9.8% wt.:wt. (Table 2).

For isolate Bb-1333, only soil moisture squared (prob > χ^2 = 0.0106, df = 1) and the interaction between temperature and soil moisture (prob > χ^2 = 0.0160, df = 1) were statistically significant. Moreover, the effects of temperature and soil moisture on mycosis were weaker than that of the other isolates under all of the tested conditions (Fig. 2), and a prob > χ^2 = 0.0359 (df = 5) was observed for the entire model. The maximum predicted proportion of mycosed puparia was 23.4% and was attained at 15.0 ºC and 11.9% wt.:wt. (Table 2).

For fungal isolates, EABb 01/33-Su and EAMa 01/58-Su, the proportion of mycosis was inversely related to the temperature, alternatively, for EAMa 01/158-Su (Fig. 4), the rate of mycosis increased with an increase in temperature. Temperature effects were linear for the aforementioned isolates, and only a quadratic component was observed for EAMa 01/58-Su. For isolate Bb-1333, the temperature did not have an effect on the rate of mycosis.

Soil moisture was directly related to the proportion of mycosis for isolate EAMa 01/58-Su (mycosis increased with an increase in soil moisture, Fig. 3). For the rest of the isolates, soil moisture had a negative quadratic effect (the rate of mycosis increased with an increase in moisture and subsequently decreased).

The maximum predicted proportion of mycosed puparia for *M. anisopliae* isolates was greater than that of both *B. bassiana* isolates (Table 2).

There was no evidence of mycosis in any of the control cadavers, which indicates the condition of the insect population.

Whilst quadratic functions adequately described the effect of temperature on the Abbott-corrected mortality of pre-imaginal *C. capitata* caused by the four fungal isolates, this effect was also accurately described by linear functions for three of the isolates, EABb 01/33-Su, EAMa 01/58-Su and EAMa 01/158-Su (Fig. 5). These three isolates showed a similar response to temperature over the evaluated range (15–35 ºC), with mortality decreasing with increasing temperature, while only a slight increase in mortality was observed for Bb-1333 isolate (Fig. 5). Besides, the estimated optimum temperature for pre-imaginal Abbott mortality was higher for Bb-1333 isolate than for the other three ones (Table 3). Logit-model moisture response varied according to isolate over the evaluated range (1.0–17.0% wt.:wt.). Thus, EAMa 01/58-Su and

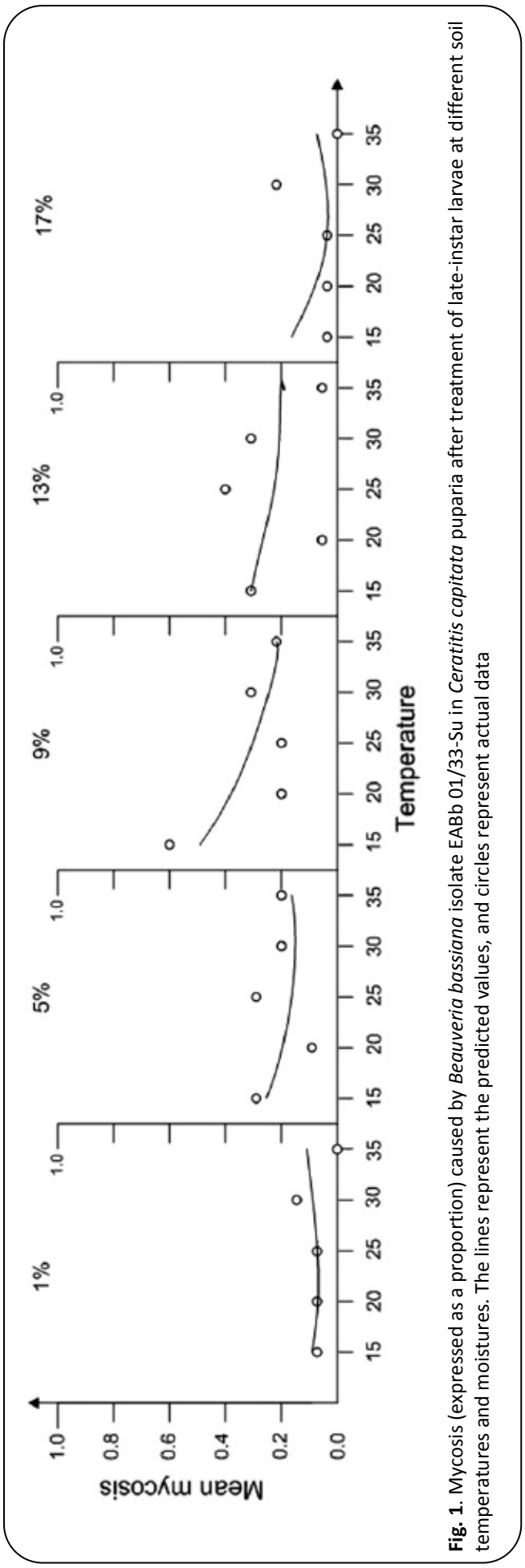

Fig. 1. Mycosis (expressed as a proportion) caused by *Beauveria bassiana* isolate EABb 01/33-Su in *Ceratitis capitata* puparia after treatment of late-instar larvae at different soil temperatures and moistures. The lines represent the predicted values, and circles represent actual data

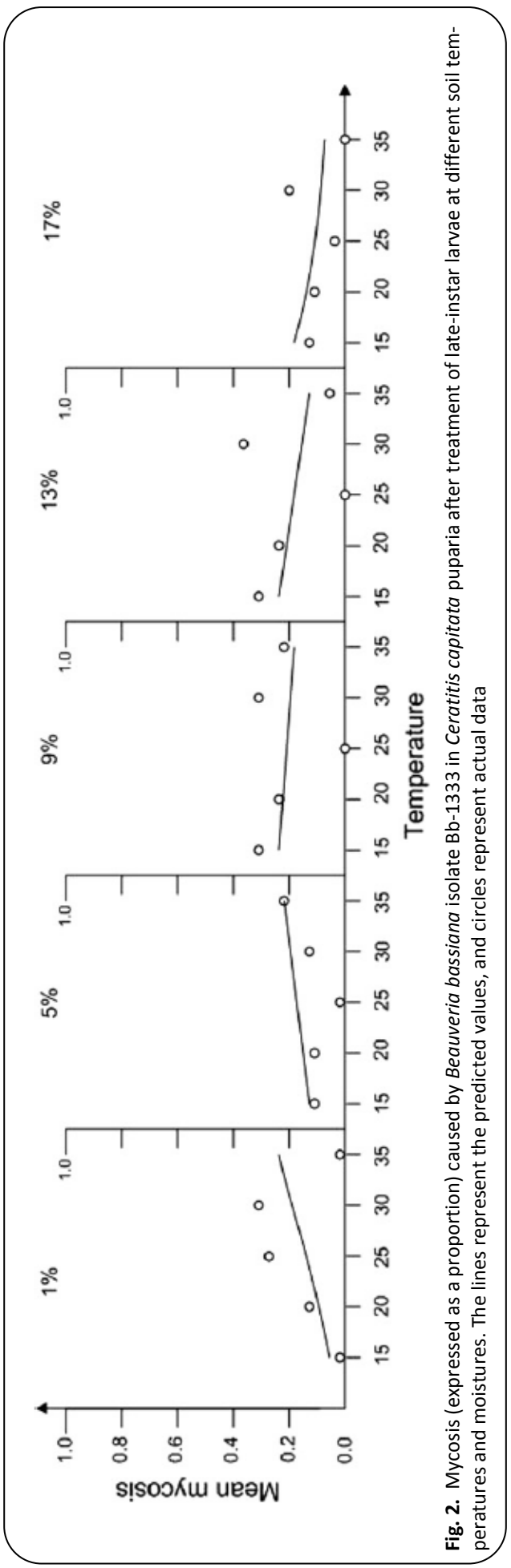

Fig. 2. Mycosis (expressed as a proportion) caused by *Beauveria bassiana* isolate Bb-1333 in *Ceratitis capitata* puparia after treatment of late-instar larvae at different soil temperatures and moistures. The lines represent the predicted values, and circles represent actual data

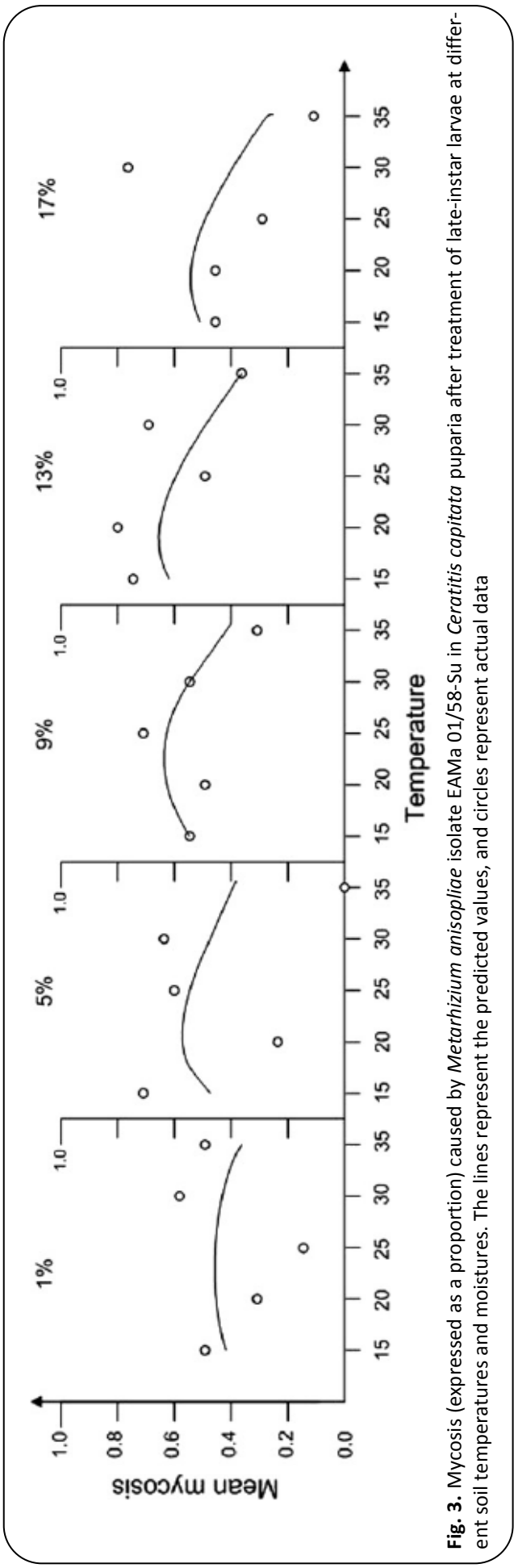

Fig. 3. Mycosis (expressed as a proportion) caused by *Metarhizium anisopliae* isolate EAMa 01/58-Su in *Ceratitis capitata* puparia after treatment of late-instar larvae at different soil temperatures and moistures. The lines represent the predicted values, and circles represent actual data

