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TESIS DOCTORAL

Combinación de diferentes estrategias de aplicación de hongos entomopatógenos para el control de *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) y *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae)

Doctoranda: Dª Gloria Arminda Resquín Romero

Directores: Prof. Dr. Enrique Quesada Moraga
Dra. Inmaculada Garrido Jurado

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TITULO: *Combinación de diferentes estrategias de aplicación de hongos entomopatógenos para el control de Spodoptera littoralis (Boisduval) (Lepidoptera, Noctuidae) y Bemisia tabaci (Gennadius) (Hemiptera:Aleyrodidae)*

AUTOR: *Gloria Arminda Resquín Romero*

Campus de Rabanales
Ctra. Nacional IV, Km. 396 A
14071 Córdoba

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TÍTULO DE LA TESIS:

Combinación de diferentes estrategias de aplicación de hongos entomopatógenos para el control de *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) y *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae)

DOCTORANDO/A:

Gloria Arminda Resquín Romero

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

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

La Doctoranda, Dña. Gloria Arminda Resquín Romero, se incorporó al Grupo de Investigación AGR 163 "Entomología Agrícola" en el año 2012, para la realización del Trabajo Profesional Fin de Máster que da opción al título de Máster en Producción, Protección y Mejora Vegetal y la posterior Tesis Doctoral.

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

- Resquín-Romero G., Garrido-Jurado, I. Quesada-Moraga, E. (2015) Combined use of entomopathogenic fungi and their extracts improves the control of *Spodoptera littoralis* (Boisduval) (Lepidoptera:Noctuidae). Biological Control 92, 101-110. DOI: http://dx.doi.org/10.1016/j.biocontrol.2015.10.007

- Resquín-Romero G., Garrido-Jurado I., Delso C., Ríos-Moreno A., Quesada-Moraga E. (2016) Transient endophytic colonizations of plants improve the outcome of foliar applications of mycoinsecticides against chewing insects. Journal of Invertebrate Pathology 136, 23-31. DOI: http://dx.doi.org/10.1016/j.jip.2016.03.003

- Garrido-Jurado I., Resquín-Romero G., Amarilla S.P., Ríos-Moreno A. Carrasco L., Quesada-Moraga E. (2016) Transient endophytic colonization of melon plants by entomopathogenic fungi after foliar application for the control of *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae). Journal of Pest Science (Aceptado)

Por tanto, se han publicado todos los trabajos en revistas pertenecientes a los dos primeros cuartiles de Journal Citation Reports (JCR) con un alto índice de impacto:

- *Journal of Pest Science* Q1 (8 de 92; IP: 2.644; JCR 2014) y *Biological Control* Q2 (25 de 92; IP: 1.635; JCR 2014) ambas del área de Entomología, que son las que presentan mayor índice de impacto en dicha área y permiten publicar un trabajo de Control de Plagas, pues las que las preceden se refieren a investigación básica sobre fisiología de insectos.
- *Journal of Invertebrate Pathology* Q1 del área de Zoología (27 de 154; IP: 2.110; JCR 2014) que es la revista más prestigiosa en esta área para exponer un trabajo de patología de invertebrados.

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

Córdoba, 5 de abril de 2016

Firma del/de los director/es

Fdo.: Prof. Dr. D. Enrique Quesada Moraga Fdo.: Dra. Dª Inmaculada Garrido Jurado

RESUMEN

Los ascomicetos mitospóricos entomopatógenos, que actúan por vía tegumentaria, han sido utilizados para el control microbiano de plagas mediante distintas estrategias que permiten el contacto de sus conidios con los insectos diana. Sin embargo, se conocen nuevos aspectos sobre su función ecológica tales como su capacidad secretora de compuestos con actividad insecticida, así como su sorprendente comportamiento como endófitos, que complementan su empleo clásico y que pueden permitir el desarrollo de nuevas estrategias de control de plagas. El empleo conjunto de estos hongos y sus extractos para el control de un insecto polífago de importancia mundial *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) es abordado en el capítulo II, donde se pone de manifiesto la existencia de variación intra- e interespecífica en la virulencia y estrategias patogénicas de las cepas de *Beauveria bassiana* y *Metarhizium brunneum* evaluadas frente a larvas del noctuido. La aplicación conjunta de cepas con distintas estrategias patogénicas junto con sus extractos a larvas del lepidóptero tuvo efecto aditivo, y alguno antagónico, de lo que depende su empleo conjunto. Los capítulos III y IV revelan la existencia de colonización endofítica transitoria tras la aplicación foliar de suspensiones de conidios de cepas de las especies mencionadas en alfalfa, tomate y melón, así como el impacto de esta colonización sobre dos fitófagos con diferentes hábitos de alimentación, uno masticador *S. littoralis* y otro picador-suctor *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae). El capítulo III muestra la contribución aditiva de la mortalidad causada por la alimentación de larvas de *S. littoralis* a expensas de material vegetal colonizado endofíticamente por cepas de *B. bassiana y M. anisopliae* con la debida al tratamiento tópico de las mismas con las suspensiones fúngicas. El origen de la mortalidad iniciada a través de la vía digestiva en larvas de este lepidóptero permanece incierto para las cepas de *B. bassiana*, pero podría estar asociado a la presencia en la planta de metabolitos fúngicos en las cepas de *M. brunneum* como denota la presencia de destruxina (dtx) A en el 11,0% de los cadáveres. El capítulo IV revela que cepas de ambas especies fúngicas pueden iniciar ciclos de infección en insectos picadores-suctores cuando se alimentan a expensas de sustrato vegetal colonizado endofíticamente, si bien, ambas especies presentan estrategias diferentes. Así, *B. bassiana* muestra una gran capacidad para colonizar el melón, incluso con efecto traslaminar, que causa la infección de ninfas de *B. tabaci* por contacto con el tegumento. Sin embargo, la mortalidad de las mismas causada por *M. brunneum*, de crecimiento mucho más localizado en la hoja, está relacionada una vez mas con la presencia de dtx A en el 43,0% de los cadáveres. Estos resultados deben ser considerados para la evaluación del impacto real de los tratamientos con hongos entomopatógenos, y abren nuevas vías en el control de plagas.

ABSTRACT

Entomopathogenic mitosporic ascomicetes have long been used for pest control by different strategies all ensuring a good probability that the target insect cuticle will come in contact with an adequate number of conidia. However, recent findings on the new ecological roles of these fungi such as insecticide compound secretion capability and their endophytic behavior could improve this classical inundative release and lead to the development of new pest control strategies. The combined use of several strains of these fungi and their extracts for the control of *Spodoptera littoralis* (Boisduval) (Noctuidae: Lepidoptera), a worldwide destructive pest, has been explored in chapter II. Intra- and interspecific variation in virulence and pathogenic strategies among *Beauveria bassiana* and *Metarhizium brunneum* strains against Egyptian armyworm larvae has been detected. The combined use of *B. bassiana* and *M. brunneum* strains with different pathogenic strategies and their crude extracts towards *S. littoralis* larvae yielded mostly additive effects, but antagonism was also encountered, therefore determining their possible combined deployment for pest control. Chapters III and IV reveal that spray application with conidial suspensions targeting chewing and sucking insect pests can be accompanied by a transient endophytic colonizations of plant (alfalfa, tomato, melon), which lead to different mortality rates on *S. littoralis* and the sweetpotato whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae). Indeed, chapter III shows that spray application of conidial suspensions of *B. bassiana* and *M. brunneum* targeting *S. littoralis* larvae in alfalfa, tomato and melon and their transient colonization produce additive effects therefore improving the control of this pest. The possible origin of such mortality in insects fed *B. bassiana*-colonized plants remains unknown, whereas this is the first study to report destruxin A traces in insects in 11.0% of the cadavers fed *M. brunneum*-colonized plants. Finally, chapter IV highlights that transient endophytic colonization of melon leaves by *B. bassiana* and *M. anisopliae* lead to different mortality rates of *B. tabaci* nymphs, whereas the origin of such mortality was different for the two fungal species. Mortality with fungal outgrowth was detected only in the *B. bassiana* treatments, with histological examination revealing translaminar behavior of this fungus coming in contact with nymphs in the abaxial surface when applied in the adaxial one. In contrast, no fungal outgrowth was detected in cadavers from the *M. brunneum* treatments, with the leaf colonizing fungus unable to come in contact with the nymphs, but with destruxin A present in 43.0% of them. The results of the present work may help in developing new pest control strategies and have to be considered to estimate the real acute impact of field sprays with entomopathogenic fungi on both chewing and sucking insects.

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CAPÍTULO I

Los hongos entomopatógenos y sus aplicaciones en el control de plagas

1. LA AGRICULTURA Y LAS PLAGAS DE INSECTOS

Las plantas captan la energía solar y la emplean para producir, a partir de moléculas inorgánicas, otras orgánicas de las que dependen todos los animales para constituir sus propios tejidos, y cuya utilización se realiza en un flujo establecido a distintos niveles tróficos. Los humanos no podrían existir sin los productos que les ofrecen las plantas, una dependencia que sin duda catalizó la aparición de la Agricultura en el Neolítico, revolución que dio origen a las primeras civilizaciones, pero también a las primeras alteraciones ecológicas.

Los paisajes agrícolas son sencillos en comparación con los naturales, con pocas especies vegetales que ocupan grandes superficies, donde se rompe el equilibrio entre los distintos eslabones de la cadena trófica, en especial entre la planta y el fitófago, y entre éste y sus enemigos naturales, siempre a favor del herbívoro. Por tanto, resulta fundamental proteger a las plantas frente al ataque de sus parásitos animales, con énfasis en los insectos, el grupo animal de mayores proporciones, cerca de un millón de especies de las que aproximadamente la mitad se alimentan de plantas o de productos de plantas (Tremblay, 1993; Speight et al., 2008).

Las plagas de insectos han sido combatidas por el hombre desde el inicio de la agricultura hace 10.000 años mediante distintos métodos culturales y con la ayuda de sustancias de origen vegetal (excepción del arsénico en China 200 a.C.), hasta que comenzaron a utilizarse los insecticidas inorgánicos en el siglo XIX, y después de la segunda guerra mundial, los insecticidas químicos de síntesis, organoclorados, organofosforafos y carbamatos. En el último tercio del siglo XX, se incorporan los piretroides y los reguladores del crecimiento de los insectos (RCI), y en definitiva, un sucesión de nuevas materias activas insecticidas como formamidinas, fenilpirazoles, neonicotinoides, etc., que en su conjunto han permitido salvar algo más del 70% de la producción agrícola potencial mundial

(Jones, 1973; Casida y Quistad, 1998; Ware y Whitacre, 2004; Oerke, 2006).

Sin embargo, el empleo de insecticidas ha acarreado una serie de efectos adversos sobre los seres vivos y el medio ambiente tales como toxicidad aguda y crónica en humanos, animales domésticos y vida salvaje, fitotoxicidad, desarrollo de plagas por especies que antes no constituían amenaza, persistencia de estos productos en el suelo y agua, así como su capacidad de transporte y contaminación medioambiental, y muy en especial, la aparición de resistencia a insecticidas, y su acción negativa sobre la artropofauna útil (Gerwick y Sparks, 2014). Más allá, la preocupación por los aspectos ecotoxicológicos y medioambientales asociados al control de plagas continúa en primera línea política y social (Altieri et al., 2012).

No en vano, desde que se inició en el año 1993 el 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. No obstante, esta criba 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. La ausencia de alternativas es uno de los factores limitantes para el desarrollo de programas de Control Integrado de Plagas bajo las premisas de sostenibilidad agrícola recogidas por la Directiva 128/2009/CE del Parlamento Europeo y del Consejo de 21 de octubre de 2009, que establece el marco de la actuación comunitaria para conseguir un uso sostenible de los plaguicidas, ya transpuestas a la legislación española mediante el Plan Nacional de Acción (Real Decreto 1311/2012 de 14 de septiembre de 2012).

Este escenario agudiza la necesidad de nuevas estrategias de control de plagas que se ajusten a los principios de la Agricultura Sostenible, entre las que han adquirido especial relevancia los bioinsecticidas a base de agentes entomófagos y

entomopatógenos, semioquímicos, e insecticidas derivados de plantas y de microorganismos (Czaja et al., 2015). No obstante, este tipo de productos ingresa poco a poco en el mercado, a escala mundial, los productos microbianos sólo representan el 3% de todos los productos fitosanitarios comercializados (Ravensberg, 2015), ya que aún deben mejorarse tanto su eficiencia como su competencia ambiental (Lacey et al, 2015).

A este respecto, los hongos entomopatógenos han recibido una atención creciente en los últimos años por sus interesantes características para el desarrollo de bioinsecticidas y de nuevas estrategias en protección de cultivos que se analizan a continuación.

2. LOS HONGOS ENTOMOPATÓGENOS

Los hongos entomopatógenos (HE) constituyen un grupo heterogéneo desde el punto de vista sistemático y también presentan diferencias en cuanto a su biología, actúan por la vía tegumentaria y su virulencia frente a insectos, en realidad frente a artrópodos, les convierte en un importante factor de regulación natural de sus poblaciones (Quesada-Moraga et al., 2007). Además, estos agentes fúngicos de control microbiano se ajustan al criterio de "búsqueda inteligente" de nuevos compuestos insecticidas de origen natural propuesto por Schulz et al. (2002), al coexistir con los insectos fitófagos en el mismo nicho ecológico, con un proceso patogénico caracterizado por la secreción de distintos metabolitos insecticidas (Quesada-Moraga et al., 2007). Finalmente, se han descrito en los últimos años nuevas y sorprendentes funciones ecológicas de los hongos entomopatógenos de gran interés para la protección de cultivos.

