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Departamento de Ciencias y Recursos Argícolas y Forestales

Los hongos entomopatógenos y sus extractos en el control de la mosca del olivo *Bactrocera oleae* (Diptera: Tephritidae)

Tesis presentada por D. **Meelad Yousef Naef** para optar al grado de Doctor Ingeniero Agrónomo

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TITULO: *Los hongos entomopatógenos y sus extractos en el control de la mosca del olivo Bactrocera oleae (Diptera: Tephritidae)*

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INFORMAN: que el trabajo **"Los hongos entomopatógenos y sus extractos en el control de la mosca del olivo *Bactrocera oleae* (Diptera: Tephritidae)"** realizado bajo nuestra dirección por el ingeniero agrónomo **D. Meelad Yousef Naef**, lo consideramos ya finalizado y puede ser presentado para su exposición y defensa como Tesis Doctoral en el Departamento de Ciencias y Recursos Agrícolas y Forestales de la Universidad de Córdoba.

Córdoba, junio de 2016

Fdo.: Prof. Dr. D. **Enrique Quesada Moraga**

Fdo.: Dra. Dña. **Inmaculada Garrido Jurado**

A todo aquel que prometió y cumplió

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Resumen

La mosca del olivo *Bactrocera oleae* es el fitófago más destructivo para el olivar por atacar al fruto, parte cosechable del cultivo, lo que origina graves pérdidas de cantidad de la producción, pero más grave, también en su calidad, donde este díptero constituye la principal amenaza biótica. Hoy en día el control de esta mosca es un verdadero desafío, tanto por la escasez de materias activas insecticidas disponibles, como por las primeras citas de aparición de resistencia a las mismas. Además, los efectos negativos de los insecticidas químicos sobre el medioambiente y los seres vivos, que han impulsado la normativa reguladora de la Unión Europea que emana a todos los estados miembros, promulgan los métodos no químicos de control de plagas, con énfasis en los bioinsecticidas. Dentro de ellos, los hongos entomopatógenos presentan gran potencial para ser usados dentro de los programas de control integrado de la mosca del olivo. Su presencia natural en el agroecosistema del olivar, su modo de acción por contacto, y su capacidad de secretar nuevas moléculas con actividad insecticida, son factores que les sitúan en primera línea mundial para el desarrollo de estrategias alternativas de control para la mosca del olivo, lo que se ha convertido en el objetivo clave de esta tesis doctoral, la evaluación de los hongos entomopatógenos y sus extractos en el control de este temible tefrítido.

El capítulo III revela la actividad insecticida *per os* de un extracto crudo de un hongo entomopatógeno frente a adultos de la mosca del olivo. El extracto crudo de la cepa EAMb 09/01-Su de *Metarhizium brunneum* causó un 80,0 % de mortalidad con 27,7 h de tiempo medio de supervivencia (TMS), concentración letal media (CL_{50}) del 49,98 % del extracto crudo, y tiempo medio de exposición de 12,26 h, con una elevada termo estabilidad y fotorresistencia. En el capítulo IV, se ha llevado a cabo un proceso de optimización de la producción del extracto crudo de la cepa EAMb 09/01-Su, utilizando como modelo la mosca de la fruta, *Ceratitis capitata* (Wied.). El extracto crudo mostró la mayor actividad después de 15 días de fermentación, con un 73,3 % de mortalidad y 25,5 horas de TMS. El pH inicial óptimo para una máxima actividad de extracto crudo en términos de mortalidad osciló entre 7 y 9. A lo anterior se le añade la compatibilidad entre el hongo entomopatógeno y su propio extracto crudo, ya que todas las

combinaciones de la suspensión fúngica con el extracto crudo tuvieron un efecto aditivo sobre la mortalidad de adultos.

El capítulo V pone de manifiesto que los tratamientos de suelo bajo la copa del olivo con la cepa EAMa 01/58-Su de *M. brunneum* dirigidos a larvas *B. oleae* de tercera edad próximas a pupación, presentan un gran potencial para reducir la población de primavera del tefrítido, aspecto muy importante dentro de un programa para su control integrado. El hongo demostró capacidad de persistir en el suelo durante el desarrollo del experimento, con concentraciones iniciales de 1.0×10^5-3.0×10^5 conidios por g de suelo, y finales que oscilaron entre $0,8 \times 10^2$ y $1,1 \times 10^3$ conidios por g de suelo. En todas las campañas del tratamiento, la densidad de la población de *B. oleae* procedente del suelo en parcelas tratadas fue un 50-70 % inferior a la de las parcelas control. Finalmente, en el capítulo VI se pone de manifiesto, tanto *in vivo* como *in vitro*, la alta compatibilidad entre la cepa EAMa 01/58-Su de *M. brunneum* y los herbicidas autorizados en el Reglamento de Producción Integrada para su uso en suelos de olivar, en especial oxifluorfeno al 24,0 %, tanto en términos de mayor crecimiento (3,3 mm por día), mortalidad de pupas (80,0 %), y mayor número de conidios viables en el suelo después del tratamiento ($1,0 \times 10^5$ conidios por g de suelo), lo que permitiría su aplicación simultanea con la cepa fúngica, aspecto clave para reducir los costes de tratamiento.

Abstract

Nowadays, there is increasing concern over dependence on chemical pesticides for olive fly *Bactrocera oleae* (Rossi) control, and a global trend to find new methods for its management. The progress of olive fly control programs achieved in recent years consisted of exploit bioinsecticides, with emphasis in those developed with base in entomopathogenic organisms, particularly mitosporic entomopathogenic fungi. These fungi infect the host via the exoskeleton, a unique mode of action among entomopathogens, which makes them an attractive alternative to chemicals for the control of this tephritid pest. Besides, they are naturally present in the olive agroecosystem, their mass production and formulation is relatively easy and they may secrete natural insecticidal compounds, all factors putting them at the forefront of the global development of alternative control strategies of *B. oleae*, key objective of the present work, investigating the potential of entomopathogenic fungi and their insecticidal compounds for the control of this key pest.

Chapter III reveals *per os* insecticidal activity of an entomopathogenic fungal extract from an entomopathogenic fungus against *B. oleae*. The crude extract of *Metarhizium brunneum* EAMb 09/01-Su strain caused 80.0 % adult mortality when administered *per os*, with average survival time (AST) of 27.7 h, LC_{50} of 49.98 % of the crude extract, and exposure time required to achieve 50 % mortality of 12.26 h. In addition, the crude extract of the strain EAMb 09/01-Su was demonstrated to be quite thermostable and photoresistant. Indeed, in chapter IV, EAMb 09/01-Su strain crude extract production is optimized in terms of fermentation period and starting pH conditions, using medfly *Ceratitis capitata* (Wied.) as model. Fermentation time significantly affected the *per os* insecticidal activity of the crude extract when it was provided to *C. capitata per os,* with the highest mortality rate (73.3 %) and the shortest AST value (25.5 h) obtained from 15-d-old cultures. The optimum initial pH for maximum crude extract activity in terms of mortality ranged between 7 and 9. Lastly, an additive effect was observed for all *M. brunneum* EAMb 09/01-Su strain suspension and crude extract combinations tested, indicating compatibility between the strain and its crude extract.

In chapter V, a long-term field experiment was conducted, with an experimental mycoinsecticide produced using the *M. brunneum* EAMa 01/58-Su strain and evaluated in selected farms in Andalusia (Spain) over 4 years. In each season, the field experiments lasted for 4-5 months, with two applications of the mycoinsecticide beneath the tree canopy at a rate of 1.0×10^5-3.0×10^5 conidia g soil^{-1}, the first one in late fall, when prepupariating larvae typically fall from the olive fruit to the soil, and the second one in early spring, when adult emergence from the soil is expected. After each application, the fungal inoculum reached basal levels varying from 0.8×10^2 to 1.1×10^3 conidia g soil^{-1}. During the four seasons, a 50-70 % reduction of adult olive fruit fly populations was observed in the treated plots compared with the untreated ones, which could help in minimizing the number of treatments, if any, within an olive fruit fly IPM strategy.

Finally, chapter VI reveals the *in vitro* and *in vivo* compatibility of the six most common herbicides applied to the soil of olive orchards with the *Metarhizium brunneum* strain EAMa 01/58-Su for controlling *B. oleae* preimaginals, with emphasis in 24.0 % oxyfluorfen, in terms of maximum growth rates in malt agar (3.3 mm d^{-1}), virulence against prepupariating third instars (80.0), and conidia viability in the treated soil (1.0 x 10^5 conidia g soil^{-1}), which could allow simultaneous application of both the fungal suspension and the herbicide therefore reducing the pesticide treatment cost.

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REVISIÓN

1. El cultivo del olivo en el mundo y en España

El olivo, *Olea europaea* L., pertenece a la familia botánica Oleaceae, que comprende especies distribuidas por las regiones tropicales y templadas del mundo. Hay unas 35 especies del genero *Olea*, si bien todos los olivos cultivados pertenecen a la especie *O. europaea L.* (var. *europaea*) y los acebuches u olivos silvestres a *O. europaea L.* (var. sylvestris) (Rapoport 2008). *O. europaea* es la única especie de la familia Oleaceae con fruto comestible. Es una de las plantas más antiguas, cuyos orígenes como cultivo podrían remontarse a 4000 a. c. en la zona de Palestina (Civantos 2008), si bien, se ha propuesto recientemente la posible existencia de varios centros de origen y diversificación (Diez et al. 2015). El hábitat del olivo se encuentra entre las latitudes 30˚ y 45˚, tanto en el hemisferio norte como en el sur, en regiones climáticas del tipo mediterráneo, caracterizadas por un verano seco y caluroso. En el hemisferio sur, el olivar está presente en latitudes más tropicales con clima modificado por la altitud (Civantos 2008) (Fig. 1).

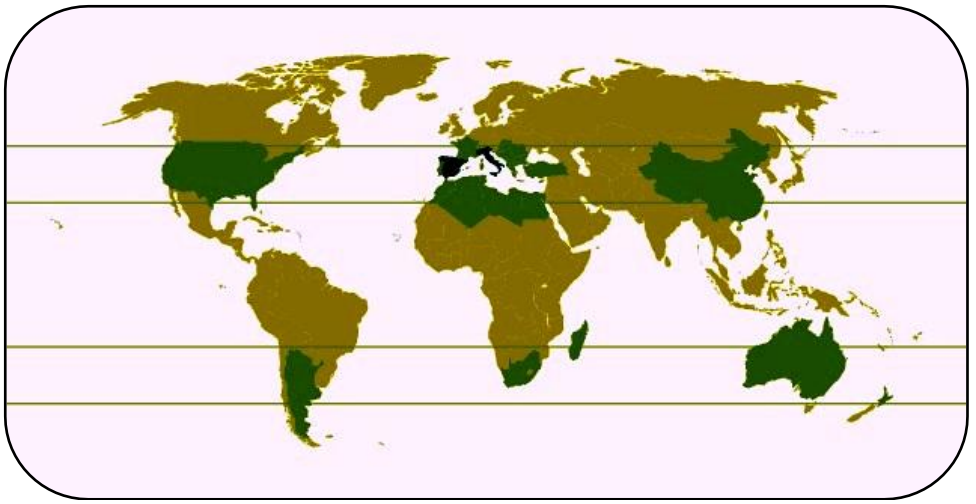

Fig. 1. Distribución mundial del cultivo del olivo. Los países en verde son aquellos en los que se cultiva el olivo. Los países en negro representan el 66 % de la producción mundial.

Según datos del Consejo Oleícola Internacional (COI), el olivar mundial está constituido aproximadamente por 1000 millones de árboles que ocupan una superficie de más de 10 millones de hectáreas. De éstas, más de un millón se dedica a la producción

de aceitunas de mesa (MAGRAMA 2014). El 98 % del total se sitúa en los países de la cuenca mediterránea, con España como país con mayor superficie, 2.593.523 hectáreas, seguido por Túnez, Italia, Turquía, Grecia, Marruecos y Siria (Fig. 2) (IOOC 2010; MAGRAMA 2014).

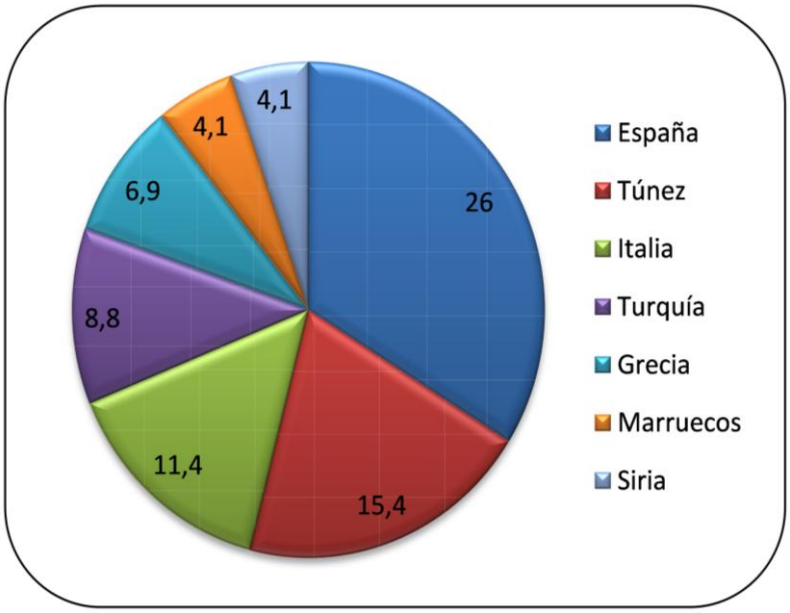

Fig. 2. Distribución (%) por países de la superficie total de olivar.

El cultivo del olivo tiene un papel muy importante en la sociedad española, tanto económico como social e incluso cultural. El olivar español está presente en 34 provincias de 13 Comunidades Autónomas. La principal zona productora se sitúa en Andalucía, zona donde el olivo es el principal cultivo, con una superficie de 1,5 millones de hectáreas, 169.459 explotaciones, y un valor de la producción de 3000 millones de euros (Fig. 3) (MAGRAMA 2014; Junta de Andalucía 2015). La superficie de olivar cultivada en Andalucía se concentra fundamentalmente en el centro y noreste de la comunidad autónoma, siendo Jaén y Córdoba las principales provincias olivareras concentrando más del 60 % de la superficie del olivar en Andalucía, seguidas por Sevilla, Granada y Málaga (Fig. 4). A pesar de ser un cultivo tradicional, se encuentra en la actualidad en fase expansiva, sobre todo con el establecimiento de plantaciones intensivas en regadío

donde el buen manejo de plagas se postula como una práctica esencial (Metzidakis et al. 2008).

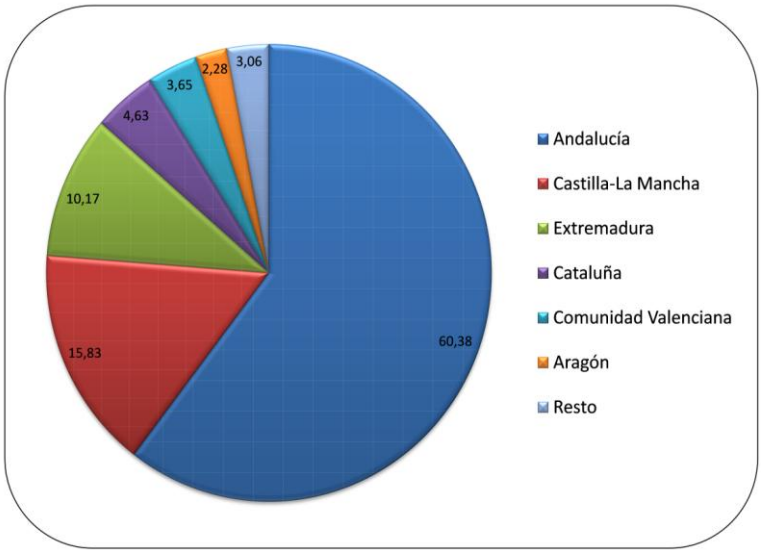

Fig. 3. Distribución (%) por comunidades autónomas de la superficie total de olivar en España.

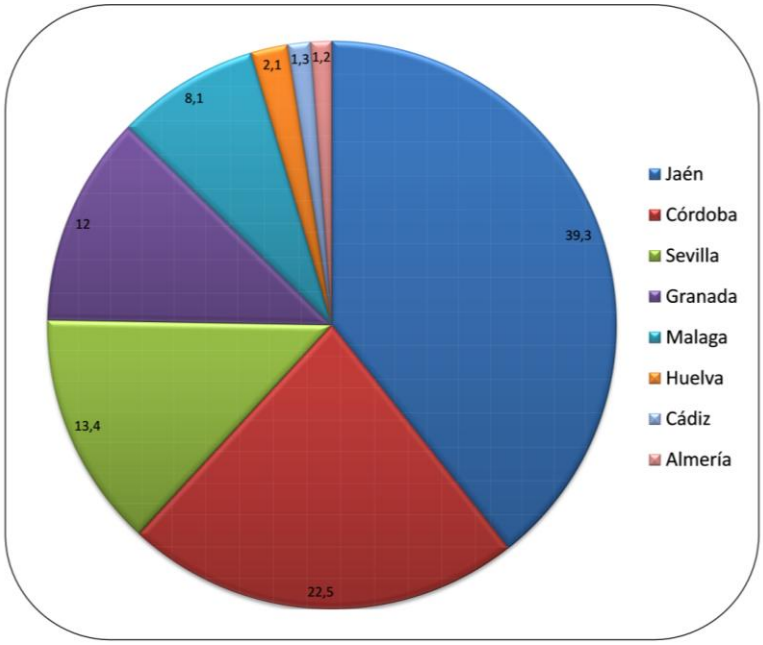

Fig. 4. Distribución (%) por provincias de la superficie total de olivar en Andalucía.

2. Insectos y ácaros perjudiciales al olivo

El olivar se encuadra dentro de los agroecosistemas perdurables, aquellos que permiten la estabilización de una abundante biocenosis en la cual se establecen relaciones de las que dependen en gran medida los equilibrios biológicos. El conjunto de especies de insectos fitófagos que utilizan el olivo como fuente de alimentación, lugar de desarrollo o para ambas funciones, supera el centenar, repartidas en número desigual por todos los órdenes donde se manifiesta este hábito alimenticio que enumeramos en disposición decreciente Hemiptera, Coleoptera, Lepidoptera, Diptera, Orthoptera, Hymenoptera y Thysanoptera (Tabla 1) (Tzanakakis 2006; Quesada-Moraga et al. 2009). Un importante número de estas especies son polífagas u oligófagas, de las que algunas, en áreas específicas, han desarrollado poblaciones o razas adaptadas al olivo, las cuales junto al pequeño grupo de especies monófagas representan la mayor amenaza para el cultivo y su medio (Tzanakakis 2006). Todas las partes de la planta son susceptibles de utilización por los insectos (Alfaro-Moreno 2005). Las plagas del olivo han cambiado muy poco desde que empezó a cultivarse y sigue siendo la mosca *Bactrocera oleae* (Rossi) la más importante (Alvarado et al. 2008).

2.1. La mosca del olivo *Bactrocera oleae* (Rossi) (Diptera: Tephritidae)

2.1.1. Morfología

La mosca del olivo es una especie monófaga, cuyo desarrollo larvario solo puede completarse en los frutos de las especies de género *Olea*, (Santiago-Álvarez y Quesada-Moraga 2007). Los adultos son pequeñas moscas que miden de 4 a 5 mm (Fig. 5). Su cabeza es ancha de tonalidad amarillenta, en la que destacan los ojos compuestos de color verde azulado y de gran tamaño. El tórax es de color amarillo con cuatro bandas grisáceas. Destaca, entre la cabeza y el tórax, una macha negra en el extremo muy característica (Alvarado et al. 2008). Las alas son hialinas con una típica mancha parda en el ápice. El abdomen es rojizo con manchas negras en las áreas laterales de los cuatro primeros segmentos con forma y dimensiones variables (Crovetti 1996). Los adultos

tienen dimorfismo sexual (Fig. 5), que se puede apreciar en el abdomen. El macho posee peines en los márgenes del urito III.

Tabla 1. Los fitófagos (insectos y ácaros) más importantes que atacan al olivar. Elaboración propia a partir de (Quesada-Moraga et al. 2009)

Orden/ subclase	Fitófago	Parte atacada del árbol				
		Raíz	Ramas y tronco	brotes	Flores	Fruto
Hemiptera	*Stictocephala bisonia Kopp & Yonke*		■			
	Saissetia oleae (Olivier)		■	■		
	Hysteropterum grylloides (F.)		■			
	Cicada barbara (Stal.)		■	■		
	Lepidosaphes ulmi L.		■			
	Parlatoria oleae Colvée		■	■		■
	Aleurolobus olivinus Silvestri			■		
	Euphyllura olivina (Costa)			■	■	
	Aspidiotus nerii Bouche			■		■
	Calocoris trivialis Costa				■	
Thysanoptera	*Liothrips oleae* (Costa)			■	■	
Coleoptera	*Vesperus xatarti* Mulsant	■				
	Anoxia villosa F.	■			■	
	Melolonta papposa Illiger	■				
	Ceramida cobosi (Báguena)	■				
	Phloeotribus scarabaeoides (Bernard)		■			
	Hylesinus oleiperda F.		■			
	Leperesinus varius F.		■			
	Sinoxylon sexdentatum Olivier		■			
	Otiorrynchus cribricollis (Gyllenhall)			■		
	Rhinchites cribipennis (Desbr)				■	
Lepidoptera	*Euzophera pinguis* Haworth		■			
	Cossus cossus L.		■			
	Zeuzera Pyrina L.		■			
	Prays oleae (Bernard)			■	■	■
	Margaronia unionalis (Huebner)			■		
Diptera	*Resseliella oleisuga* (Targioni-Tozzetti)		■			
	Dasineura oleae (F.)			■		
	Bactrocera oleae (Rossi)					■
Acarina	*Aceria (Eriophyes) oleae* Nalepa			■		

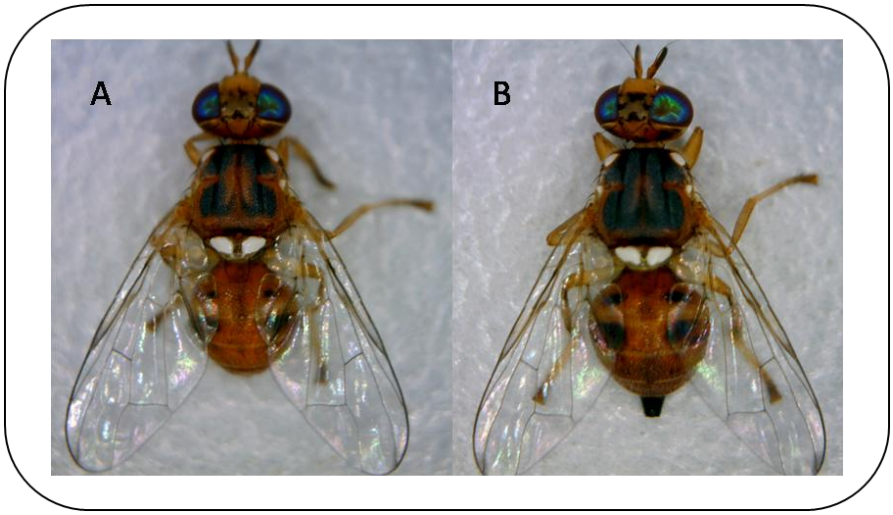

Fig. 5. Adultos de *Bactrocera oleae*. A: macho, B: Hembra. Foto: Meelad Yousef.

La hembra posee una anchura considerable en la parte central, extremidad distal puntiaguda con oviscapto bien visible, parcialmente invaginado con el urito VII de color negruzco (Tremblay 1994; Crovetti 1996). Los huevos son de color blanco, alargado y cilíndrico. Miden unos 0,7 mm de longitud por 0,2 mm de diámetro. En uno de sus extremos presentan un pedicelo, mientras que el otro es liso y redondeado sin ninguna apertura o estructura externa (Tzanakakis 2006). La superficie de los huevos es poligonal, típicamente hexagonal, reticulada o con crestas bajorrelieve (Headrick y Goeden 1998).

Las larvas recién nacidas son de color blanco o amarillo pálido y miden sobre 1 mm de longitud, aunque llegan hasta 7 a 8 mm al final de su desarrollo (Alvarado et al. 2008) (Fig. 6). 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 y liso con 11 segmentos distinguibles (Santiago-Álvarez y Quesada-Moraga 2007) (Fig. 6). El color varía en función del estado de desecación de la epidermis pasando del blanco al amarillo y puede usarse para medir la edad de la pupa (Tremblay 1994).

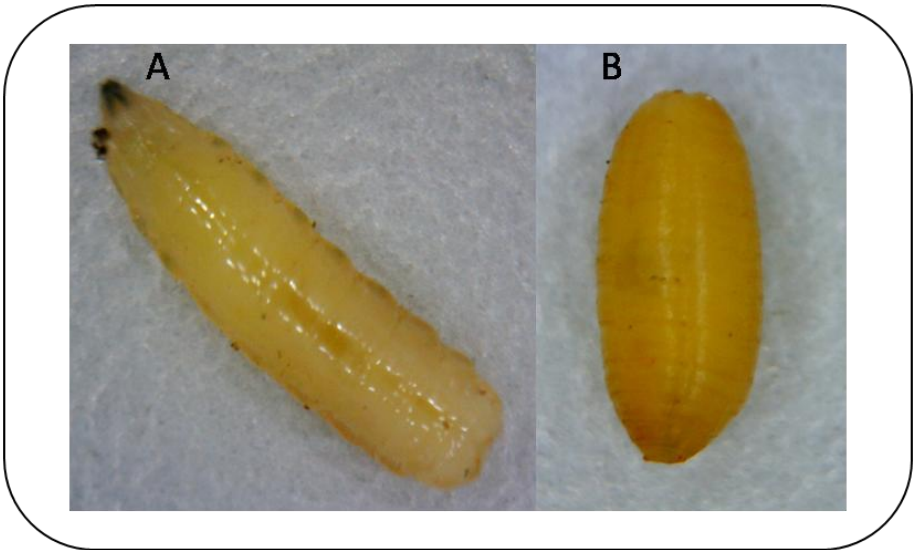

Fig. 6. Estados preimaginales de *Bactrocera oleae.* A: Larva de tercera edad, B: Pupario. Foto: Meelad Yousef.

2.1.2. Distribución geográfica

Según los nuevos avances en biología molecular, África es el origen geográfico de este fitófago (Nardi et al. 2005). Hoy en día, su distribución geográfica comprende todo el área mediterránea, el oeste asiático, India y este y sudeste de África (Fig. 7).