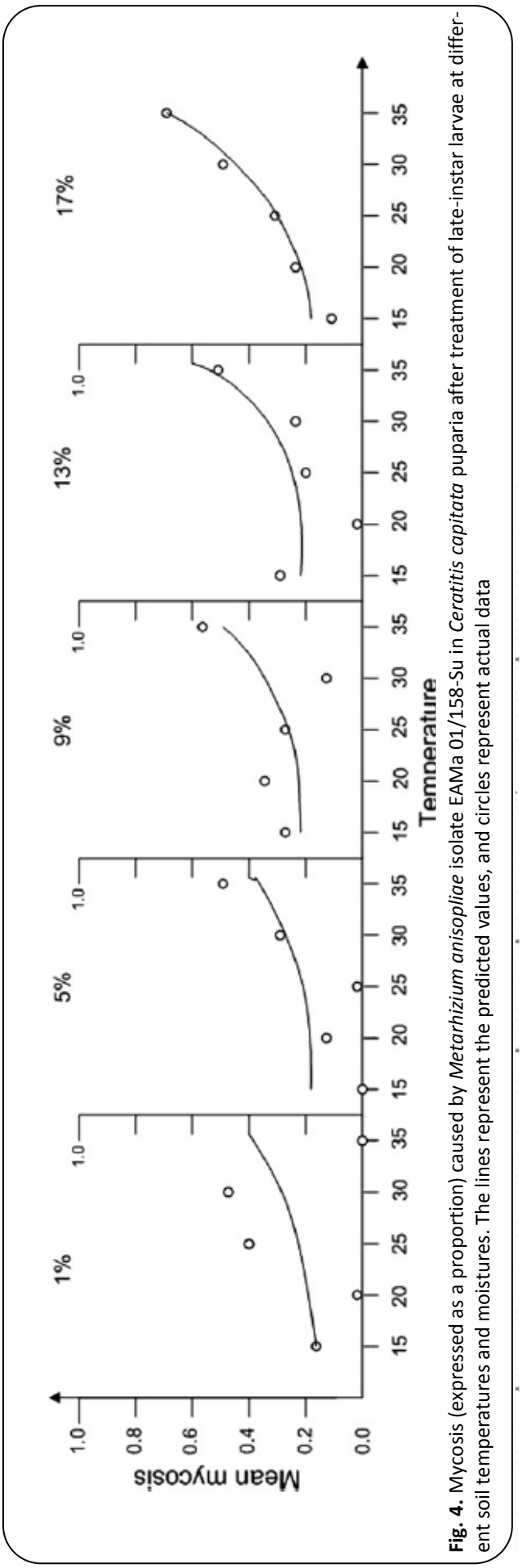

Fig. 4. Mycosis (expressed as a proportion) caused by *Metarhizium anisopliae* isolate EAMa 01/158-Su in *Ceratitis capitata* puparia after treatment of late-instar larvae at different soil temperatures and moistures. The lines represent the predicted values, and circles represent actual data

Bb-1333 isolates were adjusted to a quadratic function, fitting to an inverted parabola, with maximum prediction moisture values at 7.7 and 9.3% wt.:wt., respectively, while EAMa 01/158 and EABb 01/33-Su ones were not moisture-dependent (Table 3). Abbott preimaginal corrected mortality was linearly and positively correlated with moisture for EAMa 01/158 isolate, and fairly constant with increasing moisture for EABb 01/33-Su isolate.

4. Discussion

Abiotic factors impact the efficacy of entomopathogenic fungi (EF) conidia and affect fungal infectivity or persistence (Jaronski, 2010). Wraight et al. (2000) and Fargues et al. (2003) indicated that fungal infection was highly influenced by the conditions of the insect's habitat. Moreover, host-pathogen systems are diverse and complex (Vidal and Fargues, 2007). The survival and infectivity of EF in soil are affected by complex interactions between soil temperature and moisture (Ekesi et al., 2003; Quesada-Moraga et al., 2006b), as demonstrated in the present study.

Several studies were conducted to establish a model for the prediction of the effects of the aforementioned factors on fungal development. In general,

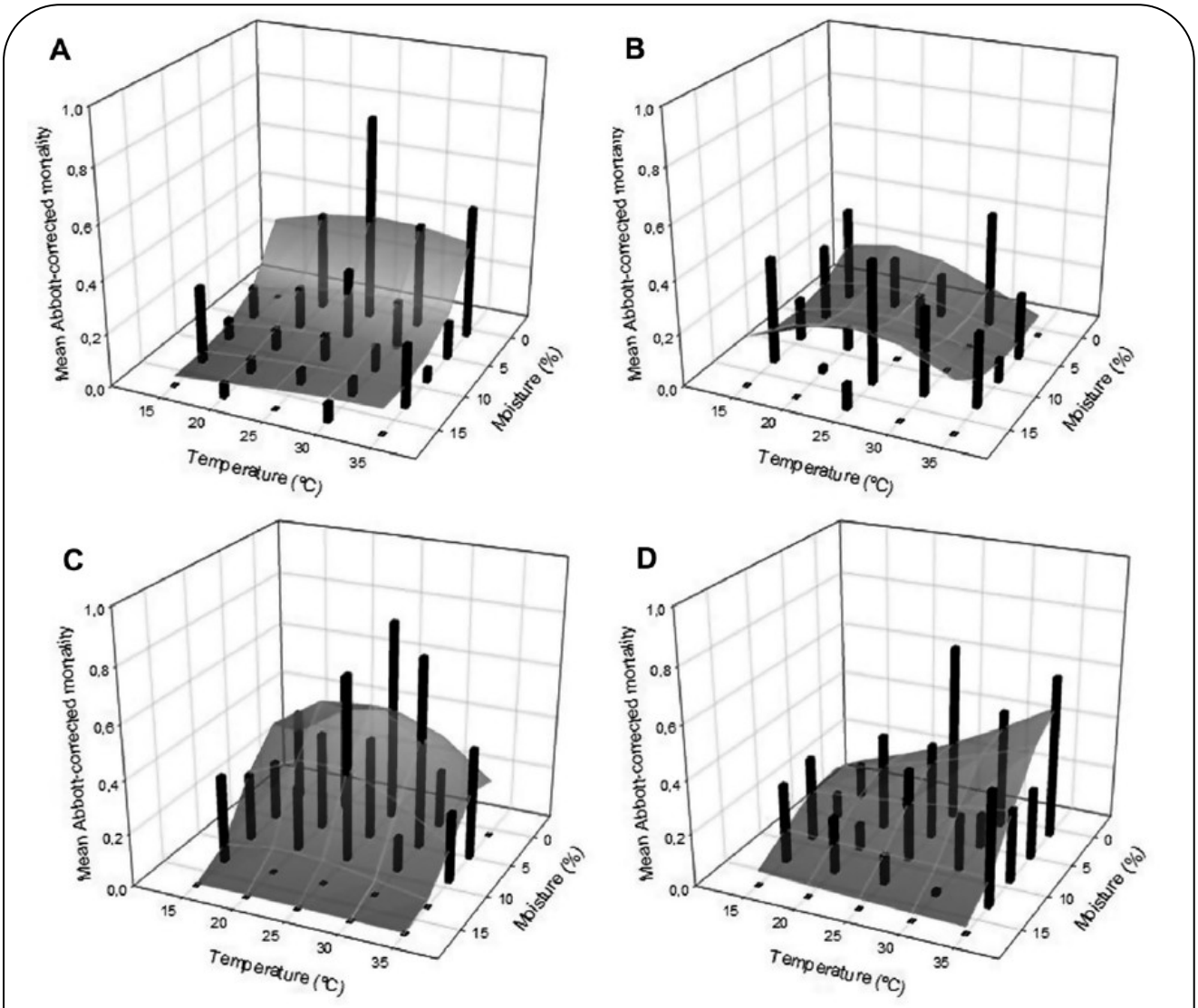

Fig. 5. Abbott-corrected mortality (expressed as a proportion) caused by *Beauveria bassiana* (A: EABb 01/33-Su; B: Bb-1333) and *Metarhizium anisopliae* (C: EAMa 01/58-Su; D: EAMa 01/158-Su) isolates in *Ceratitis capitata* puparia after treatment of late-instar larvae at different soil temperatures and moistures. The mesh plots represent the predicted values, and bars represent actual data

Table 3. Maximum predicted values for mortality of the generalized linear model for the studied isolates.

	Max. Predicted proportion of mortality					Model fit test Likelihood-ratio χ2 (df=5)		Goodness of fit test Pearson's χ2 (df=744)	
Isolate	Tempera-ture (ºC)	Moisture (% wt:wt)	Proportion of mortality	L CI	U CI	χ2	Prob> χ2	χ2	Prob> χ2
Bb-1333	35.0	9.3	0.31	0.23	0.41	21.60	0.0006	769.53	0.2509
EABb 01/33-Su	15.0	10.6	0.40	3.21	0.50	72.29	<0.0001	834.96	0.0111
EAMa 01/58-Su	19.3	7.7	0.53	0.45	0.61	153.31	<0.0001	609.34	0.9999
EAMa 01/158-Su	15.0	17.0	0.50	0.37	0.63	79.70	<0.0001	677.74	0.9603

temperature and moisture present non-linear response curves, in which the studied parameter increases to a maximum at an optimal temperature or moisture content, and then decreases rapidly (Hywel-Jones and Gillespie, 1990; Hong et al., 1997; Smits et al., 2003; Quesada-Moraga et al., 2006a).

Each fungal isolate responded differently to the interaction between soil moisture and temperature, and the rate of puparia mortality and incidence of visible mycosis on pre-imaginal stages of *C. capitata* were variable. Generally, the survival of EF conidia in soil decreased with an increase in the soil moisture content (Lingg and Donaldson, 1981). Quesada-Moraga et al. (2006b) reported that an increase in the moisture level did not translate to higher pupal mortalities when they were treated with *B. bassiana* and *M. anisopliae* conidia. Thus, although a decrease in the rate of mycosis was observed under extreme conditions (1.0% and 17.0% wt.:wt.), slight differences in the rate of mycosis were detected between the isolates. Overall, *M. anisopliae* isolates provided higher mortalities and mycosis rates than *B. bassiana* isolates. Both *B. bassiana* isolates and EAMa 01/58-Su from *M. anisopliae* showed an inverted parabolic relationship with moisture (the maximum predicted value was obtained at 9.9–11.9% wt.:wt.), while the mycosis rate of EAMa 01/158-Su from *M. anisopliae* increased linearly with an increase in the moisture content (the maximum predicted value was obtained at 17.0%). In general, the superficial fungal growth of pre-imaginal stages when they are treated with *M. anisopliae* was greater than that with *B. bassiana*. The observed increase in the rate of mycosis with an increase in soil moisture can be attributed to the fact that *M. anisopliae* conidia germinated more quickly than *B. bassiana* isolates at all moisture levels (Lazzarini et al., 2006), which produced early infections and increased the time available for sporulation. Namely, the penetration of fungal germ tubes (inward or outward from the puparium) is often restricted by the degree of puparia sclerotization (Ekesi et al., 2002; Cossentine et al., 2010).

Similarly, compared to the other isolates, soil temperature had a different effect on EAMa 01/158-Su of *M. anisopliae*. For preimaginal *C. capitata* treated with EAMa 01/158-Su, mycosis increased with an increase in temperature (the maximum predicted value was obtained at 35.0 ºC). Alternatively, for the rest of the isolates, the maximum rate of mycosis was observed at lower temperatures (the maximum predicted values were obtained at 15.0–20.1 ºC). Although the optimal germination temperature of *Metarhizium* is 22.0–30.0 ºC, germination can occur within in a wide range of temperatures (5.0–37.0 ºC) (McCammon and Rath, 1994). Hywel-Jones and Gillespie (1990) reported that *M. anisopliae* strains germinated faster than those of *B. bassiana* at 20.0–30.0 ºC; however, isolates were not analyzed at temperatures less than 20.0 ºC. In addition, rapid germination and growth may be correlated to higher virulence (Samuels et al., 1989).