2.1 Distribución y ecología

Los HE son un importante y ampliamente distribuido componente de la mayoría de los ecosistemas terrestres, con más de 750 especies descritas, procedentes de diversos hábitats como el suelo, insectos y otros artrópodos, la

filosfera (filoplano) y la endosfera (como endófitos) (Scheepmaker y Butt, 2010; Quesada-Moraga et al., 2014a; ARSEF, 2016). Esta variada procedencia lleva también asociadas distintas respuestas de los HE a los factores bióticos y abióticos a los que se ven expuestos en los ecosistemas naturales y agroforestales (Quesada-Moraga et al., 2007; Jaronski, 2010).

Entre los **factores bióticos,** l**os microorganismos del suelo** pueden afectar a la viabilidad y persistencia de los HE por fungistasis, lo que a su vez depende de otros factores relacionados con los estímulos procedentes del hospedante y del suelo que son necesarios para su germinación (Groden y Lockwood, 1991; Jaronski, 2010). Algunos **invertebrados geobiontes o geófilos**, en especial colémbolos, ácaros o lombrices, desempeñan un papel importante en la dispersión en el suelo de los conidios tras ser ingeridos por ellos. Además, la prevalencia de los HE en el suelo, está estrechamente relacionada con la presencia de insectos susceptibles a la infección (Meyling y Eilenberg, 2007; Lacey et al., 2015). Cuando los HE se encuentran en el filoplano, pueden verse afectados por la química de la superficie de **las plantas** y la emisión de volátiles (Cory y Ericsson, 2010), mientras que en el suelo, son exudados de las raíces los que pueden favorecer la presencia y competencia del hongo en la rizosfera, en especial el género *Metarhizium* (Li y Holdom, 1993).

Los principales **factores abióticos** que influyen en los HE son la radiación UV, textura del suelo, temperatura y humedad, aunque otros como el pH, capacidad de intercambio catiónico o materia orgánica también pueden influir sobre la eficacia insecticida del inóculo y persistencia del mismo (Jaronski, 2010). **La radiación UV** es la banda del espectro solar más perjudicial y mutagénica, en particular la radiación UV-B (295-320 nm) para los procesos biológicos (Smits et al., 1996). La exposición directa a la radiación solar durante cuatro horas puede inactivar conidios de la mayoría de las especies de HE (Braga et al., 2015). Sin embargo, el efecto de la radiación solar puede verse alterado por la formulación y la especie fúngica o aislado (Jaronski, 2010) Por tanto, una buena selección de aislados y el empleo de

filtros solares mejoran las aplicaciones de HE en hábitats epígeos (Chelico et al., 2006; Fargues et al., 2003).

El rango de **temperatura** óptimo para la infección, crecimiento y desarrollo de los HE varía entre 20 y 30ºC, con óptimos térmicos de crecimiento entre 23 y 28ºC, y temperaturas mínimas entre 5 y 10ºC y máximas de 34 a 36ºC (Doberski, 1981; Pedrini et al., 2007; Quesada-Moraga et al., 2007). **La humedad relativa** ha sido reconocida como un factor esencial para la germinación de los conidios pero a nivel de microhábitat en el que se va a producir la infección, hoja, suelo etc. (Fargues et al., 2003; Tseng et al., 2014). **La textura del suelo,** entendida como el tamaño de poro y distribución de partículas, **y la práctica agrícola,** también interfieren en la presencia y persistencia del inóculo en el suelo. Así, los conidios de *B. bassiana* quedan retenidos en el complejo de cambio de las arcillas, mientras que los conidios de *M. anisopliae*, más hidrofóbicos y de mayor tamaño no parecen mostrar esta asociación con ellas (Garrido-Jurado et al., 2011). Además, *B. bassiana* se encuentra de manera común en hábitats naturales, mientras que en cultivados es más probable encontrar *M. anisopliae* (Quesada-Moraga et al., 2007). Debido probablemente a que *M. anisopliae* es capaz de persistir en el suelo más tiempo que *B. bassiana* en ausencia de insectos hospedantes (Fargues y Robert, 1985; Vänninen, 1996).

2.2 Sistemática y biología

2.2.1 Sistemática

Los análisis filogenéticos recientes demuestran que dentro del reino Mycota, los hongos entomopatógenos se clasifican en dos divisiones, Entomophtoromycota y Ascomycota, donde se sitúan respectivamente los órdenes con más representantes, Entomophtorales e Hypocreales, respectivamente (Gryganskyi et al., 2012, 2013; Hibbett et al., 2007; Humber, 2012) (Figura 1).

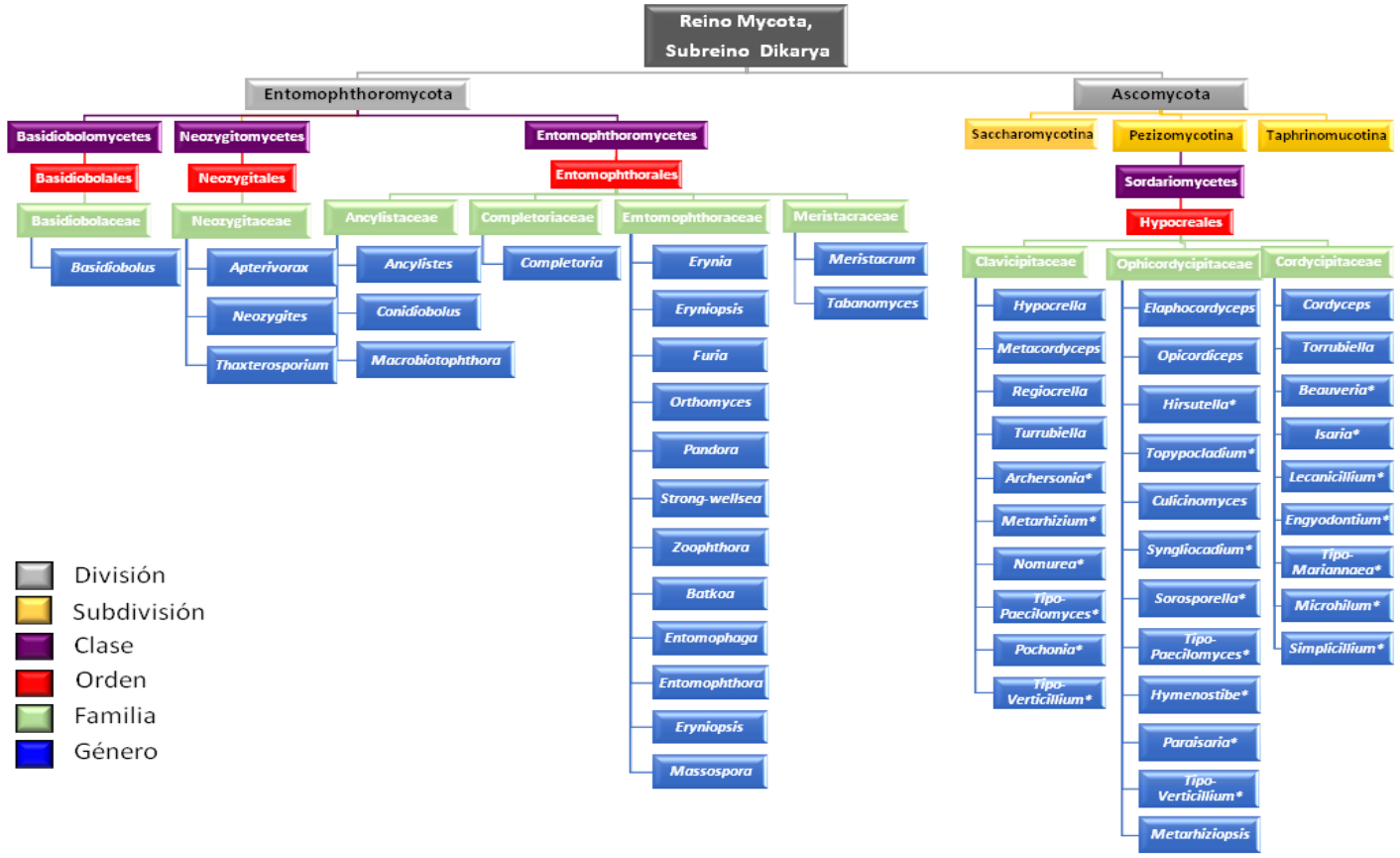

Figura 1. Clasificación sistemática de los Hongos Entomopatógenos. Los géneros entomopatógenos *Lagenidium* (Oomycetes: Lagenediales) y *Leptolegmia* (Oomycetes: Saprolegniales) pertenecientes a los oomicetos no se incluyen en el reino de los hongos. Esta clasificación se basa en la propuesta de Hibbet et al. (2007), Humber (2012), Gryganskyi et al. (2012) y las ARS collection of the entomopathogenic fungal cultures (http: //arsef.fpsnl.cornell. edu). Los géneros marcados con asterisco son anamorfos y el resto telomorfos.

Los Entomophthorales, con las familias Entomophthoraceae, Neozygitaceae, y Ancylistaceae, se caracterizan por ser biotrofos obligados, de difícil multiplicación en medio artificial, lo que limita su empleo al control biológico clásico (Keller, 2007; Pell et al., 2010).

Los Ascomicetos, con las familias Clavicipitaceae, Cordycipitaceae y Ophiocordycipitaceae, presentan buena eficacia insecticidas y son de fácil manejo y producción en masa, factibles para la comercialización como micoinsecticidas (Pell et al., 2010). Los géneros más ampliamente distribuidos son *Beauveria* Vuill. y *Metarhizium* Sorok., ambos con 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). El género *Metarhizium,* está compuesto por un complejo críptico de nueve especies, seis agrupadas en el denominado clade 1 (*M. majus, M. guizhouense, M. brunneum, M. pingshaense, M. robertsii, M. anisopliae*) por oligonucleótidos específicos de clade (Bischoff et al., 2009; Schneider et al., 2011).

2.2.2 Biología

La biología de los principales grupos de HE, ascomicetos mitospóricos y los entomophthoráceos presenta una serie de diferencias, aunque las interespecíficas se solapan a veces con las intraespecíficas.

En los ascomicetos mitospóricos, las esporas asexuales o conidios son los responsables de la infección y están dispersos por el medio donde se encuentran los insectos hospedantes. 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; logran atravesarla, gracias a la

combinación de mecanismos bioquímicos y mecánicos (Figura 2) (Ortiz-Urquiza y Keyhani, 2013). Alcanzado el hemocele, el hongo crece en forma de cuerpos hifales (Figura 2), para lo que debe vencer la respuesta defensiva del insecto, tanto celular (fagocitosis y encapsulación) (Han et al., 2013; Tseng et al., 2014) como humoral (producción de fenoloxidasa, lectinas, u otras proteinas y peptidos defensivos etc.) (Vey et al., 2001; Volkoff et al., 2003).

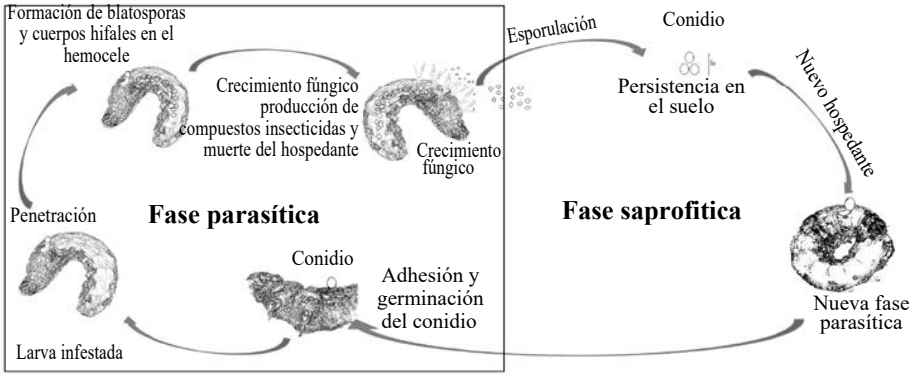

Figura 2. Proceso patogénico de los hongos los ascomicetos mitospóricos entomopatógenos

La muerte del insecto puede ser resultado de la combinación de distintas acciones del hongo como la utilización de los nutrientes, la invasión física de los diferentes órganos del hospedante y la producción de toxinas. Después de la muerte, el hongo manifiesta crecimiento saprofítico (necrotrófico), y en condiciones de humedad y temperatura favorables, las hifas emergen del cadáver, se produce la esporulación y con la liberación de los conidios se inicia un nuevo ciclo (Figura 2) (Goettel et al., 2005; Charnley y Collins, 2007).