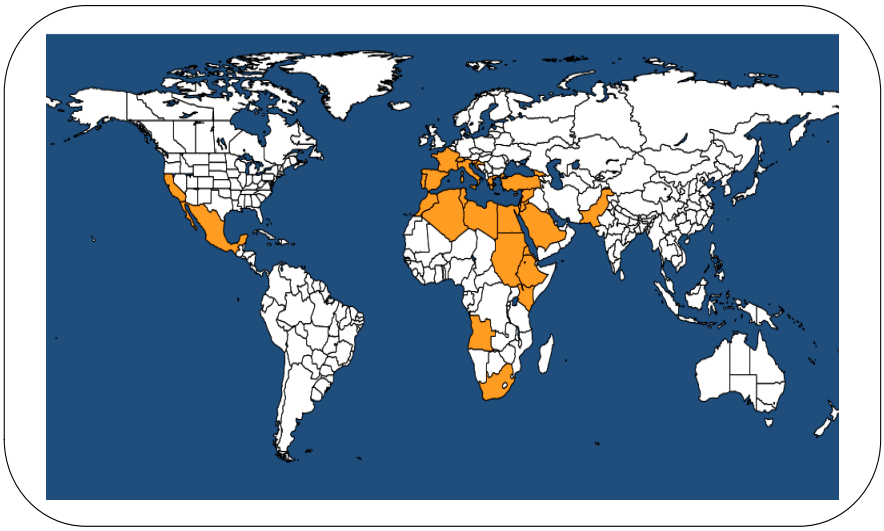

Fig. 7. Presencia de *Bactrocera oleae* en el mundo. Elaboración propia de Nardi et al. 2005 y EPPO (European Plant Protection Organization; https://gd.eppo.int/taxon/DACUOL/distribution).

En los últimos años se ha introducido en la costa oeste de Estados Unidos y noroeste de Méjico, donde ha causado problemas para el comercio internacional (Tzanakakis 2006). Dentro del área de distribución mundial del cultivo del olivo, Australia es la única región exenta del ataque de este tefrítido (Burrack y Zalom 2008).

En España, ha sido detectada en todas las comarcas olivícolas, y se establecen tres zonas de ataque: una costera que se extiende desde la provincia de Barcelona hasta Huelva donde la mosca del olivo es endémica, otra inmediata a la anterior, con ataques inconstantes, y una tercera en olivares situados en la región central que presentan daños de menor importancia (Fig. 8) (Alfaro-Moreno 2005).

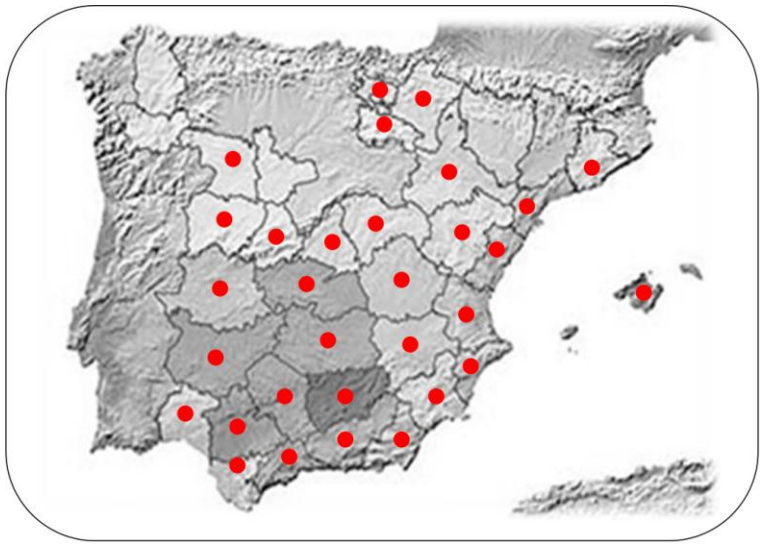

Fig. 8. Presencia de *Bactrocera oleae* en España.

2.1.3. Biología y comportamiento

La mosca del olivo es una especie polivoltina que presenta entre 2 y 5 generaciones anuales en función de las condiciones de humedad relativa y temperatura locales y regionales (Fig. 9) (Ruiz-Castro 1948; Santiago-Álvarez y Quesada-Moraga 2007). Las moscas adultas muestran una alta actividad diurna con ausencia de viento y una temperatura media de 14 a 15 ˚C. Éstas 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 continúa hasta principios de invierno (Santiago-Álvarez y Quesada-Moraga 2007).

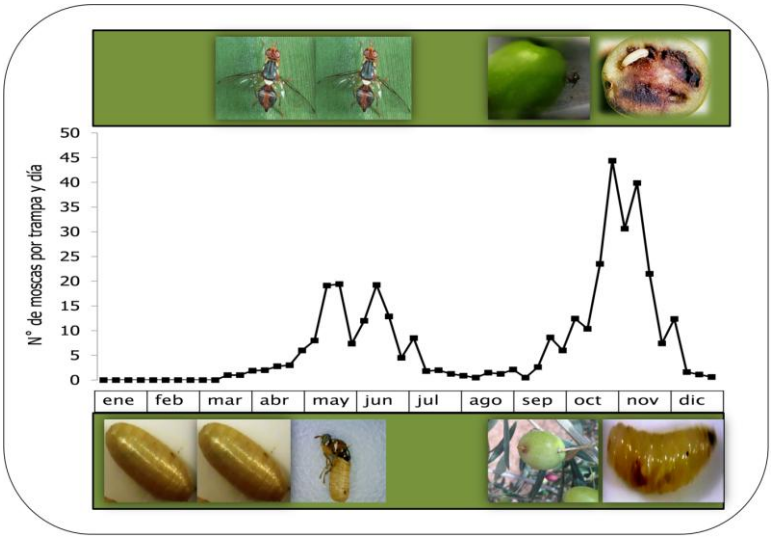

Fig. 9. Actividad de vuelo y ciclo biológico de *Bactrocera oleae* en Córdoba.

Las hembras introducen su oviscapto para atravesar la epidermis del fruto, y generalmente cada una pone 10 a 12 huevos diariamente, como norma uno por fruto, y entre 200 y 250 a lo largo de toda su vida (Weems y Nation 2009). 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 mosca o por ambas causas conjuntamente. Los periodos de oviposición duran alrededor de un mes en verano y poco más de 3 meses en invierno (Santiago-Álvarez y Quesada-Moraga 2007). La incubación de los huevos puede variar de 2 a 19 días en función de la temperatura, y tras la eclosión, la larva se alimenta del mesocarpio en el interior de una galería que hace en la pulpa de la aceituna. Al principio es estrecha y sinuosa, después se va ensanchando hasta formar una cavidad que ocupa una parte importante del fruto. Cuando llega el final de su desarrollo, hace una cámara y se

transforma en pupa en el interior del fruto, o bien caen al suelo para pupar debajo de la copa del árbol (Ruiz-Castro 1948; Alfaro-Moreno 2005; Alvarado et al. 2008).

La mayor parte de la población de la mosca del olivo (casi el 80 %) pasa el invierno como pupa enterrada en los 3 primeros cm del suelo (Dimou et al. 2003). La duración del estado de pupa oscila entre 10 días y varios meses (hasta 4) según la estación del año. Las moscas adultas aparecen con el inicio de la primavera (desde marzo hasta mayo) (De Andres-Cantero 2001). La maduración sexual de los adultos está altamente sincronizada con la disponibilidad de frutos adecuados para la puesta, donde la diapausa reproductiva juega un papel fundamental (Michelakis 1987; Torres-Vila et al. 2003).

2.1.4. Daños e importancia económica

Este tefrítido carpófago supone la mayor amenaza para el cultivo del olivar en toda su área de extensión, porque a la reducción en rendimiento unitario de producción que puede ser importante, se unen las pérdidas en calidad del aceite proveniente de aceitunas "picadas" (Daane y Johnson 2010). Las larvas se alimentan del mesocarpio, consumiendo entre 50 a 150 mg por larva según la variedad del olivo (Neuenschwander y Michelakis 1978). El impacto de la mosca del olivo varía en función del uso previsto del fruto. Se estima que este insecto es responsable del 30-40 % de las pérdidas en la producción total de aceituna en la región Mediterránea, incluso se han descrito pérdidas del 100 % de algunas variedades de mesa y hasta el 80 % del valor del aceite causadas por *B. oleae* (Zalom et al. 2003) (Fig. 10).

Por otro lado, se ha demostrado que el ataque de *B. oleae* reduce el rendimiento de aceite y altera la composición química del fruto, que afecta negativamente a muchos parámetros cualitativos del aceite de oliva como el acidez, el índice de peróxidos y la absorción ultravioleta (Gómez-Caravaca et al. 2008). Además, las aceitunas "picadas" producen aceite con un contenido reducido de compuestos fenólicos antioxidantes y alterando el perfil de los compuestos volátiles que lleva a un inaceptable mal sabor (Angerosa et al. 2004; Gómez-Caravaca et al. 2008; Gucci et al. 2012). La disminución en la calidad del aceite depende del porcentaje de frutos dañados, la etapa de desarrollo del

fruto y la variedad (Gucci et al. 2012). De hecho, se ha detectado en algunas variedades un incremento de acidez directamente proporcional al ataque del tefrítido, con valores de 0,6, 1,5 y 3,4 para niveles de ataque de aceituna del 10, 15 y 100 % (Mraicha et al. 2010). En consecuencia, el valor nutricional y las propiedades sensoriales del aceite extraído de aceitunas infestadas se ven afectados, y el producto no cumple las especificaciones legales del aceite virgen extra o virgen.

Fig.10. Daños causados por *Bactrocera oleae.* Fotos: Meelad Yousef.

2.1.5. Seguimiento de la población de la mosca del olivo y estimación de riesgo para el cultivo

El seguimiento de la población de la mosca del olivo es de gran importancia para establecer las medidas de control aplicadas (Perović et al. 2009). El porcentaje de aceitunas con picada y número de adultos por trampa y día son las dos variables que se miden en un sistema de seguimiento de la mosca del olivo. El seguimiento se realiza en el centro de la parcela de cultivo al principio del estado fenológico H ”endurecimiento de hueso” cada 15 días mientras el fruto esta en color verde mate y cada semana a partir del momento en que cambia a verde brillante. Para la estimación de daño, se seleccionan 20 árboles homogéneos. En caso de olivar de almazara, se cogen 10 frutos por árbol en

todas las direcciones en zonas donde habitualmente hay problemas de mosca y 20 frutos por árbol en zonas donde habitualmente no hay problemas de mosca. En caso de olivar de mesa el número de frutos por árbol se aumenta a 50 (MAGRAMA 2014).

Para el monitoreo de la población de la mosca, se utilizan 3 trampas McPhail cebadas con fosfato biamónico al 4 % y 3 trampas cromotrópicas con una capsula de feromona situadas en la cara sur o sureste del árbol a una altura aproximada de 1,5 m (MAGRAMA 2014).

2.1.6. El control de la mosca del olivo *B. oleae*

Las medidas de control de la mosca del olivo pretenden evitar, o reducir de manera significativa, que las hembras realicen la puesta, causa inicial de la depreciación del fruto, a la que sigue el barrenado de larvas, que disminuye la calidad del aceite. El control de *B. oleae*, que es difícil, se basa principalmente en el empleo de insecticidas químicos, bien mediante tratamientos cebo o adulticidas, o mediante tratamientos larvicidas (Alvarado 2008).

2.1.6.1. Control químico

Desde el inicio en 1993 del programa de revisión de sustancias activas en la UE, como consecuencia de la Directiva 91/414/CEE y hasta su conclusión en 2010, existió una exhaustiva criba comunitaria que supuso la aprobación de sólo un 33,8 % (378) de las 1119 sustancias consideradas. El Reglamento (CE) nº 1107/2009 de comercialización de productos fitosanitarios, que deroga la Directiva 91/414, tiene por objeto último reforzar las garantías de un alto grado de protección de la salud humana y animal y del medioambiente.

Actualmente hay dieciocho formulados para la mosca del olivo en el Registro de Productos Fitosanitarios del Ministerio de Agricultura, cuyas materias activas son deltametrin, lambda-cihalotrin, dimetoato, fosmet, imidacloprid y spinosad. La mencionada criba, y el reducido número de materias activas existentes, algunas de las cuales incluso coinciden en su modo de acción, plantea un serio problema a la hora de

combatir determinadas plagas de insectos, e incluso obliga al empleo continuo de las escasas materias activas disponibles.

De hecho, el empleo continuado de los insecticidas químicos para el control de la mosca del olivo durante más de 50 años ha causado importantes efectos sobre el ecosistema y la salud humana ya que han sido detectados residuos de pesticidas en el aceite de oliva y en ambientes donde crecen los olivos (Haniotakis 2005). Por otro lado, el empleo reiterado de un número muy limitado de materias activas ha propiciado la aparición de resistencia frente a varios organofosforados y piretroides, y más recientemente al spinosad (Margaritopoulos et al. 2008; Kakani et al. 2010; Pereira-Castro et al. 2015).

Por todo ello y por la irrupción de la directiva europea 2009/128/CE de uso sostenible de insecticidas, y su transposición para el uso sostenible de productos fitosanitarios a través del Real Decreto 1311/2012 (RD) y el Plan de Acción Nacional (PAN), y la obligatoriedad de la Gestión Integrada de Plagas desde 1 de enero de 2014, hacen imprescindible investigar en el desarrollo de metodologías alternativas para el control integrado de la mosca del olivo, que pasen a formar parte de las ya incorporadas en la guía del cultivo.

2.1.6.2. Medidas agronómicas o culturales

La aplicación de distintas prácticas agronómicas puede tener repercusión en la incidencia de la mosca del olivo. Destaca en este apartado el 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 (Quesada-Moraga et al. 2009). Por otro lado, la recolección temprana es una medida relativamente eficaz para reducir o evitar la generación de otoño que será el origen de las poblaciones del año siguiente pero es una medida difícil de aplicar, ya que su eficacia depende de las características de cada variedad y por lo tanto puede afectar directamente a la calidad del aceite resultante (Rojnić et al. 2015). Además de lo anterior, hay un repelente registrado, el **caolín** 95 % [WP] P/P, que crea una barrera física sobre la aceituna, impidiendo la oviposición del

díptero. Sin embargo, se ha demostrado que las pulverizaciones con caolín reducen el porcentaje del parasitismo de *Psyttalia concolor* ya que las hembras de este parasitoide prefieren los hospedantes no tratados (Bengochea et al. 2014a), y por otro lado, éstos tratamientos con caolín reducen la eclosión de los huevos del depredador *Chrysoperla carnea*, y actúan como repelentes bajando la actividad depredadora de sus larvas que a su vez prefieren hospedantes no tratados (Bengochea et al. 2014b). Finalmente, labrar el suelo después de la recolección puede ser útil para disminuir la población invernante de *B. oleae* (MAGRAMA 2014).

2.1.6.3. Medidas genéticas y lucha autocida

La elección varietal se ajusta a distintos factores en olivicultura, algunos de tipo agronómico, otros de tipo económico, etc., y hasta la fecha, el comportamiento varietal frente a la mosca del olivo no ha sido factor clave en los programas de mejora desarrollados, Sin embargo, tanto para el diseño de una nueva plantación, como para su futuro manejo, conviene tener presente la existencia de notables diferencias de susceptibilidad de las variedades de olivo de almazara y de mesa más ampliamente distribuidas, destacan por su susceptibilidad en las primeras 'Nevadillo blanco de Jaén', 'Lechín de Sevilla' y 'Hojiblanca', y 'Gordal Sevillana', 'Ocal' y 'Manzanilla de Sevilla' entre las segundas (Quesada-Moraga et al. 2015).

Por otra parte, la lucha autocida, con énfasis en la técnica de los **machos estériles,** se basa en la producción de un gran número de insectos en condiciones controladas de insectario, que son esterilizados mediante radiaciones ionizantes. Aunque se haya aplicado esta técnica en algunas de las especies de dípteros tefrítidos entre ellas *B. oleae*, actualmente sólo en *C. capitata* ha sido posible llevarla a los niveles de cría masiva requeridos por este método de control (Liedo et al. 2010). En el caso de *B. oleae* existen una serie de complicaciones que impiden un uso práctico de esta técnica como la extrema dificultad para criar la mosca del olivo sobre dieta artificial en el laboratorio, y en caso de éxito parcial, la baja calidad de los adultos esterilizados por irradiación, lo que reduce su competitividad de copulación frente a los adultos silvestres y produce un

apareamiento selectivo (Estes et al. 2012). Para afrontar estos problemas, se ha desarrollado por parte de la empresa Oxitec e investigadores de las Universidades de Oxford y Creta, un set de mosca del olivo con mortalidad condicional de hembras, que mueren en estado de larva, denominado "Liberación de insectos portadores de letalidad dominante RIDL®", que proporciona alta mortalidad de hembras, marcador fluorescente dominante, y esterilidad genética. Los machos de la cepa OX3097D-Bol de la mosca del olivo presentan características que vienen a solucionar los tres problemas clave para el funcionamiento de la técnica mediante irradiación: (1) son sexualmente muy competitivos con los machos silvestres; (2) presentan actividad sincrónica para el apareamiento con hembras silvestres; (3) inducen en ellas la pérdida de receptividad necesaria para esta técnica. Además, la liberación semanal de machos de esta cepa OX3097D-Bol en cajas que contienen poblaciones estables de mosca del olivo da lugar a un rápido descenso poblacional e incluso una erradicación eventual (Ant et al. 2012). El posible empleo de estas moscas transgénicas, manipuladas con un ADN sintético, ha dado lugar a una fuerte controversia en España, por lo que su empleo práctico aún parece lejano.

2.1.6.4. Medidas basadas en el empleo de semioquímicos

Se conoce desde hace tiempo que existe una feromona sexual de *B. oleae* como parte de su comportamiento de apareamiento, que emitida por las hembras vírgenes, atrae al macho para la cópula (Haniotakis 1974; Haniotakis et al. 1977). El componente principal de esta feromona sexual denominado espiroacetal fue identificado a finales de 1979 como 1,7-dioxaespiro [5.5] undecano (Baker et al. 1980). Sin embargo, también se ha demostrado que durante los períodos sin actividad reproductiva en el comienzo del verano, los machos de *B. oleae* también puede producir cantidades medibles del componente principal espiroacetal descrito anteriormente, posiblemente como una señal de agregación a la fuente de alimento (Mazomenos y Pomonis 1983).

En un contexto de gestión de plagas, el uso práctico de estas sustancias se puede dividir en dos categorías principales, seguimiento de poblaciones, donde constituyen un

elemento clave (MAGRAMA 2014), o control de las mismas, donde su eficacia es limitada (Montiel y Jones 2002).

Dentro de los métodos de control que tienen fundamento en el empleo de este semioquímico, el trampeo masivo, en general, no se ha mostrado eficaz a gran escala por varias razones, en especial, la falta de trampas de alta eficiencia de captura, la saturación de las mismas cuando las poblaciones de la mosca son elevadas, la destrucción de poblaciones de enemigos naturales que se sienten atraídos por las mismas trampas, y en definitiva, la necesidad de un elevadísimo número de trampas por hectárea que hace la técnica muy costosa (Muñoz y Marí 2012; Yasin et al. 2014).

Respecto a la técnica de atracción y muerte, se han evaluado distintos dispositivos de madera, bolsas, etc., cebados con sales de amonio o espiroacetal e impregnados con distintas materias activas, con resultados inciertos en áreas extensas, y algunos prometedores en pequeñas parcelas. Es necesario mencionar los tratamientos en bandas con distintos atrayentes e insecticidas, en general realizados con avioneta, y cuestionados por la normativa vigente. Finalmente, el espiroacetal no se adapta a los requisitos que permiten el desarrollo de la confusión sexual, pues tiene un efecto de llamada de machos y hembras a las parcelas donde se produce en concentraciones elevadas (Montiel y Jones 2002).

2.1.6.5. Control biológico

Actualmente, el control biológico se postula como medida prioritaria en olivicultura, tanto en Producción Integrada como en la Ecológica, pues su objetivo es reducir o eliminar el uso de los insecticidas convencionales y por tanto sus efectos negativos sobre el ecosistema y la salud humana (Quesada-Moraga et al. 2009). Por todo ello, 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 (Copping y Menn 2000).

El control macrobiano por medio de entomófagos, depredadores y parasitoides, ha sido objeto de muchos esfuerzos con poca eficacia hasta la fecha. Se han encontrado varios depredadores en campos de olivar entre ellos se destaca el neuróptero *Chrysoperla carnea* y algunos coccinélidos, pero debido a que los huevos de la mosca se situan en posición subepidermica, y a que la actividad alimenticia de las larvas se produce en el mesocarpio, los estados preimaginales de *B. oleae* están protegidos frente a la mayoría de los depredadores (Daane et al. 2011). Sin embargo, una vez la larva sale del fruto para pupar en el suelo debajo la copa del árbol estará expuesta a los depredadores de suelo, que son principalmente las hormigas (Orsini et al. 2007). De modo general, hasta la fecha no hay uso práctico de los depredadores para el control de la mosca del olivo, y por lo tanto, el control macrobiano se restringe al uso de los parásitoides concretamente de la familia Braconidae que proporcionan los niveles de parasitismo más altos (Neuenschwander 1982). Hoy en día hay más de 10 especies del orden Hymenoptera de distintas familias citadas como parasitoides de *B. oleae* (Tabla 2).

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). En el olivar, las especies *Psyttalia 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).

Tabla 2. Los parásitoides más importantes que atacan a la mosca del olivo. Elaboración propia a partir de (Daane et al. 2010)

Familia	Especie
Braconidae	*Psyttalia concolor* (Szépligeti) *P. dacicida* Silvestri *P. lounsburyi* (Silvestri) *P. ponerophaga* (Silvestri) *P. humilis* (Silvestri) *Utetes africanus* (Szépligeti) *Bracon celer* Szépligeti *Triaspis daci* (Szépligeti) *Fopius arisanus* (Sonan)
Eulophidae	*Achrysocharella formosa* (Westwood) *Pnigalio mediterraneus* (Ferriere y Delucchi)
Eupelmidae	*Eupelmus afer* Silvestri *Eupelmus urozonus* Dalman
Pteromalidae	*Halticoptera daci* Silvestri
Diapriidae	*Coptera silvestrii* (Kieffer)
Tormyidae	*Microdontomerus* sp.
Encyrtidae	*Tachinaephagus zealandicus Ashmead*

En el caso del **control microbiano,** las primeras experiencias en olivar fueron realizadas con la **bacteria** *Bacillus thuringiensis* (Berliner) para el control de *Prays oleae* (Yamvrias 1977). De hecho, esta bacteria se utiliza en la actualidad de forma regular para tratamientos de la generación antófaga de la polilla del olivo *P. oleae*, aunque hasta la fecha, no se han encontrado aislados de *B. thuringiensis* con notable actividad insecticida frente la mosca del olivo *B. oleae* (Alberola et al. 1999). Los **virus** han sido poco explorados, a pesar de haber mostrado gran potencial un Reovirus evaluado frente a adultos de *B. oleae* (Manousis et al. 1987). Además, el Picornavirus V y el Reovirus I son capaces de replicarse de forma natural y matar adultos de *B. oleae* y otros tefrítidos. 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). Al no existir enfermedades causadas por baculovirus y protozoos en la mosca del olivo, las posibilidades de uso de microorganismos entomopatógenos se restringen a los **hongos entomopatógenos (HE)**, en especial los del grupo de los ascomicetos mitospóricos (Hypocreales), cuyo modo de acción único, por vía tegumentaria, les sitúa en primera línea mundial para el desarrollo de estrategias alternativas de control de las plagas de tefrítidos (Castillo et al. 2000; Quesada-Moraga et al. 2006; Ekesi et al. 2007).

3. El empleo de los hongos entomopatógenos en el control de la mosca del olivo

Los hongos entomopatógenos se encuentran en dos divisiones del reino Mycota, Entomophtoromycota y Ascomycota, donde se sitúan respectivamente los órdenes con más representantes, Entomophtorales e Hypocreales (Hibbett et al. 2007; Gryganskyi et al. 2012, 2013; Humber 2012). Los Entomophthorales se caracterizan por ser biotrofos obligados, de difícil multiplicación en medio artificial, lo que limita su empleo al control biológico por conservación (Keller 2007; Pell et al. 2010). Sin embargo, los ascomicetos mitospóricos, que también regulan de forma natural las poblaciones de insectos, son de fácil manejo y producción en masa, lo que facilita su empleo por inundación como micoinsecticidas (Pell et al. 2010).

Dentro de los nuevos métodos de control de *B. oleae* eficaces y respetuosos con el medioambiente, el empleo de los HE presenta algunas ventajas, en primer lugar debido a su presencia natural en el suelo y el filoplano del olivar, pero además por su modo de acción por contacto, único dentro de los microorganismos entomopatógenos, que lleva asociados efectos directos letales, así como subletales reproductivos, e incluso capacidad de autodiseminación, sin comprometer la fauna auxiliar del olivar. Además, estos hongos constituyen una nueva fuente poco explorada de nuevas moléculas insecticidas de origen natural.

3.1. Presencia natural y diversidad en el olivar

Los ascomicetos mitospóricos entomopatógenos se encuentran de forma natural en el suelo, en las plantas, tanto en el filoplano como endófitos, y en los insectos, cuyas poblaciones regulan (Quesada-Moraga et al. 2014).

El suelo es el hábitat natural de los HE, en el que constituyen un componente importante de la microflora (Keller y Zimmerman 1989). En este sentido se ha detectado la presencia de HE en la mayoría de los suelos de ecosistemas tanto cultivados como naturales en la Península Ibérica y los Archipiélagos Balear y Canario, en especial en suelos de olivar, donde los géneros *Beauveria* Vuill. y *Metarhizium* Sorok., por este orden, son los mas característicos (Quesada-Moraga et al. 2007). Los géneros *Beauveria* Vuill. y *Metarhizium* Sorok., tienen origen monofilético dentro de las familias Cordycipitaceae y Clavicipitaceae, respectivamente (Bischoff et al. 2009; Rehner et al. 2011). El género *Beauveria* cuenta con doce especies, algunas de ellas sólo distinguibles mediante herramientas moleculares (Rehner et al. 2011), mientras que el género *Metarhizium* está compuesto por un complejo críptico de nueve especies, seis agrupadas en el denominado clade 1 por oligonucleótidos específicos del mismo, *M. majus*, *M. guizhouense*, *M. brunneum*, *M. pingshaense*, *M. Robertsii* y *M. anisopliae* (Bischoff et al. 2009; Schneider et al. 2011). Los ascomicetos mitospóricos entomopatógenos pueden permanecer en el suelo en forma de micelio en cadáveres de insectos y ácaros momificados, o en forma de esporas asexuales o conidios, donde factores edáficos como textura, pH, materia orgánica y humedad pueden afectar a su presencia y distribución (Keller y Zimmerman 1989; Quesada-Moraga et al. 2007).

A pesar de que existe una idea general y aceptada que el suelo, como su hábitat natural, y las poblaciones de insectos hospedantes a las que infectan, son las dos fuentes principales de HE, recientemente se ha puesto de manifiesto la importante presencia de éstos hongos en el filoplano, con énfasis en el del olivo, tanto convencional como ecológico, con más riqueza y diversidad de estas especies fúngicas que otros ecosistemas (Garrido-Jurado et al. 2015).

3.2. Modo de acción de los ascomicetos mitospóricos entomopatógenos

Los hongos entomopatógenos, en especial los ascomicetos mitospóricos, son únicos y relevantes dentro del elenco de microorganismos entomopatógenos por actuar por contacto, pues infectan al hospedante a través del tegumento, modo de acción complejo que limita el desarrollo de resistencia frente a ellos (Khan et al. 2012). Las esporas asexuales o conidios son los responsables de la infección y están dispersos por el medio donde se encuentran los insectos hospedantes (Fig. 11). Cuando alcanzan la cutícula se adhieren fuertemente debido a mecanismos mediados por fuerzas inespecíficas, tales como la hidrofobicidad de su pared celular, germinan, e inician cascadas de reconocimiento y activación enzimática, a lo que sigue la formación de las características de estructuras de penetración (Fig. 11). Logran atravesar la cutícula gracias a la combinación de mecanismos bioquímicos y mecánicos.