In this work, dead pre-imaginal *C. capitata* were

dissected to determine for possible mycosis of within pupae. Consequently, some of the pupae could have been killed by the fungi even if their puparia did not shown fungal outgrowth. Nonetheless, Cossentine et al. (2010) have recently reported that incidence of *B. bassiana* mycosis outside and within *Rhagoletis indifferens* puparia is significantly correlated, concluding that puparial mycosis can be used as suitable measurement of fungal virulence against tephritids. Besides, in our former work (Quesada-Moraga et al., 2006b), total mortality of pre-imaginal *C. capitata* and the one caused by micosis were very similar. Likewise, in the present work, differences between total (Abbott) pre-imaginal mortality and mortality due to mycosis by *B. bassiana* or *M. anisopliae* were in the range of 0–16% for EABb 01/33-Su isolate, of 0–13% for Bb-1333, of 0–9% for EAMa 01/58-Su and of 0–21% for EAMa 01/158-Su, which again indicates that puparia mycosis provides an appropriate measurement of entomopathogenic fungi (EF) virulence against tephritid puparia (Ekesi et al., 2005; Yee and Lacey, 2005; Ekesi et al., 2007). This is further supported by the fact that similar patterns were also detected in the general effect of temperature and soil moisture both on puparia total mortality (Abbott) and the one caused by mycosis; in both cases, as revealed by the associate statistics, the logistic model shows a good fit for all isolates, therefore representing a good reflection of the soil moisture x temperature impact on the virulence of individual fungal isolates.

The results of our laboratory experiments can be applied to successfully control *C. capitata*. In the present study, temperature and moisture tolerances of different isolates of *B. bassiana* and *M. anisopliae* were tested in a specific soil arena. The results suggest that entomopathogenic fungal isolates for pest control should have a range of temperatures and humidities that can be matched to the desired environment. Namely, the thermal and moisture requirements of entomopathogenic fungi should be similar to the environment of the target pest.

5. Acknowledgments

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CAPÍTULO VII

UNIVERSIDAD DE CÓRDOBA

Artículo

The effect of temperature and soil moisture on the development of the pre-imaginal Mediterranean fruit fly *Ceratitis capitata* (Diptera: Tephritidae)

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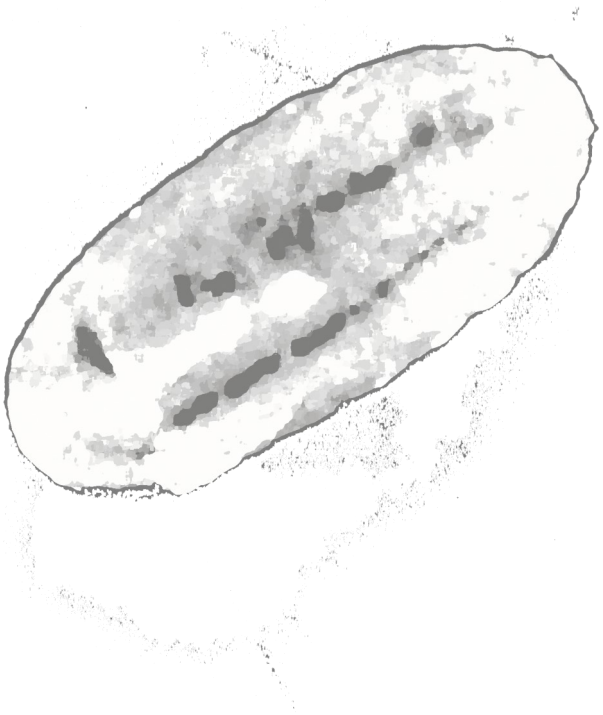

The effect of temperature and soil moisture on the development of the pre-imaginal Mediterranean fruit fly *Ceratitis capitata* (Diptera: Tephritidae)

ABSTRACT

Laboratory studies were conducted to assess the effect of soil moisture and temperature on the development of the pre-imaginal stages of *Ceratitis capitata* (Dipt.: Tephritidae). The number of days required for the immature flies to complete their development and reach the adult stage (development time, DT) were studied at five temperatures (15, 20, 25, 30 and 35ºC) and under five soil moisture regimes (–2.14 Mpa (mega Pascal) [1% (wt:wt)], -0.5 MPa [5.0% (wt:wt)], -0.47 MPa [9.0% (wt:wt)], -0.28 MPa [13.0% (wt:wt)] and -0.23 MPa [17.0% (wt:wt)]). A parametric survival model describing the effect of linear and quadratic combinations of temperature and soil moisture and their interaction on the probability of completing the development over the experimental period was used. The lognormal parametric survival model was significant for *C. capitata* pre-imaginal development, with DT_{50} (the development time for 50% of the pre-imaginal *C. capitata* to reach the adult stage) ranging from 12.8 to 32.4 days. Besides, the highest percentages of adult emergence at 30 days were obtained at 24.8ºC and intermediate soil moistures of 5.0-13.0% wt:wt. The average development time of the medfly pre-imaginals reaching the adult stage was inversely related to temperature and ranged from 7.4 to 26.1 days. This model could allow the monitoring of medfly pre-imaginal natural mortality in the soil, the prediction of adult emergence under field conditions, and therefore, the identification of suitable application times in the medfly lifecycle to achieve the maximum degree of adult and pre-imaginal control.

1. Introduction

The Mediterranean fruit fly or medfly, *Ceratitis capitata* (Wiedemann) is one of the most harmful species of Tephritidae. It is a serious pest of more than 250 species of fruit trees and causes considerable damage worldwide (Thomas et al. 2001, Morales et al. 2004). The medfly is polyvoltine, with up to seven or eight generations per year in the Mediterranean basin. To overwinter, third-instar *C. capitata* drop from the fruits to the ground, burrow in the soil, and form a puparium within the first 2–4 cm (Tremblay 1994, Alfaro-Moreno 2005). Consequently, *C. capitata* control measures may be applied to adults or prepupating larvae and puparia in the soil. Likewise, the soil stage is regarded as the most vulnerable part of the life cycle of soil-dwelling pests (Brown and Gange 1990), especially tephritids (Ekesi et al. 2007). In this context, entomopathogenic fungi have shown their potential for use in medfly control measures that target adults or pupariating larvae and puparia in the soil (Ekesi et al. 2007).

Our previous work has shown that the mitosporic ascomycetes *Beauveria bassiana* and *Metarhizium anisopliae* are frequently isolated from fruit-tree orchard soils in Spain (Quesada-Moraga et al. 2007), with several isolates of both species showing potential for the control of medfly adult and puparia (Quesada-Moraga et al. 2006, 2008). In addition, to optimize the efficacy of soil treatments, previous studies have addressed the effect of temperature and moisture (Garrido-Jurado et al. 2011a) and soil physicochemical properties (Garrido-Jurado et al. 2011c) on the availability, movement, and virulence of conidia against puparia of *C. capitata*. Research has even investigated the effect of such soil treatments on the soil-dwelling non-target arthropods (Garrido-Jurado et al. 2011b).

However, optimizing the effect of any control measure, particularly entomopathogenic fungi, against medfly adults and pre-imaginal stages would require a better understanding of the biology of pupae in the soil, a topic that remains poorly understood. A better understanding of this topic, would allow the monitoring of natural pre-imaginal mortality in the soil, the prediction of adult emergence, and therefore, the identification of suitable application time in the tephritid lifecycle to achieve the maximum degree of adult and pre-imaginal control.

Temperature and moisture are major determinants of the developmental rates and natural mortality of soil-

dwelling insect stages (Johnson et al. 2007), and they are particularly important for the pre-imaginal stages of tephritids (Fletcher and Kapatos 1983, Milward-de-Azevedo and Parra 1989, Bento et al. 2010). Nevertheless, research on these questions remains scarce due to the difficulty of replicating natural soil conditions in the laboratory.

In this study, an approach based on a lognormal parametric survival model has been used to define the effects of soil moisture and temperature on pre-imaginal medfly mortality and to understand the relationship between both factors and their interaction as influences on the development of pre-imaginal medflies in the soil.

2. Materials and Methods

2.1. Insects

Pre-imaginal *C. capitata* were obtained from a laboratory population maintained at our insectary since 2004. This population was initially was derived from the stock colony of El Encin (INIA, Madrid). The colony was reared under a photoperiod of 16:8 (L:D) h at 50-60% RH and 26 ± 2ºC. Adult flies were provided with water and an artificial diet based on a mixture of protein-hydrolysate (Yeast Hydrolysate Enzymatic, ICN Biomedicals, Aurora, Ohio, USA) and sucrose (Panreac) (1:4 wt:wt). The larvae were reared on wheat bran + sucrose + brewer's yeast + nipagin + nipasol + benzoic acid + water (300 g/kg + 75 g/kg + 36 g/kg + 2 g/kg + 2 g/kg + 2.4 g/kg + 600 ml/kg).

2.2. Experimental design

The soil used in all experiments was field collected in Córdoba, Spain. It was characterized as sandy-loam (78% sand, 17% silt, 5% clay, and 0.2 organic matter, pH 8.4). The soil was sieved (2 mm mesh), sterilized and air-dried at 26ºC for 10 days prior to use. Sterile distilled water volumes of 0.3, 1.5, 2.7, 3.9, and 5.1 ml containing 0.1% Tween 80 were added to transparent containers (80 by 80 by 55 mm), each holding 30 g of soil. These water volumes provided corresponding water potentials of –2.14 Mpa (mega Pascal) [1% (wt:wt)], -0.5 MPa [5.0% (wt:wt)], -0.47 MPa [9.0% (wt:wt)], -0.28 MPa [13.0% (wt:wt)] and -0.23 MPa [17.0% (wt:wt)], measured using a WP4 Decagon psychrometer apparatus. The soil was thoroughly mixed by shaking for 5 min to achieve a uniform distribution of the liquid. Ten pupariating late third instars of *C. capitata* were released into the soil. The containers were then placed inside a large plastic container (250 by 450 mm), which was covered with a damped filter paper that was wetted periodically (Ekesi et al. 2003). This procedure helps to maintain soil moisture conditions by limiting loss ± 3% loss of the initial soil water. Three replicates of 10 insects per replicate were used for each treatment. The containers were incubated at 15.0, 20.0, 25.0, 30.0 and 35.0ºC until adult emergence. The full experiment was repeated twice.

2.3. Statistical Analysis

A parametric survival model was used to evaluate the effect of soil moisture and temperature on the developmental rate (the percentage of individuals that fully completed their development) and the time to reach the pre-imaginal stage.

The lognormal model describes the effect of linear and quadratic combinations of temperature and soil moisture and their interaction on the probability of completing development over the duration of the experiment.

$$Prob\,(full\ development) = Normal\ dist[\log(time)\,, a + b \times Temp + c \times Soil\ moist + d \times Temp * Soil\ moist + e \times Temp^2 + f \times Soil\ moist^2, \sigma]$$

where the polynomial expression (second-order surface model) and σ are the mean and the standard deviation of the normal distribution, respectively. Other parametric survival models were also evaluated (Weibull, exponential, and loglogistic). The lognormal model furnished a better fit (lower AIC and -2loglikelihood values).

The lognormal model was fitted with JMP 9.0 (parametric survival module; SAS® Institute Inc.). The statistical significance of the global model as well as the significance of linear, quadratic and interaction (temperature and soil moisture) effects were determined by likelihood-ratio χ^2 tests (alpha level= 0.05). Values of χ^2 with probabilities lower than 0.05 indicate significant effects. The model estimates the probability or percentage of individuals that completed their development at a given time t, as well as the time $DT_{x\%}$ required for x% of the individuals to complete their development for each combination of temperature and soil moisture. The average development times (ADT, mean value of the individual development times of the group of insects) were also calculated for each combination of temperature and soil moisture. The significance of differences in ADT was tested with a log-rank test.

The combination of temperature and soil moisture values which provided the highest percentage of insects completing their development by day 30th and lowest time for DT_{25}, DT_{50}, DT_{75} and DT_{90}, was calculated using Profiler for the parametric survival module (JMP 9.0).