En las infecciones por Entomophthorales, se forman cuerpos hifales (estructuras unicelulares tipo levadura con pared celular quitinosa) que se dispersan por el hemocele, causando la muerte al hospedante debido a la utilización de sus nutrientes (Figura 3). Algunas especies de Entomophthorales producen inicialmente protoplastos circulares, con

ausencia de residuos ricos en azúcares, de cara a evitar su reconocimiento por los hemocitos del hospedante (Eilenberg, 2002; Keller, 2007). Tras la muerte del insecto, el hongo emerge del mismo, y en condiciones favorables, la esporulación o conidiogénesis tienen lugar fuera del cadáver, lo que favorece la liberación de los conidios, que inician un nuevo ciclo, y contribuyen a la transmisión del hongo. Los cadáveres de insectos muertos por Entomophthorales (Figura 3) quedan adheridos normalmente al sustrato vegetal, a veces por medio de estructuras especiales como rizoides, lo que asegura la permanencia del hongo en el medio y la dispersión de los propágulos infectivos a nuevos hospedantes (Quesada-Moraga et al., 2007; Shang et al., 2015).

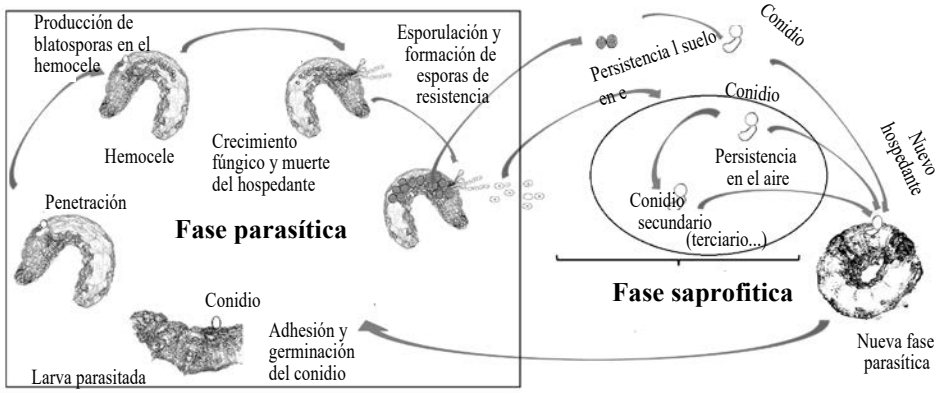

Figura 3. Proceso patogénico de los hongos Entomophthorales entomopatógenos

Los conidios de los ascomicetos mitospóricos como *Beauveria* sp. y *Metarhizium* sp., son hidrofóbicos y se dispersan de forma pasiva desde los cadáveres infectados principalmente por el viento. Los conidios de los Entomopthorales se descargan de forma activa (Figura 3) debido a fuerzas de presión hidrostática, con la excepción del género *Massospora*. Tras su descarga, los conidios de los Entomophthorales son transportadas por el viento o por insectos próximos al infectado. Si los conidios primarios de las especies de entomophthorales no alcanzan un hospedante susceptible para germinar, entonces la mayoría forman conidios de órdenes superiores que bien pueden ser descargados de forma activa (por ejemplo, *Erynia* o

Pandora), o formar conidios secundarios que no son descargados de forma activa, los capilloconidios, que se producen en largas estructuras (capilloconidióforos, por ejemplo, *Zoophthora*) (Figura 3).

Cuando existe escasez de insectos hospedantes, o las condiciones ambientales no son favorables, la mayoría de especies de entomophthorales producen esporas de reposo, clamidosporas, zigosporas o azigosporas para persistir en el suelo durante largos períodos de tiempo (Figura 3), mientras que a tal efecto, los ascomicetos mitospóricos pueden también permanecer en el suelo durante largos periodos gracias a la formación de esclerocios, clamidosporas, etc (Quesada-Moraga et al., 2007).

2.2 Estrategias para el empleo de hongos entomopatógenos en control microbiano

La presencia natural de los HE no es suficiente en la mayoría de los casos para contener el incremento de las poblaciones de insectos que afectan a las plantas cultivadas, por eso, es preciso recurrir a su manejo para aprovechar todo el potencial que encierran. Son cuatro las estrategias que podemos utilizar para el empleo eficiente de los HE en el control de las plagas de insectos: (1) conservación; (2) control biológico clásico; (3) inoculación e (4) inundación (Eilenberg, 2002).

La **estrategia de conservación** implica la modificación de algunas prácticas agronómicas para aumentar su actividad sobre la población del insecto tales como la reducción del uso de fitosanitarios, la provisión de lugares de hibernación para hospedantes alternativos, etc. En este sentido, se da especial importancia al manejo de los márgenes de los cultivos como refugio de artrópodos hospedantes de hongos entomophthoráceos para el inicio de epizootias (Pell et al., 2001).

El **control biológico clásico** promulga la introducción deliberada del HE en un hábitat nuevo para el control de una especie invasiva. Los HE

seleccionados para este fin deben ser capaces de persistir en el medio, por ejemplo, las especies de entomophthoráceos que tiene esporas de reposo, y la eficacia de la estrategia se favorece en hábitats con un cierto grado de permanencia, como por ejemplo praderas, bosques y cultivos leñosos. Además, esta estrategia requiere un alto nivel de especificidad por parte del enemigo natural y además que su respuesta a la población del hospedante se acomode al modelo densidad dependiente. Dos ejemplos claros de éxito con HE son el establecimiento del entomophthoráceo *Entomophaga maimaiga* Humber, Shimazu & Soper en los EEUU, que ha permitido el control de *Lymantria dispar* Linnaeus (Lepidoptera: Lymantriidae) en bosques del Noreste del país, y el control de *Therioaphis trifolii* f. *maculata* (Monell) (Homoptera: Aphididae) en Australia por medio del entomophthoraceo *Zoophthora radicans* importado desde Israel (Shah y Pell, 2003).

La **estrategia de inoculación** supone la aportación de cantidades bajas o medias del inóculo del hongo entomopatógeno para conseguir el control de un insecto en el medio y largo plazo, como se ha descrito con *Beauveria brongniartii* (Saccardo) Petch aplicado contra larvas de gusanos blancos en Europa (Shah y Pell, 2003).

Finalmente, la **estrategia de inundación** consiste en la aplicación del hongo, a menudo en grandes cantidades, para conseguir bajar la población del insecto en un plazo de tiempo corto, sin esperar a infecciones secundarias. En este caso el hongo se usa de manera similar a los insecticidas químicos y se emplea el término micoinsecticida (Tanada y Kaya, 1993).

Los ascomicetos mitospóricos se adaptan bien a esta estrategia porque son fáciles de producir a gran escala y formular para empleo convencional con equipos de pulverización. Las especies de HE comercializadas en la actualidad para el control de insectos y ácaros pertenecen principalmente a los géneros *Beauveria* spp., *Metarhizium* spp. y *Lecanicillium* spp., pero no únicos como se recoge en la tabla 1 (Lacey et al., 2015).

Tabla 1. Hongos entomopatógenos que han sido desarrollados para el control de insectos y ácaros. Modificada a partir de Alves et al. (2008) y Lacey et al. (2015).

Especie fúngica	Insecto diana	Producido en	Referencias
Aschersonia aleyrodis	Hemiptera (Aleyrodidae)	Rusia	Fransen, 1990; Meekers et al., 2002; McCoy et al., 2009
Beauveria bassiana sensu lato	Acari, Coleoptera, Diplopoda, Diptera, Hemiptera, Isoptera, Lepidoptera, Orthoptera, Siphonoptera, Thysanoptera	Africa, Asia, Australia, Europe, Sur y Norte de América	De la Rosa et al., 2000; Wraight et al., 2000, 2007; Chandler et al., 2005; Wekesa et al., 2005; Brownbridge et al., 2006; Labbé et al., 2009
Beauveria brongniartii	Coleoptera (Scarabaeidae)	Europa, Colombia, Isla Reunión (Francia)	Zimmermann, 1992; Keller et al., 2003; Dolci et al., 2006; Keller, 2007; Townsend et al., 2010
Conidiobolus thromboides	Acari Hemiptera, Thysanoptera	Colombia, India, Sudáfrica	Papierok y Hajek, 1997; Nielsen y Hajek, 2005; Hajek et al., 2012
Hirsutella thompsonii	Acari	India	Chandler et al., 2000, 2005; McCoy et al., 2009
Isaria fumosorosea	Acari, Diptera, Coleoptera, Hemiptera, Thysanoptera	Belgica, Colombia, Mexico, USA, Venezuela	Wraight et al., 2000, 2007; Alves et al., 2002; Zimmermann, 2008; Lacey et al., 2011
Lagenidium giganteum	Diptera (Culicidae)	USA	Kerwin y Petersen, 1997; Skovmand et al., 2007
Lecanicillium lecanii		Brazil, Colombia, Cuba, Perú	Alves et al., 2002
Lecanicillium muscarium	Acari, Hemiptera, Thysanoptera	Países Bajos, Rusia	Bird et al., 2004; Down et al., 2009; Kim et al., 2009

Tabla 1 (continuación). Hongos entomopatógenos que han sido desarrollados para el control de insectos y ácaros. Modificada a partir de Alves et al. (2008) y Lacey et al. (2015).

Especie fúngica	Insecto diana	Producido en	Referencias
Metarhizium anisopliae sensu lato	Acari, Blattodea, Coleoptera, Diptera, Hemiptera, Isoptera, Lepidoptera, Orthoptera	Africa, Asia, Australia, Europa, Sur, Central y Norte de América	De la Rosa et al., 2000; Chandler et al., 2005; Wekesa et al., 2005; Lacey et al., 2011; Jaronski y Jackson, 2012
Metarhizium acridum	Orthoptera	Australia, Sudáfrica, USA	Lomer et al, 1999, 2001; Thomas, 2000
Nomuraea reley	Lepidoptera	Colombia, Brazil, India	Alves et al., 2002; Moscardi y Sosa-Gómez, 2007; Thakre et al., 2011

Existen numerosos ejemplos de HE utilizados por inundación para el control de plagas, bien en ambientes epigeos, bien en los hipógeos. En **ambientes epigeos**, la principal estrategia natural de dispersión de los HE es el transporte de los conidios por el viento o insectos al adherirse a la superficie externa del hospedante antes de la germinación y colonización. Esta estrategia natural de dispersión es la recreada mediante las pulverizaciones de productos micoinsecticidas a las copas de los árboles y partes aéreas de plantas, y utilizada frente a la mayoría de las plagas, junto con la aplicación de cebos alimenticios o atrayentes (Quesada-Moraga y Santiago-Álvarez, 2008).

En los ambientes hipogeos también se han obtenido buenos resultados, no en vano, el suelo es el hábitat natural de los HE, en él se encuentran protegidos de cualquier factor adverso, y es donde ejercen su máximo potencial de biocontrol (Scheepmaker y Butt, 2010). Para que su empleo sea satisfactorio en el suelo deben ser de fácil aplicación en el medio natural del fitófago y de suficiente vida útil y persistencia en el medio tras la aplicación. Los HE pueden permanecer e incluso reciclarse en el suelo, por lo

que este método de aplicación posibilita reducir progresivamente el número de tratamientos tanto en el espacio como en el tiempo (St Leger, 2008; Scheepmaker y Butt, 2010).

Los HE también pueden ser aplicados mediante los tratamientos de semillas, técnica que se ha utilizado tradicionalmente para proteger de plagas y enfermedades de suelo a semillas y plántulas en desarrollo mediante el recubrimiento de estas con fungicidas e insecticidas de amplio espectro. La aparición de nuevos biopolímeros en la industria de la formulación de insecticidas ha permitido que el recubrimiento de semillas con agentes de biocontrol sea posible. Los HE competentes en la rizosfera, como es el caso de *Metarhizium* que puede establecerse en las raíces de las plántulas en desarrollo, y los endófitos, como *Beauveria* que coloniza la planta, pueden mitigar el daño producido por plagas y patógenos de suelo pero también por aquellos que se desarrollan en el interior de la planta.

2.3. Nuevas funciones ecológicas de los hongos entomopatógenos y su aplicación para el control de plagas

Hasta finales del siglo XX los retos científicos en el estudio de los HE se dirigían principalmente a su presencia natural y ecología, modo de acción y empleo práctico para el control de insectos y ácaros. Sin embargo, en el siglo XXI se han utilizado nuevas técnicas para profundizar en estos aspectos, pero además se han descrito nuevas funciones ecológicas así como su significado para la protección de cultivos, entre ellas debe destacarse su carácter endófito, su competencia en la rizosfera, donde incluso pueden promover el crecimiento vegetal, su función como antagonistas de microorganismos fitopatógenos, y su capacidad para secretar compuestos con múltiples actividades biocidas como insecticidas, antifúngicos, herbicidas y antivirales (Vega et al., 2009, Ownley et al., 2010; Han et al., 2013; Quesada-Moraga et al., 2014a; Lozano-Tovar et al., 2015; Taibon et al., 2015).

Los objetivos de esta tesis incluyen algunas de estas nuevas funciones como la capacidad secretora de compuestos insecticidas por parte de los HE así como su función como endófitos a las que dedicaremos especial atención.