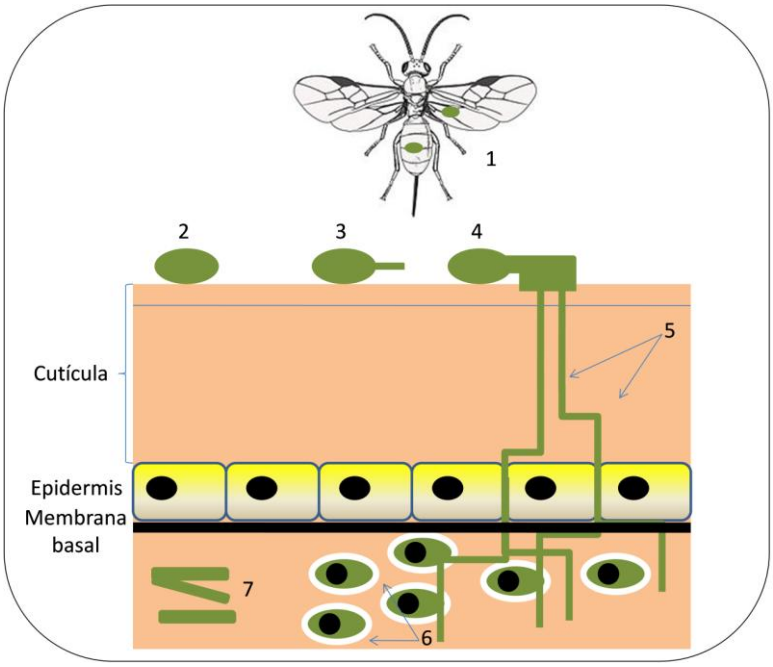

Fig. 11. Modo de acción general de un ascomiceto mitospórico: (1) contacto entre el conidio y la cutícula del insecto; (2) Adhesión de los conidios a la epicutícula (3) Germinación; (4) Penetración; (5) Hifas que atraviesan la cutícula y alcanzan el hemocele; (6) Reacción defensiva celular del insecto; (7) Cuerpos hifales en el hemocele.

El hongo, que debe vencer las respuestas defensivas celular y humoral del insecto hospedante, provoca su muerte por utilización de los nutrientes que le pone a disposición, por invasión de sus tejidos y órganos, por asfixia al desarrollarse en el sistema respiratorio, y en algunos casos, por la producción de metabolitos tóxicos. La muerte del insecto acarrea el crecimiento saprofítico 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, y contribuyen a su transmisión horizontal (Goettel et al. 2005; Charnley y Collins 2007; Quesada-Moraga y Santiago-Álvarez 2008; Vega et al. 2012).

3.3. Estrategias de aplicación de los hongos entomopatógenos para el control de la mosca del olivo

Al igual que con otros agentes de control biológico, los HE se pueden emplear dentro de cuatro estrategias de control: (1) Conservación; (2) Clásica; (3) Inoculación; (4) Inundación (Eilenberg 2002; Quesada-Moraga y Santiago-Álvarez 2008). En particular, los ascomicetos mitospóricos están bien adaptados para la estrategia de inundación, pues se han realizado grandes avances en su producción a gran escala y formulación, así como en su aplicación convencional con equipos de pulverización. En la actualidad hay disponibles en el mercado mundial más de 30 micoinsecticidas, que incluyen como materias activas hasta 13 especies fúngicas, en su mayor parte pertenecientes a los géneros *Beauveria*, *Metarhizium* y *Lecanicillium* (Lacey et al. 2015). A este respecto, se indican a continuación las principales estrategias que pueden adoptarse para el control de tefrítidos, aunque los trabajos realizados en el caso de *B. oleae* son de momento escasos.

3.3.1. Tratamientos adulticidas con suspensiones de conidios

El uso de los HE en tratamientos adulticidas con suspensiones de conidios 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. Actualmente, en el Registro de Productos Fitosanitarios del Ministerio de Agricultura existe un insecticida biológico a base de HE autorizado para el control de adultos de la mosca del olivo, se trata de un formulado del hongo *B. bassiana*. Se aplica en pulverizaciones terrestres totales, al inicio de la infestación, recomendándose la repetición de las aplicaciones. El formulado registrado es *Beauveria bassiana* 2,3 % (2,3 x 10^9 conidias viables/ml) [OD] P/V (Ruiz-Torres 2013). Los tratamientos adulticidas con estos hongos frente a la mosca del olivo han sido poco estudiados. Konstantopoulou y Mazomenos (2005), evaluaron en el laboratorio diferentes hongos entomopatógenos frente a adultos de *B. oleae* con resultados prometedores y mortalidades de hasta el 63 %. Sin embargo, la eficacia de los HE para el control de otros tefrítidos se ha revelado en numerosos trabajos para *C. capitata* (Quesada-Moraga et al. 2006; Quesada-Moraga et al. 2008), *Anastrepha ludens* (Lezama-Gutierrez et al. 2000; De la Rosa et al. 2002), *A. fraterculus* (Carneiro y Salles 1994; Destefano et al. 2005), *B. tryoni* (Carswell et al. 1998) o *Rhagoletis indifferens* (Yee y Lacey 2005).

3.3.2. Tratamientos adulticidas con extractos fúngicos

El potencial de los HE para el control de tefrítidos, hoy en día, no solo reside en su virulencia frente a estados preimaginales y adultos, sino también en su capacidad para secretar nuevas moléculas insecticidas de origen natural para el control de adultos (Yousef 2012). Durante la etapa de post-penetración, los HE secretan una gran variedad de compuestos, que sin resultar fundamentales para su desarrollo, son de gran ayuda para evitar el crecimiento de otros microorganismos en la conquista del insecto hopedante (Shwab y Keller 2008). Los HE producen tanto compuestos de bajo peso molecular (<5 kDa), denominados metabolitos secundarios como metabolitos proteinicos macromoleculares (>5-10 kDa) (Quesada-Moraga y Vey 2004; Ortiz-Urquiza et al. 2009; Golo et al. 2014). Los compuestos secretados por estos hongos se revelan como unas moléculas con gran actividad insecticida para el control de adultos en forma de pulverización-cebo o incluso en la estrategia de "atracción y muerte". Nuestros trabajos

previos ponen de manifiesto la actividad insecticida de una fracción proteínica de *Metarhizium brunneum* (formalmente *M. anisopliae*) (Met.) Sorok., para el control de adultos de la mosca mediterránea de la fruta *Ceratitis capitata* Wied. (Diptera; Tephritidae) (Quesada-Moraga et al. 2006; Ortiz-Urquiza et al. 2009), pero hasta la fecha no se ha evaluado la posible existencia de una molécula activa frente a *B. oleae*.

3.3.3. Tratamientos de suelo dirigidos a estados preimaginales

El control de los estados preimaginales de la mosca del olivo que se encuentran en el interior del fruto es una práctica muy difícil y de alto riesgo ya que, tradicionalmente, se han utilizado insecticidas organofosforados como el dimetoato para obtener una acción sistémica frente a huevos y larvas que se desarrollan dentro del fruto (Alvarado et al. 2008). Estos tratamientos larvicidas generan ciertos problemas de residuos además de presentar plazos de seguridad relativamente largos, de hasta 60 días. Sin embargo, la fase del ciclo biológoico de este tefrítido en el que las larvas de tercera edad abandonan el fruto y caen al suelo para pupar bajo la copa del árbol ofrece una opción estratégica para su control. En efecto, este es un punto crítico para el control de los tefrítidos, pues se trata de un estado invernante en el suelo, hasta ahora olvidado en las estrategias de control de la mosca del olivo, que puede permitir la reducción de la población de primavera (Ekesi et al. 2007). A tal efecto, los HE pueden ser aplicados al suelo bajo la proyección de la copa del árbol, donde los conidios, que están protegidos frente a la inactivación por los factores abióticos, en especial UV-B así como fluctuaciones de temperatura y humedad, pueden permanecer durante un largo periodo de tiempo y reducir de esta forma la población de estados preimaginales y por consiguiente la de la siguiente generación, como se ha demostrado en otros tefrítidos como *C. capitata*, *C. fasciventris*, *C. cosyra y B. invadens* (Ekesi et al. 2005; Quesada-Moraga y Santiago-Álvarez 2008; Ekesi et al. 2011). Sin embargo, esta estrategia no se ha probado contra la mosca del olivo. 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 *C. capitata* (Quesada-Moraga et al. 2006; Garrido-Jurado et al. 2011a, b).

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Justificación y objetivos

Justificación y objetivos

A pesar de que el control de la mosca del olivo ha sido objeto de numerosas investigaciones hasta la fecha aún se basa en el empleo de insecticidas químicos de síntesis en tratamientos larvicidas y adulticidas, y aun sigue siendo este díptero el que supone la mayor amenaza para el cultivo del olivo en toda su área de extensión. Sin embargo, la nueva tendencia mundial hacia la independencia del uso de los insecticidas químicos por sus daños al medioambiente y a la salud humana y la aparición de resistencia, plasmada en la directiva europea 2009/128/CE de uso sostenible de insecticidas, y en su transposición al ordenamiento español en el Plan Nacional de Acción, hace imprescindible el desarrollo de medios alternativos no químicos y su empleo como estrategia fundamental de control de plagas en programas de Gestión Integrada. Dentro de los nuevos métodos de control de *B. oleae* eficaces y respetuosos con el medioambiente, el empleo de hongos entomopatógenos del grupo de los ascomicetos mitospóricos es el que presenta mayores ventajas. Su presencia natural en los agroecosistemas, su modo de acción único dentro de los microorganismos al actuar por contacto, la posibilidad y facilidad relativa de su producción en masa, la capacidad de producir compuestos con actividad insecticida por ingestión, y por último el nulo efecto sobre los artrópodos no diana, son factores que les sitúan en primera línea mundial para el desarrollo de estrategias alternativas de control de plagas. Por todo ello, explotar al máximo el potencial insecticida tanto de los hongos entomopatógenos como de sus extractos insecticidas para el control de plagas ha sido el empeño de nuestro grupo de investigación durante los últimos años. Trabajos realizados en el seno del grupo de investigación PAIDI AGR 163 “Entomología Agrícola” de la Universidad de Córdoba han constatado: (1) La gran presencia natural de los hongos entomopatógenos de los géneros *Beauveria* y *Metarhizium* en el suelo y filoplano de olivar de la Península Ibérica, con énfasis en Andalucía, así como la influencia de las prácticas agronómicas sobre la misma (Quesada-Moraga et al. 2007; Garrido-Jurado et al. 2015); (2) La eficacia de suspensiones fúngicas de cepas autóctonas de ambos géneros para el control de adultos de moscas de las frutas y de larvas próximas a pupación y puparios de las mismas en aplicaciones al

suelo (Quesada-Moraga et al. 2006; Quesada-Moraga et al. 2008; Garrido-Jurado et al. 2011b, c); (3) La ausencia de impacto negativo de estos tratamientos de suelo en un olivar frente a la artropofauna edáfica del mismo. (Garrido-Jurado et al. 2011a).

Por tanto, los objetivos específicos de este trabajo son:

1**.** Determinar el potencial de las cepas EAMb 09/01-Su y EAMa 01/58-Su del hongo entomopatógeno *Metarhizum brunneum* Petch para el control microbiano de estados preimaginales y adultos de la mosca del olivo *B. oleae*, así como la actividad insecticida de su extracto crudo frente a adultos de la especie en el laboratorio.

2. Optimizar la producción del extracto crudo de la cepa EAMb 09/01-Su *M. brunneum*, con el fin de mejorar su actividad insecticida.

3. Evaluar la eficacia de la cepa EAMa 01/58-Su frente adultos y preimaginales de la mosca del olivo en el campo con el objetivo de establecer el uso de este hongo como estrategia de control biológico sostenible y eficaz frente a la mosca del olivo, mediante aplicaciones dirigidas a estados preimaginales del tefrítido en el suelo de olivar durante varias campañas agrícolas.

4. Evaluar *in vitro* e *in vivo* la compatibilidad de la aplicación conjunta de la cepa EAMa 01/58-Su y los diferentes herbicidas autorizados para su posible uso en suelos de olivar en Producción Integrada en Andalucía.

Los resultados relativos al primer objetivo se recogen en el capítulo 3, que comprende el manuscrito "Biocontrol of *Bactrocera oleae* (Diptera: Tephritidae) with *Metarhizium brunneum* and its extracts" publicado en Journal of Economic Entomology 106(3) (2013) 1118-1125. Los resultados del segundo objetivo se recogen en el capítulo 4 en el manuscrito "One *Metarhizium brunneum* strain, two uses to control *Ceratitis capitata* (Diptera: Tephritidae)" publicado en Journal of Economic Entomology 107(5) (2014) 1736-1744. El tercer objetivo queda recogido en el capítulo 5 dentro del manuscrito "Reduction of adult olive fruit fly populations by targeting preimaginals in the soil with the entomopathogenic fungus *Metarhizium brunneum*", publicado en Journal of Pest Science DOI 10.1007/s10340-016-0779-y. El cuarto objetivo corresponde al capítulo 6 de esta Tesis Doctoral "Compatibility of herbicides used in olive orchards with a

Metarhizium brunneum strain used for the control of preimaginal stages of tephritids in the soil" publicado en Journal of Pest Science 88 (2015) 605-612.

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ARTÍCULO

Este capítulo es una versión adaptada del artículo

BIOLOGICAL AND MICROBIAL CONTROL

Biocontrol of *Bactrocera oleae* (Diptera: Tephritidae) With *Metarhizium brunneum* and Its Extracts

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Biocontrol of *Bactrocera oleae* (Diptera: Tephritidae) with *Metarhizium brunneum* and its extracts

Abstract

The susceptibility of preimaginal and adult olive fruit fly, *Bactrocera oleae* (Gmelin) (Diptera: Tephritidae), to a strain of the mitosporic ascomycete *Metarhizium brunneum* (Petch) (Hypocreales: Clavicipitaceae) and the insecticidal activity of its crude extract to olive fruit fly adults were investigated. Strain EAMb 09/01-Su caused 60 % mortality to *B. oleae* adults, with average survival time (AST) of 8.8 d. In soil treatments against pupariating third-instar larvae, preimaginal *B. oleae* mortality reached 82.3 %, whereas preimaginal mortality targeting puparia was 33.3 %. The crude extract of EAMb 09/01-Su strain caused 80.0 % adult mortality when administered *per os*, with AST of 27.7 h. The crude extract was demonstrated to be quite thermostable and photoresistant. These results indicate that *M. brunneum* EAMb 09/01-Su strain and its crude extract show potential to be used in an integrated pest management olive fruit fly management strategy targeting both adults and preimaginals.

Keywords Biological control, Secondary metabolite, Microbial control, Crude extract, Olive fruit fly

1. Introduction

The olive (*Olea europaea* L.) tree is planted in allregions of the globe located between 30 and 45 latitude of the two hemispheres, in which it counts among the most important oil-producing crops (IOOC2010). Spain is the leading country in olive production, with > 300 million olive trees spread over 2.580 million ha (21 % of the world total and 51 % of the EuropeanUnion total). Spain is also the largest producer of table olives and oil, with 30 and 40 % of production, respectively, > 80 % of which is from Andalusia (MARM 2011).

There are more than a hundred species feeding or developing on olive tree, polyphagous, oligophagous, and a small group of monophagous species representing the

greatest threat to the crop and its environment (De Andrés Cantero 2001; Tzanakakis2006). The harvestable portion of the crop is the fruit and is used to produce oil or for table consumption. Therefore, although the general health of the tree is the concern of every good grower, the protection of the fruit is a practice of critical importance. The fruit of the olive tree is attacked by a diverse set of species, whereas the species that poses the greatest threat to the olive crop throughout its range is the olive fruit fly, *Bactrocera oleae* (Diptera: Tephritidae) (De Andrés Cantero 2001; Alfaro 2005; Tzanakakis 2006). This tephritid causes a reduction in oil quality, along with a reduction in the yield per unit, that together account for approximately €800 million/yr, with an annual expenditure on control measures of €100 million (Haniotakis 2005). There have even been reported losses of 100 % of some table varieties and up to 80 % of oil value due to *B. oleae* (Zalom et al. 2003).

The females of this species thrust their ovipositor to penetrate the skin of the fruit, usually depositing a single egg inside the fruit. After hatching, the larva feeds on the mesocarp, and when it reaches the end of its development, it forms a chamber and becomes a pupa inside the fruit or falls to the ground to pupate beneath the tree. This species completes between two and five generations per year (Santiago-Álvarez and Quesada-Moraga 2007). The feeding of the larvae at the expense of the olive pulp results in a damage of both quantity (by decreasing the amount of the crop) and quality (by facilitating the entry of fungi that affect the quality and stability of the resulting oil) (Santiago-Álvarez and Quesada-Moraga 2007; Genç and Nation 2008).

The economic importance of this dipteran pest has led to the assessment of most available tools in controlling the field against *B. oleae*, without practical results in most cases. Currently, the use of insecticides is the most widely used measure of control, either by bait adulticide treatments or by larvicidal treatments. Other strategies against *B. oleae* have a much more limited application; these strategies include mass trapping and preventative spraying with kaolin (physicalbarrier) and copper (Belcari et al. 2005; Haniotakis 2005; Johnson et al. 2006; Daane and Johnson 2010).

Insecticide treatments, not being selective when applied over large areas and throughout much of the foliage, cause severe effects on ecosystems (Ruano et al. 2001; Haniotakis 2005; Pascual et al. 2010). These effects are coupled with the loss of efficiency through the development of resistance, particularly due to the repeated and abused use of a very limited number of active materials. Already, some populations of *B. oleae* have been found to be resistant to dimethoate and other organophosphates, pyrethroids, and more recently spinosad (Kakani et al. 2008, 2010; Margaritopoulos et al. 2008).

Therefore, it is essential to investigate the development of alternative methodologies for an integrated control of the olive fruit fly, emphasizing biological control through entomophagous predators and parasitoids (research to date has devoted much effort with little effect) or microbes to exploit the full potential of entomopathogenic microorganisms.

To date, *Bacillus thuringiensis* strains with adequate insecticidal activity against *B. oleae* are not available for practical use (Navrozidis et al. 2000). In the absence of baculovirus diseases and protozoa in the olive fruit fly, the possibilities of using entomopathogenic microorganisms are restricted to entomopathogenic fungi, whose unique tegumentary mode of action puts them at the forefront of the global development of alternative control strategies of tephritids (Castillo et al. 2000; Quesada-Moraga et al. 2006b; Ekesi et al.2007); however, their use against *B. oleae* has not yet been studied. Furthermore, there is potential for the use of the mitosporic entomopathogenic ascomycetes for tephritid control; not only are they virulent against both preimaginal and adult flies but also they secrete new molecules with natural insecticidal properties for the control of adults (Konstantopoulou et al. 2006; Ekesi et al. 2007). Our previous studies demonstrate the use of the insecticidal activity of a protein fraction of *Metarhizium anisopliae* (Met.) Sorok. in the control of the adult Mediterranean fruit fly *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) (Quesada-Moraga et al. 2006b; Ortiz-Urquiza et al. 2009). However, to date, we have not evaluated the possible existence of an active molecule against *B. oleae*. The aim of this study was to evaluate the potential of a strain of the ascomycete mitosporic *Metarhizium brunneum*

(Petch) (Hypocreales: Clavicipitaceae) to infect and suppress preimaginal and adult *B. oleae* as well as to evaluate the insecticidal activity of the crude extract of this fungus against adults of the species. This strain was selected based on previous unpublished studies that indicate its high virulence against *C. capitata*.

2. Materials and methods

2.1. Insects

Wild *B. oleae* were obtained from naturally infested fruit collected from September to December 2011 in the olive groves that enclose the experimental farm "La Alameda del Obispo" belonging to Instituto de Investigación y Formación Agraria y Pesquera (Junta de Andalucía) in Córdoba, Spain. The infested olives were placed in round cages of 115 by 50 mm (40-50 olives in each cage). The cages were maintained in an environmental chamber programmed at 25 ± 2 ˚C, 50-60 % RH, and photoperiod of 16:8 (L:D) h. The puparia from pupariating larvae that left the olives to attain the pupal stage on the cage floor were collected every 7 d and placed in methacrylate round dishes (30 mm) and in Petri plates (55 mm) (an average 10-15 puparia in each plate) containing a layer of cotton. Two milliliters of distilled water was added to the cotton to keep high-humidity conditions to promote adult emergence. However, puparia were placed in the 30-mm dishes to avoid direct contact with watered cotton, which promotes growth of saprotrophic fungi.

2.2. Fungal origin

The *M. brunneum* strain EAMb 09/ 01-Su was isolated in 2009 from the soil of a meadow forest system in Castilblanco de los Arrollos, Seville, Spain (location 37˚ 42´ N, 5˚ 59´ W). This isolate from the culture collection at the Department of Agricultural and Forestry Sciences, University of Córdoba, has been deposited in the Spanish Type Culture Collection under accession CECT 20784.

2.3. Fungal preparation

To prepare the inoculums for the experiments, slant cultures of the isolates were subcultured on malt agar (BioCult B. Laboratories, Madrid, Spain) for 12 d at 25 ˚C in darkness. The Petri plates were sealed with Parafilm. Conidial suspensions were prepared by scraping conidia from the Petri plates into a sterile aqueous solution of 0.1 % Tween 80. This initial suspension was sonicated for 5 min and then filtered through several layers of cheesecloth to remove mycelia mats and thus to collect pure conidia. The conidial suspension used for the bioassays was adjusted by diluting the conidia with 0.1 % Tween 80 to a fungal concentration of 1.0×10^8 conidia per ml. The number of conidia was estimated using a Malassez hemocytometer (Marienfeld Laboratory Glassware, Lauda-Koenigshofen, Germany). The suspension was stored in a refrigerator at 4 ˚C for no longer than 24 h.

2.4. Initial pathogenicity assay of EAMb 09/01-Su strain against newly emerged *B. oleae* adults

Cold anesthetized newly emerged adults were treated with 1 ml of a 1.0×10^8 conidia per ml suspension by using a Potter tower (Burkard Rickmansworth Co. Ltd., United Kingdom), resulting in coverage of 1.57×10^5 conidia cm^{-2} for a standard tank of 1.54 ± 0.06 mg cm^{-2} and 0.7 bars of pressure. The controls were treated with the same volume of a sterile aqueous solution of 0.1 % Tween 80. The treated adults were placed in methacrylate cages (80 by 80 by 60 mm; Resopal, Alcalá de Henares, Madrid, Spain) with covers containing a circular hole (20 mm in diameter) covered with a net cloth; a liquid diet (0.1 g of hydrolyzed protein and 0.4 g of sucrose with 1.5 ml of distilled water) was provided every 48 h. The bioassay was conducted at 26 ± 2 ˚C, 50-60 % RH, and a photoperiod of 16:8 (L:D) h. Mortality was monitored for 12 d. Dead flies were removed daily to prevent horizontal transmission of the fungal inoculum. The dead flies were immediately surface sterilized with 1 % sodium hypochlorite followed by three rinses with sterile distilled water. They were then placed on sterile wet filter paper in sterile Petri plates that were sealed with Parafilm and kept at room temperature to be

inspected for fungal outgrowth on the cadavers (Quesada-Moraga et al. 2006a). Each treatment was replicated five times with five adult flies per replicate.

2.5. Initial pathogenicity assays of EAMb 09/01-Su strain against *B. oleae* preimaginals

To evaluate the pathogenicity of *M. brunneum* against *B. oleae* preimaginals, two bioassays were performed. In the first bioassay, we used pupariating larvae. Bioassay cages similar to those used for the adult bioassays were prepared with 30 g of soil that covered the entire floor. The soil used in this study was collected from a farm in Córdoba and was characterized as sandy loam (78.0 % sand, 17.0 % silt, 5.0 % clay, and 0.2 % organic matter) with a pH of 8.3. The soil was sieved (2-mm mesh) and stored in a dry place at ≈25 ˚C. The soil was then sterilized at 121 ˚C for 20 min and dried in an oven at 105 ˚C for 24 h. One milliliter of fungal suspension containing 1.0×10^8 conidia per ml and 1.7ml of sterile distilled water containing 0.1 % Tween 80 were added to the bioassay cages to attain a water potential of -0.01 MPa (9.0 % [wt:wt] measured with a Decagon WP4 psychrometer, Decagon Devices, Pullman, WA). The bioassay was arranged as a randomized complete block design with two treatments, fungal isolate and control, and four replicates per treatment. Puparia were gathered daily until a reasonably sized population (63 puparia) was achieved. The experimental unit was a transparent plastic box (80 by 80 by 60 mm). After the treatment, the cages were covered with Parafilm membrane with a 20 mm hole in the center for ventilation and they were placed in a plastic box (330 by 250 by 140 mm) that was covered with damp filter paper that was moistened periodically to maintain a ±3 % loss of the initial soil water content. The boxes were placed at 25 ˚C until adult emergence (Garrido-Jurado et al. 2011). Puparia that failed to emerge were placed as indicated with adults to be inspected for fungal outgrowth.

In the second pathogenicity bioassay, *B. oleae* puparia were immersed in a fungal suspension for 10 s. The control was immersed in sterile distilled water containing 0.1 % Tween 80. There were three replicates of each treatment, fungus and control, and 10 puparia per replicate. The bioassay end point was four days after the first adult

emergence in the control. Puparia that failed to emerge were removed and prepared as described previously for the diagnosis of cause of death (Quesada-Moraga et al. 2006b).

2.6. Production of fungal extract of EAMb 09/01-Su strain in liquid culture

The fungal extract was produced in a liquid medium consisting of 40 g of glucose and 20 g of mycological peptone per liter. A primary culture of this medium inoculated with 1 × 10^7 conidia per ml of the *M. brunneum* strain (EAMb 09/01-Su) was cultivated on a rotatory shaker at 110 rpm for 4 d at 25 ˚C. Then, 2 ml of the primary culture was transferred to flasks containing 250 ml of liquid medium. As described above, the secondary culture was cultivated for 15 d before removing the mycelia by filtration through filter paper (Whatman No. 3Chr filter paper, Whatman, Kent, United Kingdom), and this extract was centrifuged at 9000 rpm for 20 min and concentrated 15 times in a continuous flow chamber at 30 ˚C (Quesada-Moraga and Vey 2004; Ortiz-Urquiza et al. 2010).

2.7. Evaluation of insecticidal activity of EAMb 09/ 01-Su strain crude extract against newly emerged *B. oleae* adults

The insecticidal activity of EAMb 09/ 01-Su crude extract was tested in a diet test. One-day old male and female flies were collected randomly, placed in cages (80 by 80 by 50 mm; Resopal, Alcalá de Henares, Madrid, Spain), and fed an artificial diet and crude extract (test suspension: 0.4 g of sucrose and 0.1 g of protein hydrolysate per 1.5 ml of distilled water) on a 1.5-ml centrifuge tube cap. Controls were treated with an equivalent volume of the liquid medium with the above proportions of sucrose and protein hydrolysate (control suspension). Five replicates of ten insects each were used for the control and the treatments, totaling 100 insects for the full experiment. For both treated and control insects, the microtube cap was filled with a 100-µl aliquot of test or control suspension. The experiment was conducted at 26 ± 2 ˚C, 60 ± 5 % RH, and a photoperiod of 16:8 (L:D) h. The number of dead flies was recorded at 24 h.