3. Results

The lognormal survival model was significant for *C. capitata* pre-imaginal development for the range of 15.0-35.0ºC (χ^2=362.27, df=5, $P>\chi^2<0.0001$). The values of the parameters estimated from the model were: a=10.439, std error=0.4790; b=-0.649, std error=0.0394; c=-0.077, std error=0.0272; d=0.0013, std error=0.0008; e=0.013, std error=0.0008; f=0.0031, std error=0.0010 and σ=0.643, std error=0.0257. Temperature had significant quadratic and linear effects on the development time (DT) and the percentage of insects which completed their development, being the quadratic effect the most significant of the model (χ^2=319.03, df = 1, $P > \chi^2 < 0.0001$ for T^2; χ^2=78.99, df = 1, $P > \chi^2 < 0.0001$ for T). Soil moisture had also significant quadratic and linear effects on DT, although they were not as relevant as those from temperature, with lower χ^2 values in the likelihood ratio tests (χ^2=9.57, df = 1, $P > \chi^2 = 0.0020$ for SM^2; χ^2=7.68, df = 1, $P > \chi^2 = 0.0056$ for SM). The interaction between temperature and soil moisture was not significant (χ^2=2.78, df = 1, $P > \chi^2 = 0.0953$ for T*SM).

The lognormal parametric survival model also specified the percentage of pre-imaginal *C. capitata* that completed their development at a given time (t) under different soil moisture and temperature regimes. At 30 days (PCD30), end of the experiment, (Fig. 1), the percentages predicted by the model ranged from 32.08% (22.87-42.56) at 15.0ºC and 1.0% wt:wt to 90.65% (87.28-93.31) at 25.0ºC and 5.0% wt:wt (Table 1). The PCD30 increased with increasing temperature and soil moisture reached a maximum and then showed a steady decrease, being the highest PCD30 predicted for the model of 91.57% (88.32-94.07) at 23.5ºC and 7.0% wt:wt.

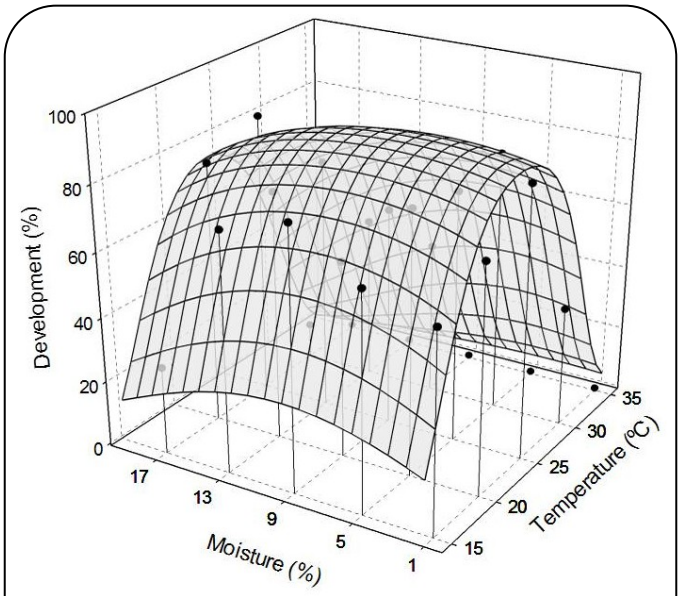

Fig. 1. Percentages of pre-imaginal *Ceratitis capitata* that completed their development at 30 days under different soil moisture and temperature regimes. The mesh plot represents predicted values, and the points represent actual data

Also, the lognormal parametric survival model provided the time required to complete the development for a x% of the individuals under different soil moisture and temperature regimes. Fig. 2 shows the development time (DT) values for 25, 50, 75 and 90% of pre-imaginal *C. capitata* to attain the adult stage. The DT_{50} values (development time for 50% of the pre-imaginal *C. capitata* to attain the adult stage) ranged from 12.8 days at 25.0ºC and 5.0% wt:wt to 32.4 days at 30.0ºC and 17.0% wt:wt, respectively. Generally, DT was lower at intermediate soil moisture (5.0, 9.0 and 13.0%) and temperature (20.0 and 25.0ºC) values. The lowest DTs predicted for the model for 25, 50, 75 and 90 % of pre-imaginal *C. capitata* to attain adult stage were 8.02 (7.23-8.90), 12.37 (11.22-13.64), 19.10 (17.23-21.17), and 28.22 (25.13-31.71) days at 23.5ºC and 7.0% wt:wt., respectively.

In general, the ADT values decreased with increasing temperature, ranging from 7.4 days at 30.0ºC and 5.0% wt:wt to 26.1 days at 15.0ºC and 1.0% wt:wt. Significant differences in the ADT of pre-imaginal *C. capitata* occurred at different soil moistures at extreme temperature conditions [15.0 ºC (χ^2=20.52, df=4, *P*<0.001) and 30.0 ºC (χ^2=34.99, df=4, *P*<0.001), (Table 2)] whereas no significant differences in ADT between soil moisture values were detected at 20.0ºC (χ^2=3.85, df=4, *P*=0.427) or 25.0ºC (χ^2=2.34, df=4,*P*=0.674).

Table 1. Percentage of pre-imaginal *Ceratitis capitata* predicted by the model that finish their development at 30 days under different soil moisture and temperature regimes

Moisture (% wt:wt)	Temperature (ºC)				
	15	20	25	30	35
	Percentage (95% confidence limits)	Percentage (95% confidence limits)	Percentage (95% confidence limits)	Percentage (95% confidence limits)	Percentage (95% confidence limits)
1	32.09 (22.87 – 42.56)	81.07 (74.88 – 86.22)	87.95 (82.94 – 91.83)	65.96 (56.53 – 74.49)	8.02 (3.72 – 15.31)
5	40.83 (33.41 – 48.59)	85.78 (81.90 – 89.06)	90.65 (87.28 – 93.31)	69.67 (63.16 – 75.60)	8.96 (4.99 – 14.89)
9	43.81 (36.44 – 51.40)	86.51 (82.49 – 89.84)	90.47 (86.88 – 93.28)	67.76 (61.08 – 73.91)	7.49 (4.10 – 12.68)
13	40.63 (32.99 – 48.64)	83.62 (79.39 – 87.24)	87.31 (83.32 – 90.58)	59.86 (52.51 – 66.88)	4.50 (2.11 – 8.70)
17	31.72 (22.23 – 42.59)	75.71 (68.49 – 81.92)	79.27 (72.40 – 85.02)	45.28 (34.90 – 55.99)	1.76 (0.54 – 4.78)

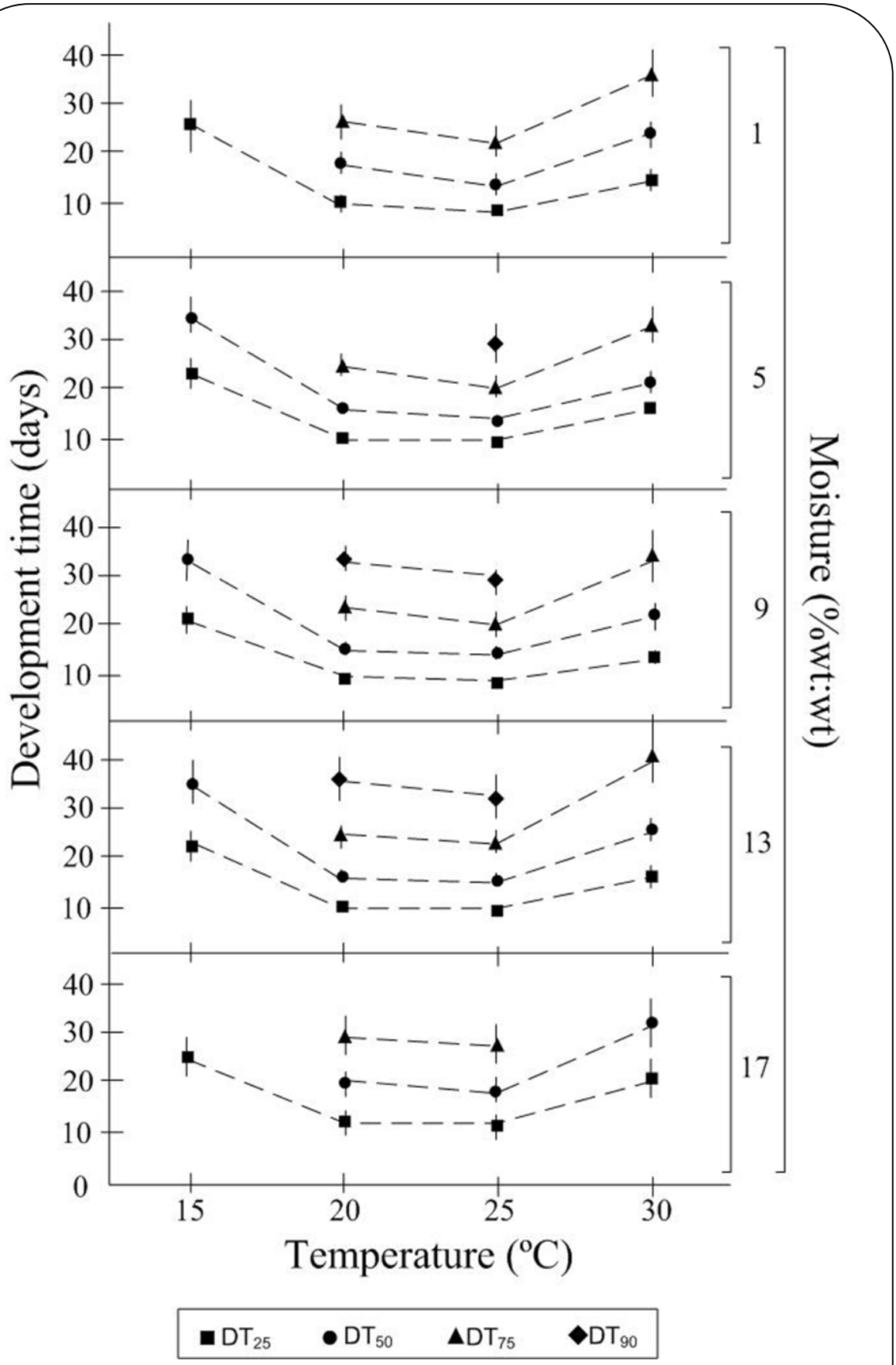

Fig. 2. Development time (mean ± standard error) of pre-imaginal *C. capitata* exposed to different soil moisture and temperature regimes. Points represent the time required to complete development (squares, circles, triangles and diamonds correspond to 25, 50, 75 and 90% of the pre-imaginal *C. capitata*, respectively)

Table 2. Average development time for pre-imaginal *Ceratitis capitata* under different soil moisture and temperature limited at 30 days

Moisture (%)	Temperature (ºC)			
	15	20	25	30
1	26.10 ± 0.18	14.80 ± 0.18	11.30 ± 0.13	7.90 ± 0.06
5	25.83 ± 0.19	14.40 ± 0.11	10.97 ± 0.14	7.43 ± 0.09
9	25.23 ± 0.13	14.27 ± 0.14	11.27 ± 0.13	7.77 ± 0.08
13	25.77 ± 0.19	14.07 ± 0.13	11.07 ± 0.14	7.67 ± 0.09
17	25.77 ± 0.10	14.13 ± 0.13	11.27 ± 0.14	7.93 ± 0.06

4. Discussion

The study of pre-imaginal *C. capitata* in relation to the soil can provide better forecasts for enhancing medfly integrated pest management, but information regarding fruit fly development as a function of soil conditions is scarce. The studies by Crovetti et al. (1986), Duyck and Quilici (2002) and Duyck et al. (2004) reported only on separate temperature- and moisture-related effects on the emergence of adult fruit flies. Linear models provided a poor fit at both low and high temperature extremes. In addition, these studies do not address DT, a key value for monitoring medfly populations. In this study, both soil temperature and humidity conditions have been considered within the same lognormal parametric survival model. This approach has allowed better predictions of DT and adult emergence prediction over the range of 15.0-35.0ºC and 1.0.-17.0.% wt:wt.