2.3.1 Secreción de compuestos insecticidas por los hongos entomopatógenos

Al explicar el modo de acción de los HE se ha constatado que poseen la capacidad de secretar compuestos tóxicos durante el ciclo de infección. Estos compuestos pueden tener distintas propiedades bioactivas como son insecticida, acaricida, nematicida, antibiótica, antifúngica, citotóxica, mutagénica o ser factores de virulencia de estos agentes de control (Quesada-Moraga et al., 2014a). Resulta vital conocer el modo de acción de dichos compuestos ya que pueden presentar un riesgo para los organismos vivos o por el contrario pueden tener un gran valor para el sector de los agroquímicos o incluso de la industria farmacéutica. Estos compuestos bioactivos de origen fúngico se clasifican en:

a) Metabolitos secundarios de bajo peso molecular (menos de 5000 Da) producidos principalmente por los géneros *Beauveria, Metarhizium, Tolypocladium, Paecylomices* y *Cordyceps*, que incluyen **poliquétidos** (oosporeina, cordiol C, diorcinol, violaceol-I y -II, naftopirano, emodina, bassianolona, cefalosporolidos, aurovertinas, micestericinas, esfingofunginas, flavovirinas), **péptidos no ribosomales** (leucinostatinas, culicininas, efrapeptinas, diketopiperazinas, bassiatina, beauverolidas, destruxinas, cyclosporinas, beauvericina, enniatinas, bassianolida, farinosonas, ophiocordina), **terpenoides** (paecilomycinas, ascochlorinas, aphidicolina, metarhizinas, ácido helvólico) y **otros** como la cordycepina o la familia de las maleimidas (Molnar et al., 2010). De entre todos los compuestos descritos anteriormente, son sin duda las destruxinas los metabolitos secundarios más frecuentes y numerosos. Estos compuestos cíclicos hexadepsipéptidos de un ácido-hidroxi y cinco aminoácidos residuales se describieron por primera vez

en 1961. Su nombre deriva de "destructor", especie del hongo *Oospora destructor* (Metschn.) Delacr. (= *Isaria destructor* Metschn.) del que se aislaron por primera vez estos metabolitos (Pedras et al., 2002). El género *Metarhizium* es conocido por producir frecuentemente estos metabolitos secundarios de actividad insecticida y fitotóxica, así como efectos subletales en la reproducción de aquellos insectos que sobreviven al tratamiento (Amiri et al., 1999; Hu and St Leger, 2002; Pedras et al., 2002; Lozano-Tovar et al., 2015). El interés por las destruxinas deriva principalmente de su potencial como factores de virulencia para el control biológico de plagas de insectos (Skrobek et al., 2008; Han et al., 2013; Tseng et al., 2014; Taibon et al., 2015).

b) Proteínas de alto peso molecular como la Hirsutelina A (15 KDa) producida por el hongo *Hirsutella thompsonii* Fisher (Mazet y Vey, 1995), o la Bassiacridina (60 KDa) producida por *Beauveria bassiana* (Quesada-Moraga y Vey, 2003). Recientemente se ha detectado y caracterizado una nueva proteína insecticida secretada por *Metarhizium* sp. que ha recibido el nombre de proteína(s) secretada(s) tóxica para insectos 1 (SIT1), que induce melanización y muerte celular en el insecto (Ortíz-Urquiza et al., 2009; 2013). El extracto bruto del aislado EAMa 01/58-Su de *M. brunneum* está compuesto por tres proteínas/péptidos de 15/15/11 KDa que han mostrado una gran toxicidad *per os* frente dípteros y lepidópteros, pero su completa purificación aún no se ha podido llevar a cabo (Ortiz-Urquiza et al., 2009; Quesada-Moraga et al., 2006a; Resquín-Romero, 2016).

2.3.2 Los entomopatógenos como endófitos e interacciones tróficas

En sentido amplio, los endófitos son microorganismos que viven en los tejidos vegetales durante todo o parte de su ciclo de vida, sin causarles síntomas aparentes (Schulz y Boyle, 2005). No fue hasta finales del siglo XX cuando se observó por vez primera el establecimiento de *B. bassiana* en el interior de tallos de maíz (*Zea mays* L.), y su desarrollo para la protección

sistémica frente al barrenador *Ostrinia nubilalis* (Lepidoptera: Pyralidae) (Bing y Lewis, 1992; Wagner y Lewis, 2000). Este hecho impulsó el interés por la función de los HE como endófitos, más allá de la interacción directa con los insectos, que se ha ido ampliando con el tiempo a un gran número de especies vegetales y fúngicas (Tabla 2) (Vega et al., 2008; Roy et al., 2010; Quesada-Moraga et al., 2014a).

Tabla 2. Especies de plantas colonizadas de manera natural y artificial por hongos entomopatógenos endófitos. Modificada de Quesada-Moraga et al. (2014a) y actualizada

Especie fúngica	**Inoculación**	**Plantas hospedantes**	**Referencias**
Arthrobotrys oligospora	Artificial	*Hordeum vulgare*, *Solanum lycopersicum*	Bordallo et al., 2002
Arthrobotrys dactyloides	Artificial	*Hordeum vulgare*	López Llorca et al., 2006
Beauveria bassiana	Natural	*Abies beshanzuensis*; *Ammophila arenaria*, *Carpinus carolinana*, *Caffea arabica*, *Dactylis glomerata*, *Datura stramonium*, *Elymus farctus*, *Espeletia* spp., *Eucalyptus globulus*, *Gossypium hirsutum*, *Papaver somniferum*, *Pinus montícola*; *Pinus radiata*, *Pinus sylvestris*, *Quercus ilex*, *Theobroma gileri*, *Zea mays*, *Schizachne purpurascens*	Bills y Polishook, 1991; Collado et al., 1999; Ganley y Newcombe, 2006; Quesada-Moraga et al., 2006b; Sánchez et al., 2007, 2008, 2011; Thomas et al., 2008; Vega et al., 2008 Giordano et al., 2009 Yuan et al., 2011 Brownbridge et al., 2012; Miles et al., 2012; Behie et al., 2015
	Artificial	*Coffea arabica*, *Corchorus olitorius*, *Musa* sp., *Papaver somniferum*, *Phoenix dactylifera*, *Pinus radiata*, *Sorghum bicolor*, *Theobroma cacao*, *Zea mays*, *Lycopersicon esculentum*, *Triricum aestivum*, *Gossypium hirsutum*, *Cynara scolymus*, *Vitis vinifera*, *Phaseolus vulgaris*, *Nicotiana tabacum*, *Triticum sativus*, *Glycine max*, *Brassica napus*, *Oryza sativa*	Bing y Lewis, 1992; Wagner y Lewis, 2000; Posada y Vega, 2005; Gómez Vidal et al., 2006; Posada et al., 2007; Akello et al., 2007; Tefera y Vidal, 2009; Biswas et al., 2012; Brownbridge et al., 2012; Castillo-Lopez et al., 2014; Guesmi-Jouini et al., 2014; Qayyum et al., 2015; Jaber, 2015; Behie et al., 2015; Castillo-Lopez y Sword, 2015; Lohse et al., 2015; Mantzoukas et al., 2015; Russo et al., 2015; Sánchez-Rodríguez et al., 2015

Tabla 2 (continuación). Especies de plantas colonizadas de manera natural y artificial por hongos entomopatógenos endófitos. Modificada de Quesada-Moraga et al. (2014a) y actualizada

Especie fúngica	Inoculación	Plantas hospedantes	Referencias
Bionectria ochroleuca	Artificial	*Cynara scolymus*	Guesmi-Jouini et al., 2014
Clonostachys rosea	Natural	*Coffea arabica*, *Quercus myrsinifolia*	Collado et al., 1999 Vega et al., 2008; Shirouzu et al., 2009
Cordyceps memorabilis	Natural	*Eucaliptus globulus*	Sanchez Marquez et al., 2011
Cordyceps sinensis	Natural	*Holcus lanatus*, *Theobroma gileri*	Thomas et al., 2008; Sánchez et al., 2010
Lecanicillium dimorphum, *Lecanicillium* cf. *psalliotae*		*Phoenix dactylifera*	Gómez Vidal et al., 2006
Lecanicillium lecanii	Natural	*Ammophila arenaria*, *Carpinus caroliniana*, *Dactylis glomerata*, *Elymus farctus*, *Gossypium hirsutum*	Bills y Polishook, 1991; Sánchez et al., 2007, 2008; de Souza Vieira et al., 2011
Hirsutella aphidis	Natural	*Lolium perenne*	Autores, sin publicar
Hypocrea lixii	Artificial	*Allium cepa*	Muvea et al., 2015
Isaria farinosa	Natural	*Pinus sylvestris*	Bordallo et al., 2002
Isaria fumosorosea	Artificial	*Sorghum bicolor*	Mantzoukas et al., 2015
Metarhizium anisopliae	Natural	*Cynodon dactylon*	Autores, sin publicar
	Artificial	*Solanum lycopersicum*	García et al., 2011
Metarhizium robertsii	Natural	*Aster vimineus*, *Hieracium pratense*, *Solidago altissima*	Behie et al., 2015
	Artificial	*Panicum virgatum*, *Phaseolus vulgaris*, *Sorghum bicolor*	Sasan y Bidochka, 2012; Behie et al., 2015; Mantzoukas et al., 2015

Tabla 2 (continuación). Especies de plantas colonizadas de manera natural y artificial por hongos entomopatógenos endófitos. Modificada de Quesada-Moraga et al. (2014a) y actualizada

Especie fúngica	**Especie fúngica**	**Especie fúngica**	**Especie fúngica**
Metarhizium brunneum	Natural	*Schizachne purpurascens*	Behie et al., 2015
Metarhizium guizhouense	Natural	*Solidago altissima*	Behie et al., 2015
Nematoctonus robustus	Artificial	*Hordeum vulgare*	López Llorca et al., 2006
Paecilomyces sp.	Natural	*Carpinus caroliniana*, *Dactylis glomerata*, *Holcus lanatus*, *Musa acuminata*, *Oryza sativa*	Sánchez et al., 2007, 2010; Vega et al., 2008
Plectosphaerella cucumerina	Natural	*Phaseolus vulgaris*, *Cynodon dactylon*, *Ammophila arenaria*, *Elymus farctus*	Autores, sin publicar; Sánchez et al., 2010
Pleurotus djamor	Artificial	*Hordeum vulgare*	López Llorca et al., 2006
Pochonia chlamydosporia	Artificial	*Hordeum vulgare*, *Solanum lycopersicum*, *Schizachne purpurascens*	Bordallo et al., 2002 Behie et al., 2015
Purpureocillium lilacinum	Artificial	*Gossypium hirsutum*	Castillo-Lopez et al., 2014; Castillo-Lopez y Sword, 2015
Tolypocladium cylindrosporum	Natural	*Festuca rubra*, *Holcus lanatus*	Sánchez et al., 2010

Resulta difícil precisar el contexto evolutivo de este carácter endófito asociado a unos hongos especializados en infectar insectos, e incluso si es previo o posterior. Se ha postulado que el género *Metarhizium* pudo haber evolucionado a partir de adaptaciones ecológicas en el área de la rizosfera que le permitieron colonizar las raíces de las plantas y compartir hasta un 16% de identidad de su transcriptoma con otros hongos endófitos de plantas (Vega et al., 2009; Gao et al., 2011). El suelo se considera el reservorio natural de los HE y por tanto parte de dicha adaptación a endófitos pudo realizarse en el, sin embargo los HE también han sido aislados en hábitats muy diversos como el filoplano de varias especies de cultivos y flora arvense

(Meyling et al., 2011; Sasan y Bidochka, 2012; Garrido-Jurado et al., 2015). No en vano, es precisamente en la parte aérea de las plantas (hojas y tallos) donde se encuentran con mayor frecuencia los HE endófitos (Posada et al., 2007; Vega et al., 2009).

La colonización de la planta por parte de los HE podría ocurrir a partir de los conidios depositados en el filoplano y en otros casos del suelo a través de las raíces. De hecho, tanto *Beauveria* como *Metarhizium* forman asociaciones íntimas con una gran variedad de plantas y han demostrado ser capaces de movilizar nitrógeno y de recibir carbono (carbohidratos) de las plantas hospedantes (Behie et al., 2012; 2013; Sasan y Bidochka, 2012).

2.3.2.1 Aplicación de hongos entomopatógenos endófitos

Las técnicas utilizadas para la inoculación artificial de plantas son la pulverización foliar, inyección del tronco, adición al suelo o tratamiento de semillas, pero todas ellas presentan diferencias que determinarán el éxito del tratamiento, entendido como la ocurrencia o no de la colonización sistémica y su extensión (Tefera and Vidal, 2009).

La penetración de los HE en los tejidos de las plantas se realiza a través de los estomas o por medio de la penetración directa mediante la actuación de la enzima MAD2 que permite la adhesión a la planta (Wang and St. Leger, 2007). Posteriormente, el movimiento se produce en los espacios intercelulares siguiendo la vía del apoplasto (Landa et al., 2013).

En comparación con los insecticidas convencionales, el uso de los HE como endófitos tiene la ventaja de que son necesarias menores cantidades de inóculo, al encontrarse el insecto confinado en el interior. Además, la planta confiere protección al inóculo frente a factores abióticos y bióticos que limitarían su uso en condiciones de campo (Akello et al, 2008; Backman y Sikora, 2008). La mayoría de estos estudios sólo han completado la primera etapa, es decir, la introducción en la planta, aunque en algunos de ellos se ha

indicado además, que la colonización endofítica de la planta por hongos entomopatógenos afecta a la supervivencia y al desarrollo de los insectos (Bing y Lewis, 1992; Akello et al, 2008; Quesada-Moraga et al, 2009). Por último, cabe señalar que los HE pueden transmitirse de manera vertical a la descendencia de las plantas colonizadas vía semilla (Quesada-Moraga et al., 2014b).