B.oleae adult survival was determined to the highest concentration of crude extract (10:0). Newly emerged adults were fed according to the bioassay method described above. Since the beginning of the photophase in the rearing chamber, the control and treated insects were fed for 48 h with a mortality register every 3 h. The bioassay was arranged with five replicates, with five insects per replicate. The entire procedure was repeated after 45 d with a new extract and a new generation of adult flies, gathering in total 50 insects for the full experiment.

2.8. Biological activity of EAMb 09/01-Su strain crude extract against newly emerged *B. oleae* adults

The insecticidal activity of the crude extract was tested against adults in a diet test. For this experiment, a diet was prepared containing 0.4 g of sucrose and 0.1 g of protein hydrolysate per 1.5 ml of distilled water, and the fraction of the crude extract was added as indicated in Table 1.

Two controls were used: one control was treated with an equivalent volume of the liquid medium with the above-mentioned proportions of sucrose and protein hydrolysate, and the other control was treated with an equivalent volume of the water with the above-mentioned proportions of sucrose and protein hydrolysate. Five replicates of five insects each were used for the control and the treatments, totaling 175 insects for the full experiment. For treated and control insects, the microtube cap was filled with a 100-µl aliquot of test or control suspension, respectively. The experiment was conducted at 26 ± 2 ˚C, 60 ± 5 % RH, and a photoperiod of 16:8 (L:D) h. The number of dead flies was recorded at 24 h.

2.9. Effects of *B. oleae* adult exposure time on EAMb 09/01-Su strain crude extract insecticidal activity

The effect of exposure time on the chronic insecticidal activity of the crude extract of EAMb 09/01-Su was studied using an experimental protocol that included five exposure times and the controls. One-day-old male and female flies were collected randomly and

placed in the bioassay cages. Control groups were fed with 1.5-ml microtube caps containing 100 µl of a liquid artificial diet consisting of 0.4 g of sucrose and 0.1 g of yeast hydrolysate per 1.5 ml of distilled water.

Table 1. Crude extract (15 d of fermentation) concentration range used in *per os* bioassay against *B. oleae* adults

Concn [1] (extract: water)	Vol (µl) (extract: water)
10:0	1500:0
7:3	1050:450
6:4	900:600
4:6	600:900
2:8	300:1200

[1] Concentration was defined using a 0-10 rating scale, i.e. 10:0, 10 parts of extract and 0 part of water and 2:8, 2 parts of extract and 8 parts of water.

In contrast, the treatment groups were acutely exposed to 100 µl of crude extract containing the same proportions of sucrose and yeast hydrolysate. Starting at the beginning of the photophase in the rearing chamber, both control and treatment groups were fed for 1, 3, 6, 9, and 24 h. After the groups were fed as described above, a liquid diet lacking treatment was offered to the flies until the end of the experiment. The total experimental time was 48 h, and mortality data were recorded every 24 h.

For every exposure time, five replicates consisting of five insects each were used, totaling 150 flies for this experiment. The mean time of exposure (MET), that is, the exposure time causing 50 % of mortality, was calculated by correlating the time with the mortality values reached at every time of exposure.

2.10. Effect of temperature treatments on EAMb 09/ 01-Su strain crude extract insecticidal activity against newly emerged *B. oleae* adults

The crude extract was incubated at two temperatures: 60 ˚C for 2 h and 120 ˚C for 20 min. The treated solutions were offered to adults as previously described. Control flies were fed the liquid medium with the same temperature treatments. Five replicates of five insects each were used for treatments and controls, totaling 100 insects for the full experiment. The experiment was conducted and monitored at 26 ± 2 ˚C, 60 ± 5 % RH, and a photoperiod of 16:8 (L:D) h.

2.11. Effect of irradiance on EAMb 09/01-Su strain crude extracts insecticidal activity against newly emerged *B. oleae* adults

To evaluate the effect of ultraviolet (UV) radiation on the insecticidal activity of the crude extract, it was exposed for 2, 4, and 6 h at UV-B (920 mW/cm^2). The treated solutions were offered to *B. oleae* adults as described previously. Controls flies were fed the liquid medium, water, and crude extract without UV-B irradiance treatment. Five replicates of five insects each were used for treatments and controls, totaling 150 insects for the full experiment. The experiment was conducted and monitored at 26 ± 2 ˚C, 60 ± 5 % RH, and a photoperiod of 16:8 (L:D) h.

2.12. Statistical analysis

Mortality data were analyzed using the one-way analysis of variance (Statistix 9.0, Analytical Software, Tallahassee, FL); Tukey's honestly significant difference (HSD) test was used to compare means. If data did not conform to a normal distribution, the following transformation was used:

$$\text{arc} = 180 \times \text{arcsen}\left\{\left(\frac{dead\ insects}{100}\right)^{0.5}\right\} \times \frac{360}{2 \times \text{Pi}}$$

The values of average survival times obtained by the Kaplan-Meier method and compared using the log-rank test were calculated with SPSS 15.0 Software for Windows

(SPSS, Inc., Chicago, IL). The mean lethal concentration (LC_{50}) was estimated by Probit analysis (Finney 1971), as performed with SPSS 15.0.

3. Results

3.1. Initial pathogenicity assay of EAMb 09/01-Su strain against newly emerged *B. oleae* adults

There was a significant effect of the fungal treatment with *M. brunneum* ($F_{1,7}$ = 12, P < 0.001) on total adult mortality, with a mean value of 60 % compared with uninoculated adult flies, for a mortality of 6.66 % (Table 2).

Table 2. Pathogenicity of *M. brunneum* EAMb 09/01-Su strain against *B. oleae* newly emerged adults

Treatment	Total mortality (mean ± SE)%[1]	Mortality with fungal outgrowth (mean ± SE)%[1]	Kaplan-Meier survival analysis	
			AST (d, mean ± SE)[2]	95% CI
M. brunneum	60.00±10.95a	38.78±5.41a	8.80±0.72a	7.39-10.21
Control water	6.66±6.66b	0b	12.00±0.00b	-

[1] Means within columns with the same letter are not significantly different (P ≤ 0.05) according to the Tukey's HSD test.
[2] AST limited to 12 d. Means within columns with the same letter are not significantly different (P ≤ 0.05) according to the log-rank test.

The fungal treatment also had a significant effect on mortality with fungal outgrowth ($F_{1,7}$ = 28.78, P <0.001), reaching 38.75 %, with no signs of fungal outgrowth in the controls. The AST of adults treated with *M. brunneum* EAMb 09/01-Su strain was 8.8 d, significantly shorter than that of the controls (12 d; Table 2).

3.2. Initial pathogenicity assays of EAMb 09/01-Su strain against *B. oleae* preimaginals

The treatment with the EAMb 09/01-Su strain targeting pupariating third-instar larvae had a significant effect on *B. oleae* preimaginal survival ($F_{1,7}$ = 22.78, P = 0.0031), with 82.27 % of preimaginals unable to reach the adult stage compared with 35.45 % in the controls, with a significant ($F_{1,7}$ = 8.55, P = 0.0265) percentage of nonviable preimaginals, 15.8 %, showing fungal outgrowth (Table 3). Although the fungal treatment targeting *B.*

oleae puparia did not cause a significant mortality (33.3 %) compared with the controls (13.3 %) ($F_{1,5}$ = 1.8, P = 0.25), 20 % of them showed fungal outgrowth (Table 3).

Table 3. Pathogenicity of the *M. brunneum* EAMb 09/01-Su strain against *B. oleae* third-instar pupariating larvae and puparia

Treatment	Pupariating larvae		Puparia	
	Mortality (mean ± SE)%	With fungal growth (mean ± SE)%	Mortality (mean ± SE)%	With fungal growth (mean ± SE)%
M. brunneum	82.27±10.26a	15.80±6.21a	33.33±1.20a	20.00±1.15a
Control water	35.45±4.24b	0b	13.33±0.88a	0a

[1] Means within columns with the same letter are not significantly different (P ≤ 0.05) according to the Tukey's HSD test.

3.3. Evaluation of insecticidal activity of EAMb 09/ 01-Su strain crude extract against newly emerged *B. oleae* adults

The crude extract of *M. brunneum* EAMb 09/01-Su strain (after 15 d of fermentation) exhibited a highly significant insecticidal effect on adult *B. oleae* ($F_{1,9}$ = 468.33, P < 0.001), with 70.16 % mortality after 24 h of treatment compared with mortality in the controls that was 0 and 1.52 % in the liquid medium and water controls, respectively. In a further experiment with a new batch of insects and a crude extract obtained from new inoculum, *B. oleae* adult mortality reached 80 % after 48 h, with an AST of 27.72 h (95 % confidence interval, 22.07-33.36 h) (Fig. 1).

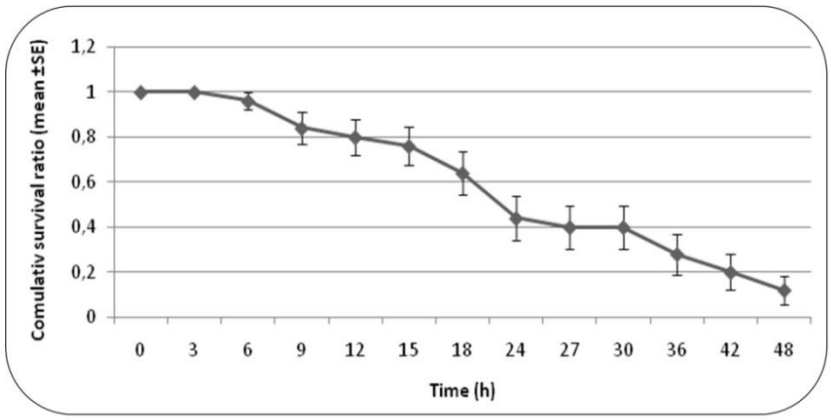

Fig. 1. Cumulative survival ratio (mean ±SE) of *B. oleae* adults fed a diet treated with the EAMb 09/01-Su crude extract after 15 d of fermentation.

3.4. Biological activity of EAMb 09/01-Su strain crude extract against newly emerged *B. oleae* adults

The mortality of adults fed for 24 h with EAMb 09/01-Su strain crude extract was dose related, ranging from 32.0 to 64.0 % (Table 4).

Table 4. Percentage of mortalityof newly emerged *B. oleae* adults after being fed for 24 h with EAMb 09/01-Su crude extract at different concentrations

Concentration[1]	Mortality (mean ± SE)[1]
20	32.00±8.00
40	40.00±8.94
60	56.00±9.79
70	64.00±7.48
100	60.00±6.32
Significance	Linear**
CV (%)[2]	24.58

**P < 0.01.
[1] It is indicated the percentage of crude extract after being water diluted. Control mortality was zero and was not included in the analysis.
[2]Coefficient of Variance.

These dose-related mortality values were submitted to concentration-mortality response regression analysis (Probit); the regression coefficient was 1.23 and the $\chi 2$ value was not significant (1.028, with 3 df), indicating good fit of the regression line. The LC_{50} valuewas 49.98 % crude extract.

3.5. Effect of *B. oleae* adult exposure time on EAMb 09/01-Su strain crude extract insecticidal activity

The exposure time significantly affected the adult mortality caused by the crude extract at 48 h ($F_{4,24}$ = 16.44, P < 0.001) in a dose-dependent manner (Table 5). The highest mortality value, 92.0 %, and the lowest AST, 30.7 h, were obtained after 24 h of exposure to the crude extract. The mortality values recorded for each exposure time were fitted to the following first-degree linear equation: Y = 3.6062X + 5.7863 (R^2 = 0.99),

where Y is the percentage of mortality and X is the exposure time. The period of exposure required to achieve 50 % mortality was 12.26 h.

Table 5. Percentage of mortality and AST of newly emerged *B. oleae* adults exposed to EAMb 09/01-Su crude extract for various exposure times and then transferred to control diet

Exposure time (h)	Mortality (48h) (mean ± SE)%[1]	Kaplan–Meier survival analysis	
		AST (h) (mean ± SE)[2]	95% CI
1	12.0±4.89	46.08±1.59a	42.95-49.21
3	12.0±8.00	46.08±1.59ac	42.95-49.21
6	28.0±8.00	43.20±2.07ad	39.14-47.26
9	40.0±6.32	42.24±2.16bce	38.00-46.48
24	92.0±4.89	30.72±2.20f	26.40-35.04

Means within columns with the same letter are not significantly different (P ≤ 0.05) according to the log-rank test.
[1] Control mortality was zero and was not included in the analysis.
[2] AST limited to 48 h.

3.6. Effect of temperature treatments on EAMb 09/ 01-Su strain crude extract insecticidal activity against newly emerged *B. oleae* adults

The temperature treatment had a significant effect on the insecticidal activityof the crude extract of EAMb 09/01-Su strain ($F_{2,14}$ = 4.91, P < 0.001). Although a significant reduction in adult mortality, from 60 % to 48 %, was observed after incubation of the crude extract for 20 min at 120 ˚C, its activity was not significantly reduced after incubation at 2 h at 60 ˚C (72 % adult mortality).

3.7. Effect of irradiance on EAMb 09/01-Su strain crude extract insecticidal activity against newly emerged *B. oleae* adults

The UV-B irradiance exposure was significantly ($F_{5,29}$ = 25.66, P < 0.001) affected EAMb 09/01-Su strain crude extract insecticidal activity, with adult mortalities of 60, 60, 48, and 56 % after 0, 2, 4, and 6 h at UV-B (920 mW/cm^2) exposure, respectively, with no mortality in the two controls, medium, and water.

4. Discussion

The results of this work indicate high susceptibility of the preimaginal- and adult-state *B. oleae* to the effects of conidia and the crude extract obtained from *M. brunneum* EAMb 09/01-Su strain. Adult mortality reached 60 % at the dose we used, whereas the AST was 8.8 d, helping to reduce the adult population in the field before the first egg laying, a critical moment of insect attack (Santiago-Álvarez and Quesada-Moraga 2007). To further contribute to this goal, the fungal inoculum may be disseminated by adults before being killed (Quesada-Moraga et al. 2008). Similar results have been obtained by Konstantopoulou and Mazomenos (2005), who, in evaluating different entomopathogenic fungi against adults of *B. oleae*, obtained mortalities between 57.8 and 63.3 % after 14 d of treatment. However, these mortalities were obtained by walking bioassay on fungal colonies, where exposure time to conidia is higher than in spraying bioassay. Besides Siebeneicher et al. (1992) reported that some application methods of conidial suspension could be more effective than others because they cover more insect body area. The similarity between both results is striking, considering the different methods used. Only Mahmoud (2009) used an application method similar to the method used in this work. With three commercial products of entomopathogenic fungi, Mahmoud (2009) obtained mortalities in the range of 47.2-70.4 % after 15 d of treatment. These mortalities are quite similar to ours, even if our product was not a commercial product.

Preimaginal stages of tephritids, particularly puparia, are less susceptible to entomopathogenic fungi (Ekesi et al. 2007). According to Vanninen et al. (1999), stages of insects living in the soil may have developed high levels of resistance to infection by natural selection because entomopathogens, especially fungi, are widespread in soil. Furthermore, the cuticle of the third stage larvae remains in the tephritids to form the puparium conferring a barrier to penetration and output of these fungal agents of microbial control. Despite such apparent mechanisms conferring resistance to fungal infection in the third instars and puparium, our results indicate high efficacy of the strain EAMb 09/01-Su against the pupariating third instars (82.3 % mortality) but less so against

puparia (33.3 %). De la Rosa et al. (2002) found that the strains of *B. bassiana* effective against adults were not pathogenic against *Anastrepha ludens* (Loew) puparia, whereas Cossentine et al. (2010) also obtained highly virulent strains of *B. bassiana* against the adult western cherry fruit fly, *Rhagoletis indifferens* Curran, but low efficacy against its larvae and puparia. Investigations of Kaaya and Munyinyi (1995) in tse-tse fly (Diptera: Glossinidae; Glossina Wiedemann) also indicated low susceptibility of puparia to entomopathogenic fungi. In previous studies, only the EAMa 01/58-Su strain of *M. anisopliae* from our collection of indigenous strains and the Bb-1333 strain of *B. bassiana*, originating from adults of *B. oleae*, have shown greater virulence against tephritid puparia used in this work (Quesada-Moraga et al. 2006b). Therefore, strain EAMb 09/01-Su of *M. brunneum* shows potential against *B. oleae* in treatments against adults in aerial part of the tree and against third-instar pupariating larvae and puparia in soil beneath the tree canopy. However, the fact that we used sterile soil in our work should be taken into account in future studies on controlling pupariating larvae in the soil environment.

The crude extract of the strain EAMb 09/01-Su of *M. brunneum* also has *per os* insecticidal activity against adults of *B. oleae*, as already observed with the crude protein extract of strain EAMa 01/58-Su (Ortiz-Urquiza et al. 2009). Specifically, the value of AST was 27.7 h and 80 % mortality against *B. oleae*. These results place this extract among the most toxic against tephritid adults derived from both plants and microorganisms (Konstantopoulou and Mazomenos 2005; Quesada-Moraga et al. 2006a; Zapata et al. 2006) and represent the first report of an extract from an entomopathogenic fungus with activity against *B. oleae*.

Adult mortality was directly related to crude extract dose, with a mean lethal concentration LC_{50} of 49.9 % crude extract. Dosages are expressed in terms of crude extract dilution. Dose-related insecticidal effects have been observed before with other fungal secreted secondary metabolites, such as destruxins, efrapeptins, and cordycepin (Amiri et al. 1999; Bandani et al. 1999; Kim et al. 2002; Konstantopoulou et al. 2006),

Photorhabdus toxins (Blackburn et al. 1998; Gerritsen et al. 2005), a few plant-derived compounds (Zapata et al. 2006), and spinosad (Raga and Sato 2005).

It is interesting to highlight the acute activity of the crude extract after 15 d of fermentation of *M. brunneum* EAMb 09/01-Su strain against *B. oleae*, with a mortality of 50 % after 12.2 h of exposure. These results are in the range of those reported in our previous work for the crude extract of the strain EAMa 01/58-Su of *M. anisopliae* against laboratory *C. capitata* adults, 8.3 h (Ortiz-Urquiza et al. 2010), these insects are very seldom more susceptible than field-collected insects such as those used in this work. This observation shows the potential of this extract for use as a bait-spray or even to develop a new "lure-and-kill" olive fruit fly control strategy.

Another aspect to highlight in this study is the photo-resistance and thermo-stability of the crude extract of the *M. brunneum* EAMb 09/01-Su strain that seems to guarantee its environmental competence. The exposure at 60 ˚C for 2 h did not significantly reduce the insecticidal activity of the extract (72.0 % mortality among adults of *B. oleae*); on the contrary, the exposure tended to increase the activity. Furthermore, exposure to 120 ˚C for 20 min only reduced the insecticidal activity of the extract by 20 %. These results are slightly different from those obtained in our previous work (Ortiz-Urquiza et al. 2009) that showed a significant reduction in the insecticidal activity of the EAMa 01/58-Su strain after exposure at 120 ˚C for 20 min; this reduction is undoubtedly related to the proteinaceous nature of this active compound. Our results also reveal the photostability of EAMb 09/01-Su *M. brunneum* crude extract, which again highlights its potential for olive fruit fly control, as UV-B exposure limits the practical use of numerous natural insecticides. This aspect is even more relevant if the heatstroke conditions prevailing in the olive tree distribution areas are considered. Further studies regarding purification of the active fraction (or compound) and its mode of action are being carrying out. The use of the pure active fraction could highly reduce the exposure time needed to achieve substantial mortality as observed in our previous studies (Ortiz-Urquiza et al. 2009). Besides, we are exploring different lure and kill devices to optimize the active adult intake ratio to accelerate the speed of kill.

In conclusion, this study reveals the potential of the EAMb 09/01-Su *M. brunneum* strain for the microbial control of *B. oleae* adults in aerial sprays and pupariating third-instar larvae and puparia in soil treatments beneath the tree canopy. In addition, the crude extract of this isolate also shows potential to be used against the tephritids as a new insecticidal compound of natural origin. Presently, we are investigating the combined use of the fungus and its extract, and we have progressed in the purification of the active fraction of the extract.

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ARTÍCULO

Este capítulo es una versión adaptada del artículo

BIOLOGICAL AND MICROBIAL CONTROL

One *Metarhizium brunneum* Strain, Two Uses to Control *Ceratitis capitata* (Diptera: Tephritidae)

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One *Metarhizium brunneum* strain, two uses to control *Ceratitis capitata* (Diptera: Tephritidae)

Abstract

We determined the virulence and insecticidal activity of the hypocrealean fungus *Metarhizium brunneum* (Petch) strain EAMb 09/01-Su and its crude extract against Mediterranean fruit fly *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) and we evaluated the combined use of the fungus with its crude extract. We also determined the effect of fermentation time, temperature, and initial pH of the *M. brunneum* culture medium on the insecticidal activity of the crude extract. When *C. capitata* adults were sprayed with a conidial suspension, the strain EAMb 09/01-Su caused 100 % mortality with a mean lethal time (LT_{50}) of 5.6 d and mean lethal concentration (LC_{50}) of 2.84×10^7 conidia per milliliter. Fermentation time significantly affected the lethality of the crude extract when it was provided to *C. capitata per os*. The highest level of mortality (73.3 %) and the shortest median survival time (25.5 h) was obtained from 15-d-old cultures. The crude extract was demonstrated to be thermostable, given that the mortality was > 50 % at 48 h when the extract had been heated to 100 ˚C for 3 h. Lastly, the optimum initial pH for maximum crude extract activity in terms of mortality ranged between 7 and 9. Additivity was observed for all *M. brunneum* EAMb 09/01-Su strain crude extract combinations tested, indicating compatibility between products. We concluded that the *M. brunneum* EAMb 09/01-Su strain is a promising tool for med fly control alone or in combination with its crude extract.

Keywords *Metarhizium brunneum*, Crude extract, Tephritidae, Biological control, Combined use

1. Introduction

The global trend to reduce the widespread use of chemical pesticides to minimize effects on humans, nontarget animals, and the environment is embodied in the most recent European phytosanitary regulations, which promote the use of nonchemical

control methods. These regulations bring some urgency to thesearch for alternative, biologically based methods of pest control that are effective, economically viable, and compatible with existing legislation (Montoya and Cancino 2004; Magaña et al. 2007). Entomopathogenicfungi (EF) can infect their host through the cuticle, putting them at the forefront of the global developmentof alternative biocontrol strategies for tephritid prepupariating larvae, puparia, and adults, as reported for *Beauveria* spp. and *Metarhizium* spp. (Ekesi et al. 2002, 2003; Konstantopoulou and Mazomenos 2005; Quesada-Moraga et al. 2006b, 2008; Santiago-Álvarezand Quesada-Moraga 2007; Ali et al. 2010; Yousef et al. 2013).

In addition to their role as microbial control agents, EF have been shown also as a rich source of structurally novel biologically active substances (Isaka et al. 2005). Although various studies have been dedicated for searching natural products derived from EF for insect control, the production of novel insecticide compounds from these organisms remains poorly explored (Ortiz-Urquiza et al. 2010b). Insecticidal compounds can be either high-molecular-weight proteins (Quesada-Moraga and Vey 2003, 2004; Ortiz-Urquiza et al. 2009, 2010a), or low-molecular-weight secondary metabolites (Amiri et al. 1999; Liu et al. 2004; Rohlfsand Churchill 2011). One of the main inherent weakness of EF that has prevented them from meeting expectations is their relatively slow kill speed compared with chemicals. Several strategies have been devised to solve this problem such as strain selection, formulation, manipulation of application parameters, etc., and more recently biotechnological improvement of the fungal strains by engineering overexpression of virulence factors (St Leger et al. 1996; Wang and St Leger 2007) and the exploitation of synergistic interactions between fungal strains and low doses of chemical insecticides (Gosselin et al. 2009; Paula et al.2011). However, the possible combined use of EF and their insecticidal compounds have not been explored as an alternative additional strategy to date. The objectives of this study were to evaluate 1) the virulence of one strain of *Metarhizium brunneum* Petch (Hypocreales: Clavicipitacea) against adult *Ceratitis capitata* Weidmann (Diptera: Tephritidae), 2) the insecticidal activity of its crude extract, and 3) to determine what fermentation parameters

optimized the insecticidal activity of the crude extract. Finally, we were interested in examining the potential of the combined use of the fungal strain and its extract to maximize the effectiveness of the mitosporic ascomycete.

2. Materials and methods

2.1. Insects

Adult *C. capitata* were obtained from a stock colony maintained at the Department of Agricultural and Forestry Sciences of the University of Cordoba (Córdoba, Spain).A colony of flies was maintained in an environmental growth chamber at 25 ± 2 ˚C, 50-60 % relative humidity (RH), and a photoperiod of 16:8 (L:D) h. Adult flies were provided with water and a standard artificial diet consisting of 40 g protein hydrolysate (ICN Biomedicals, Aurora, OH) and 10 g of sucrose (Panreac, Barcelona, Spain). Larvae were reared until pupation on an artificial diet consisting of 300 g wheat bran, 75 g of sucrose, 38 g of brewer's yeast, 2 g of nipagin, 2 g of nipasol, and 2.4 g of benzoic acid dissolved in 600 ml of distilled water per kilogram of diet.

2.2. Fungal strain

M. brunneum strain EAMb 09/01-Su was isolated in 2009 from the soil of a holm oak (*Quercus ilex* L.) forest in Castilblanco de los Arroyos, Seville, Spain (location 37˚ 42´ N, 5˚ 59´ W). This isolate belongs to the culture collection at the Department of Agricultural and Forestry Sciences of the University of Córdoba, and it is also deposited in the Spanish Type Culture Collection under accession number CECT 20784. The EF-α sequence for *M. brunneum* EAMb 09/01-Su is deposited in the GenBank nucleotide sequence database with accession number KJ158746.

2.3. Propagation of the fungal strain

To prepare inoculums for the experiments, cultures of the isolate were subcultured on malt agar (BioCult Laboratories, Madrid, Spain) in Petri plates for 12 d at 25˚C in darkness. Conidial suspensions were prepared by scraping conidia from the Petri plates

into a sterile 0.1 % Tween 80 aqueous solution that was then sonicated for 5 min and filtered through several layers of cheesecloth to remove mycelial mats and to collect pure conidia. The concentration of the conidial suspension was determined by direct counting using a Malassez chamber (Blau Brand, Wertheim, Germany). The viability of the conidia was checked before preparation of suspensions by germinating test samples in liquid Czapek-Dox broth plus 1 % (wt:vol) yeast extract medium.

In all experiments, germination rates were > 90 %. The suspension was stored in a refrigerator at 4 ˚C for no longer than 24 h.