Lognormal parametric survival models have not previously been used to model insect pest development. The model used in this work offers advantages not provided by the linear and degree-day models such as considering linear and quadratic effects of temperature and soil moisture and their interaction (response surface model), and estimations of number of days required for pre-imaginal medflies to reach the adult stage at different temperature and moisture regimes without the need to accumulated degree days. Thus, monitoring soil moisture and temperature would allow the prediction of the onset of adult emergence and ultimately the optimization of the timing of pre-imaginal and adult control operations.

The lognormal parametric survival model has calculated an optimum temperature for percentage of pre-imaginal *C. capitata* that finished their development of 24.8ºC. This value is higher than the 22.0ºC temperature reported by Crovetti et al. (1986). The calculated lower threshold value of 12.5Cº is likewise higher than the 11ºC value provided by Crovetti et al. (1986) and Duyck et al. (2004). It is probable that this difference is due to the lack of temperature-moisture interaction in these previous models. In addition, this interaction could also explain the conflicting results obtained by several previous studies for the effect of soil moisture on the percentage of pre-imaginal fruit flies reaching the adult stage. These previous studies reported increasing mortality with increased (Eskafi and Fernandez 1990, Bento et al. 2010) or decreased (Milward-de-Azevedo and Parra 1989, Hulthen and Clarke 2006) soil moisture, respectively. In this context, the soil texture must also be considered because the number of flies emerging from the soil appears to be positively correlated with the percentage of porous space in the soil and the percentage of water saturation of the soil (Eskafi and Fernandez 1990, Garrido-Jurado et al. 2011c).

In general, the ADT values were lower than the

DT_{50}. The distribution of the percentage of pre-imaginal *C. capitata* that finished their development over the time is negatively skewed. That is, the better conditions for 50% adult emergence appeared to be associated with longer development time. These DT_{50} ranged from 12.8 to 32.4 days for 20 to 30 ºC. Long period of time under constant soil moisture and temperature will be favourable to reach the adult stage.

In the experiments conducted in this study soil moisture and temperature were kept constants. Log-normal parametric survival model proposed in this study represents an early, but important, step toward developing more complex model that describe the effect of temperature and soil moisture on the development of pre-imaginal *C. capitata* under variable regimens of temperature and soil moisture. Even so, the results of our investigation should improve the forecasts for enhancing *C. capitata* integrated pest management.

5. Acknowledgements

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CAPÍTULO VIII

Este capítulo es una versión adaptada del artículo

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Effects of soil treatments with entomopathogenic fungi on soil dwelling non-target arthropods at a commercial olive orchard

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Effects of soil treatments with entomopathogenic fungi on soil dwelling non-target arthropods at a commercial olive orchard

ABSTRACT

Recent studies have shown the potential of entomopathogenic fungi (EF) for the biocontrol of tephritid puparia in soil treatments beneath the tree canopy. The soil is the natural ecosystem of these fungi, and the soil environment provides protection against UV. Furthermore, the fungi may recycle in the soil, providing a long term control of the puparia. However, such soil applications could have a negative impact on non-target soil arthropod fauna. In particular, ants play an important role in olive grove soil fauna because they are biological indicators of the soil condition. Thus, the aims of this study were to (i) determine the persistence of *Metarhizium anisopliae* EAMa 01/58-Su isolate in the soil when applied beneath olive trees for controlling olive fly puparia, (ii) elucidate its possible effect on non-target soil dwelling arthropod communities and (iii) evaluate the effect of soil treatment with *M. anisopliae* and *Beauveria bassiana* on *Tapinoma nigerrimum* colonies. Our results indicate that the soil ecosystem favors the persistence of this autochthonous isolate, maintaining levels of 10^5 $CFUg^{-1}$ of soil. This level allows long-term protection of the crop against the olive fly puparia. Furthermore, in pitfall traps, no infected insects were found in field as a result of the fungal treatment. The most abundant arthropods trapped were formicidae species. In laboratory assays, no significant differences in mortality were found between fungal treatments and control. The mean mortality values were between 41.0% and 64.7%. However, a significant reduction in average survival time was observed with 16.9 days in control to 15.6 days in the treatment with *B. bassiana* and 14.8 days in the treatment with *M. anisopliae*. Furthermore, there were no significant differences in ant activity before and after fungal treatment. The soil samples were also evaluated to determinate if these ants distribute conidia from treated soils to non-treated soils without being able to notice the presence of EF.

1. Introduction

The olive fly *Bactrocera oleae* Gmelin is the main pest of the olive crop and it is largely responsible for around 60% of the losses due to insects (Mazomenos et al., 1997). The predominant method of olive fly control has been through the use of conventional insecticides targeting adults, but the continued use of such products has been questioned in the last years. It is well known that the wasteful over-use of insecticides (i.e. dimethoate) poses a threat for the public health and it increases environmental side effects such as insecticide resistance of target pests (Skouras et al., 2007), which is detrimental to the natural enemies, with the subsequent appearance of secondary pests (Katsoyannos, 1985; Santos et al., 2010). For this reason, there are considerable efforts to develop alternative for olive fruit fly control, promoting agricultural strategies of low environmental impact, such as integrated production and organic farming. As an alternative to chemical control there is resurgence in the use of microbial insecticides for biological control of tephritids, particularly entomopathogenic fungi (EF). They infect their host through the cuticle, so they must not be ingested to be effective, offering an excellent opportunity for a successful control of soil-dwelling stages of insects. The soil is the natural ecosystem of EF as its environment provides protection against UV along with optimal conditions of temperature and moisture. Furthermore, the fungi may survive in the soil through recycling in insects or roots (St. Leger, 2008), may provide a long-term strategy for the puparia control. Recent research shows the potential of EF in soil inoculation targeting pupariating larvae and puparia (Ekesi et al., 2003; Quesada-Moraga et al., 2006).

Although at isolate level, entomopathogenic mitosporic ascomicetes may have different levels of specificity, at species level, these fungi show low specificity for their insect host (Hesketh et al., 2010; Jaronski, 2010), so these soil applications could have a negative impact on beneficial insects, such as parasitoids and predators, which regulate phytophagous population (Ekesi et al., 2005; Pearson and Callaway, 2005). The

best way to address this is to perform impact assess-ment on the olive orchard arthropod fauna. Ants are the most abundant element in olive orchards and play an important role in olive grove soil fauna because they are biological indicators of the soil condition (Redolfi et al., 2005).

Several studies have shown the susceptibility of various ant species to EF (Santos et al., 2007a), but no works have studied ants in the habitat conditions that we are presenting here. *Tapinoma nigerrimum* Nylander is considered one of the most abundant ant species in olive groves (Redolfi et al., 1999). It is a generalist species used in activity pattern studies (Lopez et al., 1997; Redolfi et al., 2002), and it nests in the soil where EF is present. This species is considered omnivorous, collecting sugar secretions exuded by insects but also predating on some insects, some of which are pests (Morris et al., 2002).

The aim of this study was to determine the side effect of soil treatment with *Metarhizium anisopliae* (Metsch.) Sorok. and *Beauveria bassiana* (Balsamo) Vuill. on soil dwelling non-target arthropods at a commercial olive orchard, with an emphasis on ants. This paper describes pre- and post-contact responses of ants to EF in terms of activity as well as their mortality and fungi transmission potential.

2. Materials and methods

2.1. Fungal strains and cultivation

Metarhizium anisopliae strain EAMa 01/58-Su from the culture collection at the Department of Agricultural and Forestry Sciences and Resources (AFSR) of the University of Cordoba (Spain) was originally isolated from the soil of a wheat crop at Hinojosa del Duque (Cordoba, Spain). *Beauveria bassiana* strain EABb 01/103-Su was isolated from a forest soil in Sevilla (Spain). *Metarhizium anisopliae* EAMa 01/58-Su strain was selected for evaluating the effect of soil treatment on soil dwelling non-target arthropods while the combination of the *M. anisopliae* strain and the *B. bassiana* EABb 01/103-Su strain were used for studying the effects of soil treatments on *Tapinoma nigerrimum* colonies under laboratory conditions. These strains were selected because they have been shown in our previous work to be the most virulent against both adult and puparia of *C. capitata* and *B. oleae* (Quesada-Moraga et al., 2006; Garrido-Jurado, 2008). Slant monoconidial cultures of the strains were grown on Malt Agar (MA) at 25 ºC in darkness, then were lyophilized in the LyoQuest laboratory freeze dryer (Telstar, Barcelona, Spain), and stored at -80 ºC (Humber, 1996).

Both strains were cultured on rice in polypropylene bags. After fungal growth, conidia were collected by sieving and suspended in sterile aqueous 0.1% Tween 80 solution. The conidial concentration was quantified in a Malassez chamber and adjusted to 1.0 x 10^8 conidia ml^{-1}. The viability of the conidia was verified before the preparation of suspensions by germinating tests in liquid Czapek-Dox broth plus 1% (w/v) yeast extract medium. In all the experiments, germination rates were higher than 90%.

2.2. Effects of field soil treatment on soil dwelling non-target arthropods

2.2.1. Experimental site

The study was conducted at Antequera, Málaga (37º 01'N, 4º 41'W) on an organic olive orchard with spontaneous plant cover in July and August 2007 and

2009 with the same plots in both years. The mean air temperatures were 24.0 ºC in 2007 and 27.5 ºC in 2009, while the soil temperature was constant for both years (the temperature ranged from 26–29ºC). The olive orchard was 400 ha with trees spaced 10 m x 10 m and contained the olive varieties Picual, Hojiblanca, Arbequina and Cornicabra. The study site was divided into two square 1 ha sub-fields approximately 400 m apart. One sub-field was the fungi treated plot and the other was the control plot (water treated). Each plot has 100 drip-irrigated olive cv. 'Hojiblanca'.

2.2.2. Monitoring of fungus persistence

The soil beneath the canopy of the trees was sprayed with a suspension of conidia of *M. anisopliae* EAMa 01/58-Su strain at the rate of 2.5 x 10^{11} conidia per ha during the second week of June in both years. Before fungal treatment, 10 completely randomized soil samples were collected to determine the natural presence of entomopathogenic fungi in the soil. Finally, the *Galleria* Bait Method was applied to each soil sample to isolate entomopathogenic fungi (Zimmermann, 1986).

After spraying with the treatment, 10 trees were randomly selected to evaluate the inoculum presence in soil. For this purpose soil samples were collected beneath the canopy following the same procedure as mentioned above at 0, 7, 14, 21, 28, 35, 42, 49 and 56 days after treatment was applied. To assess the conidial density in each sample, the number of colony forming units (CFU) per gram of dry soil was determined by Sabouraud glucose agar chloramphenicol (0.5 g/l) in Petri dishes (Goettel and Inglis, 1996). One gram of the homogenized sample of soil was added to 9 ml of sterile distilled water, and shaken for 20–60 min. After homogenization, aliquots of 100 μl were spread onto the medium. In some cases, it was necessary to dilute the soil solution before spreading.