2.3.2.2 Interacciones tritróficas planta-hongo entomopatógeno endófito-insecto fitófago

Conviene remarcar en este punto que los HE actúan por contacto, invaden a sus hospedantes directamente a través del exoesqueleto o tegumento, tanto en especies provistas de aparato bucal masticador que se alimentan, de manera ectófita o endófita, de los tejidos de las plantas, Orthoptera, Coleoptera, Diptera, Lepidoptera e Hymenoptera, como en aquellas provistas de aparato bucal picador suctor y que extraen los fluidos (fitomizos), Thysanoptera y Hemiptera (Dolinski y Lacey, 2007; Quesada-Moraga y Santiago-Álvarez, 2008).

El descubrimiento de la capacidad endofítica de los hongos entomopatógenos ha permitido la protección sistémica de las plantas frente a insectos crípticos barrenadores, un hito en la historia de la patología de invertebrados. A este respecto permanecen inéditos los efectos directos e indirectos que las plantas y los insectos fitófagos pueden ejercer sobre los HE endófitos (Figura 4), aunque trabajos realizados con otros hongos endófitos revelan efectos promotores e inhibidores tanto de las sustancias aleloquímicas como de las estructura de los tejidos vegetales en la parte epigea de la planta (Brown et al., 1995; Inyang et al., 1998; Baverstock et al., 2005; Hountondji et al., 2005; Cory y Ericsson, 2010), y también en la hipogea en la rizosfera, donde los HE con competencia pueden tener efectos opuestos atrayentes y repelentes de insectos geobiontes (Kepler y Bruck,

2006; St. Leger, 2008).

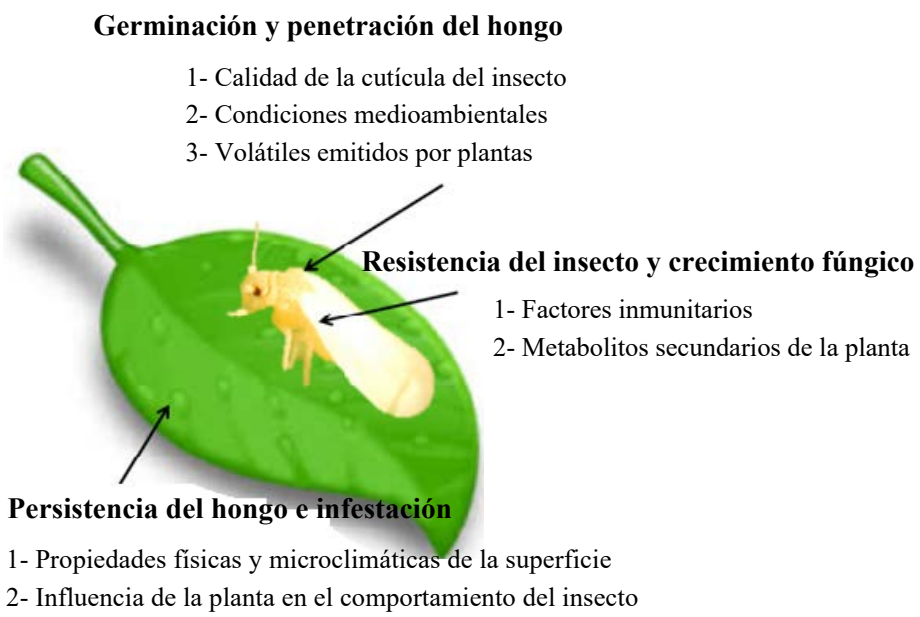

Figura 4. Efectos directos e indirectos mediados por la planta en la relación del hongo con el insecto.

Pero sin duda, uno de los aspectos más fascinantes aun no esclarecido es la posible ocurrencia de un proceso patogénico cuando un insecto masticador o picador-chupador se alimenta de una planta endofíticamente colonizada por un hongo entomopatógeno, situaciones ambas donde el inóculo debería entrar en contacto con el tegumento por una ruta inusual, la del tubo digestivo. Además, resulta importante dilucidar si existe algún grado de colonización endofítica transitoria en tratamientos por pulverización realizados para el control de insectos masticadores y chupadores, así como el efecto de la misma sobre la eficacia global del tratamiento, aspectos todos recogidos en los objetivos de esta tesis.

Para abordar estos objetivos se han seleccionado dos especies de insectos de gran importancia para la agricultura mediterránea bajo abrigo y en

campo abierto, una masticadora, la "rosquilla negra" *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae), otra picadora-chupadora, la "mosca blanca del tabaco" *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae).

2.4 Los insectos fitófagos

2.4.1 *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae)

Este fitófago se alimenta de más de 87 especies de plantas de 40 familias, ocho de las cuales, Leguminosae, Solanaceae, Malvaceae, Moraceae, Compositae, Gramineae, Chenopodiaceae y Cruciferae, reúnen a más del 50% de sus hospedantes conocidos.

2.4.1.1 Morfología, biología y distribución

El adulto se caracteriza por ser una mariposa de color marrón blanquecino con dos manchas circulares oscuras en la parte central de las alas anteriores y de aproximadamente 30-40 mm de envergadura (Figura 5A). Con temperaturas cálidas la maduración de los adultos se produce inmediatamente después de la emergencia, mientras que si la temperatura baja el periodo de preoviposición abarca de 2 a 11 días (El-Sayes y Kaschef, 1977). Las hembras ovipositan entre 100-300 huevos en masas agrupadas denominadas "plastones", dispuestos normalmente en tres subcapas en el envés de las hojas preferiblemente del tercio superior de la planta (Figura 5B). El huevo mide de 0,5 a 0,6 mm y es de coloración verduzca. La mortalidad de huevos es muy baja y eclosionan de 3-5 días después de la puesta (Amate et al., 2000). Las larvas neonatas se alimentan al principio de parénquima de las hojas hasta completar la primera ecdise (Figura 5C). Estas pasan por 6 estadios larvarios, durante los cuales sigue alimentándose de parénquima, de las nervaduras y limbos de las hojas (Figura 5D-I). Los primeros estadios muestran un hábito de alimentación gregario, mientras que del cuarto en adelante se vuelven solitarios (Alford, 2007). La larva de último

estadio puede llegar a los 45 mm de longitud y adquiere una coloración negruzca. Este es el estado en el que inverna, pues en el suelo se desarrolla lentamente durante el invierno y pupa en primavera. La pupa mide entre 15 y 20 mm de longitud y es de color rojizo, el insecto permanece entre 8 y 10 días en ese estado (Figura 5J-L) (Amate et al., 2000). El tiempo necesario para completar el ciclo varía en función de la temperatura, si bien está comprendido entre 2 y 5 semanas (Salama et al., 1970).

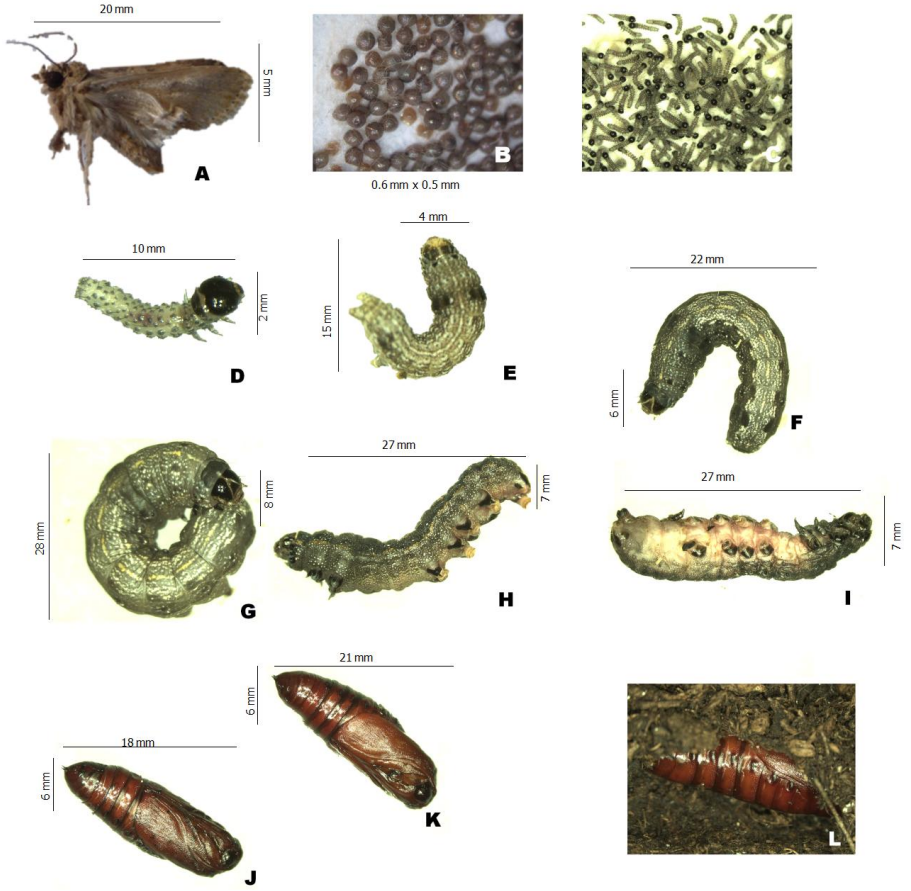

Figura 5. Etapas de desarrollo de *Spodoptera littoralis*. A) Adulto, B) Huevo, C y D) Larvas de primer estadio (L_1), E) Larva de segundo estadio (L_2), F) Larva de tercer estadio (L_3), G) Larva de cuarto estadio (L_4), H) Larva de quinto estadio (L_5), I) Larva de sexto estadio (L_6), J) Pupa hembra, K) Pupa macho y L) Pupa enterrada en el suelo.

Spodoptera littoralis se distribuye por una amplia zona geográfica que incluye parte de Europa, Medio Oriente, África y una parte de Asia Occidental (Figura 6). Origina cuantiosas pérdidas en cultivos tropicales y subtropicales de África, Asia y la Cuenca Mediterránea. En Europa se encuentra ampliamente distribuida por toda España, si bien también aparece en el sur de Portugal, Francia, Grecia e Italia (Alford, 2007; EPPO, 2016).

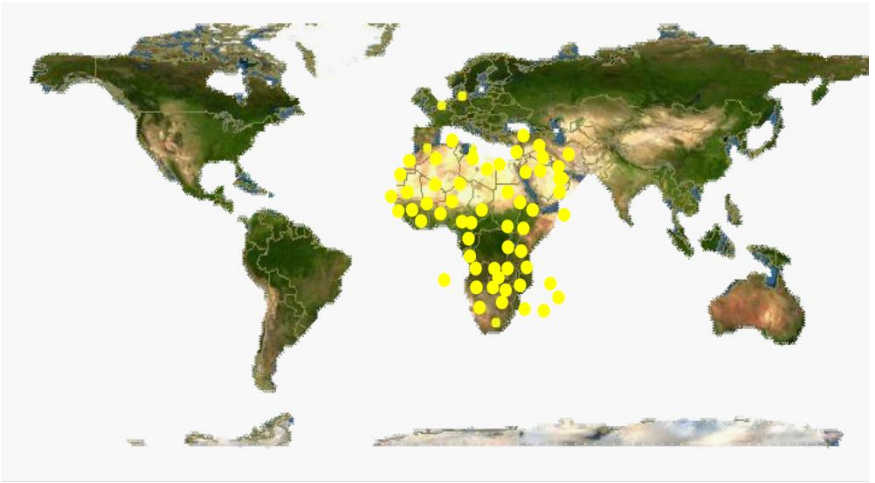

Figura 6. Presencia de *Spodoptera littoralis* en el mundo. Elaboración propia a partir de EPPO (2016).

2.4.1.2 Daños

Los daños causados a las plantas se deben a una alimentación formando ventanas en los primeros estadios y libre en los estadios superiores, lo que origina defoliaciones en los cultivos. Además puede alimentarse de flores, tallos y frutos en desarrollo. Los daños se producen de noche, mientras las larvas permanecen en el suelo durante el día debajo de hojas secas, hierba, etc. (Alford, 2007).

2.4.1.3 Control

El uso de insecticidas químicos sigue siendo la principal forma de control de este fitófago, sin embargo su empleo excesivo en los últimos 30

años ha provocado la aparición de resistencia a muchas de las materias activas registradas (El-Sheikh, 2015). Para superar estos problemas generados con los insecticidas convencionales de amplio espectro, se ha generado una búsqueda exhaustiva de nuevos insecticidas con otro modo de acción, como productos que alteren el desarrollo normal del fitófago (análogos de la hormona juvenil, inhibidores de la síntesis de la quitina…) o derivados de productos naturales (extractos vegetales, de microorganismos…) (Pineda et al., 2006; El-Sayed, 2011; Shalaby et al., 2013). Por otro lado, la bacteria entomopatógena *Bacillus thuringiensis* es muy utilizada, tanto en formulaciones aplicadas sobre el cultivo para el control del fitófago por ingestión como en transgénesis de plantas que expresen las toxinas Cry1A yVip3BR (Gayen et al., 2015; Moussa et al., 2016). Dentro del control microbiano, los baculovirus juegan un papel importante el control de *S. littoralis*, aunque presentan como principales problemas una baja virulencia en larvas de último estadio y sensibilidad a la radiación UV-B (El-Menofy et al., 2014). Todas estas medidas de control pueden ser utilizadas en combinación con trampas lumínicas o con feromonas para el monitoreo de adultos.