2.4. Virulence assay of EAMb 09/01-Su strain against newly emerged *C. capitata* adults

Newly molted adults (< 24 h) that had previously been cold anesthetized were treated with a Potter tower (Burkard Manufacturing Co. Ltd, Rickmansworth, the United Kingdom), with a deposit of 1.54 ± 0.06 mg cm^{-2} and a 0.7 bars of pressure. One milliliter of conidial suspension was used for each replicate, with three replicates per treatment, 10 adult flies each. The following five concentrations were used: 10^5, 10^6, 10^7, 10^8, and 10^9 conidia per milliliter. The controls were treated with the same volume of a sterile aqueous solution of 0.1 % Tween 80. The treated adults were placed in metacrylate cages (8 by 8 by 6 cm; Resopal, Alcalá de Henares, Madrid, Spain) containing a circular hole (2 cm in diameter) covered with a net cloth; a liquid diet (0.1 g of hydrolyzed protein and 0.4 g sucrose with 1.5 ml of distilled water) was provided every 48 h. The bioassay was conducted at 26 ± 2 ˚C, 50-60 % RH, and a photoperiod of 16:8 (L:D) h. Mortality was monitored for 12 d. Dead flies were removed daily to prevent horizontal transmission of the fungus and were immediately surface-sterilized with 1 % sodium hypochlorite for 1 min, followed by three rinses with sterile distilled water for 1 min each. They were then placed on sterile wet filter paper in sterile Petri plates that were sealed with Parafilm and kept at 25 ˚C to be inspected for fungal outgrowth on the cadavers (Quesada-Moraga et al. 2006a). The whole experiment was repeated after 45 d with a new fungal suspension and a new generation of *C. capitata* adult flies.

2.5. Production of EAMb 09/01-Su strain fungal extract in liquid culture

The crude fungal extract was produced using a two-step protocol. First, a primary culture of EAMb 09/01-Su was produced by adding 1 ml of a 10^7 conidia per ml suspension to 100 ml Erlenmeyer flasks containing 25 ml of the liquid medium G40:P20 (40 g glucose and 20 g mycological peptone per liter of distilled water). These primary culture flasks were maintained on a rotary shaker at 110 rpm for 4 d at 25 ˚C. Two-milliliter aliquots of this primary culture were added to a 1000 ml flasks containing 250 ml of the above-described liquid medium and were maintained under the same conditions for either 10, 11, 12, 13, 14 15, or 16 d, with three flasks per day. Then, mycelia were removed from the 10-16 d-old liquid fermentations via vacuum filtration through a Whatman No. 3 Chr filter paper (Whatman, Kent, UnitedKingdom). This extract was centrifuged at 9000 rpm for 20 min at 4 ˚C, and the supernatant was concentrated 15 times in a continuing flow chamber at 30 ˚C (Quesada-Moraga and Vey 2004; Ortiz-Urquiza et al. 2010a,b). The concentrated supernatant was stored at -20 ˚C until use.

The rate of biomass (milligram dry biomass per milliliter liquid medium) and protein (microgram soluble protein per milliliter liquid medium) production in each fermentation time was measured. The biomass was collected and placed in Petri dishes and dried in an oven at 70 ˚C for 48 h, and the dry weight was determined. The soluble protein content was determined by the Bradford assay (Bradford 1976) using bovine serum albumin (Merk-Schuchardt, Hohenbrunn, Germany) as the standard. Absorbance at 595 nm was used to estimate the protein content.

2.6. Effect of fermentation time on the insecticidal activity of the EAMb 09/01-Su strain crude extract against newly emerged *C. capitata* adults

The crude extract from each fermentation time, from 10 to 16 d, was collected as described above, and the insecticidal activity of each extract was evaluated against *C. capitata* adults. Recently emerged adults (< 24 h) were placed in metacrylate cages (8 by 8 by 6 cm) containing a circular hole (2 cm in diameter) covered with a net cloth, with nine replicates of 10 adults for each replicate. Control groups were fed with 1.5-ml

microtube caps containing 100 µl of a liquid artificial diet consisting of 0.4 g of sucrose and 0.1 g of protein hydrolysate per 1.5 ml of distilled water. In contrast, the treatment groups were fed 100 µl of crude extract containing the same proportions of sucrose and protein hydrolysate. The experiment was conducted at 26 ± 2 ˚C, 60 ± 5 % RH, and a photoperiod of 16:8 (L:D) h, and the mortality was recorded every 3 h during 48 h. After this trial, the extract fermented for 15 d was selected for the remaining experiments. The entire procedure was repeated after 45 d with a new fungal extracts and a new generation of *C. capitata* adult flies.

2.7. Insecticidal activity of EAMb 09/01-Su strain crude extract fermented for 15 d against newly emerged *C. capitata* adults

This is a test to determine how the concentration of the 15-d-old crude extract added to artificial diet affects survival of newly emerged adult flies. For this experiment, a diet was prepared containing 0.4 g of sucrose and 0.1 g of protein hydrolysate per 1.5 ml of distilled water, and the crude extract was added at concentrations of 10, 30, 50, 70, and 100 %.

Two controls were used. One was treated with an equivalent volume of the liquid culture medium with the above proportions of sucrose and protein hydrolysate, and the other was treated with an equivalent volume of water with the above proportions of sucrose and protein hydrolysate. Three replicates of 10 insects each were used for the controls and the treatments. For treated and control insects, the microtube cap was filled with a 100 µl of aliquot of test or control suspension, respectively. The experiment was conducted at 26 ± 2 ˚C, 60 ± 5 % RH, and a photoperiod of 16:8 (L:D) h. The number of dead flies was recorded at 24 and 48 h. The experiment was repeated after 45 d with a new fungal extract and a new generation of *C. capitata* adult flies.

2.8. Effect of exposure time of the EAMb 09/01-Su strain crude extract against *C. capitata* adults

The effect of exposure time on the insecticidal activity of the crude extract of EAMb 09/01-Su was studied using an experimental protocol that included six exposure times and the controls. One-day-old male and female flies were collected and randomly placed in the bioassay cages. The control groups were fed with 1.5-ml microtube caps containing 100 µl of a liquid artificial diet consisting of 0.4 g of sucrose and 0.1 g of protein hydrolysate per 1.5 ml of distilled water. In contrast, the treatment groups were exposed to 100 µl of crude extract containing the same proportions of sucrose and protein hydrolysate. Starting at the beginning of the photoperiod in the bioassay chamber, both the control and treatment groups were fed for 1, 3, 6, 9, 12, and 24 h. After the groups were fed as described above, a liquid diet lacking crude extract was offered to flies until the end of the experiment. The total experimental time was 48 h, and mortality data were recorded every 24 h. Three replicates consisting of 10 insects each were used for every exposure time. The exposure time causing 50 % mortality (mean exposure time) was calculated by correlating the time with the mortality values obtained for every exposure time. The experiment was repeated after 45 d with a new extract and a new generation of *C. capitata* adult flies.

2.9. Effect of temperature treatment on the insecticidal activity of the EAMb 09/01-Su strain crude extract against newly emerged *C. capitata* adults

The crude extract was incubated at four temperatures (25, 50, 75, and 100 ˚C) and three exposure times for each temperature (1, 2, and 3 h). The treated crude extract was offered to adults with the same proportions of sucrose and protein hydrolysate (0.4 g of sucrose and 0.1 g of protein hydrolysate per 1.5 ml of distilled water). Control flies were fed the nonfungus-inoculated liquid fermentation medium with the same temperature treatments. Three replicates of 10 adults each were used for treatments and controls, which were placed in the metacrylate cages previously described, and the various treated extracts were offered in accordance with the procedure already described. The

experiment was conducted and monitored at 26 ± 2 ˚C, 60 ± 5% RH, and a photoperiod of 16:8 (L:D) h. Mortality was recorded at 24 and 48 h. The experiment was repeated after 45 d with a new extract and a new generation of *C. capitata* adult flies.

2.10. Effect of initial pH of the culture medium on the insecticidal activity of the EAMb 09/01-Su strain crude extract against newly emerged *C. capitata* adults

To study the effect of the initial pH of the culture medium on the insecticidal activity of the crude extract, a liquid medium of 40 g glucose and 20 g mycological peptone per liter was prepared, and the initial pH of the liquid medium was adjusted in a range of 5.5-10 by addition of solutions of either 1N HCl or 1N NaOH. Three 500-ml flasks that contained 125 ml of the liquid medium were used for each pH and inoculated with 2 ml of the primary culture and were maintained on a rotary shaker at 110 rpm for 15 d at 25 ˚C. The extracts of each pH were obtained. Three replicates of 10 *C. capitata* adults each were used for each extract and control, which were placed in the metacrylate cages where the extract were offered in accordance with the procedure described above. The final pH, rate of biomass, and protein were measured for each initial pH condition. Mortality was recorded at 24 h. The entire procedure was repeated after 45 d with a new extracts and a new generation of adult flies.

2.11. Combined treatment of the entomopathogenic fungus and its extract against newly emerged *C. capitata* adults

To evaluate the types of effects (synergistic, additive, or antagonistic) that can be produced by the combined use of the *M. brunneum* EAMb 09/01-Su strain and its crude extract, two concentrations (LC_{50} and LC_{90}) of the fungal suspension were prepared on the basis of the above virulence bioassay. These fungal suspensions were applied using a Potter tower (Burkard Manufacturing Co. Ltd, Rickmansworth, United Kingdom) in combination with three exposure times (1, 2, and 3 h) of the crude extract which was prepared and applied *per os* as described above. The crude extract was applied directly after the fungal suspension. The treatments were as follows: T1: LC_{90} of the fungal

suspension and 1 h exposure to the crude extract; T2: LC_{90} of the fungal suspension and 2 h exposure to the crude extract; T3: LC_{90} of the fungal suspension and 3 h exposure to the crude extract; T4: LC_{50} of the fungal suspension and 1 h exposure to the crude extract; T5: LC_{50} of the fungal suspension and 2 h exposure to the crude extract; T6: LC_{50} of the fungal suspension and 3 h exposure to the crude extract; T7: LC_{50} of the fungal suspension (spray); T8:LC_{90} of the fungal suspension (spray); T9: 1 h exposure to the crude extract; T10: 2 h exposure to the crude extract; T11: 3 h exposure to the crude extract; T12: water spray; T13: *per os* supply of growth medium.

The bioassay was conducted as described for the virulence bioassay. Mortality was monitored for 11 d. Dead flies were treated as described above to be inspected for fungal outgrowth. Each treatment was replicated three times with 10 adult flies per replicate. The whole experiment was repeated after 45 d with a new fungal suspension and extract and a new generation of adult flies.

2.12. Statistical analysis

The mean lethal concentration (estimated concentration required to kill 50 % of the test insects, LC_{50}) was estimated by Probit analysis (Finney 1971), and the mean lethal time (estimated time to kill 50 % of the insects, LT_{50}) was determined using the Probit analysis method for correlation data (Throne et al. 1995). Regression of time and dry biomass and soluble protein production during fermentation was performed by the program Statistix 9.0. Mortality data were analyzed using one-way analysis of variance (analysis of variance, Statistix 9.0 [Analytical Software 2008]), and the Tukey's honestly significant difference (HSD) test was used to compare means. A Kruskal-Wallis one-way nonparametric AOV analysis for the fermentation time data were performed using the program Statistix 9.0 (Analytical Software 2008). The values of average survival times (ASTs) were obtained by the Kaplan-Meier method and compared using the log-rank test calculated with SPSS 15.0 software for Windows (SPSS Inc., Chicago, IL). The data were analyzed to determine whether there was a synergic, additive, or antagonistic effect between the isolate and its extract. These assessments were made using a chi-square

test (Koppenhöfer and Fuzy 2008). The expected interaction mortality value (M_E) was calculated using the formula $M_E = M_S + M_C (1 – M_S/100)$, where M_S is the observed Abbott corrected mortality (OACM) caused by the application of fungal suspension, and M_C is the OACM caused by the *per os* administration of the crude extract alone. The results from the χ2 tests $\chi2 = (M_{SC} – M_E)^2/M_E$) were compared with the χ2 table value for 1 *df*. If the calculated χ2 value was less than that of the χ2 table, there was an additive effect between the two agents. If the calculated χ2 value was greater than the value in the χ2 table and $M_{SC} – M_E > 0$, the interaction was considered to be significant and synergistic.If the calculated χ2 value was less than the value of the χ2 table and $M_{SC} – M_E < 0$, the interaction was considered to be significant and antagonistic.

3. Results

3.1. Virulence assay of EAMb 09/01-Su strain against newly emerged *C. capitata* adults

The mean mortality at 6 and 12 d post inoculation ranged from 13.3 to 63.3 % and from 20 to 100 %, respectively, with control mortality values of 6.7 and 10 % at 6 and 12 d, respectively. *M. brunneum* outgrew from 100 % of cadavers in the 10^9 conidia per ml treatment, 92.3 % in the 10^8 conidia per ml treatment, 80 % in the 10^7 conidia per ml treatment, and from 33.3 and 42.8 % in the 10^5 and 10^6 conidia per ml treatments, respectively.

These mortality values were subjected to Probit regression analysis. The regression coefficient (slope ± SE) was 0.54 ± 0.08, and the chi-square value was not significant (χ2 = 6.6, with 3 df), indicating a good fit of the regression line, with a LC_{50} value of 2.84×10^7 conidia per ml.

The cumulated mortality values were also subjected to Probit analysis, providing not significant chi-square values (slope ± SE = 4.3 × 1.1, χ2 = 1.35, with 2 df; slope ± SE = 9.9 ± 1.4, χ2 ± 0.77, with 2 df), respectively, indicating a good fit of the regression lines, with LT_{50} values of 6.6 and 5.6 d for 10^8 and 10^9 conidia per ml, respectively.

3.2. Effect of fermentation time on the insecticidal activity of EAMb 09/01-Su strain crude extract against newly emerged *C. capitata* adults

The increase in dry biomass and soluble protein in the crude extract of the strain in medium G40:P20 was linear with fermentation time (R^2 = 0.97 and R^2 = 0.98; P < 0.001, respectively). The dry biomass was greatest on day 11 with 22.9 mg dry biomass per ml, and the protein content was greatest on day 15 with 369.3 µg soluble protein per ml. The fermentation time had no significant effect on the insecticidal activity of the crude extract against adult flies ($F_{6,20}$ = 1.26, P = 0.334). Although the highest mortality (83.3 %) was recorded for the extract after 13 d of fermentation, shorter survival time was recorded for the extract after 15 d of fermentation (Table 1).

Table 1. Insecticidal activity of EAMb 09/01-Su crude extract produced with different fermentation times against newly emerged *C. capitata* adults

Fermentation time (d)	Mortality (48 h) (mean ± SE)%[1]	Kaplan-Meier survival analysis	
		AST (h) (mean ± SE)[2]	95% CI
10	60±0a	38.3±2.4 a	33.7-42.9
11	63.3±13.3a	33.5±2.7 ab	28.2-38.8
12	66.7±12.0a	32.4±2.8 abc	26.9-37.8
13	83.3±6.7a	28.6±3.1 bcd	22.6-34.6
14	70±5.7a	33.5±2.8 ade	28.1-38.9
15	73.3±3.3a	25.5±3.2 bcef	19.2-31.8
16	56.7±8.8a	32.7±3.3 adf	26.2-39.2

[1] Control mortality was zero and was not included in the analysis. Means within columns with the same letter are not significantly different (P ≤ 0.05) according to a Kruskal-Wallis AOV followed by pairwise comparison of Mean Ranks.
[2]AST limited to 48 h.

3.3. Insecticidal activity of EAMb 09/01-Su Strain 15 days crude extract against newly emerged *C. capitata* adults

The mortality of adults fed on the crude extract (15 days fermentation) for 24 and 48 h was concentration related, ranging from 30 to 63.3 % and from 53.3 to 80 %, respectively.

These concentration-related mortality values were subjected to Probit regression analysis. After 24 h exposure, the regression coefficient (slope ± SE) was 0.89 ± 0.3, and

the χ2 value was not significant (χ2 = 0.089, with 3 df), indicating good fit of the regression line. The LC_{50} value was 40.54 % crude extract.

3.4. Effect of exposure time of the EAMb 09/01-Su strain crude extract against *C. capitata* adults

Exposure time significantly affected adult mortality ($F_{6,20}$ = 21.71, P < 0.001; Table 2).

Table 2. Percentage of mortality and AST of newly emerged *C. capitata* adults exposed to the EAMb 09/01-Su strain crude extract for various times and then transferred to control diet

Exposure time (h)	Mortality (48 h) (mean ± SE)%	Kaplan–Meier survival analysis	
		AST (h) (mean ± SE)[1]	95% CI
0	6.7±3.3	47.2±0.8a	45.7-48.7
1	16.7±8.8	45.6±1.5ab	42.7-48.5
3	36.7±6.7	41.3±2.0b	37.3-45.2
6	50±5.8	38.4±2.2bc	34.1-42.5
9	70±5.8	35.2±2.2cd	30-38.8
12	76.7±8.8	34.4±2.3de	30.8-39.6
24	83.3±3.3	32±2.1de	27.9-36.1

[1]AST limited to 48h. Means within columns with the same letter are not significantly different (P ≤ 0.05) according to the log-rank test.

The highest mortality value, 83.3 %, and the lowest AST, 32 h, were obtained after 24 h of exposure to the crude extract. Similarly, the lowest mortality value, 16.6 %, and the highest AST, 45.6 h, were obtained after 1 h of exposure to the crude extract. The exposure required to achieve 50 % mortality (mean exposure time) was 6 h (Table 2).

3.5. Effect of temperature treatment on the insecticidal activity of the EAMb 09/01-Su strain crude extract against newly emerged *C. capitata* adults

Both the temperature treatment ($F_{3,35}$ = 5.65, P = 0.005) and the exposure time to each temperature ($F_{2,35}$ = 7.93, P = 0.0025) had a significant effect on the insecticidal activity of the crude extract (24-h exposure) with mortality values of *C. capitata* adults ranged between 42.2 and 55.5 % and between 39.1 and 51.6 %, respectively, whereas the interaction betweenboth factors was not significant ($F_{6,35}$ = 1.8, P = 0.1462). The relationship found between mortality and temperature was linear (L*) and quadratic (Q**), while the relationship between mortality and exposure time was strongly linear

(L***). The same trend was observed at 48 h of exposure, which also showed significant differences between temperatures ($F_{3,35}$ = 4.49, P = 0.0116) and exposure times ($F_{2,35}$ = 4.39, P = 0.0168 with mortality values of *C. capitata* adults ranging between 62.2 and 78.8 % andbetween 60.8 and 73.3 %, respectively, although againthe interaction between both factors was not significant ($F_{6,35}$ = 1.46, P = 0.2156; Fig. 1).

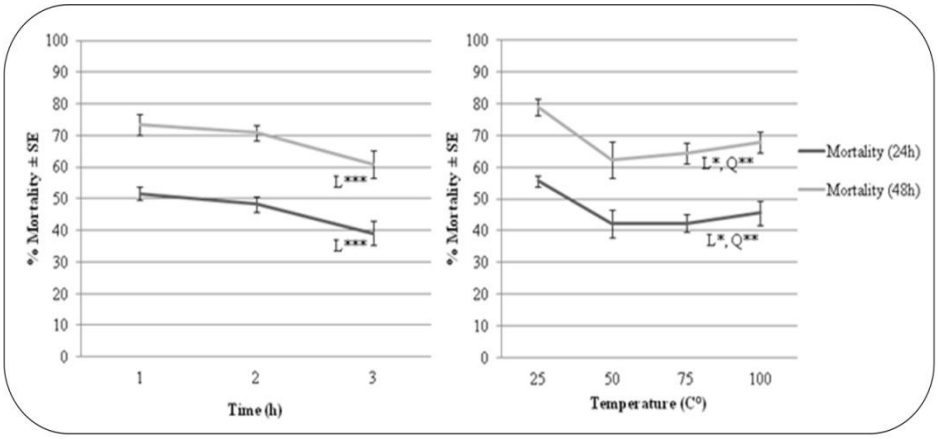

Fig. 1. Effects of temperature and exposure time on the insecticidal activity of the crude extract (mean ± SE) from the *M. brunneum* EAMb 09/01-Su strain (15 days of fermentation) against newly emerged adults of *C. capitata*. Mortality at 24 and 48 h. L= Linear, Q= quadratic, * P <0.05, ** P <0.01, *** P <0.001.

3.6. Effect of initial pH of the culture medium on the insecticidal activity of the EAMb 09/01-Su strain crude extract against newly emerged *C. capitata* adults

The pH of the culture medium had a significant effect on both the fungal biomass production ($F_{7,23}$ = 12.08, P < 0.001), which significantly decreased with increasing pH values, and protein content ($F_{7,23}$ = 69.95, P < 0.001), which increased with increasing pH values (Table 3), and on the pH final values ($F_{7,23}$ = 50.35, P < 0.001). In general, the pH tended to drop during fermentation, with most final pH values in the range of 4-4.5, except those from flasks with initial pH 9 and 10, which remained > 6. Furthermore, the pH of the culture medium had a significant effect on the insecticidal activity of the crude extract ($F_{7,23}$ = 3.17, P = 0.026). Although mortality increased with increasing initial pH, reaching 93 %, there were no significant differences in mortality for extracts with pH between 6 and 10 (Table 3).

Table 3. Influence of the initial pH of the culture medium G40: P20 on the protein, biomass production, insecticidal activity, and final pH of the crude extract of the fungus *M. brunneum* EAMb 09/01-Su strain obtained from 15 days of fermentation

Initial pH	Biomass mg per ml (mean ± SE)	Protein µg per ml (mean ± SE)	Mortality (24h) (mean ± SE)%	Final pH (mean ± SE)
5.5	22.7±0.9a	79.7±7.5d	60±5.8b	4.07±0.03b
6	20.5±0.8ab	95±4.2cd	76.7±3.3ab	4.21±0.06b
6.5	19.1±0.5bc	107.3±3.8cd	76.7±6.7ab	4.06±0.03b
7	19.5±1.1bc	122±4.9cd	93.3±3.3a	4.07±0.03b
7.5	17.9±0.3bcd	115.3±6.5cd	76.7±8.8ab	4.25±0.11b
8	16.1±0.4cd	139.3±4.1c	80±5.8ab	4.36±0.01b
9	17.9±0.5bcd	243.7±10.7b	90±5.8a	6±0.23a
10	15.3±0.4d	294.3±19.8a	76.7±3.3ab	6.68±0.30a

Control mortality was zero and was not included in the analysis. Means within columns with the same letter are not significantly different ($P \leq 0.05$) according to the Tukey HSD test.

3.7. Combined effect of treatment with the entomopathogenic fungus and its extract against newly emerged *C. capitata* adults

The combined use of EAMb 09/01-Su strain with its crude extract had a significant effect on the mortality of *C. capitata* adults ($F_{10,32} = 45.92$, $P < 0.001$). Higher mortalities were registered for combined treatments when compared with noncombined treatments. The maximum mortality was achieved with the combination of 1, 2, and 3 h of exposure to the crude extract and the LC_{90} of the fungal suspension (100 % mortality in the T1, T2, and T3 groups after 11 d of treatment). The noncombined treatments had mortality values between 26.6 and 73.3 % for T9 (1 h of exposure to the crude extract) and T8 (spraying with the LC90 of the fungal suspension), respectively (Table 4).

The *M. brunneum* outgrowth from the cadavers was independent of the combined treatment, with significant differences between both treatments with the fungal suspension (LC_{90} and LC_{50}; $F_{10,32} = 106$, $P < 0.001$).

The mortality with fungal outgrowth values ranged between 91.6 and 100 % for the T1, T2, T3, and T8 treatments performed with the LC_{90} of the fungal suspension, and between 52.3 and 62.8 % for the T4, T5, T6, and T7 treatments with the LC_{50} of the fungal suspension (Table 4).

All combinations of the fungal suspension and the crude extract had an additive effect on *C. capitata* adults based on the chi-square test (Table 4).

Table 4. Susceptibility and response of newly emerged *C. capitata* adults to treatment with different combinations of the EAMb 09/01-Su strain and its extract

Treatment[1]	Total mortality (11d) (mean ± SE)%	Mortality with fungal outgrowth (mean ± SE)%	$\chi^2_{calculated}$ (*df* = 1)	$\chi^2_{table\ value}$[2]	Type of effect
T1	100±0a	96.6±3.3a	4.77	10.83*	Additive
T2	100±0a	100±0a	4.77	10.83*	Additive
T3	100±0a	100±0a	3.05	3.84**	Additive
T4	63.3±6.6c	62.8±4.3b	0.10	3.84**	Additive
T5	70.3±5.7bc	52.3±2.3c	1.48	3.84**	Additive
T6	80±0bc	62.5±7.2bc	2.13	3.84**	Additive
T7	46.6±3.3d	56.6±3.3bc	-	-	-
T8	73.3±3.3bc	91.6±8.3a	-	-	-
T9	26.6±3.3e	0d	-	-	-
T10	26.6±6.6e	0d	-	-	-
T11	40.0±5.7d	0d	-	-	-

Control mortality was zero and not included in the analysis. Means within columns with the same letter are not significantly different (P ≤ 0.05) according to the Tukey HSD test.

[1] T1: LC_{90} of the fungal suspension (FS) and 1 h exposure to the crude extract (ETCE). T2: LC_{90} of the FS and 2 h ETCE. T3: LC_{90} of FS and 3 h ETCE. T4: LC_{50} of the FS and 1 h ETCE. T5: LC_{50} of the FS and 2 h ETCE. T6: LC_{50} of the FS and 3 h ETCE. T7: Pulverization with the LC_{50} of the FS. T8: Pulverization with the LC_{90} of the FS. T9: 1 h ETCE. T10: 2 h ETCE. T11: 3 h ETCE. T12: control water spray; T13: control *Per os* supply of growth medium.

[2] Chi-square table value: * p ≤ 0.05, ** p ≤ 0.01.

4. Discussion

This study reports the high virulence of the *M. brunneum* EAMb 09/01-Su strain against *C. capitata*a dults and the high insecticidal activity of its crude extract against the same pest when applied alone or in combination. Adult mortality reached 100 % at the highest fungus concentration assayed (10^9 conidia per ml), with LT_{50} of 5.6 d, which would help to reduce the adult population in the field before the first egg laying as far as the oviposition begins between 5 and 10 d after adult emergence (Alfaro-Moreno 2005). Meanwhile, adults may disseminate the fungal inoculums before they are killed (Quesada-Moraga et al. 2008). Likewise, the spread of the fungal inoculum is further

promoted by the fact that this *M. brunneum* strain grew from all insect cadavers treated with the concentration 10^9 conidia per ml and from 92.3 % of the insect cadavers treated with the concentration 10^8conidia per ml.

The crude extract of the *M. brunneum* EAMb 09/01-Su strain also showed *per os* insecticidal activity against *C. capitata* adults, although it depended on the period of fermentation. Specifically, the lowest value of AST (25.5 h) was obtained with an extract after 15 dof fermentation with a mortality rate of 73.3 % against *C. capitata* adults. The higher insecticidal activity of the extracts obtained from later stages of fungal fermentation reveals that the molecules responsible for such activity could be secondary metabolites, and particularly destruxins, whose insecticidal activity has been described before (Wang et al. 2004).

Adult mortality was directly related to the dose of the crude extract, with a mean lethal concentration (LC_{50}) of 40.5 %. Dose-related insecticidal effects have been observed for other fungal secreted secondary metabolites, such as destruxins, efrapeptins, and cordycepin (Amiri et al. 1999; Bandani and Butt 1999) and for fungal secreted proteins (Quesada-Moraga et al. 2006a; Ortiz-Urquiza et al. 2009). Furthermore, the crude extract exhibited chronic insecticidal activity, although at least 6 h of feeding were required to reach 50 % mortality. In our previous work on the crude soluble protein extract of the *M. brunneum* EAMa 01/58-Su strain (formally *Metarhizium anisopliae*) against *C. capitata* adults, the exposure time required to reach 50 % mortality was 8.3 h (Ortiz-Urquiza et al. 2010a). This observation shows the potential use of this extract as a bait-spray or even in the development of a new “lure and kill” medfly control strategy. The temperature and exposure time had a significant effect on the insecticidal activity of this extract. The mortalityof the adults was > 50 % at 48 h even in extreme temperature and exposure time conditions (100 ˚C during 3 h), showing the thermostability of the extract.These results are slightly different from those obtained in our previous works (Ortiz-Urquiza et al. 2009; Quesada-Moraga et al. 2006a), which showed a significant reduction in the insecticidal activity of the EAMa 01/58-Su strain after exposure at 120 ˚C

for 20 min; this reduction is undoubtedly related to the proteinaceous nature of its active compound.