2.2.3. Collection of arthropods

Arthropod population in each sub-field was sampled using pitfall traps (75 mm diameter by 100 mm deep) every two weeks after the initial treatment, during July and August 2007 and 2009 with a total of four samplings per year. Twenty pitfall traps were randomly located on each sub-field. The traps were buried with the lip of the container flush with the soil surface. Each trap contained 125 ml soaped water to keep the insects inside. Traps were removed after 2 days and carried to the laboratory for counting and identification of insects. The insects were identified to the taxonomic level of order and the total number of each taxon recorded (Barrientos, 2004). However, the family Formicidae was identified to the taxonomic level of genus due to its abundance. Determination of possible fungal infection in the collected specimens was performed with a humid chamber. Before being placed in the humid chamber the arthropod specimens were washed off with sterilized water to remove soil particles. They were then placed in 1% sodium hypochlorite for 3 min and rinsed twice with sterilized water. After that the insects were placed on a damp sterilized filter paper in Petri dishes that were kept at 25 ºC. Fungi were prepared with lactophenol cotton blue and they were examined by contrast microscopy (magnification, 100x).

2.3. Effects of soil treatments on Tapinoma nigerrimum *colonies under laboratory conditions*

2.3.1. Sampling of ants

Twenty-eight nests of *Tapinoma nigerrimun* were

sampled in ''Arenales de San Pedro'' Granada (37º 18'N, 3º 39'W). More than 100 workers were captured per nest and nests were considered different when separated by more than 20 m.

Ants were transferred to plastic cages ringed with Fluon® to prevent their escape. They were maintained in these cages for one month at insectary conditions (25 ± 2 ºC, 60 ± 5% RH and 16 h/day length) to become acclimatized to the new environment. Honey was used as the standard diet twice a week, and lacewings larvae, aphids or *Ephestia kuehniella* (flour moth) eggs were provided once a week as a protein source.

The ants were placed in experimental nests to perform the bioassay. These ones consisted in two clear plastic cages linked by a plastic tube. The smaller cage was considered the artificial nest (Fig. 1) and was supplied with water. The bigger cage was considered the feeding chamber, like their external world, and it contained 150 g of soil (modified from Redolfi et al., 2002).

2.3.2. Experimental procedures

2.3.2.1. Pathogenicity of B. bassiana *and* M. anisopliae *against* T. nigerrimum. Ants from 20 different nests were exposed to the fungal entomopathogens *B. bassiana* and *M. anisopliae* (n = 10) in the experimental nests. Eight different nests served as the control. In each treatment group, the nests were treated with 13.5 ml of a 1.0 x 10^8 conidia/ml fungal suspension adjusted by diluting conidia with 0.1% aqueous solution of Tween 80. In the control group, the nests were treated with the same volume of a sterile aqueous solution of 0.1% Tween 80. The experiment was continued for 20 days. Dead ants were removed daily and they were place in humid chamber. They were then placed on sterile wet filter paper in sterile Petri dishes that were sealed with Parafilm® and kept at 25 ºC. They were later inspected for development of mycosis on the cadavers.

2.3.2.2. Activity of T. nigerrimum. Ant activity level is often an important factor when examining the effects of changes in ecosystems. These changes in spatial pattern resulting from altered behavior of ants are used as biological indicators. The activity of the ants was observed before and after the treatment. The actual number of ants going across the link tube of both sides of the experimental nest was observed for 5 min every 12 h. Activity measures taken place in the middle of the tube represented by a circle in Fig. 1. This method was continued for 11 days before the

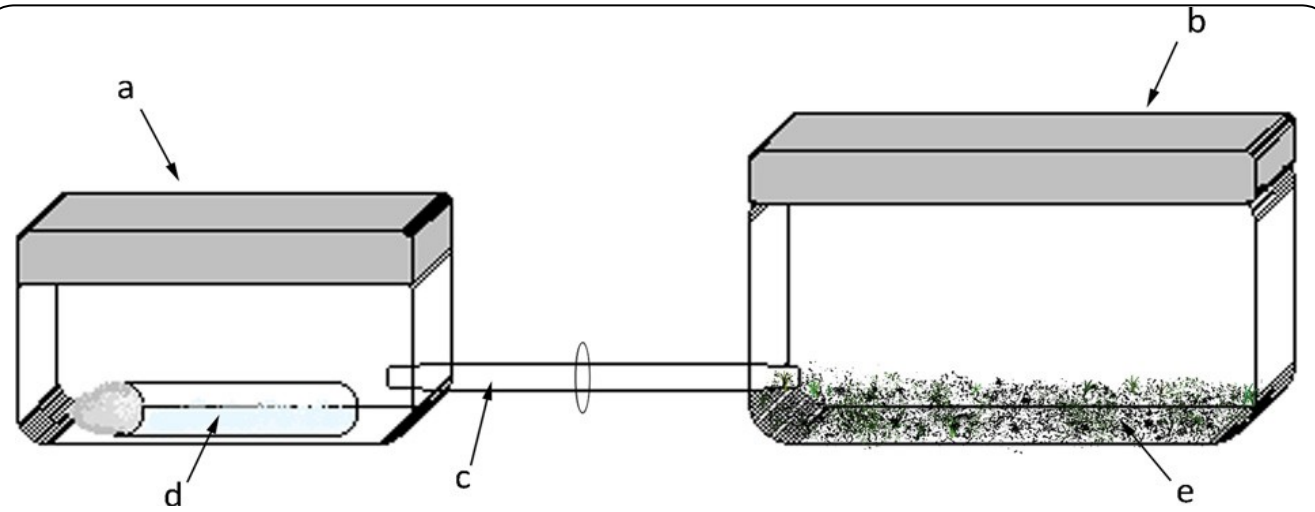

Fig. 1. Experimental nest (a) nest (80 x 150 x 80 mm), (b) feeding chamber (100 x 200 x 120 mm), (c) linker tube (80 x 10 mm), (d) water, and (e) 150 g soil

treatment and 11 days after the treatment.

2.3.2.3. Fungal dispersal by T. nigerrimum. The potential of these ants to distribute conidia from treated soil to non-treated soil was evaluated. An annex with sterile soil was linked to the treated experimental nests, and ants were allowed to cross from one side to the other. Soil samples from the non-treated nest were collected every 2 days and they were examined by the CFU method to determine the presence of fungi, as it was explained in 2.2.2.

2.4. Statistical analysis

Values of CFU g^{-1} soil were Log_{10} transformed make the data linear. After confirming that all data were normally distributed, a one-way analysis of covariance (ANCOVA) was performed to compare the linear regressions of the inoculum density decline in soil (Rumbos et al., 2008). The log transformation of CFU in 2009 was used as the dependent variable and the log transformation of CFU in 2007 was used as the covariate.

The Kruskal–Wallis one-way non-parametric AOV analysis for pitfall traps data in both treated and control fields was performed using the program Statistix 9.0 (Analytical software 2008). Likewise, the mortality data were analyzed using one-way analysis of variance (ANOVA) while Tukey's honest significant difference (HSD) test was used to compare means.

The cumulative mortality response across the assessment period was analyzed using the Kaplan–Meier survival analysis. A general linear model for repeated measurement was used to analyze the activity of *T. nigerrimum* before and after treatment. These analyses were performed using the SPSS 15.0 for Windows (SPSS Inc. 2006).

3. Results

3.1. Monitoring of fungus persistence

Two autochthonous fungal species were obtained prior to treatment through the *Galleria* Bait Method. While *B. bassiana* was obtained from all the analyzed soil samples, *M. anisopliae* was found only in one soil sample at 10^4 CFU g^{-1} of soil, coexisting with *B. bassiana*.

During both years of the study, EAMa 01/58-Su densities decreased over time after the treatment application (Fig. 2). The reduction in CFU numbers compared to initial densities ranged from 37.54% in 2007 to 28.11% in 2009, 56 days after the treatment application. In 2007, the regression coefficient describing the linear decline of EAMa 01/58-Su density over time was -0.0025 (Fig. 2). However, in 2009 the linear decline of the EAMa 01/58-Su density in the soil over time was described by the regression coefficient of -0.0022. The analysis of covariance revealed no significant differences ($F_{1,8} = 0.95$, $P = 0.362$) between the regression coefficients of the linear regressions describing the dynamics of EAMa 01/58-Su isolate in the different years of treatment.

3.2. Effects of field soil treatment on soil dwelling non-target arthropods

The total numbers of arthropods collected during each year of the study were 6766 and 5572 for 2007 and 2009, respectively. They were divided into 16 orders, 12 of which belonged to the Insecta class. Hymenoptera (particularly Formicidae) was the domi-

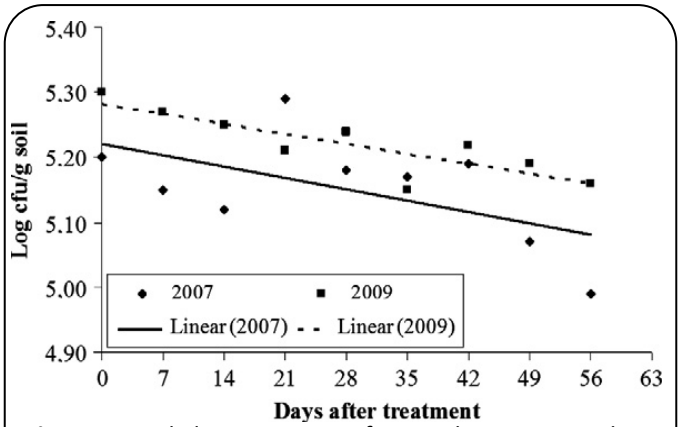

Fig. 2. Conidial persistence of *Metarhizium anisopliae* strain EAMa 01/58-Su after soil application in the olive orchard in years 2007 and 2009 over 56 days. The inoculum recovered is expressed as colony forming units (CFU)

nant taxa in terms of number of individuals from all subfields in both years. A total of 63.61% of all organisms captured in the first year and 88.48% in the second year belonged to this taxa. *Cataghyphis* and *Pheidole* account for 31.61–22.56% and 17.47–43.02%, respectively, of the Formicidae captures, followed by Messor with 13.82– 19.26% (Table 1).

The Acariformes, Cephalostigmata, Orthoptera, Embioptera, Blattodea, Mantodea, Thysanoptera, Neuroptera, and Lepidoptera orders were excluded from the statistical analysis due to the lower catches. These orders were found in very limited numbers and were therefore not considered representative.

For the number of captures, the Kruskal–Wallis one-way analysis showed a significant difference between the years of treatment ($H = 67.14$, $P < 0.001$). A decrease in total number of individuals (from 6766 to 5572) was observed during the second year throughout the entire experimental field.