2.4.1.3.1 Los hongos entomopatógenos en el control de *Spodoptera littoralis*

Furia virescens (Thaxter) Humber es el único hongo entomophthoraceo que ha causado infección natural en larvas de *S. littoralis*, pero no se ha podido obtener un control satisfactorio del fitófago (Martins et al., 2005). Sin embargo, los ascomicetos mitospóricos entomopatógenos sí que han sido empleados frente a este, incluso algunos formulados comerciales del HE *Isaria fumosorosea* han dado muy buenos resultados aplicados solos o en combinación con insecticidas químicos, extractos de plantas u otros microorganismos (El-Hawary y Abd El-Salam, 2009; Zemek et al., 2012). Además, algunos extractos fúngicos también han mostrado tener

actividad insecticida aplicados *per os* frente a larvas de este fitófago (Quesada-Moraga et al., 2006a).

2.4.2 *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae)

Bemisia tabaci se describió hace más de 100 años, sin embargo fue a partir de la década de los 80 cuando se convirtió en un fitófago de interés mundial tras el desplazamiento de su zona de origen a 54 países (De Barro et al., 2011). Se considera una especie compleja y de gran variabilidad genética, que se ha expresado con la existencia de un complejo de biotipos (De Barro et al., 2005), aunque recientemente se ha sugerido modificar a un complejo de especies (De Barro et al., 2011). Estos biotipos se diferencian en base a características bioquímicas o polimorfismos moleculares y difieren en su rango de hospedantes, en la capacidad para causar daños en la planta, atracción por enemigos naturales, expresión de resistencia y capacidad para transmitir virus fitopatógenos (Perring, 2001; Horowitz et al., 2005).

2.4.2.1 Morfología, distribución y biología

El adulto, de 1-2 mm de longitud, es de color amarillo con el cuerpo y alas revestidos de una cera pulverulenta y su periodo de vida puede llegar hasta 5 semanas. Una sola hembra puede poner entre 200 y 400 huevos durante toda su vida. Los huevos, amarillos-verdosos, son colocados en el envés de las hojas, por un pedúnculo corto. Al eclosionar estos, las ninfas recién emergidas pasan a succionar la savia de las hojas (Suarez-Gonzalez et al., 2015). En la fase inicial, las ninfas N_1 son móviles y pueden desplazarse unos 1 a 2 mm en la hoja, mientras que el resto de estadios ninfales, N_2, N_3 y N_4, permanecen inmóviles en la superficie del envés (Figura 7). En ocasiones hay un siguiente estadio ninfal que presenta un color amarillo-rojizo es denominado pupa y de él emerge el adulto en una semana aproximadamente (Baig et al., 2015).

B. tabaci se encuentra ampliamente distribuida en regiones tropicales, subtropicales, y zonas templadas (Figura 8) (Cheek y Macdonald, 1994).

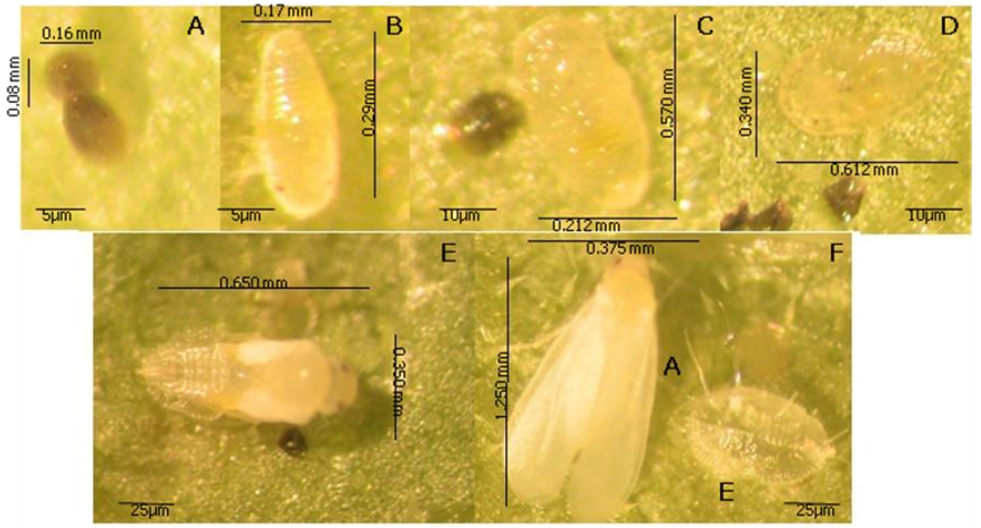

Figura 7. Etapas de desarrollo de *Bemisia tabaci*. A) Huevo (H), B) Ninfa de primer estadio (N_1), C) Ninfa de segundo estadio (N_2), D) Ninfa de tercer estadio (N_3), E) Pupa (N_4), F) Adulto (A), Exuvio (E).

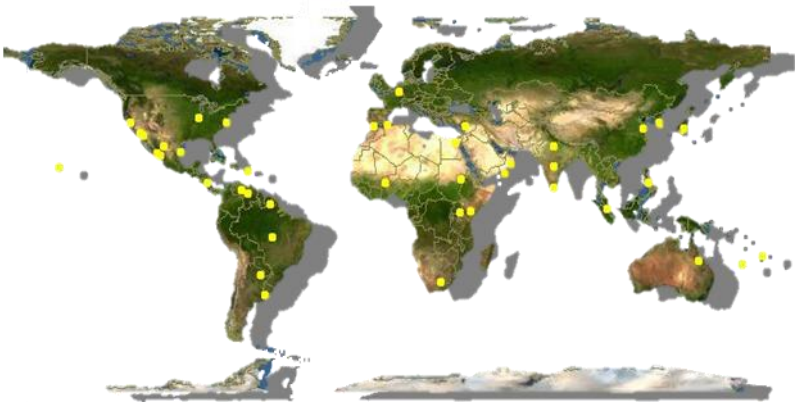

Figura 8. Presencia de *Bemisia tabaci* en el mundo. Elaboración propia a partir de EPPO (2016).

2.4.2.2 Daños

B. tabaci es un fitófago de gran importancia económica por la magnitud de los daños causados y el difícil control de la misma debido a su amplia gama de hospedantes, más de 600, y breve tiempo entre generaciones debido principalmente a que afecta cultivos de invernadero (Oliveira et al.,

2001; Global Invasive Species Database, 2016). Un adulto de mosca blanca puede consumir en un día seis veces su peso y un inmaduro ocho veces, debido a su metabolismo y a la constante excreción de líquido (Suarez-Gonzalez et al., 2015). Además, es un importante vector en la transmisión de virus, más de 150 virus, especialmente begomovirus como es el caso del *Tomato Yellow Leaf Curling Virus* (TYLCV), y puede causar pérdidas económicas de hasta 100% en cultivos de solanáceas en muchas regiones tropicales y subtropicales (Pico et al., 1996; Gomez et al., 2012; Shadmany et al., 2013).

2.4.2.3 Control

La aplicación foliar de insecticidas químicos ha sido la principal herramienta de control utilizada frente a moscas blancas, sin embargo las características morfológicas y ecológicas de estos insectos han impedido un control satisfactorio de los mismos, al estar localizados en el envés de las hojas y en las partes inferiores de la planta, además de tener su tegumento recubierto de sustancias cerosas que impide o limita la penetración del insecticida (Horowitz e Ishaaya, 1996). El uso intensivo de estos productos ha provocado el desarrollo de poblaciones resistentes a insecticidas tan importantes como los neonicotinoides y algunos reguladores del crecimiento. Para evitar la selección de biotipos resistentes se recomiendan frecuentes cambios de ingredientes activos en las aplicaciones. Sin embargo, el control con insecticidas no solo se ha visto difícultado por la aparición de resistencia, sino también por los efectos adversos que estos manifiestan para el medio ambiente y la salud pública. Esto ha estimulado estudios sobre estrategias para el control integrado de las plagas de estos insectos en las cuales el control biológico juega un importante papel (Horowitz e Ishaaya, 1996).

Probablemente, el empleo de enemigos naturales sea el método más efectivo para controlar *B. tabaci* (Horowitz et al., 2011). Se han descrito

varias especies de himenópteros parasitoides y de coccinélidos depredadores entre las 123 especies de insectos capaces de controlar a este fitófago, lista que continúa en aumento en los últimos años. Sin embargo, en su uso también hay que tener en cuenta la preferencia del enemigo natural por la mosca blanca frente a la planta hospedante, pues hay varias especies de hemípteros zoofitófagos que se comercializan para el control *B. tabaci* como son *Macrolophus caliginosus* en el norte de Europa y *Nesidiocoris tenuis* en la cuenca mediterránea (Horowitz et al., 2011). Las principales especies de parasitoides recogidas en varias partes del mundo para el control de estas mosca blancas son *Encarsia lutea, E. formosa, E. inaron*, *E. partenopea, Eretmocerus mundus* y *Er. eremicus*.

Debido a su modo de alimentación (succión directa de la savia) las moscas blancas no son susceptibles a muchos patógenos de insectos, es decir los virus y las bacterias, que normalmente son adquiridos por ingestión del tejido foliar del hospedante contaminado. Por el contrario los hongos entomopatógenos, infectan a sus hospedantes por penetración directa a través tegumento sin necesidad de ser ingeridos.

2.4.2.3.1 Los hongos entomopatógenos en el control de *Bemisia tabaci*

B. tabaci es muy susceptible a la mayoría de los HE descritos para el control de moscas blancas en general. Entre los géneros utilizados se encuentran *Beauveria*, *Isaria* y *Lecanicillium*, algunos de ellos base de productos comerciales autorizados para su control (Tabla 3) (Horowitz et al., 2011). En el control microbiano de moscas blancas mediante hongos entomopatógenos pueden utilizarse dos estrategias básicas. La primera es la técnica inoculativa, donde se procura establecer gradualmente el patógeno en el área para potenciar el control biológico natural. Estas introducciones inoculativas son de gran éxito en los cultivos perennes ya que las

condiciones favorecen a la supervivencia de enemigos naturales de los insectos fitófagos. La segunda es la técnica inundativa, se basa en la utilización masiva del entomopatógeno, que tiene por finalidad la reducción inmediata de la población del insecto fitófago. Estas estrategias son definidas por una serie de factores, que incluyen el método de introducción del patógeno, el desarrollo poblacional del insecto, características del patógeno y el nivel de daño provocado por el insecto. Esta técnica está recomendada en cultivos de ciclo corto como en el caso de las hortalizas, donde se hace difícil el establecimiento y desarrollo de las poblaciones de enemigos naturales en niveles deseados (Gerling, 1990)..

Tabla 3. Productos comerciales para el control de moscas blancas en el mundo. Modificado de Stansly y Natwick (2011).

Especie fúngica	Producto	Empresa
Beauveria bassiana	BotaniGard	Futureco Bioscience, S.A.U.
	Naturalis-L	Agrichem, S.A.
	Bassi WP	Comercial Quimica Massó, S.A.
Isaria fumosorosea	Agro Biocontrol *Beauveria*	Agro Biocontrol
	Bea-Sin	Agrobiologicos de Noroeste S.A. de C.V.
	PFR-97	Certis
	PreFerRal	Biobest N.V.
Lecanicillium lecanii	Pae-Sin	Agrobiológicos de Noroeste S.A. de C.V.
	Agro Biocontrol *Verticillium*	Agro Biocontrol
	Mycotal	Koppert Biological Systems

Los hongos entomopatógenos como agentes de biocontrol para moscas blancas se han utilizado en todos los estados del desarrollo de estos insectos. Se observa una baja tasa de infección en los huevos y adultos tratados con la gran mayoría de las especies fúngicas, si bien los estadios ninfales son altamente susceptibles. Los géneros *Beauveria* e *Isaria* son los que han mostrado, en general, mayor eficacia en control de moscas blancas tanto en condiciones de campo como en invernadero, si bien la planta hospedante es determinante en dicha eficacia (Huang et al., 2010; Quesada-Moraga et al., 2006b; Santiago-Alvarez et al., 2006). Esta eficacia se ha visto mejorada mediante la combinación de estos hongos con otros enemigos naturales como *I. fumosorosea* y *E. formosa,* cuya aplicación conjunta ha producido el 90%

de mortalidad sin el empleo de insecticidas químicos (Avery et al., 2008). Por tanto, se hace necesaria la búsqueda de nuevas cepas que produzcan mortalidades superiores en huevos y adultos de mosca blanca, o bien indagar en las posibles combinaciones con otros métodos de control.

3. OBJETIVOS DE LA TESIS

3.1. Evaluar la actividad insecticida del extracto crudo de varias cepas de hongos entomopatógenos y su compatibilidad en tratamientos simultáneos con los conidios de las mismas.