To further optimize the production of the EAMb 09/01-Su crude extract, we evaluated the effect of the initial pH of the culture medium on the insecticidal activity of the extract, which is considered one of the most important factors in the optimization of the fermentation conditions (Hu et al. 2006). Mortality increased with increasing initial pH to 93 and 90 % at pH 7 and 9, respectively, with high mortality caused by the extracts secreted from medium with other initial pH values. The production of insecticidal compounds occurs at different initial pH values, but the best values were 7 and 9. The higher mortalities produced at pH 7 and 9 may be due to the secretion of destruxins as previously reported (Hu et al. 2006; Liu et al. 2007).

The compatibility between EF and chemical insecticides (Morales-Rodriguez and Peck 2009; Huang et al. 2013), natural insecticides (Gosselin et al. 2009; Hernández et al. 2012), or even fungal compounds (Hu et al. 2007) was previously tested in other studies. However, this is the first report dealing the compatibility of one entomopathogenic fungal strain and its own crude extract for pest control. The mortality of *C. capitata* adults was higher for combined treatments than when those treatments were applied individually, exhibiting an additive effect. The interaction between biological control agents and chemical or natural products can have different responses (Purwar and Sachan 2006). However, to achieve stronger synergistic effects, both products usually are applied at different times (Ansari et al. 2004) or at higher concentrations (Gosselin et al. 2009). The additive effect found in all fungus-extract combinations gives the option of using the LC_{50} of the fungal suspension and 1 h of exposure to the crude extract as an effective control for *C. capitata* adults with less risk to natural enemies in the environment.

In conclusion, this study reveals the potential of the *M. brunneum* EAMb 09/01-Su strain for the microbial control of *C. capitata* adults in aerial sprays with conidia. In addition, the crude extract of this isolate also shows potential to be used against tephritids as a new insecticidal compound of natural origin. Furthermore, the combined

use of the EAMb 09/01-Su strain and its extract enhances the short-term protection by the extract and provides long-term suppression of subsequent generations through the persistence of the fungus in the environment. Presently, we are investigating the field application of EF and their crude extracts for the control of tephritid fruit flies, and we are progressing in the purification of the active fraction of the extract to identify the compound responsible for the insecticidal activity.

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ARTÍCULO

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ORIGINAL PAPER

Reduction of adult olive fruit fly populations by targeting preimaginals in the soil with the entomopathogenic fungus *Metarhizium brunneum*

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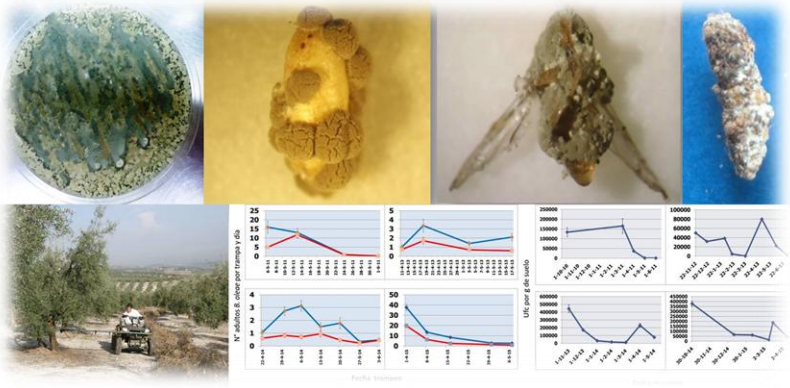

Reduction of adult olive fruit fly populations by targeting preimaginals in the soil with the entomopathogenic fungus *Metarhizium brunneum*

Abstract

The primary research focus of the present work has been to evaluate the effectiveness of soil applications with *Metarhizium brunneum* EAMa 01/58-Su strain beneath the tree canopy targeting *Bactrocera oleae* (Rossi) preimaginals to reduce the adult fly population. In a first series of laboratory bioassays, the virulence of the strain against puparia and adults was ascertained, with LC_{50} values of 1.0×10^7 and 7.0×10^6 conidia ml^{-1} respectively. In a second long-term field experiment, an experimental mycoinsecticide was produced using this strain and evaluated in selected farms in Andalusia (Spain) over 4 years. In each season, the field experiments lasted for 4-5 months, with two applications of the mycoinsecticide beneath the tree canopy at a rate of 1.0×10^5-3.0×10^5 conidia g $soil^{-1}$, the first one in late fall, when prepupariating larvae typically fall from the olive fruit to the soil, and the second one in early spring, when adult emergence from the soil is expected. After each application, the fungal inoculum reached basal levels varying from 0.8×10^2 to 1.1×10^3 conidia g $soil^{-1}$. During the four seasons, a 50-70 % reduction of adult olive fruit fly populations was observed in the treated plots compared with the untreated ones, which could help in minimising the number of treatments, if any, within an olive fruit fly IPM strategy.

Keywords *Bactrocera oleae*, *Metarhizium brunneum*, integrated management, soil application, tephritid puparia

Key messages

- *B. oleae* populations are difficult to be managed due to both, the low number of available active ingredients and the development of resistance to most of them.
- Strain EAMa 01/58-Su of *Metarhizium brunneum* is a promising tool to be used in an integrated olive fruit fly management strategy.

- Soil sprays of fungal suspension of this strain beneath the tree canopy targeting preimaginals in winter led to a 50-70 % reduction in the *B. oleae* adult population emerging on the following spring

1. Introduction

Olive grove is one of the most important crops in the Mediterranean basin, and plays an important role in economical, cultural and social areas (Loumou and Giourga 2003). The Mediterranean basin alone provides 98 % of the total acreage for olive tree culture and 97 % of total olive production (MAGRAMA 2014). Despite being a traditional crop, olive cultivation is again on the upswing, especially because of the establishment of intensive irrigated plantations where an adequate pest management is considered essential (Metzidakis et al. 2008). The fruit of the olive tree is attacked by a diverse set of species, whereas the monophagous olive fruit fly *Bactrocera oleae* is the most destructive to the olive crop worldwide (Daane and Johnson 2010). The entry of this tephritid fly into California and Mexico has caused problems for international trade since 1998 (Nardi et al. 2005; Neokosmidi et al. 2005; Yokoyama 2012).

The olive fruit fly is a multivoltine species with 2 to 5 generations per year depending on the local and regional temperature and humidity conditions (Ruiz-Castro 1948). *B. oleae* females lay their eggs inside the fruits. After egg hatching, larvae feed on the mesocarp and develop through three instars destroying and consuming the pulp and facilitating the development of secondary organisms. As in most tephritid species, at the end of the third stage, the larva pupates in the olive fruit or jumps to pupate on the ground (Santiago-Álvarez and Quesada-Moraga 2007; Kakani et al. 2010). An increasing number of third instar larvae leave the olive fruit to pupate in the soil from mid-autumn onwards (Dimou et al. 2003). The harmful effects caused by *B. oleae* on olive production are mainly the premature olive drop, the alimentary process of the larvae inside the fruit flesh which ranged from 50 to 150 mg per larva, depending on olive variety, and decrease of oil quality depending on the percentage of damaged fruits, fruit development stage, and variety (Neuenschwander and Michelakis 1978; Gucci et al.

2012). However, in some varieties, the increasing acidity is directly proportional to the *B. oleae* attack (Mraicha et al. 2010). For more than fifty years, *B. oleae* control has been based on the abusive use of chemical insecticides (Haniotakis 2005). The overuse of these chemicals caused severe effects on ecosystems and human health, and also it is difficult to deal with the *B. oleae* populations due to the development of resistance (Margaritopoulos et al. 2008; Kakani et al. 2010; Pascual et al. 2010; Hsu et al. 2015). Regarding other control measures, the efficacy of mass-trapping depends on a number of variables including the trap type, trap density and deployment, attractant(s) and their formulations, insecticides (in toxic traps) as well as the methods of application, the degree of orchard isolation, the size of the protected orchard, local environmental factors (temperature and relative humidity), biological factors (pest population density in the protected orchard, tree size, fruit variety size, and fruit load), cultural factors (irrigation, pruning, and soil fertilization), and the number of years the method has been applied in the same orchard. Together, these factors limit the use of traps primarily to monitoring of pest populations (Muñoz and Marí 2012; Yasin et al. 2014). Furthermore, many studies have demonstrated that kaolin and copper salt sprays reduce parasitism rates by Psyttalia females and negatively affect other arthropod communities at the soil level (Bengochea et al. 2014). For all that, nowadays, there is increasing concern over dependence on chemical pesticides, and a global trend to find new methods for pest control. The progress of pest control programs achieved in recent years consisted of exploit the potential of entomopathogenic organisms, particularly the entomopathogenic fungi (EF) (Khan et al. 2012). However, their effectiveness against *B. oleae* has not yet been well studied. Konstantopoulou and Mazomenos (2005) study, shed light on the use of EF against *B. oleae*, by evaluating different EFs against adults of olive fly, our previous studies revealed the pathogenecity of *Metarhizium brunneum* against adults and preimaginals of *B. oleae* (Yousef et al. 2013).

The soil is the natural habitat of EFs and is generally considered a favorable environment for fungal microbial control since it provides protection from environmental extreme conditions, which increases the persistence of the conidia and their ability to

thrive and provide a long-term strategy for the puparia control (Ekesi et al. 2007; Zimmermann 2007; Vega et al. 2009; Garrido-Jurado et al. 2011a, b, c). Thus, the soil-dwelling stage of tephritid flies offers an excellent opportunity for an effective management, nevertheless this manage strategy has been little explored (Ekesi et al. 2007). Around 80 % of the third instar prepupating olive fruit fly larvae pupate approximately 3 cm below the soil surface (Dimou et al. 2003). Therefore, soil treatment beneath the host trees becomes an important strategy of fruit fly suppression and eradication programs to reduce the fruit fly larvae and puparia population (CDFA 1993; Mohamad et al. 1979). Furthermore, fungal application targeting tephritid fruit flies preimaginals in the soil can be compatible with the classic biological control in which *Psyttalia spp* larval parasitoids are field released (Ekesi et al. 2005; Daane et al. 2015). Our laboratory researches have showed that the strain EAMa 01/58-Su of *M. brunneum* is highly virulent against adults and preimaginal stages of tephritids (Quesada-Moraga et al. 2006; Yousef et al. 2015). Furthermore, this strain has demonstrated high compatibility with herbicides used in olive orchards (Yousef et al. 2015), and no significant negative direct or indirect impact on the soil dwelling arthropod population (Garrido-Jurado et al. 2011a). Therefore, primary research focus of the present work has been to evaluate the effectiveness of soil applications with *Metarhizium brunneum* EAMa 01/58-Su strain beneath the tree canopy targeting *Bactrocera oleae* (Rossi) preimaginals to reduce the adult fly population. Prior to the field tests, the virulence of this strain against *B. oleae* adults and puparia in the laboratory was ascertained.

2. Materials and methods

2.1. Fungal strain, cultivation and inoculum production

Metarhizium brunneum strain EAMa 01/58-Su (formally *M. anisopliae*) 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). This strain was deposited with accession number CECT 20764 in the Spanish collection of culture types (CECT) located at

the University of Valencia (Spain). Slant monoconidial cultures of the strain were grown on Malt Agar (MA) (BioCult B. Laboratories, Madrid, Spain) at 25 ˚C in darkness, then were lyophilized in the LyoQuest laboratory freeze dryer (Telstar, Barcelona, Spain), and stored at 80 ˚C (Humber 1997). The inoculums for the laboratory experiments were prepared using slant culture that subcultured on MA in Petri plates for 12 d at 25 ˚C in darkness. Then, conidia were scraped from the Petri plates into a sterile 0.1 % Tween 80 aqueous solution that was then sonicated for 5 min and filtered through several layers of cheesecloth to remove mycelial mats and to collect pure conidia. The concentration of the conidial suspension was determined by direct counting using hemocytometer (Malassez chamber) (Blau Brand, Wertheim, Germany). For the field applications, mass production of conidia was prepared before each soil treatment, in which, conidia were produced on rice in polypropylene bags. After fungal growth, sieving collected conidia were formulated with talcum powder 1:4 (w/w) ratio (conidia: talc powder). The viability of the conidia was verified before the preparation of suspensions by germinating tests in Sabouroud Dextrode Broth medium (BioCult B. Laboratories, Madrid, Spain). In all the experiments, germination rates were higher than 90 %.

2.2. Insects used in the laboratory bioassays

Adults and puparia of *B. oleae* used in the laboratory bioassays were obtained from naturally infested fruit collected from September to December in the Córdoba area. The infested olives were placed and maintained as described by Yousef et al. (2013) to obtain the puparia and adults. The lack of a well-developed laboratory breeding methods of the olive fruit fly in our facilities and the partial maintenance of insect from field-collected olives made impossible evaluating the strain against prepupariating larvae.

2.3. Virulence bioassay of the EAMb 01/58-su strain against newly emerged adults and puparia of *B. oleae* in the laboratory

To evaluate the virulence of the EAMa 01/58-Su strain against *B. oleae* adults, four concentrations of this strain were prepared in a sterile aqueous solution that contained

0.1 % Tween 80 [10^5, 10^6, 10^7, and 10^8 conidia ml^{-1}] as well as a control without fungus spores. Then, newly emerged *B. oleae* adults that had previously been cold anesthetized were treated with a Potter tower (Burkard Rickmansworth Co. Ltd., Rickmansworth, UK) using a deposit of 1.54 ± 0.06 mg cm^{-2} and 0.7 bars of pressure. One millilitre of conidial suspension was used for each replicate, with three replicates per treatment (7-12 adult flies each). The treated adults were placed in methacrylate cages (80 × 80 × 60 mm; Resopal, Alcalá de Henares, Madrid, SP) with covers containing a circular hole (20 mm in diameter) covered with a net cloth. A liquid diet (0.1 g of hydrolysed protein and 0.4 g of sucrose with 1.5 ml of distilled water) was provided every 48 h. The bioassay was conducted at 26 ± 2 ˚C, with 50-60 % RH and using a photoperiod of 16:8 (L:D) h. Mortality was monitored for 10 d. Dead flies were removed daily to prevent horizontal transmission of the fungal inoculum. The dead flies were immediately surface-sterilized with 1 % sodium hypochlorite followed by three rinses with sterile distilled water for 1 min each. They were then placed on sterile wet filter paper in sterile Petri plates, sealed with parafilm, and kept at 25 ˚C to be inspected for fungal outgrowth on the cadavers (Quesada-Moraga et al. 2006). The entire experiment was repeated three times with a new fungal suspension and a new generation of *B. oleae* adult flies each time.

To evaluate the virulence of EAMa 01/58-Su strain against *B. oleae* puparia, four concentrations of this strain were prepared as described above [10^5, 10^6, 10^7, and 10^8 conidia ml^{-1}] as well as a control without fungus spores. Then, the *B. oleae* puparia were immersed in a fungal suspension for 10 s. Control puparia were immersed in sterile distilled water containing 0.1 % Tween 80. There were three replicates of each treatment, fungus and control, and 10 puparia per replicate. The bioassay end point was four days after the first adult emergence in the control. Puparia that failed to emerge were removed and prepared as described previously for a diagnosis of the cause of death. The entire experiment was repeated two times with a new fungal suspension and a new generation of *B. oleae* puparia each time.

2.4. Study sites, experimental design, and field application of the EAMa 01/58-Su strain

Field experiments were conducted to evaluate the bioefficacy of the *M. brunneum* EAMa 01/58-Su strain against *B. oleae* preimaginals. The experiments took place from 2010 to 2015 (Table 1). All the experimental fields were conventional olive orchards, and surrounded by conventional ones. The integrated pest management is applied as authorized by the Spanish Ministry of Agriculture, Food and Environment (MAGRAMA 2014).

Table 1. Experimental sites in the study

Year	Site	Surface Ha	Variety	Olive age (years)	Geographical coordinates
2010-11	Illora (Granada)	27.97	Picual	35-40	37º 20' 46" N 3º 52' 22" O
2012-13	Montefrío (Granada)	10.23	Picual	25-30	37º 23' 12" N 3º 58' 21" O
2013-14	Castro del Río (Córdoba)	13.46	Picual	30-35	37º 41' 9.5" N 4º 29' 38.7" O
2014-15	Castro del Río (Córdoba)	13.46	Picual	30-35	37º 41' 9.5" N 4º 29' 38.7" O

The geographical coordinates of the study sites are shown in Table 1. In all experiments, the sites were divided into four square ha sub-fields (≈120 olive tree each). Two of these sub-fields were the fungi-treated plots and the others were the control plots (Online Resource 1). In all years of the study (except the first one), soil applications of this fungus were performed twice, once in autumn to (October-November) target prepupating third-instar olive fruit fly larvae that exit from fruits to the ground to pupate beneath the tree and spend the winter in the pupal stage (Santiago-Álvarez and Quesada-Moraga 2007) and once in spring (April-May) to target the emerging adults. In Illora (Granada) 2010, only the autumn soil application was conducted. In all the production years, the soil beneath each tree canopy (≈12 m^2 per tree) in the olive orchards was sprayed with one liter of *M. brunneum* EAMa 01/58-Su strain suspension (which contained 1 g of conidia or 1×10^9 conidia) using the same herbicide application atomizer each year. This spraying equipment consists of a quad (500 cc) fitted out with 1

pump, 1 bar with 4 standard flat fan nozzles (03-Blue) situated 50 cm from each other, and a 75-litre tank with an agitator for correct product mixing. In the control plots, the soil beneath each tree canopy was sprayed with one litre of aqueous solution containing 0.1 % Tween 80 (Online Resource 2).

2.5. Monitoring of EAMa 01/58-Su strain persistence in the soil after treatments

In all field experiments and before fungal treatment, 6 completely randomized soil samples were collected using a soil corer (Ø 5 cm) to a depth of 15 cm to determine the natural presence of entomopathogenic fungi in the soil according to Goettel and Inglis (1996). In all experiments and after fungal treatment, 6 trees were randomly selected to evaluate the inoculum presence in soil. For this purpose, soil samples were collected monthly beneath the canopy following the same procedure as mentioned above for 10 months after treatment was applied. To avoid contamination between samples, soil corer was sterilized in 70 % alcohol, rinsed in tap water and dried with a paper towel before being used in another replicate orchard. To assess the conidial density in each sample, the number of cfu per gram of dry soil was determined by Sabouraud Chloramphenicol Agar medium in Petri dishes (Goettel and Inglis 1996). One gram of the homogenized soil sample was added to 9 ml of sterile distilled water and then stirred with a rotary shaker (P-selecta shakers, Barcelona, Spain) at 12 rpm for 60 min. After homogenization, three dilutions were prepared from each sample (1:10, 1:100, 1:1000), and aliquots of 100 µl were spread onto the medium. Precipitation data were obtained from the climatological stations operated by Junta de Andalucía (Red de Alerta e Informacíon Fitosanitaria-RAIF 2016).

2.6. Efficacy of EAMa 01/58-Su strain soil treatments against *B. oleae*

To evaluate the effect of the field fungal treatments on the *B. oleae* population, we compared the adult population dynamics in both treated and control plots, with emphasis on the moment of adult emergence, which is a critical moment in the treatment evaluation. A combination of pheromone yellow and McPhail traps were used

to estimate the number of emerged adults, in order to capture the majority of the olive fly adults emerged from the soil since the yellow traps are more attractive to males and McPhail traps are more attractive to femals (unpublished data). In each plot (treated and control), 15 yellow traps and 6 McPhail traps baited with diammonium phosphate 4 % were randomly distributed and inspected weekly to count the number of *B. oleae* adults. The data presented represent the total number of captured insects in both types of the traps. To improve the count accuracy, each plot (both treated and control) was surrounded with 120 Olipe traps (one per tree) to prevent, insofar as was possible, the entry of adults from other farms (Online Resource 3). These traps were baited with protein-based attractant, which is the most effective for the olive fruit fly (Ruiz-Torres 2010)

2.7. Statistical analysis

The mean lethal concentration (the estimated concentration required to kill 50 % of the test insects, LC_{50}) was estimated by Probit analysis (Finney 1971), and the mean lethal time (the estimated time required to kill 50 % of the insects, LT_{50}) was determined using the Probit analysis method for correlation data (Throne et al. 1995) using SPSS 15.0 software for Windows. The area under the *B. oleae* flight curves (AUBFC) in treated and control plots was calculated by trapezoidal integration method of SAS (Campbell and Madden 1990). Then, the values of AUBFC were $\log_{10}$ transformed and subjected to factorial analysis of variance using Statistix 9.0 program (Analytical Software 2008). The same program was used to analyze data and to obtain means and standard errors of both fungal densities in the soil after treatments and *B. oleae* population densities in treated and control plots.

3. Results

3.1. Virulence assay of the EAMa 01/58-Su strain against newly emerged adults and puparia of *B. oleae* in the laboratory

The fungal treatment significantly influenced both the total *B. oleae* adult mortality ($F_{4,44}$ = 124.29, P < 0.001), with mean mortality ranging between 24.4 and 95.2 % compared with control mortality of 1.1 %, and mortality with fungal outgrowth ($F_{4,44}$ = 156.72, P < 0.001), with fungal outgrowth in 91.8, 39.1, 11.3 and 11.2 % of the cadavers for 10^8, 10^7, 10^6 and 10^5 conidia ml^{-1}, respectively.

The mortality values were subjected to Probit regression analysis. The regression coefficient (slope ± SE) was 1.32 ± 0.2, and the χ^2 value was not significant (χ^2 = 2.62, with 1 df), indicating a good fit of the regression line at an LC_{50} value of 7 × 10^6 conidia ml^{-1}. Furthermore, the cumulated mortality values from the treatments of 10^7 and 10^8 conidia ml^{-1} were also subjected to Probit regression analysis with LT_{50} of 8 and 6.2 days, respectively, and chi-square values that were not significant [(slope ± SE = 5.0 ± 1.5, χ^2 = 0.016, with 1 df), (slope ± SE = 6.8 ± 0.69, χ^2 = 3.7, with 2 df)], indicating a good fit of the regression lines.

Fungal treatment with the EAMa 01/58-Su strain also had a significant effect on *B. oleae* puparial survival ($F_{3,23}$ = 17.75, P < 0.001), with mortality values that ranged between 31.6 % and 68.3 % compared with a control mortality of 8.3 %. A significant percentage ($F_{3,23}$ = 22.59, P < 0.001) of the puparia were nonviable (6.6 % to 38.3 %) and showed fungal outgrowth. Again, the mortality values were subjected to Probit regression analysis. The regression coefficient (slope ± SE) was 0.52 ± 0.13, and the χ^2 value was not significant (χ^2 = 0.006, with 1 df), indicating a good fit of the regression line, with an LC_{50} value of 1 × 10^7 conidia ml^{-1}.

3.2. Monitoring EAMa 01/58-Su strain persistence in the soil after treatments

In all experiments, the previous monitoring of *Metarhizium* species in the experimental plots yielded the absence of autochthonous strains. The EAMa 01/58-Su densities decreased over time after the treatment applications. Fig. 1 shows the time

course of *M. brunneum* EAMa 01/58-Su strain concentration in the soil after treatments and its relation with the monthly precipitation and date of fungus application. In all treatment years, the fungus persisted in the soil after treatment. After the autumn treatment of 2010-2011 (the only treatment in this production year), the fungus persisted in the soil approximately one year; at that point the fungal concentration in the soil was (6.1×10^2 conidia g $soil^{-1}$). After the treatments performed in the autumn of 2012-2013 and 2013-2014, the fungus persisted in the soil until the spring treatment, approximately 4 months; at that point the fungal concentrations in the soil were 0.8×10^2 and 1.1×10^3 conidia g $soil^{-1}$, respectively. Precipitation levels were almost similar in all years of treatment. Rainfall occurring from January to March (after the first fungal soil application) was higher than the one from April to May (after the second fungal soil application) every year (Fig.1).

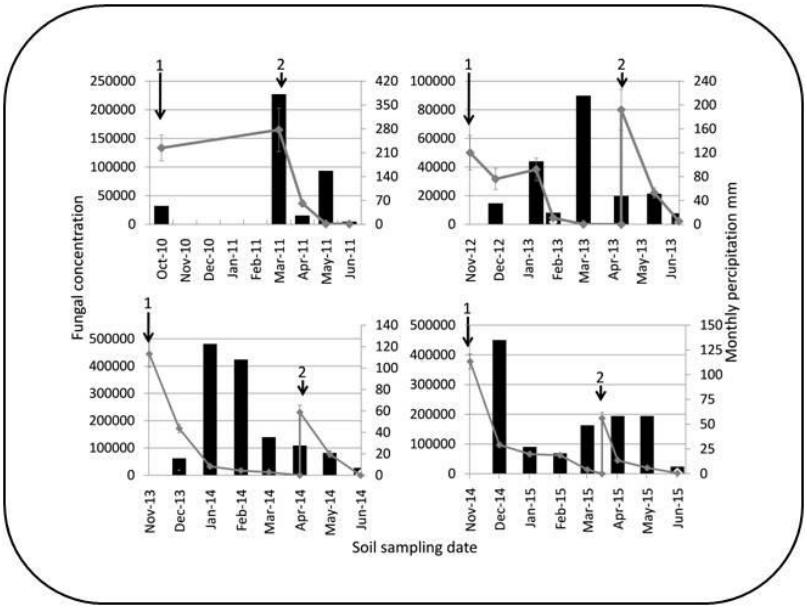

Fig. 1. Time course of *M. brunneum* EAMa 01/58-Su strain concentration in the soil after treatments (line), and monthly precipitation registed during the period (bars). 1: first fungal soil application, 2: second fungal soil application.

3.3. Efficacy of soil treatments beneath the tree canopy with EAMa 01/58-Su strain targeting preimaginals

The soil treatments with the *M. brunneum* EAMa 01/58-Su strain targeting *B. oleae* preimaginals reduced the *B. oleae* population in the treated plots compared with controls ($F_{1,15}$ = 4.9, P < 0.05) (Fig. 2). In all treatment years, there was a significant reduction in the *B. oleae* adult population emerging from the treated plots compared to the control plots. In 2010-2011, the maximum capture registered at the beginning of adult emergence was 5 flies per trap/day in the treated plots compared with 16 flies per trap/day in the control plots. In 2012-2013, the maximum capture registered at the beginning of adult emergence was 1.7 flies per trap/day compared with 3.3 flies per trap/day in the control plots. In 2013-2014, the same capture trend was registered at the beginning of adult emergence in spring, with 1.4 flies per trap/day in the treated plots and 6.2 flies per trap/day in the control plots.