Furthermore, there were significant differences between treatments (2007: $H = 8.03$, $P = 0.004$ and 2009:$H = 7.17$, $P = 0.007$). In both years, the total number of individuals caught in the fungal treatment sub-field was higher than the total number of individuals caught in the control sub-field. There were significant differences between the orders trapped each year ($H = 93.52$, $P < 0.001$ in 2007 and $H = 84.11$, $P < 0.001$ in 2009). In 2007, one dominant order in addition to ants was Isopoda. A total of 27.5 and 52.9 individuals were captured per trap for the control and treated subfield, respectively. The remaining orders

Table 1. Pitfall captures of arthropods taxa from an organic olive orchard at Antequera (Málaga)

Taxa collected			2007			2009		
Order	Family	Genus	Captures per trap		Percent total of captures	Captures per trap		Percent total of captures
			Control	Treated		Control	Treated	
Araneae			4.0	5.8	2.90	3.1	4.3	2.64
Acariformes			0.1	0.7	0.22	1.0	1.4	0.84
Isopoda			27.5	52.9	23.74	0.4	2.0	0.86
Cephalostigmata			0.0	0.1	0.01	0.0	0.1	0.02
Orthoptera			0.6	0.5	0.33	0.5	0.2	0.23
Embioptera			0.1	0.0	0.03	0.1	0.1	0.04
Blattodea			1.2	2.6	1.11	0.3	1.0	0.45
Mantodea			0.2	0.1	0.06	0.0	0.0	0.00
Isoptera			0.0	0.0	0.00	0.3	0.0	0.09
Hemiptera			1.9	2.8	1.39	3.3	6.5	3.50
Thysanoptera			0.1	0.4	0.13	0.0	0.0	0.00
Neuroptera			0.0	0.1	0.01	0.1	0.2	0.07
Lepidoptera			0.3	0. 5	0.21	0.2	0.1	0.07
Diptera			2.9	6.7	2.82	1.9	1.7	1.26
Hymenoptera			80.8	141.1	65.58	114.8	133.4	89.05
	Formicidae		77.7	137.6	63.61	113.9	132.6	88.48
		Messor	16.0	30.8	13.82	16.3	37.4	19.26
		Plagiolepis	1.1	1.0	0.55	3.7	2.0	2.05
		Cataglyphis	41.1	65.9	31.61	21.6	41.3	22.56
		Pheidole	19.4	39.7	17.47	70.6	49.3	43.02
		Camponotus	0.3	0.0	0.07	0.0	0.0	0.00
		Tapinoma	0.0	0.0	0.00	1.5	1.3	0.97
		Crematogaster	0.1	0.2	0.09	0.3	1.5	0.63
Coleoptera			2.7	2.3	1.46	0.7	1.8	0.88

Classification according to Hickman et al., 1999

used for the statistical analysis appeared infrequently. Only 1.9–4.0 individuals per trap were captured in the control and 2.3–6.7 in the treated sub-field. Two dominant taxa (>3.0 individuals per trap in both treatments) appeared throughout both experimental subfields during the second year. These were Araneae and Hemiptera.

The Formicidae family was analyzed separately because of the higher catch. Only *Messor*, *Plagiolepis*, *Cataglyphis* and *Pheidole* genera were considered for this analysis as the catches of the remainder were erratic. There were significant differences in the Kruskal – Wallis one-way analysis between the years ($H = 5.23$, $P < 0.001$). In 2007, a total of 4293 ants were collected throughout all the experimental subfields. However, an increase in the total number of ants (4841) was observed during the second year.

Furthermore, there were significant differences between the treatments in 2007 ($H = 7.03$, $P = 0.008$). The total number of ants collected in the fungal treatment sub-field was higher than the total number of ants collected in the control sub-field. There were significant differences between the genera trapped each year (2007: $H = 99.65$, $P < 0.001$ and 2009: $H = 120.03$, $P < 0.001$). In 2007, one genus was dominant, which was *Cataglyphis* with 41.1 individuals per trap in the control and 65.9 in the treated sub-field. During 2007, the remaining genus appeared at minor frequencies. In 2009, *Pheidole* was the dominant genus with 70.6 ants per trap in the control and 49.3 in the treated sub-field. As a result of the high presence of this genus there were no significant differences between the control and the treated sub-fields.

3.3. Effects of soil treatments on Tapinoma nigerrimum *colonies under laboratory conditions*

3.3.1. Pathogenicity of B. bassiana *and* M. anisopliae *against* T. nigerrimum

No significant differences were found on workers mortality between fungal treatments and control (Table 2, $F_{2,27} = 2.34$, $P = 0.13$). The mean values ranged between 41.1% and 64.7%. The average survival times (AST) of each treatment are shown in Table 2. No significant reduction in AST was observed between treatments (from 16.9 days in control to 15.6 days in treatment with *B. bassiana* and 14.8 days in treatment with *M. anisopliae*). The fungal treatment did not cause a significant reduction of the survival ratio of ants when compared to the control (Fig. 3). The three curves progress very similarly showing a decrease between 10 and 13 days.

3.3.2. Activity of T. nigerrimum

In both controls and fungally challenged ants, significant differences were detected in their activity before and after treatment ($F_{1,25} = 18.07$, $P < 0.001$), with an activity decrease over time. The workers through the linker tube counted before the treatment for control, *B. bassiana* isolate and *M. anisopliae* isolate were

Table 2. Pathogenicity of *Beauveria bassiana* and *Metarhizium anisopliae* to *Tapinoma nigerrimum* workers

		Kaplan-Meier survival análisis	
	Mortality[a], % (mean ± SE)	**AST[b], days (mean ± SE)**	**95% CI**
Control[c]	41.1 ± 8.8 a	16.9 ± 0.2 a	16.5-17.3
EABb 01/103-Su[c]	48.6 ± 8.5 a	15.1 ± 0.3 a	14.6-15.6
EAMa 01/58-Su[c]	64.7 ± 9.3 a	14.8 ± 0.3 a	14.3-15.3

[a] Data in the same column followed by the same letter are not significantly different (a = 0.05) according to the Tukey's HSD test. [b] Average survival time (AST) limited to 20 d.

[b] Data in the same column followed by the same letter are not significantly different (a = 0.05) according to the log-rank test.

[c]Control: 8 replicates with 100 ants per replicate. EABb 01/103-Su and EAMa 01/58-Su: 10 replicates with 100 ants per replicate.

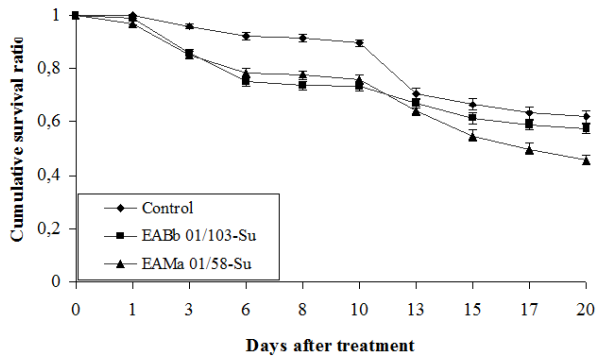

Fig.3. Cumulative survival ratio (mean ± SE) of *Tapinoma nigerrimum* adults exposed to soil treated with *Beauveria bassiana* (EABb 01/103-Su) and *Metarhizium anisopliae* (EAMa 01/58-Su) or untreated soil

4.3, 3.9 and 4.8, while after treatment were 3.6, 4.1 and 3.5 respectively. Nonetheless, the fungal treatment had no detectable effect on ants ($F_{2,25}$ = 0.256, P = 0.78).

3.3.3. Fungal dispersal by T. nigerrimum

Soil samples from the annex artificial nests were collected and examined by the CFU method to determine the presence of fungi. In all plates, the absence of EF was observed. This finding was caused by a lack of inoculum dispersal by the ants.

4. Discussion

The pitfall collections represented a broad diversity of olive orchard dwelling arthropods. The olive grove supported an abundant group of edaphic arthropods that was numerically dominated by ants. In accordance with previously reported results, the composition of soil fauna in the Iberian Peninsula is similar to that obtained in our study with the most abundant taxa being Formicidae (Morris and Campos, 1999; Santos et al., 2007b). All ants genera collected are frequently found in olive orchards nesting under the tree canopy or exclusively in open zones (Redolfi et al., 1999). The results of the two applications to the same field plot showed that the EAMa 01/58-Su isolate, which have been recovered up to 10^5 CFU g^{-1} soil during the experiment, did not have a significant effect on the arthropods as there were more individuals collected in the treatment subfield than in the control subfield during both years. Furthermore, there were no infected individuals. Parker et al. (1997) performed a *B. bassiana* soil application and obtained 5% infected individuals. However, it was not possible to confirm whether the infected arthropods were a result of the treatment or due to the natural presence of *B. bassiana* in that forest soil.

The pathogenicity of EF against ants was performed with the *T. nigerrimum* species. Although it is considered one of the most abundant olive ants (Morris and Campos, 1999; Redolfi et al., 1999), it was not found into pitfall traps, due to seasonality of this species (Retana et al., 1990); in Southern Spain is mainly found in spring time while the experiment was performed in summer. Several species of ants are susceptible to EF (Siebeneicher et al., 1992; Rodrigues et al., 2010), but no effects were detected for any of the fungi evaluated under the conditions of this study. Ants have evolved many effective defense mechanisms against pathogens. One of these common defenses used by ants is grooming. Ants spend more time on grooming (self and allo-grooming) in the presence of fungi as they remove the conidia directly from the exoskeleton (Siebeneicher et al., 1992; Richard and Errard, 2009; Reber et al., 2011). Furthermore, entomopathogenic fungi can be inhibited by antimicrobial secretions of the ants' metapleural and mandibular glands (Bot et al., 2002; Rodrigues et al., 2009). Beattie et al. (1986) suggested that these secretions are not decisive in the survival of the host while the innate immune system or inducible antibiotic peptides in the haemolymph are key factors im-

pairing the fungal infection. Ants are known to be able to carry conidia of EF on the cuticle or infrabuccal pellets (Bird et al., 2004; Pagnocca et al., 2008), which is an important factor in the dispersal of this fungi. A lack of significant interaction between ants and EF could be attributed to the previously mentioned mechanisms. However, it was previously reported as an antagonist of non-sterile soil (Pereira et al., 1993). Soil antagonism against EF was further evidenced by the lack of development of the fungus on fire ants killed (Pereira et al., 1993) or in soil (Siebeneicher et al., 1992). This antagonism may affect conidia in the soil by inhibiting germination and penetration to insects. Based on the lack of infection among the trapped arthropods and the low level of infection among treated nests, our results suggest that these EF could be applied to olive orchard soil without a significant negative direct or indirect impact on the soil dwelling arthropod population.

5. Acknowledgments

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CAPÍTULO IX

DISCUSIÓN

Discusión

A lo largo de los distintos capítulos de esta Tesis Doctoral se ha puesto de manifiesto el potencial de los ascomicetos mitospóricos entomopatógenos (AME), *Beauveria bassiana* y *Metarhizium anisopliae*, como agentes de control de estados pre-imaginales de dos tefrítidos de gran importancia económica en España, *Ceratitis capitata* o mosca mediterránea de la fruta y *Bactrocera oleae* o mosca del olivo. Hay que destacar que el éxito de estos agentes de control microbiano depende de su virulencia probada frente al fitófago y de su "competencia ambiental" (Jackson y O'Callaham, 1997), que determina la presencia en el medio de cantidades de inóculo suficientes para ejercer el máximo poder insecticida (Ekesi et al., 2007). Los resultados de esta Tesis Doctoral pueden permitir el desarrollo de una estrategia de control de tefrítidos mediante tratamientos de suelo de estados pre-imaginales con AME, pues en ella se constata su presencia natural en el suelo, su virulencia frente al estado diana, su competencia ambiental, e incluso información relevante para optimizar el momento de la aplicación.

Para ello, en el principio de esta andadura decidimos seleccionar 57 aislados de *B. bassiana* de la micoteca de nuestro Grupo de Investigación con distintos orígenes geográficos y hábitats de aislamiento (suelo/ insecto), a los que sometimos a análisis filogenéticos de modelos de inserción de intrones y factor de elongación EF1-α. El estudio comparativo de las secuencias de nucleótidos de los genes que codifican para ARNr permite analizar relaciones filogenéticas en un amplio rango de niveles taxonómicos (Márquez et al., 2006). De hecho, diversos trabajos de caracterización de los géneros *Beauveria* y *Metarhizium* se centran en el estudio de los intrones del grupo I de los genes SSU o LSU del ADNr (Neuveglise et al., 1997; Wang et al., 2003). Sin embargo, la identificación de aislados a nivel de especie es más eficiente empleando el factor de elongación EF1-a (Rehner et al., 2011). La secuenciación del extremo 3' de la LSU de los distintos aislados evaluados de *B. bassiana* en esta Tesis Doctoral, ha mostrado sólo 3 de los 4 posibles patrones de inserción de intrones descritos previamente con 125 aislados de la misma especie fúngica (Wang et al., 2003), debido quizás al menor número de los mismos empleados en nuestro estudio. Mientras que el análisis con el EF1-α muestra cinco y tres subgrupos filogenéticos para *B. bassiana* y *B. pseudobassiana*, respectivamente. El estudio conjunto de ambas distribuciones filogenéticas nos ha permitido demostrar el origen monofilético y la transmisión vertical de los intrones insertados en la posición 4. En general, no hemos encontrado correlaciones entre los datos moleculares y los insectos hospedantes, aunque si una tenue correlación con su origen geográfico.