3.2. Estudiar la posible existencia y extensión de colonización endofítica en tratamientos por pulverización sobre alfalfa, tomate y melón para el control de un insecto masticador *Spodoptera littoralis* y en su caso, el efecto de esta colonización sobre el noctuido y su contribución a la eficacia global del tratamiento.

3.3. Estudiar la posible existencia y extensión de colonización endofítica en tratamientos por pulverización sobre melón para el control de un insecto picador-chupador, *Bemisia tabaci*, y en su caso, el efecto de esta colonización sobre él y su contribución a la eficacia global del tratamiento.

El objetivo 3.1. se corresponde con el manuscritoResquín-Romero, G., Garrido-Jurado, I., Quesada-Moraga, E. 2016. Combined use of entomopathogenic fungi and their extracts improves the control of Spodopteralittoralis (Boisduval) (Lepidoptera:Noctuidae). BIOLOGICAL CONTROL 92: 101-110. Biological Control es una revista Q2 del área de Entomología.

El objetivo 3.2. se corresponde con el manuscrito Resquín-Romero, G., Garrido-Jurado, I., Delso, C., Ríos-Moreno, A., Quesada-Moraga, E. 2016. Transient endophytic colonizations of plants improve the outcome of foliar applications of mycoinsecticides against chewing insects. JOURNAL OF INVERTEBRATE PATHOLOGY. 136: 23-31. La revista Journal of InvertebratePathology es una revista Q1 del área de Zoología.

El objetivo 3.3. se corresponde con el manuscrito Garrido-Jurado, I., Resquín-Romero, G., Amarilla, S.P., Ríos-Moreno, A., Carrasco, L., Quesada-Moraga, E. 2016. Unravelling the effects of temporally colonization of plants after foliar application of mycoinsecticides for the control of sucking insects. JOURNAL OF PEST SCIENCE (Aceptado). Journal of Pest Science es una revista Q1 del área de Entomología.

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CAPÍTULO II

Este capítulo es una versión adaptada del artículo: Combined use of entomopathogenic fungi and their extracts for the control of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae), que se encuentra publicado en Biological Control en el quartil Q2 del área Entomología (posición 25 de 92) del JCR

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Combined use of entomopathogenic fungi and their extracts for the control of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae)

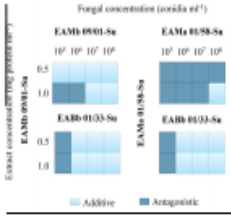
G. Resquín-Romero [a,b], I. Garrido-Jurado [a], E. Quesada-Moraga [a,*]

[a] Department of Agricultural and Forestry Sciences, ETSIAM, University of Cordoba, Campus de Rabanales, Edificio C4 Celestino Mutis, 14071 Cordoba, Spain
[b] School of Agrarian Sciences of the National University of Asunción, Paraguay

HIGHLIGHTS

- Isolates of *Beauveria* and *Metarhizium* genera were pathogenic against *S. littoralis* larvae.
- The most of their crude extracts were toxic for *S. littoralis* larvae.
- Spray application of crude extracts did not cause mortality on *S. littoralis* larvae.
- Combined application of EF and their extracts obtained additive effect at high doses.

GRAPHICAL ABSTRACT



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ABSTRACT

Both the virulence and insecticidal activities of the crude extracts of 26 isolates of the entomopathogenic mitosporic ascomycetes *Metarhizium* sp. and *Beauveria* sp. (Ascomycota, Hypocreales) were evaluated against the second-instar larvae of *Spodoptera littoralis* (Boisduval) (Lepidoptera, Noctuidae), a very harmful polyphagous insect pest. Although all isolates caused infection when the second-instar *S. littoralis* larvae were immersed in the conidial suspensions, only four isolates of *Beauveria* (EABb 01/33-Su, EABb 01/88-Su, EABb 01/103-Su, and 3155) and one isolate of *Metarhizium* caused >50% mortality of the larvae, with average survival times (ASTs) of 9.67 and 8.73 days for the isolates EABb 01/33-Su and EABb 01/88-Su, which caused the highest mortality rates of 78.33% and 75.00%, respectively. The LC_{50} and LT_{50} values were 5.69×10^6 conidia ml^{-1} and 6.76 days for EABb 01/33-Su and 1.05×10^7 conidia ml^{-1} and 7.02 days for EABb 01/88-Su. By contrast, the crude extracts obtained from the *Metarhizium brunneum* EAMb 09/01-Su and EAMa 01/58-Su isolates caused the highest mortality rates of 80.00% and 66.66% and the lowest ASTs of 5.13 and 4.43 days, respectively. Topical application of the crude extracts did not cause mortality. Combined treatments with fungal suspensions of the isolates EAMb 09/01-Su and EAMa 01/58-Su and their extracts caused higher mortality rates than the single isolates and extracts, with the increases occurring in a dose-dependent manner, and with mortality rates reaching 100% for the EAMb 09/01-Su isolate and its extract at 1 mg ml^{-1} and 76% for the EAMa 01/58-Su isolate and its extract at 1 mg ml^{-1}. The combination of the maximal concentrations of both fungi and crude extract had an additive effect on larvae, resulting in 100% mortality for the combination of the extract EAMb 09/01-Su with the strains EABb 01/33-Su and EAMb 09/01-Su. These results show the potential of certain entomopathogenic fungal isolates for use in an integrated *S. littoralis* management strategy targeting larvae, as well as the potential of the combined use of entomopathogenic fungi and their extracts.

Combined use of entomopathogenic fungi and their extracts for the control of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae)

Resquín-Romero, G.[a,b], Garrido-Jurado, I.[a], Quesada-Moraga, E.[a]

[a] Department of Agricultural and Forestry Sciences, ETSIAM, University of Cordoba, Campus de Rabanales, Edificio C4 Celestino Mutis, 14071 Cordoba, Spain

[b] School of Agrarian Sciences of the National University of Asunción, Paraguay

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Both the virulence and insecticidal activities of the crude extracts of 26 isolates of the entomopathogenic mitosporic ascomycetes *Metarhizium* sp. and *Beauveria* sp. (Ascomycota, Hypocreales) were evaluated against the second-instar larvae of *Spodoptera littoralis* (Boisduval) (Lepidoptera, Noctuidae), a very harmful polyphagous insect pest. Although all isolates caused infection when the second-instar *S. littoralis* larvae were immersed in the conidial suspensions, only four isolates of *Beauveria* (EABb 01/33-Su, EABb 01/88-Su, EABb 01/103-Su, and 3155) and one isolate of *Metarhizium* caused >50% mortality of the larvae, with average survival times (ASTs) of 9.67 and 8.73 days for the isolates EABb 01/33-Su and EABb 01/88- Su, which caused the highest mortality rates of 78.33% and 75.00%, respectively. The LC_{50} and LT_{50} values were $5.69x10^6$ conidia ml^{-1} and 6.76 days for EABb 01/33-Su and $1.05x10^7$ conidia ml^{-1} and 7.02 days for EABb 01/88-Su. By contrast, the crude extracts obtained from the *Metarhizium brunneum* EAMb 09/01-Su and EAMa 01/58-Su isolates caused the highest mortality rates of 80.00% and 66.66% and the lowest ASTs of 5.13 and 4.43 days, respectively. Topical application of the crude extracts did not cause mortality. Combined treatments with fungal suspensions of the isolates EAMb 09/01-Su and EAMa 01/58-Su and their extracts caused higher mortality rates than the single isolates and extracts, with the increases occurring in a dose-dependent manner, and with mortality rates reaching 100% for the EAMb 09/01-Su isolate and its extract at 1mg ml^{-1} and 76% for the EAMa 01/58-Su isolate and its extract at 1mg ml^{-1}. The combination of the maximal concentrations of both fungi and crude extract had an additive effect on larvae, resulting in 100% mortality for the combination of the extract EAMb 09/01-Su with the strains EABb 01/33-Su and EAMb 09/01-Su. These results show the potential of certain entomopathogenic fungal isolates for use in an integrated *S. littoralis* management strategy targeting larvae, as well as the potential of the combined use of entomopathogenic fungi and their extracts.

1. INTRODUCTION

Lepidopteran pests cause billions of dollars in crop loss worldwide each year, making them one of the most dangerous classes of agricultural pests (Fan et al., 2012). The cotton leafworm *Spodoptera littoralis* (Boisduval) (Lepidoptera, Noctuidae) is one of the most destructive phytophagous pests because it can attack numerous economically important crops in either protected or open fields in the Mediterranean region and the Middle East, particularly in Cyprus, Israel, Malta, Morocco, Italy, Greece, and Spain (Hatem et al., 2009; Azab et al., 2001; Ahmad, 1988; Blackford et al., 1997; Champion et al., 1997; Salama et al., 1971). The life cycle of this insect can be completed in approximately 5 weeks. Females lay egg masses (100-300 eggs each) on the lower surfaces of leaves of host plants. After hatching, larvae feed in groups at night and in the early morning, and they pass through six instars before pupating in soil (Amate et al., 2000; Salama et al., 1971). *Spodoptera littoralis* attacks most vegetable crops, including tomato, pepper, eggplant, lettuce, artichoke, strawberry and asparagus, but it also damages ornamental plants and herbs (Lanzoni et al., 2012).

The overuse and reliance on chemical insecticides for controlling *S. littoralis* has resulted in the emergence of insecticide resistance and cross resistance (Ghribi et al., 2012). In addition, *Bacillus thuringiensis*-resistant field populations have been detected in several countries (Assaeedi et al., 2011). Therefore, the development of new, environmentally safe and sustainable alternatives has become an urgent need for integrated control programs for the cotton leafworm, with emphasis on biological control through entomophagous predators, parasitoids, and microorganisms.

Entomopathogenic fungi (EF), in particular entomopathogenic mitosporic ascomycetes, have been shown to successfully control a large variety of lepidopteran pests (Quesada-Moraga et al., 2013; Schulte et al., 2009; Devi et al., 2005; Vänninen and Hokkanen, 1997). Their unique tegumentary mode of action and horizontal transmissibility have been used for the development of alternative control strategies in recent years (Quesada-Moraga and Santiago-Álvarez, 2008; Quesada-Moraga et al., 2006; Wraight et al., 2000). Moreover, EF have been shown to contribute not only as controllers of insect populations but also as producers of novel insecticidal compounds (Isaka et al., 2005). These insecticidal compounds can be either highmolecular weight proteins or low-molecular-weight secondary metabolites, both showing high mortality and antifeedant properties against lepidopteran insects (Amiri et al., 1999; Quesada-Moraga et al., 2006).

The combined use of EF and synthetic insecticides or natural compounds has been proposed as a strategy to improve their efficacy (Hu et al., 2007) by reducing the lethal times (Sharififard et al., 2011) or enhancing the virulence of the fungi (Zurek et al., 2002). To date, the interactions between EF and chemical insecticides or even fungal compounds have been addressed for lepidopteran pest control (Ribeiro et al., 2012; Cazorla and Morales Moreno, 2010; Hu et al., 2007; Devi et al., 2004). However, the effect of combining treatments of EF and of their own extracts has not previously been tested for lepidopteran pest control. The aim of this study was to evaluate the biological activities of 26 isolates from different species of EF and the insecticidal activities of their crude extracts and to evaluate combined treatments of EF and their crude extracts against *S. littoralis* larvae, supported by the findings of previous studies of pathogens and toxins demonstrating positive results against Lepidopteran pests (Lastra et al., 1995).

2. MATERIALS AND METHODS

2.1. *Spodoptera littoralis* (Boisduval) (Noctuidae: Lepidoptera) rearing

S. littoralis larvae used in this study were obtained from a stock colony at the Department of Agricultural and Forestry Sciences of the University of Córdoba (Spain). These insects were originally collected in the field from various crops. They were maintained in an environmental chamber programmed at 26 ± 2 ºC, 70 ± 5% RH, and photoperiod of 16:8 (L:D) h (Poitout and Bues, 1974). Adults were placed in oviposition chambers consisting of a cylindrical filter paper (150x120 mm) closed at both ends with a layer of filter paper. Cotton moistened with a 10% honey solution was placed inside, in a small container. This cotton was replaced every two days to prevent possible contamination and fermentation. Each chamber contained 5 pupae of each sex. The chambers were observed daily to monitor and collect the egg clusters, which were externally disinfected by immersion in 10% formalin for 10 min and then washed three times with sterile water to remove any formalin residues. The disinfected egg clusters were then placed on pieces of filter paper to remove water. Filter papers containing 12 or 15 egg clusters were placed at the bases of plastic containers (6×10^6 mm^3) with perforated covers. Approximately 2400 to 3000 eggs were placed in each container. The larvae were reared until pupation on an artificial diet consisting of alfalfa meal (85 g), brewer's yeast (34 g), wheat germ (32 g), agar-agar (18 g), casein (14 g), ascorbic acid (4.5 g), benzoic acid (1.3 g), nipagin (1.1 g), 10% formaldehyde (5 ml) and distilled water (800 ml) (Santiago-Álvarez, 1977). The pupae were removed and separated by sex using a stereomicroscope (Nikon SMZ800, Japan), after which they were classified by size and vigour and transferred to oviposition chambers under the insectary conditions described above.