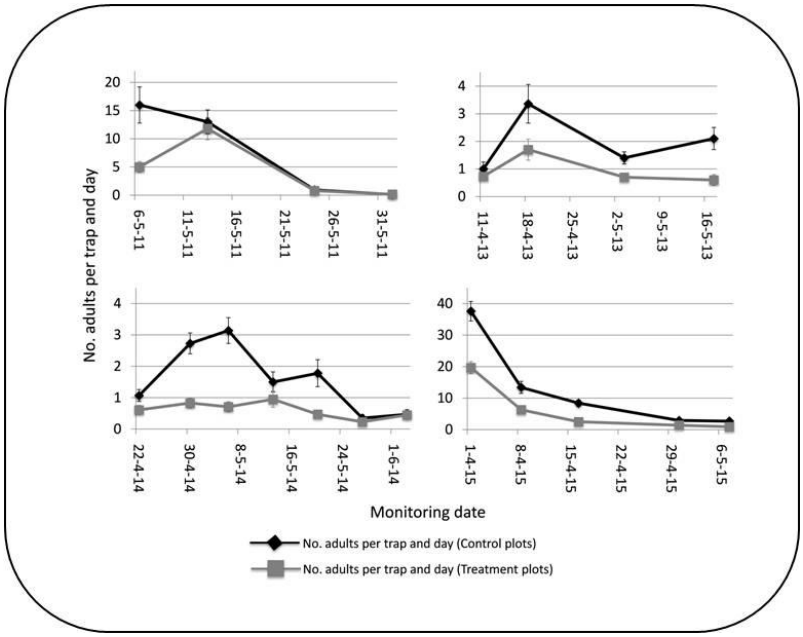

Fig. 2. *B. oleae* population densities emerged from soil in treated and control plots after *M. brunneum* EAMa 01/58-Su strain soil application beneath the tree canopy.

4. Discussion

Olive groves cover a significant proportion of the land area in the Mediterranean region. Indeed, in Spain alone, more than 2.5 million hectares (MAGRAMA 2014) are dedicated to olive groves. Thus, the appropriate management of olive groves also has positive effects on biodiversity and the environment (Beaufoy 2009). The management of olive fruit flies is currently almost exclusively based on the use of chemical insecticides, which are harmful to humans, the environment, and non-target organisms (Pontikakos et al. 2012). To tackle these problems, we present a biological, efficient, economically viable and environmentally friendly method to control the olive fruit fly using the EAMa 01/58-Su strain of *M. brunneum* as a soil treatment. This strain demonstrated high virulence against both adults and puparia of *B. oleae* in laboratory bioassays. As we know, the natural occurrence of EF in the soil is considered to be relatively low (Scheepmaker and Butt 2010). Therefore, repeated applications of fungi might fill this gap. In this study, field experiments using the *M. brunneum* EAMa 01/58-Su strain were conducted to develop a viable field application strategy to control *B. oleae*. The high *B. oleae* adult mortality in the laboratory bioassays, which reached 95.2 % at a concentration of 10^8 conidia ml^{-1} with an LT_{50} of 6.2 days, an LC50 of 7×10^6 conidia ml^{-1}, and a 91.8 % rate of fungal outgrowth from cadavers, would help to reduce the adult population in the field before the first egg laying, as oviposition begins between 5 and 10 d after adult emergence (Alfaro-Moreno 2005). To further contribute to this goal, the fungus demonstrated high virulence against *B. oleae* puparia in laboratory bioassays that reached 68.3 % with an LC_{50} of 1×10^7 conidia ml^{-1} and a 38.3 % rate fungal outgrowth from nonviable puparia. To this end, it has to be underlined that virulence of entomopathogenic fungi toward prepupariating larvae (with no protection) is always higher than the one against puparia (in which the pupae is protected by the exuvium of the third instar) (Ekesi et al. 2007; Yousef et al. 2013). These results make this fungus an excellent candidate for field use in biological control of the olive fly. Our previous studies have demonstrated the effective use of EF for controlling olive fruit fly (Yousef et al. 2013) and Mediterranean fruit fly (Quesada Moraga et al. 2006; Yousef et al. 2014), but

with lower mortality values than were achieved by this strain. Generally, tephritid puparia are less susceptible to the effects of EF than adults (De la Rosa et al. 2002; Ekesi et al. 2007; Yousef et al. 2013), but the EAMa 01/58-Su strain has previously achieved both a higher *C. capitata* puparia mortality and a higher fungal outgrowth from cadavers compared with all evaluating strains (Quesada Moraga et al. 2006). Moreover, adults may disseminate the fungal inoculum before they die from it (Quesada-Moraga et al. 2008). Therefore, field experiments using soil applications were performed over a 4-year period to evaluate the field bioefficacy of this strain for the biological control of *B. oleae* and to establish an environmentally friendly *B. oleae* control method, which scientists have sought in recent years for control of tephritids (Ekesi et al. 2007). In all the production years of the treatment, *M. brunneum* EAMa 01/58-Su strain persisted in the soil until the second application (4-5 months), a length of time sufficient to infect most of the *B. oleae* pre-imaginals dropping to the soil for pupation. Even if the fungal density in the soil were to decrease considerably after yearly treatments, the fungal concentration recovered from the soils after 4-5 months were 0.8×10^2 to 1.1×10^3 conidia g soil^{-1} close to the natural background concentration registered in bulk soil (Bruck 2010; Scheepmaker and Butt 2010). For registration purposes, the risks concerning the persistence of entomopathogenic fungi being developed as biological control agents have to be evaluated according to EU legislation, which requires the decline of BCAs to acceptable background levels unless related risks are acceptable (Scheepmaker and Butt 2010). To this end, as observed in this work, it has been demonstrated for several species of *Metarhizium* and *Beauveria* that natural densities are relatively low and introduced strains of these fungi decreased gradually in time as determined by edaphic, biotic, climatic, and cultural factors (Scheepmaker and Butt 2010; Garrido-Jurado et al. 2011b). In addition, it has been shown the decline of the fungal content in the soil to be also influenced by application method and propagule carrier (Inglis et al. 2001).

The same trend was found in our laboratory bioassays, in which fungal concentration in the soil decreased from the theoretical applied titre to 3.3×10^6 conidia g soil^{-1} 15 days after application (Yousef et al. 2015). The persistence of EF propagules is higher in sterile

soils than in non-sterile soils (Zelger and Schweigkofler 2004). On one hand, the possible germination of the conidia and subsequent death may be related to this decrease. On the other hand, even if the cfu is an appropriate method for spore counting in a soil suspension, it may also have limitations such as underestimations, and the method often results in wide variances from the mean values and yields a relatively narrow countable range in a standard Petri dish (Sutton 2012). Moreover, conidial survival in the soil is affected by agrochemicals and environmental factors (Benz 1987). Soil texture can influence the persistence of entomopathogenic fungi (Ekesi et al. 2011). Generally, retention of *Metarhizium* conidia in the surface layer is higher in sandy than in clayey soils (Garrido-Jurado et al. 2011b). All the soils in this study contained high levels of clay, which may further explain the decline in conidial density of the EAMa 01/58-Su strain of *M. brunneum*. On the other hand, the rainfall occurred after autumn and spring applications could have affected the movement of conidia through the soil profile since intensive rainfall can further contribute the reduction of conidia concentration by washing them off of the soil (Thompson et al. 2006; Furlong and Pell 1997; Garrido-Jurado et al. 2011b). Although soil moisture has been reported as important abiotic factor influencing the efficacy of EF against soil-dwelling insect pests, conidial germination in soil will occur at virtually all moisture level but a given fungal strain will have an optimum level (McCoy et al. 1992). Beyond this, our previous research showed that the pathogenicity of the strain EAMa 01/58-Su against medfly puparia is not significantly affected by soil properties (Garrido-Jurado et al. 2011b).

During four years of EAMa 01/58-Su soil applications, this strain demonstrated high efficacy against olive fruit fly. The soil application of this strain targeting pupariating larvae and puparia caused a high reduction (50 % to 70 %) in the *B. oleae* population emerging during spring from the soil of treated plots compared to controls plots. To the best of our knowledge, this is the first study that presents—on a practical level—an effective biological method for controlling olive fruit fly based on the use of EF. Ekesi et al. (2011), demonstrated that the combined use of *M. anisopliae* and GF-120 spinosad bait as soil treatments is effective in suppressing fruit flies on mango trees with only one

fungal application coupled with weekly spinosad applications. However, the weekly applications of spinosad increase the application costs. To this it may add that some populations of *B. oleae* have been found to be resistant to spinosad (Kakani et al. 2010). The EF strain used in our study demonstrated high capacity to control olive fruit fly population with just two treatments per year and to persist in the treated soil, providing long-lasting control of *B oleae*. There are no comparable field studies in the literature on the use of EF as a soil application beneath olive tree canopies. Although the use of EF for pest control has been widely studied, knowledge of their effective use in field conditions has remained elusive (Shahid et al. 2012). This study and our previous studies have revealed the following: 1) the compatibility of EAMa 01/58-Su with the most of herbicides used in olive orchards allows its simultaneous application together with herbicides in an atomizer tank for simultaneous treatment beneath the tree canopy. This reduces the application costs of the fungus and herbicides (Yousef et al. 2015); 2) this strain can be applied to the soil of olive orchards for control of *B. oleae* with no negative effects on the soil-dwelling arthropod population (Garrido-Jurado et al. 2011a), and 3) this strain is perfectly adapted to Mediterranean soil conditions (Garrido-Jurado et al. 2011b, c). We conclude that the application of the *M. brunneum* EAMa 01/58-Su strain to the soil of olive orchards beneath the tree canopy is an efficient biological control method that can be used in an integrated olive fruit fly management strategy in either conventional or organic olive orchards at regional or national scope. Furthermore, the use of this strain can reduce the negative effects of chemical insecticides used to date to control the olive fruit fly on both the ecosystem and on human health. The 50-70 % reduction of the adult olive fruit fly spring population reported in this work is a key factor in diminishing the possible need for additional treatments within an IPM strategy that conserves both the environment and biodiversity in the olive agroecosystem and promotes olive oil quality by reducing pesticide residues.

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6. References

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ORIGINAL PAPER

Compatibility of herbicides used in olive orchards with a *Metarhizium brunneum* strain used for the control of preimaginal stages of tephritids in the soil

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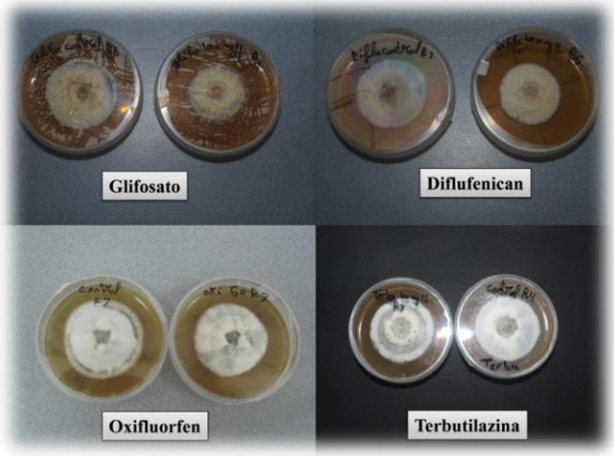

Compatibility of herbicides used in olive orchards with a *Metarhizium brunneum* strain used for the control of preimaginal stages of tephritids in the soil

Abstract

In vitro and *in vivo* studies were developed to evaluate the compatibility of the six most common herbicides applied to the soil of olive orchards with the *Metarhizium brunneum* strain EAMa 01/58-Su for controlling *Ceratitis capitata* preimaginals. The fungus demonstrated high *in vitro* compatibility with the six active ingredients in malt agar medium, with growth rates (a) ranging between 2.5 mm d^{-1} (glyphosate + terbuthylazine) and 3.3 mm d^{-1} (oxyfluorfen). This compatibility was also revealed *in vivo* by assaying the fungus (1.0×10^8 conidia g $soil^{-1}$) toward medfly prepupariating larvae in soil containing herbicides. Even if there was a decrease in the *M. brunneum* level in the soil up to 10^4–10^5 conidia g $soil^{-1}$ 15 days after inoculation, mortality rates, which ranged between 70-80 %, did not differ significantly from the control, except the ones observed in soils that contained glyphosate and its herbicide combinations, in which a significant 50 % reduction of virulence was detected. These results reveal a general compatibility of *M. brunneum* with the most common herbicides applied to the soil of olive orchards, but a mixture of the fungus in the atomizer tank for simultaneous treatment beneath the tree canopy is recommended for all active ingredients except glyphosate.

Keywords *Metarhizium brunneum,* Herbicides, Compatibility, *Bactrocera oleae,* Fly preimaginals, *Ceratitis capitata, in vivo*

Key messages

The *Metarhizium brunneum* strain EAMa 01/58-Su demonstrated high *in vitro* and *in vivo* compatibility with the herbicides applied to the soil of olive orchards. A mixture of the fungus in the atomizer tank for simultaneous treatment beneath the tree canopy is recommended for all active ingredients except glyphosate. The best combination was 24 % oxyfluorfen with the EAMa 01/58-Su strain, which produced higher fungal growth, higher pupal mortality and a higher number of viable conidia in the soil after treatment.

1. Introduction

Tephritid fruit flies are among the most important pests around the globe and cause a substantial loss of agricultural products (Purcell 1998; Chang et al. 2012). The monophagous *Bactrocera oleae* and the polyphagous *Ceratitis capitata* are the most destructive of all known Tephritid species (Liquido et al. 1991; Alfaro-Moreno 2005). Historically, since the beginning of the 20th century, bait and insecticide sprays have been used to control adult flies in aerial treatments (Moreno and Mangan 2000), but there is increasing interest in soil treatments beneath the tree canopy targeting preimaginals (Ekesi et al. 2007). In recent years, scientists have sought environmentally friendly medfly control methods, such as microbial control with entomopathogenic fungi (Ekesi et al. 2007). Several species of entomopathogenic fungi (EF) have been developed as benign alternatives to the synthetic chemical insecticides that have been withdrawn because of the risks they pose to humans and the environment and because pests have developed resistance to them (Shah and Pell 2003). EF occur naturally in the soil, which is a favorable environment for fungal microbial control because it provides protection from environmental extremes, increasing the persistence of the conidia and their ability to thrive and providing a long-term strategy for the control of tephritid puparia (Ekesi et al. 2007; Zimmermann 2007; Vega et al. 2009; Garrido-Jurado et al. 2011a, b, c). Therefore, EF are well adapted to being used for controlling soil-dwelling pests (Klingen and Haukeland 2006; Cossentine et al. 2010). Prepupating third-instar olive fruit fly larvae drop from fruits to the ground to pupate beneath the tree and spend the winter in the pupae stage (Santiago-Álvarez and Quesada-Moraga 2007). Thus, this soil-dwelling stage of tephritid flies presents an excellent opportunity for effective management (Ekesi et al. 2007).

However, the success of a pest control program using EF depends on conidia survival in the field environment (Benz 1987). Conidia survival may be affected by either environmental factors or biopesticides and/or chemical products used to protect the crop plants. The effects of factors such as geographical location and altitude, habitat type (natural or cultivated), subhabitat type (cropping systems in cultivated soils or type of

natural habitat) and soil composition on the occurrence of EF in the soil have been studied, and *M. anisopliae* was found to be more common in cultivated soils (Queada-Moraga et al. 2007). Moreover, Garrido-Jurado et al. (2011c) investigated the effects of abiotic factors in the soil on the virulence of entomopathogenic fungi. Furthermore, several studies have determined the effects of acaricides, fungicides, insecticides and herbicides on EF (De Oliveira and Neves 2004; Mochi et al. 2005; Alizadeh et al. 2007; Rashid et al. 2010; González et al. 2011; Clear and Kos 2012; Khan et al. 2012). Pesticides are anthropogenic factors with either a synergistic or antagonistic influence on pests or their pathogens (EF), which affects their insecticidal activity (Benz 1987; Kouassi et al. 2003). A lack of negative effects on EF would enable their successful use in the integrated production of olives. In our laboratory research, we previously demonstrated the effective use of the fungus *M. brunneum* against the preimaginal stages of tephritid fruit flies (Quesada-Moraga et al. 2006; Yousef et al. 2013). To complement our laboratory experiments, we performed field experiments by applying *M. brunneum* to the soil of olive orchards for the control of the preimaginal stages of *B. oleae*. Therefore, knowing the effects of the chemical herbicides applied to olive crops on EF is important for any future joint applications and for determining management techniques for agroecosystems that permit the compatible use of this pathogen with agricultural defense strategies (Mochi et al. 2005). The objective of the present study was to analyze any toxic effects of the active ingredients present in the herbicides used in olive orchards on the EF *M. brunneum* strain EAMa 01/58-Su. We used both *in vitro* assays to determine the effects of herbicides on fungal growth parameters and *in vivo* assays to determine the effects of combining this fungus with herbicides on the pathogenicity of the fungus to the preimaginals of *Ceratitis capitata*.

2. Materials and methods

2.1. Entomopathogenic fungus

The *Metarhizium brunneum* strain EAMa 01/58-Su, obtained 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, SP). Previous work had shown this strain to be virulent against both adults and puparia of *C. capitata* and *B. oleae* (Quesada-Moraga et al. 2006; Garrido-Jurado 2008). Slant monoconidial cultures were grown on malt agar (MA) at 25 ˚C in darkness and were then lyophilized in the LyoQuest laboratory freeze dryer (Telstar, Barcelona, SP) and stored at -80 ˚C. The strain was cultured on rice in polypropylene bags. After fungal growth, conidia were dry collected by sieving and were suspended in a sterile aqueous solution containing 0.1 % Tween 80. The conidial concentration was assessed according to the colony-forming unit (cfu) method (Goettel and Inglis 1996). The viability of the conidia was verified before suspension preparation using germination tests in Sabouraud dextrose broth medium (BioCult B. Laboratories, Madrid, SP). In all the experiments, germination rates were higher than 90 %.

2.2. Insects

The bioassays were performed using the Mediterranean fruit fly (*C. capitata*) as a model of olive fruit fly (*B. oleae*). Prepupating third-instar larvae of *C. capitata* were obtained from a stock colony maintained at the Department of Agricultural and Forestry Sciences of the University of Cordoba (Spain). The flies were reared in a rearing chamber (16 h light:8 h dark photoperiod, 50–60 % relative humidity and 25 ± 2 ˚C). Adult flies were provided with water and a standard artificial diet consisting of 40 g yeast hydrolysate (ICN Biomedicals, Aurora, Ohio, USA) and 10 g sucrose (Panreac, Barcelona, SP). Larvae were reared until pupation on an artificial diet consisting of 300 g wheat bran, 75 g sucrose, 38 g brewer's yeast, 2 g nipagin, 2 g nipasol and 2.4 g benzoic acid dissolved in 600 ml distilled water per kg of diet (Albajes and Santiago-Álvarez 1980).

2.3. Herbicides

The herbicides used in the current work are presented in Table 1. Four active ingredients (a.i.) from six different formulations were selected according to the guidelines for the integrated production of olives in Andalusia (Junta de Andalucía 2013).

Eight concentrations of each herbicide were used for the *in vitro* experiments, while the recommended field concentration of each herbicide was used for *in vivo* experiments. Commercial herbicide formulations were used in all experiments, and dosages were calculated according to the active ingredient composition of the formulations. Therefore, not only the active ingredients were examined, but also the formulations, so the data reflect their possible effects.

Table 1. Herbicides used in the compatibility assays

AI[1]	Commercial name	Formulation[2]	Time of use	Dose (L ha^{-1})
1	24% Oxyfluorfen	24% CE	Pre-emergence	2-4
2	Medina	50% CS	Pre-emergence	2
3	Mamut	50% CS	Pre-emergence	0.75
4	Glifochem-36	36% CS	Post-emergence	3
5	Mamut + Glifochem-36	50% CS + 36% CS	Pre and post-emergence	1.5-7
6	Glifochem-36 + Medina	36% CS + 50% CS	Pre and post-emergence	2.85

[1] AI (Active ingredient): 1: 24 % Oxyfluorfen, 2: 50 % Terbuthylazine, 3: 30 % Diflufenican, 4: 12 % Glyphosate, 5: 4 % Diflufenican + 16 % Glyphosate, 6: 18 % Glyphosate + 34.5 % Terbuthylazine.
[2]CS - concentrate solution, CE - concentrate emulsion.

2.4. *In vitro* experiment for compatibility

The compatibility of six formulations of authorized herbicides containing 4 a.i. (24 % oxyfluorfen, 50 % terbuthylazine, 30 % diflufenican, 12 % glyphosate, 4 % diflufenican + 16 % glyphosate, 18 % glyphosate + 34.5 % terbuthylazine) with the EF was evaluated. *In vitro* experiments were conducted to determine the effects of the a.i. on the mycelial growth of the ascomycete mitosporic entomopathogenic fungus *M. brunneum* strain EAMa 01/58-Su. To prepare the desired doses of a.i., two 2-l Erlenmeyer flasks containing sterilized base medium (MA) (BioCult B. Laboratories. SP) were placed in a heat bath at 45 ˚C under aseptic conditions in a laminar flow chamber, and different

sterile deionized water dilutions of the a.i. were added. Then, the media were manually stirred to form a homogeneous dilution and poured into Petri dishes (10 ml per dish). For each a.i., seven geometrically increasing doses were prepared [1, 5, 10, 50, 100, 500, and 1,000 mg of a.i. per litre of culture medium (ppm)] as well as a control dose without a.i. Five replicates were used for each a.i. and dose. Fungal discs of 5 mm were obtained from actively growing colonies (4 days) and introduced into a hole of the same size in the middle of each plate. The plates were incubated at 25 ± 2 ˚C in the dark, and two perpendicular diameters were measured from day 2 to day 10 to obtain the corresponding measures of mycelia inhibition.

2.5. *In vivo* compatibility of *M. brunneum* with herbicides

In vivo experiments were conducted to evaluate the effect of the six formulations on the pathogenicity of the fungus *M. brunneum* in soil. The soil used in this study was collected from a farm in Córdoba and was characterized as sandy loam (78 % sand, 17 % silt, 5 % clay and 0.2 % organic matter) with a pH of 8.3. The soil was sieved (2 mm mesh) and stored in a dry place at 25 ˚C. Before use, the soil was sterilized at 121 ˚C for 20 min and dried in an oven at 105 ˚C for 24 h. Then, 30 g of soil was distributed into each bioassay methacrylate cage (80 × 80 × 60 mm; Resopal, Alcalá de Henares, Madrid, SP) so that the entire floor was covered. For each bioassay cage, 2.7 ml of suspension (1.7 ml 0.1 % Tween 80 in sterile distilled water, 1 ml 1 × 10^8 conidia ml^{-1} fungal suspension and the recommended field doses of each herbicide) was added to attain a water potential of -0.47 MPa[9.0 %(wt:wt), as measured by a Decagon WP4 psychrometer (Decagon Devices, Pullman, WA)] (Garrido-Jurado et al. 2011b). Two controls (water only and fungal suspensionwithout herbicides) were used. Three replicates of ten prepupating larvae each were used for each treatment. After treatment, the cages were covered with Parafilm® (Pechiney Plastic Packaging Co., Chicago, IL, USA), leaving a 20-mm hole in the center for ventilation, and were then placed in aplastic box (330 × 250 × 140 mm) covered with damp filter paper that was moistened periodically to maintain a 3 % loss of the initial soil water content. The boxes were placed at 25 ˚C until adult emergence. The

bioassay end point was 4 days after the first adult emergence from the control (Yousef et al. 2013). Pupae that failed to emerge were surface sterilized in 1 % sodium hypochlorite followed by three rinses with sterile distilled water. They were then placed on sterile wet filter paper in sterile Petri dishes sealed with Parafilm® and kept at room temperature for inspection for fungal outgrowth on the cadavers (Quesada-Moraga et al. 2006). The whole experiment was repeated after 45 days with a new fungal suspension and a new *C. capitata* population.

2.6. Presence of *M. brunneum* in the soil at the completion of the experiment

At the end of the testing period, each cage was evaluated for fungal inoculum in the soil. Soil samples were collected from the bioassay cages. To assess the conidial density in each sample, the number of cfu per gram of dry soil was determined using Sabouraud-chloramphenicol agar medium in petri dishes (Goettel and Inglis 1996). One gram of the homogenized soil was added to 9 ml of sterile distilled water and stirred with a rotary shaker (P-Selecta shakers, Barcelona, SP) at 12 rpm for 60 min. After homogenization, three dilutions were prepared from each sample (1:10, 1:100, and 1:1,000), and 100-µl aliquots were spread onto the medium.

2.7. Statistical analysis

Two statistical programs [SigmaPlot 12.0 for Windows (Systat Software Inc. 2011) and JMP 8 (SAS Institute Inc. 2008)] were used to analyze the data from the *in vitro* experiments. The radial growth data were fitted by regression analysis. The radial measurements (from day 2 to 10) were fitted to a linear model:

$$Y_{(mm\ \ diameter\ )} = v \times t_{(incubation\ \ \ days\ )} + B$$

The linear regression slope (v) indicates the growth rate (velocity in mm per day) for a particular herbicide; (B) is the y intercept. The effects of the herbicides on the fungal growth rates (V) were evaluated using a three-parameter logistic function (3PL) according to Zwietering et al. (1990), which is given by:

$$Y = \frac{a}{1 + \left(\frac{x}{c}\right)^b}$$

Here ‘‘a’’ is the fastest fungal growth rate, ‘‘b’’ is the intensity of the fungal growth decrease, and ‘‘c’’ is the medium lethal concentration (LC_{50}). The LC_{10} and LC_{20} for each herbicide were calculated with the same software. For the *in vivo* bioassays, mortality data were analyzed using one-way analysis of variance (Statistix 9.0, Analytical Software 2008); Tukey’s honest significant difference (HSD) test was used to compare means. Data for the fungal persistence in the soil were also analyzed using one-way analysis of variance.

3. Results

3.1. *In vitro* experiment for compatibility

Figure 1 shows the mycelial growth rates (mm day^{-1}) of the fungus *M. brunneum* in media (MA) containing different concentrations of herbicide a.i. Different parameters were used to evaluate the effects of the a.i. on the mycelia growth of *M. brunneum* (Table 2).

Table 2. Parameters used to evaluate the effects of the a.i. of herbicides used in olive orchards on the mycelial growth of *M. brunneum* strain EAMa 01/58-Su on agar media

Herbicide[1]	Highest radial growth	growth rate (slope)	LC_{50}	LC_{10}	LC_{20}
1	3.3088	0.5117	1443.9	19.8	96.8
2	3.2474	9.5397	1222.8	971.4	-
3	2.7124	0.4883	59876.6	666.5	-
4	2.6606	0.6088	3292.6	89.5	338.5
5	2.5819	0.5756	7683	169.9	707.5
6	2.5157	0.3841	9279.9	30.8	252.3

[1]: 1: 24 % Oxyfluorfen, 2: 50 % Terbuthylazine, 3: 30 % Diflufenican, 4: 12 % Glyphosate, 5: 4 % Diflufenican + 16 % Glyphosate, 6: 18 % Glyphosate + 34.5 % Terbuthylazine.

The mycelial growth rates obtained from the compatibility bioassays for all a.i. ranged from 2.51 for 18 % glyphosate + 34.5 % terbuthylazine to 3.3 for 24 % oxyfluorfen. The

LC_{50} of the a.i. were elevated and ranged between 1,222.8 and 59,876.6 ppm for 50 % terbuthylazine and 30 % diflufenican, respectively. The LC_{20} and LC_{10} values were similar (Table 2).