Esta gran diversidad filogenética de los aislados españoles de *B. bassiana* podría deberse no solo a que han sido obtenidos a partir de insectos o del suelo, sino que los obtenidos de suelo, la mayoría, proceden de distintos agroecosistemas y regiones geográficas. Las condiciones del medio determinan en gran medida la presencia, diversidad, virulencia etc., de los AME (Jaronski, 2010), por lo que su empleo satisfactorio en el suelo depende del conocimiento de su comportamiento en el mismo.

En el capítulo 5 se pone de manifiesto la importancia de la textura del suelo y el pH sobre la eficacia de las especies *B. bassiana* y *M. anisopliae* para el control de estados pre-imaginales de *C. capitata*. Ambas especies fúngicas presentan comportamientos

diferentes para las distintas texturas y pH del suelo, tanto en el ensayo de adsorción y arrastre como en el de columnas de suelo. Dichos experimentos revelan que los conidios de *B. bassiana* quedan retenidos en el complejo de cambio de las arcillas, lo que puede deberse a la gran diversidad de carbohidratos presentes en la superficie de estos propágulos infectivos, que pueden formar puentes de hidrógeno con otras superficies hidrofóbicas o hidrofílicas como las arcillas (Holder y Keyhani, 2005; Wanchoo et al., 2009). Sin embargo, conidios más hidrofóbicos y de mayor tamaño como los de *M. anisopliae* (Ignoffo et al., 1977; Jeffs et al., 1999) no parecen mostrar esta asociación con ellas. Además, hemos constatado que la capa superficial de suelo mantiene entre el 70-90% de los conidios aplicados, en función de la textura, pues los suelos arcillosos retienen más conidios que los arenosos. Pero, se ha podido ir más allá al comprobar que la bajada de pH en el suelo producido por la adición de cloruro cálcico ($CaCl_2$) afecta a la retención de conidios, en especial en los suelos alcalinos, donde esta se incrementa al intercambiar H^+ con el Ca^{2+} de la solución. Estos datos, con base en un conocimiento previo de la textura y pH del suelo, son determinantes para optimizar las aplicaciones de AME al mismo, tanto la concentración de inóculo necesaria como el número de aplicaciones.

Los ensayos de patogeneicidad realizados con las especies *B. bassiana* y *M. anisopliae* frente a larvas de tercera edad próximas a pupación de *C. capitata* revelan que textura y pH no tienen efecto sobre la infectividad de los conidios, sin embargo, la temperatura y la humedad del suelo si son determinantes sobre la misma. En este sentido, destacamos que cada aislado evaluado responde de manera diferente a estos dos nuevos factores. En general, los aislados de *M. anisopliae* proporcionaron valores mayores de mortalidad y de micosis que los de *B. bassiana*. Para ambas especies, la virulencia frente a estados pre-imaginales de *C. capitata* guardó una relación en forma de parábola invertida tanto con la humedad, con un descenso en condiciones extremas (1.0 y 17.0% p:p), como con la temperatura, con valores máximos a temperaturas cercanas a los 15.0-20.0ºC. No obstante, el aislado EAMa 01/158-Su mostró una pauta de comportamiento linear y positiva, también extensiva a la temperatura.

La aplicación al suelo del inóculo fúngico para el control de los estados pre-imaginales de *C. capitata* y otros tefrítidos como la mosca del olivo debe realizarse antes de que se produzca la caída al suelo de la larva de tercera edad próxima a pupación, aunque también se ha puesto de manifiesto la eficacia de la estrategia cuando ocurre la salida de los adultos, que se impregnan con los conidios en el suelo. Este escenario requiere un correcto conocimiento de la biología del tefrítido en el suelo, para poder predecir la salida final del adulto, y optimizar así el momento de esta segunda aplicación. En este trabajo se aplica por primera vez un modelo paramétrico lognormal de supervivencia al desarrollo de un insecto, en este caso para relacionar el efecto de la temperatura y humedad del suelo sobre la duración y supervivencia de la fase pre-imaginal de *C. capitata*. Nuestros datos revelan que el desarrollo completo en el suelo de la fase pre-imaginal de *C. capitata* hasta la emergencia del adulto está influenciado en gran medida por la temperatura y humedad del suelo. El incremento de ambos factores se traduce en un aumento del porcentaje de individuos de *C. capitata* que alcanzan el estado adulto, que es máximo a 23.5ºC y 7.0% p:p, 91.57% de emergencia, condiciones en las que también se observan los tiempos de desarrollo más cortos para el 25, 50, 75 y 90% de insectos

que llegan a completarlo, con 8.0, 12.4, 19.1 y 28.2 días, respectivamente. .

Tanto la investigación incluida en el capítulo 6, como la del capítulo 7 parecen reforzar la hipótesis ya planteada en trabajos anteriores (Quesada-Moraga et al., 2006; Eldesouki-Arafat, 2007), de que la baja susceptibilidad de estados pre-imaginales de *C. capitata* a AME en condiciones de alta humedad en el suelo puede ser debida bien a su efecto sobre la viabilidad y adherencia de los conidios a la cutícula, bien a su mayor retención por las partículas del mismo, o bien a su efecto indirecto sobre la susceptibilidad del hospedante. Tanto la regresión logística múltiple aplicada en el capítulo 6 para evaluar el efecto de humedad y temperatura sobre la virulencia de AME frente a pre-imaginales de *C. capitata*, como el modelo paramétrico lognormal de supervivencia aplicado al desarrollo pre-imaginal de *C. capitata*, suponen un gran avance en el desarrollo de herramientas estadísticas necesarias para la predicción y planificación de calendarios de aplicación de AME frente a tefrítidos.

La última parada en la senda trazada por esta Tesis Doctoral se encuentra en la evaluación del efecto de tratamientos de suelo con AME para el control de pre-imaginales de *B. oleae* sobre la artropofauna edáfica no diana en una plantación comercial de olivo. Pudimos comprobar que la aplicación en campo del aislado EAMa 01/58-Su no afectó a la artropofauna ni en abundancia ni en diversidad, mientras que los niveles de inóculo durante el experimento se mantuvieron en torno a 10^5 CFU por gramo de suelo, 100 veces superiores a los niveles naturales (Scheepmaker y Butt, 2010). De acuerdo con la legislación europea existente (Directiva 91/414/EEC del Consejo relativa a la comercialización de productos fitosanitarios; Directiva 2001/36/EC que modifica la anterior en los anexos II y III en lo que se refiere a documentación e información de una materia activa o producto fitosanitario a base de microorganismos; Directiva 2005/25/EC que modifica a la primera en el anexo VI en lo que se refiere a los productos fitosanitarios que contienen microorganismos) estos niveles de inóculo en el suelo serían aceptables al no suponer un problema en la artropofauna. En este capítulo, hemos prestado especial atención a las hormigas, por considerarse elementos bioindicadores en diferentes ecosistemas. Los ensayos realizados con las especies fúngicas *B. bassiana* y *M. anisopliae* sobre *Tapinoma nigerrimum* en laboratorio, revelan que no se produce ningún efecto de estos AME sobre esta especie de hormigas . Aunque otras especies si han mostrado susceptibilidad a AME (Siebeneicher et al., 1992; Rodrigues et al., 2010), *T. nigerrimum*, que es una de las más abundantes en el olivar del sur de España, no se vio afectada. Tanto la mortalidad como los tiempos medios de supervivencia de los distintos tratamientos con AME no difirieron del testigo, además de mantenerse similar entre los tratamientos la actividad del hormiguero antes y después de la aplicación de los AME. Por último, comprobamos que estas hormigas no dispersan el inóculo, lo que favorece la aplicación localizada que se pretende con estos micoinsecticidas en campo.

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CAPÍTULO X

CONCLUSIONES

Conclusiones

A lo largo de los distintos capítulos de esta Tesis Doctoral se han obtenido una serie de conclusiones que se enumeran de forma resumida a continuación:

1. Los aislados autóctonos de *Beauveria bassiana* utilizados en el estudio presentan una elevada diversidad genética entre sí, aunque la mayoría de ellos se sitúan dentro de un subgrupo filogenético más amplio con representantes de todo el mundo. En general, no se observa relación entre la distribución molecular en base a su modelo de inserción de intrones y el factor de elongación EF1-α, y el origen geográfico y características climáticas de los ecosistemas muestreados.

2. Los factores abióticos, textura y pH del suelo, influyen en la disponibilidad y el movimiento de los conidios de *B. bassiana* y *Metarhizium anisopliae* en el mismo, pero no sobre su virulencia frente a estados pre-imaginales de *Ceratitis capitata*. Los conidios *B. bassiana* y *M. anisopliae* quedan retenidos en suelos arcillosos o arenosos, respectivamente, en cuyo caso disminuye su disponibilidad. En ambas especies, el 90% de los conidios quedan retenidos en la capa superficial del suelo, con diferencias en función de la textura del mismo y en relación directa con la fracción arcilla. Así, la acidificación del suelo favorece la retención de conidios, en especial cuando el suelo es alcalino.

3. En general, los tratamientos de suelo con *M. anisopliae* fueron más efectivos para el control de estados pre-imaginales de *Ceratitis capitata* que los realizados con *B. bassiana* en las distintas condiciones de humedad y temperatura. No obstante, el modelo de regresión logística múltiple aplicado para determinar los efectos de estos factores abióticos sobre la eficacia de cada aislado en el suelo reveló diferencias de comportamiento inter e intraespecíficas. La mortalidad debida al aislado EAMa 01/158-Su de *M. anisopliae* mantuvo una relación directa con la temperatura y la humedad del suelo, mientras que el aislado EAMa 01/58-Su de esta especie, y los aislados EABb 01/33-Su y Bb-1333 de *B. bassiana*, se mostraron menos virulentos en condiciones de humedad extrema (1.0 y 17.0% (p:p)) y temperaturas superiores a 20.0ºC.

4. El modelo paramétrico lognormal de supervivencia aplicado para modelizar el desarrollo pre-imaginal de *C. capitata* en el suelo mostró que el máximo porcentaje de individuos que alcanzan el estado adulto (91.57%) se produce a 23.5ºC y 7.0% (p:p) de humedad. A estas mismas condiciones se producen los tiempos de desarrollo más cortos para el 25, 50, 75 y 90% de los insectos que alcanzan el estado adulto, con 8.0, 12.4, 19.1 y 28.2 días, respectivamente.

5. Los tratamientos de suelo con el aislado EAMa 01/58-Su de *M. anisopliae* en un olivar comercial no tuvieron efecto significativo ni en la abundancia ni en la diversidad de la artropofauna edáfica del mismo, con niveles de inóculo en el suelo por debajo del umbral que refleja la normativa europea vigente. En el laboratorio, la aplicación al suelo de este aislado y del EABb 01/103-Su de *B. bassiana* no tuvo un efecto significativo ni en la mortalidad ni en la actividad de comunidades de hormigas *Tapinoma nigerrimum*. Estas hormigas ni siquiera mostraron capacidad para dispersar el inóculo fúngico.