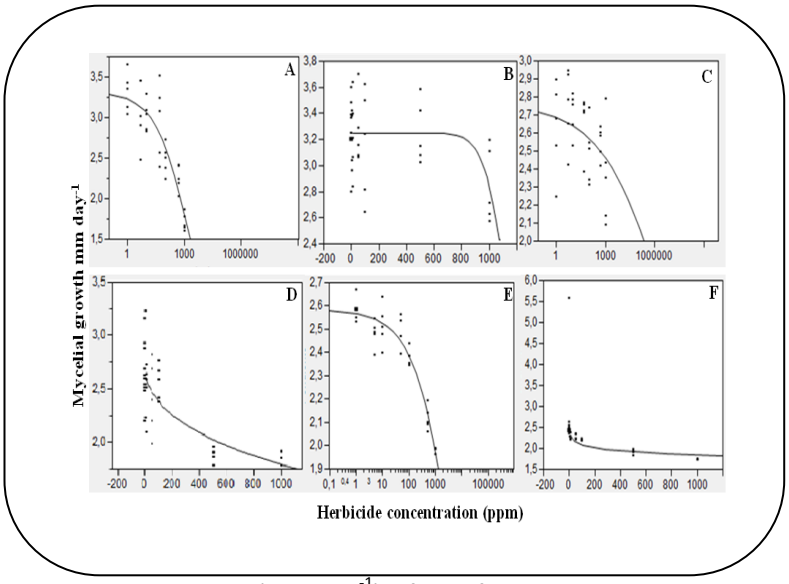

Fig. 1. Growth rates (mm day^{-1}) of the fungus *M. brunneum* strain EAMa 01/58-Su combined with different a.i. of herbicides, as estimated by the slope of the line regression. A: 24 % oxyfluorfen, B: 50 % terbuthylazine, C: 30 % diflufenican, D: 12 % glyphosate, E: 4 % diflufenican + 16 % glyphosate, F: 18 % glyphosate + 34.5 % terbuthylazine.

3.2. *In vivo* compatibility of *M. brunneum* with herbicides

Treating the EAMa 01/58-Su strain, which targets pupariating third-instar larvae in the soil, with different herbicides had a significant effect on *B. oleae* preimaginal survival ($F_{7,47}$ = 42.65, P < 0.001). All treatments were statistically equal to the fungi treatment control except the treatments that contained glyphosate; treatment with 18 % glyphosate + 34.5 % terbuthylazine yielded 45.0 % preimaginal mortality; treatment with 16 % gliphosate + 4 % diflufenican yielded 45.0 %; treatment with 12 % glyphosate yielded 50.0 % mortality. However, the highest preimaginal mortality, 80.0 %, resulted from treatment with 24 % oxyfluorfen, compared with 15.0 % mortality for the water control (Table 3). Fungal treatment also had a significant effect on mortality with fungal outgrowth ($F_{7,47}$ = 21.67, P < 0.001).

Table 3. Pathogenicity of the *M. brunneum* strain EAMa 01/58-Su against *C. capitata* third-instar pupariating larvae in soil containing different herbicides

Treatment	Pupariating larvae	
	Mortality (mean ± SE) %[1]	With fungal growth (mean ± SE) %[1]
Glyphosate 18 % + Terbuthylazine 34.5 %	45.0±4.2c	18.3±3.0b
Glyphosate 16 %+ Diflufenican 4 %	45.0±4.2c	20.0±3.6b
Glyphosate 12 %	50.0±2.5c	16.6±2.1b
Terbuthylazine 50 %	66.6±2.1b	30.0±0.0a
Diflufenican 30 %	71.6±1.6ab	31.6±3.0a
Oxifourfen 24%	80.0±2.5a	35.0±2.2a
Control fungi	75.0±3.4ab	30.0±2.5a
Control water	15.0±4.2d	0c

[1] Means within columns with the same letter are not significantly different (P ≤ 0.05) according to the Tukey HSD test.

All treatments were statistically equal to the fungi treatment control except treatments that contained glyphosate, which yielded the lowest percentages of fungal outgrowth (16.6 to 20.0 %), and treatment with 24 % oxyfluorfen, which yielded the highest percentage of fungal outgrowth (35.0 %). There were no signs of fungal outgrowth in the water treatment control (Table 3).

3.3. Presence of *M. brunneum* in the soil at the completion of the experiment

The presence of *M. brunneum* in the soil was analyzed at the end of the experiment (15 days). *M. brunneum* was not detected in the water control treatment, but it was variably detected in the treated soils.

We found significant differences in the concentration of *M. brunneum* between the different treatments ($F_{7,47}$ = 15.88, P < 0.001). Higher concentrations of *M. brunneum* were obtained from the control treatment and the 24 % oxyfluorfen treatment (6.6×10^4 and 5.0×10^4 ufc g soil^{-1}, respectively), while lower concentrations of *M. brunneum* were obtained from the glyphosate treatments, ranging between 6.6×10^3 and 2.3×10^4 ufc g soil^{-1} for 18 % glyphosate + 34.5 % terbuthylazine and 12 % glyphosate, respectively (Fig. 2).

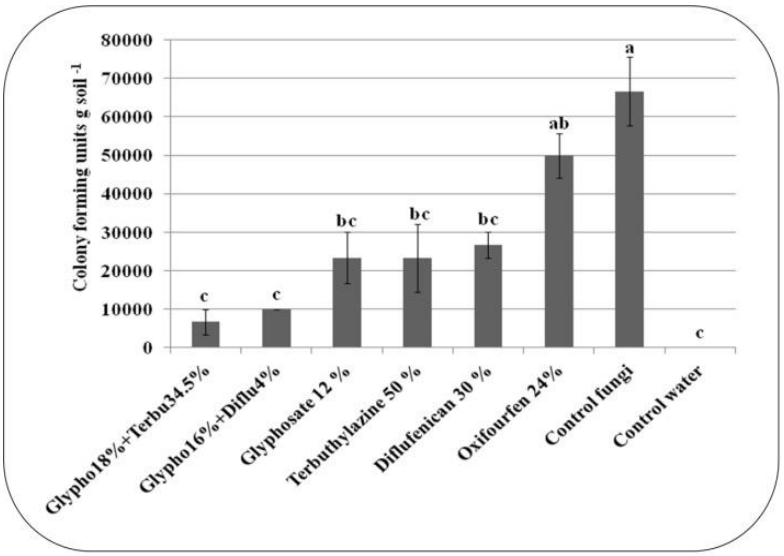

Fig. 2. Presence of *M. brunneum* in the soil (mean ± SE) at the end of the experiment (15 days).

4. Discussion

Identification of both the biotic and abiotic factors that affect EF conidia survival in soil environments is important for the application of entomopathogenic fungi to the soil for insect pest control (Scheepmaker and Butt 2010; Jaronski 2010; Bruck 2010). The *M. brunneum* EAMa 01/58-Su strain has successfully controlled tephritids when applied to soil in laboratory assays (Quesada-Moraga et al. 2006; Garrido-Jurado et al. 2011b, c). Similarly, this strain persists in the soil of olive orchards long enough to control the olive fly puparia without affecting nontarget arthropods (Garrido-Jurado et al. 2011a). However, to ensure effective field management, we must determine how it interacts with other management techniques, such as herbicides. The current work presents the effects of herbicides applied to the soil of olive orchards on the fungal growth and pathogenicity of *M. brunneum* strain EAMa 01/58-Su against *C. capitata* prepupating third-instar larvae. The presence of the EF in the soil was also detected at the end of the experiment. The fungus demonstrated high compatibility with the a.i. used in the *in vitro* assays; the maximum fungal growth rate ranged between 2.51 and 3.3 mm day^{-1}. Treatments with glyphosate yielded lower growth rates, indicating that glyphosate affects fungal growth and has possible synergistic effects when applied with other a.i.,

such as terbuthylazine and diflufenican. Our results are consistent with those of other *in vitro* studies that investigated the fungicidal effects of glyphosate and its formulations on EF. A study by Morjan et al. (2002) investigated the effects of seven glyphosate formulations on EF mycelial growth in solid media and found that the fungicidal properties of glyphosate formulations varied among fungal species but that *M. anisopliae* was susceptible to all glyphosate formulations. Furthermore, glyphosate influences metabolite secretion, including the production of aflatoxin B-1 by an *Aspergillus* strain isolated from soil (Barberis et al. 2013). EF susceptibility to herbicide application may differ depending on the concentration, as reported for *Beauveria bassiana*, in which mycelial growth and sporulation inhibition increased with herbicide concentration (Todorova et al. 1998; Clear and Kos 2012). In this study, not all of the assayed herbicides inhibited fungal growth, and 24 % oxyfluorfen yielded the highest growth rate of 3.3 mm day^{-1}, indicating possible compatibility with the *M. brunneum* EAMa strain 01/58-Su for simultaneous application to olive orchards.

In addition, the *in vivo* assays demonstrated that *M. brunneum* strain EAMa 01/58-Su had a high pathogenicity against prepupating third-instar larvae of *C. capitata* in combination with different herbicides. Higher mortality percentages were obtained from combined treatment with 24 % oxyfluorfen, supporting the simultaneous use of this herbicide with this strain. The experiments were a 'worst case' scenario (high concentrations with uniform incorporation into the soil), and so adverse effects seen in the experiment may not translate into adverse effects in operational use. However, the objective of this bioassay was to evaluate the direct effect of the herbicides on the fungus in similar realistic conditions, so it was necessary to remove biotic soil factors (sterile soil) that affect the viability of the fungus to estimate the true effect of the herbicide on it. To the best of our knowledge, no previous *in vivo* studies on the compatibility of EF with herbicides applied to olive orchards have been reported. However, Mochi et al. (2006) studied the pathogenicity of *M. anisopliae* against *C. capitata* in soil containing different pesticides, including one herbicide (ametrin), and found that pesticide application affected the pathogenic activity of *M. anisopliae* against

C. capitata to a small extent. Similarly, Zain et al. (2013) determined the *in vitro* and *in vivo* adverse effects of glufosinate-ammonium and glyphosate on fungal growth and the development of soil fungi in oil palm plantations. The concentration of the fungus in the treated soil 15 days after treatment ranged from 6.6×10^3 to 5.0×10^4 conidia g soil^{-1}, similar to the findings of other studies performed in the bulk soil (Bruck 2010; Scheepmaker and Butt 2010), even if the decrease in the number of conidia counts in treated soil was considerable from the theoretical applied titer of 3.3×10^6 conidia g soil^{-1}. This decrease may be related to a possible germination of the conidia and subsequent death, or directly with the cfu method, because, even if the cfu is an appropriate method for spore counting in a soil suspension (limit of quantification established in 30 cfu per plate diminishing the data variance), it may also have different limitations such as the underestimations and often very wide variances of the mean values and the relatively narrow countable range on a standard Petri dish (Sutton 2012). This is the first study to consider the effect of herbicides on the entomopathogenic fungus shelf-life, as persistence indicates viable conidia. These data revealed both the potential of the fungus for long-lasting control of fruit fly preimaginals and the relatively high compatibility of this fungus with the majority of the herbicides used in olive orchards, with low effects on the number of cfu at the end of the experiment.

Our study indicates that the *M. brunneum* strain EAMa 01/58-Su can be applied together with herbicides in the atomizer tank for simultaneous treatment beneath the tree canopy, which reduces the application costs of the herbicides and fungus. The best combination was 24 % oxyfluorfen with the EAMa 01/58-Su strain, which produced higher fungal growth, higher pupal mortality and ahigher number of viable conidia in the soil after treatment. Therefore, this article contributes significantly to current field research in olive fly control and identifies the possibility of simultaneous use of this EF strain with 24 % oxyfluorfen for the integrated production of olives.

5. Author contribution

EQM and IGJ conceived and designed the research. MY conducted the experiments. IGJ and MY analyzed the data. All authors wrote, read and approved the manuscript.

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UNIVERSIDAD D CORDOBA

DISCUSIÓN

Discusión

El control de la mosca del olivo *Bactrocera oleae* (Rossi), que es el principal factor biótico de reducción de la calidad del aceite de oliva, se sostiene aún en el empleo de insecticidas químicos en tratamientos larvicidas o adulticidas. Las medidas agronómicas o culturales pueden contribuir a paliar la incidencia del fitófago, mientras que los semioquímicos son de obligado empleo en el seguimiento de poblaciones, pero de limitada eficacia para el control de las mismas. La lucha autocida con base en la técnica de los machos estériles, y los recientes desarrollos asociados a la obtención de razas transgénicas de *B. oleae* no parecen aportar una solución a corto y medio plazo. Por otra parte, la susceptibilidad varietal al ataque de este temible díptero no se encuentra aún entre los criterios clave que impulsan los programas de mejora del cultivo (Daane et al. 2010; Estes et al. 2012; Bengochea et al. 2014; Yasin et al. 2014).

Este escenario complejo no está exento de dificultades, por una parte la escasez de materias activas insecticidas disponibles, consecuencia de los cambios asociados a la nueva política europea de productos fitosanitarios, Reglamento (CE) nº 33/2008 de la Comisión, antesala del Reglamento (CE) nº 1107/2009 del Parlamento Europeo y del Consejo, relativo a la comercialización de productos fitosanitarios, y de la otra, la esperada consecuencia, las primeras referencias de aparición de resistencia a las mismas. Además, los principios que emanan de la Directiva 2009/128/CE, del Parlamento Europeo y del Consejo, por la que se establece el marco de la actuación comunitaria para conseguir un uso sostenible de los plaguicidas, transpuesta por el Real Decreto 1311/2012, de 14 de septiembre, por el que se establece el marco de actuación para conseguir un uso sostenible de los productos fitosanitarios, exaltan los efectos negativos de los insecticidas químicos sobre el medioambiente y los seres vivos, y promulgan los métodos no químicos de control de plagas, con énfasis en los bioinsecticidas.

Trabajos realizados en los últimos 15 años ponen de manifiesto que los ascomicetos mitospóricos entomopatógenos presentan propiedades sobresalientes para ser desarrollados como bioinsecticidas para el control de tefrítidos tales como presencia natural en los agroecosistemas frutícolas, olivar incluido, modo de acción por contacto, y

su capacidad de secretar nuevas moléculas con actividad insecticida frente a adultos (Quesada-Moraga et al. 2006a; Ortiz-Urquiza et al. 2009; Garrido-Jurado et al. 2011a, b, c).

La presente tesis doctoral pone de manifiesto el potencial de los hongos entomopatógenos para su empleo en programas de control integrado de la mosca del olivo, tanto en tratamientos aéreos frente a adultos, con suspensiones de conidios o extractos fúngicos, como en tratamientos de suelo dirigidos a estados preimaginales, potencial que ya había sido puesto de manifiesto para otros tefrítidos (Quesada-Moraga et al. 2006a; Ekesi et al. 2011).

Las cepas EAMb 09/01-Su y EAMa 01/58-Su del hongo entomopatógeno *Metarhizium brunneum* fueron elegidas a base de estudios previos realizados en el seno de nuestro Grupo de Investigación que revelan el potencial insecticida del extracto crudo de la cepa EAMb 09/01-Su y la patogeneicidad y virulencia de la cepa EAMa 01/58-Su, ambos frente a la mosca mediterránea de la fruta *Ceratitis capitata* Wied. (Quesada-Moraga et al. 2006a; Portis 2011). En los capítulos III y IV de esta tesis se ha evaluado la actividad insecticida del extracto crudo de la cepa EAMb 09/01-Su frente a la mosca del olivo, así como la influencia sobre la misma de distintos parámetros de la fermentación (duración, pH del medio cultivo, etc..). En el capítulo V se ha estudiado la virulencia de la cepa EAMa 01/58-Su frente a adultos y estados pre-imaginales de la mosca del olivo, y el potencial de esta cepa para reducir en pleno campo la población de primavera del tefrítido al ser aplicada al suelo, debajo de la copa del olivo, dirigida a los estados preimaginales de la mosca del olivo. Finalmente, el capítulo VI refleja la compatibilidad de esta cepa con la mayoría de los herbicidas autorizados para su aplicación a suelos de olivar para poder dar recomendaciones sobre su empleo conjunto.

El extracto bruto de la cepa EAMb 09/01-Su de *M. brunneum* presenta actividad insecticida *per os* frente a adultos de *B. oleae*, aunque ésta depende del periodo de fermentación, como ya se ha observado con el extracto proteínico bruto de la cepa EAMa 01/58-Su (Ortiz-Urquiza et al. 2009). En concreto, el valor más bajo de tiempo medio de supervivencia se obtuvo al aplicar un extracto de 15 días de fermentación, 27,7

horas y 80 % de mortalidad frente a *B. oleae*, resultados que sitúan a este extracto entre los más tóxicos frente a adultos de tefrítidos tanto procedentes de plantas como microorganismos, y primera cita de un extracto de hongo entomopatógeno con actividad frente a *B. oleae* (Konstantopoulou y Mazomenos 2005; Quesada-Moraga et al. 2006b; Zapata et al. 2006). La actividad insecticida de este extracto obtenido en etapa tardía del crecimiento fúngico (15 días de fermentación) y valores iníciales de pH entre 7 y 9 delata que las moléculas responsables de tal actividad podrían ser metabolitos secundarios, lo que se ha constatado recientemente en nuestro laboratorio al relacionar esta actividad con las destruxinas A y A2 presentes en su extracto crudo (Lozano-Tovar et al. 2015); estos hongos se caracterizan por su alta diversidad en la secreción de metabolitos secundarios (Vey et al. 2001; Moon et al. 2008).

La concentración letal media CL_{50} del extracto bruto de 15 días de fermentación de la cepa EAMb 09/01-Su de *M. brunneum* frente a adultos de *B. oleae* fue del 49,9 % de su extracto crudo, cuya fotorresistencia y la termoestabilidad también han sido puestas de manifiesto en esta tesis. Más allá, la exposición a 60 ˚C durante dos horas no disminuyó su capacidad insecticida (72,0 % de mortalidad sobre adultos de *B. oleae*), e incluso la exposición a 120 ˚C durante 20 min solo redujo la actividad insecticida del extracto en un 20 %, reducción inferior a la detectada por Ortiz-Urquiza et al. (2009) en la fracción proteínica del extracto bruto de la cepa EAMa 01/58-Su frente a *C. capitata*. La exposición del extracto crudo de la cepa EAMb 09/01-Su a radiación UV-B (920 mW/cm^2) durante 2, 4 y 6 h no causó ninguna alteración sobre su la actividad insecticida, aspecto relevante, pues este factor limita el empleo práctico de numerosos productos insecticidas de origen natural. Por otro lado, se ha demostrado por vez primera el uso combinado del hongo entomopatógeno y su extracto crudo, lo que mejoraría la protección a corto plazo por el extracto y proporcionaría supresión a largo plazo de las generaciones posteriores debido a la posible transmisión vertical y horizontal del inóculo.

La compatibilidad entre los hongos entomopatógenos y los insecticidas químicos (Huang et al. 2013), insecticidas de origen natural (Gosselin et al. 2009; Hernández et al. 2012), o incluso compuestos de secretados por hongos (Hu et al. 2007) ha sido estudiada

previamente. Sin embargo, este es el primer trabajo sobre la compatibilidad de una cepa de hongos entomopatógenos y su propio extracto crudo para el control de plagas. La mortalidad de adultos de *C. capitata* fue mayor para los tratamientos combinados que cuando se aplicaron los tratamientos de forma individual, exhibiendo un efecto aditivo en todas las combinaciones hongo-extracto, lo que permitiría recomendar el empleo de la CL_{50} de la suspensión fúngica y 1 h de exposición al extracto crudo para minimizar los posibles riesgos ambientales asociados a ambos.

Un aspecto clave de la presente tesis doctoral es la evaluación de la cepa EAMa 01/58-Su del hongo entomopatógeno *M. brunneum* para el control de la mosca del olivo desde el laboratorio hasta su aplicación en pleno campo durante cuatro años. La aplicación de esta cepa en el campo se realizó después de una fase de evaluación en el laboratorio, capítulo V, donde el 95,2 % de los adultos de *B. oleae* murieron tras su pulverización con una suspensión de conidios, 91,8 % de los cuales mostraron crecimiento fúngico. El tiempo letal medio (TL_{50}) fue de 6,2 días, y concentración letal media (CL_{50}) de 7×10^6 conidios por mililitro, lo cual permitiría reducir la población adultos en campo antes de la primera puesta, momento crítico de ataque del insecto (Santiago-Álvarez y Quesada-Moraga 2007). A esto, debemos unir un alto porcentaje de esporulación en cadáveres, lo que contribuye a la autodiseminación del hongo en la población del tefrítido (Quesada-Moraga et al. 2008). Para contribuir aún más a este objetivo, los tratamientos de suelo contra larvas de *B. oleae* de tercera edad próximas a pupación causaron una mortalidad de pupas del 68,3 %, con 38,3 % de ellas con crecimiento fúngico y CL_{50} de 1×10^7 conidios por mililitro, lo que revela una actividad más alta de esta cepa frente a *B. oleae* que frente a otros tefrítidos (Quesada-Moraga et al. 2006a).

Por otro lado, en el capítulo V, se pone de manifiesto en ensayos de campo durante cuatro campañas agrícolas la eficacia de los tratamientos de suelo con la cepa EAMa 01/58-Su de *M. brunneum* debajo de la copa del árbol, dirigidos a las larvas de tercera edad que caen al suelo para pupar, con una reducción de la población de adultos de primavera del 50 a 70 % en las parcelas tratadas comparadas con las parcelas testigo.

Nuestro trabajo muestra por primera vez un método biológico y eficaz a nivel práctico frente a la mosca de olivo, y no hay estudios de campo comparables en la literatura sobre la aplicación del hongo dirigida al suelo bajo la copa de los árboles. El tratamiento de suelo con insecticidas bajo la copa de los árboles forma una parte importante de los programas de control de estados preimaginales de los tefrítidos (Ekesi et al. 2007). Ekesi et al. (2011) aplicaron una cepa de *M. anisopliae* en combinación con el spinosad al suelo para el control de *B. invadens* en el cultivo de mango y lograron bajar la población del tefrítido procedente del suelo tratado comparado con el control. Los tratamientos semanales del spinosad elevan el coste del tratamiento lo que no resultaría viable económicamente para el agricultor. Sin embargo, la cepa usada en este estudio durante cuatro años se aplica solo dos veces al año y nos proporciona un control eficaz a largo plazo. Por otro lado, la compatibilidad de la cepa EAMa 01/58-Su con la mayoría de los herbicidas autorizados en Producción Integrada puesta de manifiesto en el capítulo VI de la tesis podría permitir la aplicación simultánea de este hongo con el herbicida en el mismo tanque lo que reduce el coste de aplicación. En efecto, la cepa EAMa 01/58-Su es compatible *in vivo* e *in vitro* con la mayoría de los herbicidas autorizados en el reglamento de Producción Integrada del olivar (Consulta el 31/10/2011), con el oxifluorfeno al 24 % como la combinación más compatible tanto en términos de crecimiento fúngico, como de mortalidad de pupas e incluso número de conidios viables en el suelo después del tratamiento. Otra ventaja de esta cepa es que está perfectamente adaptada a las condiciones climáticas del Mediterráneo y no tiene efectos sobre la artropofauna del olivar (Garrido-Jurado et al. 2011a, c).

En conclusión, el uso de este hongo entomopatógeno en aplicaciones dirigidas a la base del árbol es una herramienta eficaz para ser utilizada dentro de un programa de control integrado de la mosca del olivo en olivares tradicionales o ecológicos, lo que puede permitir no solo el control de adultos antes de la puesta, sino mas importante, una notable reducción de la población de primavera del díptero como consecuencia de los tratamientos de suelo, debajo de la copa, dirigidos a los estados preimaginales. De esta forma, el empleo de esta cepa fúngica aporta una poderosa herramienta para

proteger el cultivo frente a la mosca del olivo, de una forma mas sostenible, minimizando el uso de insecticidas químicos, y por tanto el riesgo en el ecosistema y la salud humana, y preservando la calidad y seguridad del aceite de oliva, al preservar el fruto de cualquier residuo.

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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. La conclusión primera corresponde al capítulo 3, que comprende el manuscrito "Biocontrol of *Bactrocera oleae* (Diptera: Tephritidae) with *Metarhizium brunneum* and its extracts" publicado en Journal of Economic Entomology 106(3) (2013) 1118-1125. Las conclusiones 2 y 3 corresponden al capítulo 4, que incluye el manuscrito "One *Metarhizium brunneum* strain, two uses to control *Ceratitis capitata* (Diptera: Tephritidae)" publicado en Journal of Economic Entomology 107(5) (2014) 1736-1744. Las conclusiones 4 y 5 proceden del capítulo 5, manuscrito "Reduction of adult olive fruit fly populations by targeting preimaginals in the soil with the entomopathogenic fungus *Metarhizium brunneum*", publicado en Journal of Pest Science (2016) DOI 10.1007/s10340-016-0779-y. La sexta conclusión corresponde al capítulo 6 de esta Tesis Doctoral "Compatibility of herbicides used in olive orchards with a *Metarhizium brunneum* strain used for the control of preimaginal stages of tephritids in the soil" publicado en Journal of Pest Science 88 (2015) 605-612.

1.- La cepa EAMb 09/01-Su de *M. brunneum* fue patogénica frente a los adultos y estados preimaginales de *B. oleae*. Además, su extracto crudo, que es termo- y foto- resistente, presenta una gran actividad crónica y aguda lo que permitiría su desarrollo para el control de adultos de la mosca del olivo en tratamientos de pulverización-cebo o incluso mediante la estrategia de "atracción y muerte".

2.- La actividad del extracto crudo de la cepa EAMb 09/01-Su de *M. brunneum* es mayor cuando el medio de cultivo se inicia con pH elevado (7-9), y se incrementa con el tiempo de fermentación, en etapas tardías de crecimiento fúngico, con valores máximos a los 15 días, lo que indica que las moléculas responsables de tal actividad podrían ser metabolitos secundarios.

3.- El efecto aditivo que se encuentra en todas las combinaciones hongo-extracto de la cepa EAMb 09/01-Su de *M. brunneum* permitiría utilizar la CL_{50} de la suspensión fúngica y una hora de exposición al extracto crudo para un control efectivo de adultos de tefrítidos.

4.- La cepa EAMa 01/58-Su de *M. brunneum* fue muy virulenta frente a los adultos y estados preimaginales de *B. oleae* con tiempo letal medio (TL_{50}) de 6,2 días y concentración letal media de 7×10^6 conidios por mililitro para los primeros, y $1{,}0 \times 10^7$ conidios por mililitro para los segundos, lo cual permitiría reducir la población adultos en campo antes de la primera puesta, momento crítico de ataque del insecto.

5.- La aplicación en campo durante cuatro campañas agrícolas de la cepa la cepa EAMa 01/58-Su de *M. brunneum* al suelo bajo la copa de los árboles, dirigida a las larvas de tercera edad que abandonan el fruto y saltan al suelo para pupar se presenta como una estrategia eficaz para ser utilizada dentro de un programa de control integrado de la mosca del olivo, pues permite reducir la población de primavera del insecto en un 50-70 %.

6.- La cepa EAMa 01/58-Su de *M. brunneum* ha demostrado ser compatible con la mayoría de los herbicidas autorizados en producción integrada de olivar, lo que permitiría su empleo conjunto con herbicidas en el depósito del atomizador para un tratamiento simultáneo debajo de la copa del árbol, aspecto relevante para reducir el coste de aplicación. La mejor combinación de la cepa fue con oxifluorfeno al 24 %, tanto en términos de crecimiento micelial, como de viabilidad de conidios en el suelo y mortalidad de pupas.