2.2. Fungal isolates and preparation

The 26 fungal isolates used in this study were obtained from the culture collection of the Department of Agricultural and Forestry Sciences (AFS) of the University of Córdoba (Table A1). They were selected based on various criteria, including fungal species (*Beauveria bassiana*, *B. pseudobassiana*, *Metarhizium anisopliae*, *M. brunneum*, *M. robertsii*) and habitat of isolation (soil, plant, and insect). All of the isolates were obtained from monosporic cultures and maintained lyophilised at -80 ºC. To prepare inoculums for the experiments, slant cultures of the lyophilised isolates were sub-cultured on malt agar for 15 d at 25 ºC in darkness. The Petri plates were sealed with Parafilm® (Pechiney Plastic Packaging Co., Chicago, IL). Conidial suspensions were prepared by scraping conidia from the Petri plates into a sterile aqueous solution of 0.1% Tween 80. These initial suspensions were sonicated for 5 min and then filtered through several layers of cheesecloth to remove mycelial structures.

The germination rates were evaluated in Petri plates with water agar as the substrate at 25 ºC and 12 h after inoculation, with consistent rates of above 90% (Goettel and Inglis, 1997). The conidial suspensions used for the bioassays were adjusted by diluting the conidia with 0.1% Tween 80 to a final concentration of $1.0x10^8$ conidia ml^{-1}. The number of conidia was estimated using a Malassez chamber.

Table A1. Identity of the fungal isolates from the culture collection at AFS, Department of the University of Cordoba assayed against *Spodoptera littoralis* larvae

Isolates	Fungal species	Origin	Ecology	Habitat
EABb 01/88-Su	*B. bassiana*	Vila Velha de Ficalho (Portugal)	Sunflower crop	Soil
EABb 01/103-Su	*B. bassiana*	Constantina (Sevilla)	Holm oak forest	Soil
EABb 09/51-Su	*B. brongniartii*	Castilblanco de los Arroyos (Sevilla)	Holm oak dehesa	Soil
EABb 09/16-Su	*B. pseudobassiana*	Bobadilla (Málaga)	Organic olive orchard	Soil
EABb 09/18-Su	*B. pseudobassiana*	Bobadilla (Málaga)	Organic olive orchard	Soil
EAMr 09/01-Su	*M. robertsii*	Castilblanco de los Arroyos (Sevilla)	Holm oak dehesa	Soil
EAMa 01/58-Su	*M.brunneum*	Hinojosa del Duque (Córdoba)	Wheat crop	Soil
EAMa 05/03-Su	*M. anisopliae*	Posadas (Córdoba)	Traditional olive orchard	Soil
EAMa 09/15-Su	*M. anisopliae*	Bobadilla (Málaga)	Organic olive orchard	Soil
EAMb 09/01-Su	*M. brunneum*	Castilblanco de los Arroyos (Sevilla)	Holm oak dehesa	Soil
EAMb 09/02-Su	*M. brunneum*	Castilblanco de los Arroyos (Sevilla)	Holm oak reforestation	Soil
EAMb 09/03-Su	*M. brunneum*	Castilblanco de los Arroyos (Sevilla)	Holm oak dehesa	Soil
EABb 01/12-Su	*B. bassiana*	Cerro Perea (Sevilla)	Wheat crop	Soil
EABb 01/33-Su	*B. bassiana*	El Bosque (Cádiz)	Traditional olive orchard	Soil
EABb 07/06-Rf	*B. bassiana*	Catral (Alicante)	*Rhynchophorus ferrugineus*	Insect
EABb 04/01-Tip	*B. bassiana*	Ecija (Sevilla)	*Iraella luteipes*	Insect
1764	*B.bassiana*	Fuente Roldán (Salamanca)	*Dactylis glomerata* of sulfur source	Plant
2773	*B. bassiana*	El Cabaco (Salamanca)	*Dactylis glomerata* of Quercus forest	Plant
1923	*B. bassiana*	Esteiro (Coruña)	*Ammophila arenaria* of coastal dune	Plant
3158	*B.bassiana*	Esteiro (Coruña)	*Ammophila arenaria* of coastal dune	Plant
183	*B.bassiana*	Puerto Honduras (Cáceres)	*Holcus lanatus* of chestnut forest	Plant
1222	*B.bassiana*	Casas del Conde (Salamanca)	*Cynosurus echinatu* Quercus forest	Plant
3155	*B.bassiana*	Puerto Honduras (Cáceres)	*Holcus lanatus* of chestnut forest	Plant
3154	*B.bassiana*	Puerto Honduras (Cáceres)	*Holcus lanatus* of chestnut forest	Plant
3111	*B. bassiana*	Vilarube (Coruña)	*Elymus farctus* of coastal dune	Plant
EABb 09/07-Fil	*B. bassiana*	Castilblanco de los Arroyos (Sevilla)	Holm oak dehesa	Plant

2.3. Initial pathogenicity assay for entomopathogenic fungal isolates against *Spodoptera littoralis* larvae

Second-instar (L_2) *S. littoralis* larvae were treated with the 26 isolates. The larvae were immersed in 5 ml of conidial suspension for 1 min, and then each larva was individually placed in a bioassay cage (28x13 mm). Each larva was fed with an alfalfa disc. The control larvae were immersed in sterile

water containing 0.1% Tween 80. Larval mortality was recorded every two days for 18 days. Each treatment was replicated three times, with 20 L_2 larvae per replicate. The bioassay was conducted at 26 ± 2 ºC, 70 ± 5% RH, and a photoperiod of 16:8 (L:D) h. The dead larvae were immediately surface sterilised with 1% sodium hypochlorite, followed by three rinses with sterile distilled water. They were then placed on sterile wet filter paper in Petri plates sealed with Parafilm® and kept at room temperature (25 ± 2 ºC). After five days, fungal outgrowth on the surface of the insect cuticle was observed using a light microscope (Nikon, Japan). The entire experiment was performed twice, using independent batches of *S. littoralis* larvae and fungal inoculums each time. The bioassay was incubated under a photoperiod of 16:8 h (L:D) with 50-60% RH and a temperature of 26 ± 2 ºC. Mortality data (total mortality, mortality with fungal outgrowth and mortality due to other causes) were analysed using one-way ANOVA and orthogonal contrast to compare means using Statistix 9.0 (Analytical software, 2008) and JMP 8.0 (SAS Institute Inc., 2008). Six orthogonal hierarchical contrasts were performed, comparing (1) control vs. all treatments, (2) *Beauveria* vs. *Metarhizium* genera, (3) *B. bassiana* vs. *B. pseudobassiana* and *Beauveria brongniartii*, (4) *B. pseudobassiana* vs. *B. brongniartii*, (5) *M. brunneum* vs. *M. robertsii* and *M. anisopliae*, and (6) *M. robertsii* vs. *M. anisopliae*. Data were transformed using arcsine transformation. The cumulative mortality response across the assessment period and the average survival times (ASTs) were analysed with the Kaplan-Meier survival test using SPSS 19.0 for Windows (IBM Company, 2010).

2.4. Virulence assay of entomopathogenic fungal isolates against *Spodoptera littoralis* larvae

Based on the abovementioned initial bioassay, the two *B. bassiana* isolates that produced the highest mortality rates (EABb 01/33-Su and EABb 01/88-Su) and the two *M. brunneum* isolates (EAMa 01/58-Su and EAMb

09/01-Su) that showed high mortality by unknown causes were selected for evaluation of their virulence against L_2 *S. littoralis* larvae because high mortality due to unknown causes can indicate that the fungi produce toxic compounds. Groups of larvae were directly immersed in 5 ml of the different conidial suspensions for 1 min. Five concentrations of conidia were used, including $1.0x10^5$, $1.0x10^6$, $1.0x10^7$, $1.0x10^8$, and $1.0x10^9$ conidia ml^{-1}, and sterile 0.1% Tween 80 aqueous solution was used as a negative control. Three pseudo-replicates with 10 larvae each were used for each concentration, and the whole experiment was repeated twice with different fungal inoculums. Larval mortality was recorded every two days for 18 days. Dead larvae were processed as described above for inspection for fungal outgrowth, and the plates were maintained under the same conditions as in the previous experiment for five days.

The median lethal concentration (LC_{50}) values were estimated from the infection-confirmed mortality data by probit analysis (Finney, 1971), Probit parallelism test regression lines for all as sayed isolates were generated using χ^2 goodness-of-fit tests. Relative median potencies and their 95% confidence intervals were calculated for the different treatments when their slopes did not differ significantly (Finney, 1971). The estimated time to kill 50% of the insects (LT_{50}) was determined using the PROBIT analysis method for correlated data (Throne et al., 1995). Statistical analyses were performed using SPSS 19.0 for Windows (IBM Company, 2010).

2.5. *Per os* insecticidal activity of crude extracts *against Spodoptera littoralis* larvae

The fungal extracts of the 26 isolates were prepared in Adamek's liquid medium, consisting of 40 g of glucose, 40 g of yeast extract, and 30 g of corn steep liquor per litre of distilled water (Adamek, 1963). A primary culture of this medium inoculated with $1x10^7$ conidia ml^{-1} of each fungal

isolate was cultivated in a rotatory shaker (OVAN Multimix, Badalona, Spain) at 110 rpm for 4 d at 25 ºC (Fuguet and Vey, 2004). For large-scale growth of each fungus, 2 ml of the primary culture was transferred into a 1 L Erlenmeyer flask containing 250 ml of the same medium and cultured in the same manner for 7 days before the mycelia were removed by filtration through Whatman No.3 chromatography filter paper (Whatman, Kent, UK). Then, the solutions were filtered twice, once with a syringe GF-prefilter (5 µm) and once with a 0.45 µm syringe filter (Sartorius, Goettingen, Germany). The soluble protein concentration was determined by the Bradford assay (Bradford, 1976), using bovine serum albumin as a standard (Merck-Schuchardt, Hohenbrunn, Germany). Absorbance at 595 nm was measured to estimate the protein concentrations in the crude extracts. For the bioassays, these crude extracts were adjusted to 1 mg of protein ml^{-1}. Finally, the crude extracts were stored at -20 ºC until use. *Per os* toxicity of the crude soluble extracts secreted by the 26 fungal isolates was tested using alfalfa leaf disc tests. Newly molted second-instar larvae were individually placed in plastic cups (30 mm diameter) and fed on alfalfa leaf discs (5 mm diameter). Each disc was treated with 10 µl of crude extract at 1 mg protein ml^{-1}. The controls were treated with 10 µl of desalted Adamek's liquid medium. For both the treated and control insects, the alfalfa leaf discs were replaced daily for 5 days. Three pseudoreplicates of 30 larvae each were used for each treatment and for the control. The larvae were kept at room temperature (25±2 ºC) under a photoperiod of 16:8 h (L:D) at 50-60% RH during the experiment.

Mortality data were analysed using one-way ANOVA and orthogonal contrast to compare means using Statistix 9.0 (Analytical software, 2008) and JMP 8.0 (SAS Institute Inc., 2008). Six orthogonal hierarchical contrasts were performed: (1) control vs. all treatments, (2) *Beauveria* vs. *Metarhizium* genera, (3) *B. bassiana* vs. *B. pseudobassiana* and *B. brongniartii*, (4) *B. pseudobassiana* vs. *B. brongniartii*, (5) *M. brunneum* vs. *M. robertsii* and *M.*

anisopliae, and (6) *M. robertsii* vs. *M. anisopliae*. If the data did not conform to a normal distribution and homogeneity of variance, arcsine transformation was used. The cumulative mortality response across the assessment period and the average survival times (ASTs) were analysed using the Kaplan-Meier survival test in SPSS 19.0 for Windows (IBM Company, 2010).

2.6. Evaluation of the insecticidal activity of spray applications of the crude extracts against *Spodoptera littoralis*

Crude extracts from five fungal isolates (3154, 3155, 3158, EAMa 01/58-Su, EAMb 09/01-Su) were evaluated. L_2 *S. littoralis* larvae were treated with 1 ml of a crude extract adjusted to 1 mg protein ml^{-1}, using a Potter tower (Burkard Rickmansworth Co. Ltd. UK) with a standard tank of 1.54 ± 0.06 mg cm^2 and 0.70 bars of pressure. The controls were treated with the same volume of Adamek's liquid medium. The treated larvae were placed in cages of 28 x 13 mm and were fed with a fresh alfalfa disc every 24 h. The bioassay was incubated under a photoperiod of 16:8 h (L:D) with 50-60% RH and a temperature of 26 ± 2 ºC. Mortality was monitored for 5 days.

Mortality data were analysed using one-way ANOVA, and the LSD test was used to compare means, using Statistix 9.0 (Analytical software, 2008). Data were transformed using arcsine transformation.

2.7. Combined treatments with entomopathogenic fungi and their extracts

For the combined treatment, the isolates EAMb 01/09-Su and EAMa 01/58-Su with their respective crude extracts were evaluated, as well as the isolate EABb 01/33-Su with the crude extracts of both of the above fungi. The isolate EABb 01/33-Su produced higher mortality in the initial experiment, but it did not produce any insecticidal compounds. Therefore, the working assumption is that the combination of this isolate with the better crude extracts could improve the combined effect of the two isolates with

their own extracts. The crude extracts were produced as described in subheading 2.5 and were adjusted to 0.5 and 1 mg protein ml^{-1}, and five concentrations of conidial suspensions were used ($1.0x10^5$, $1.0x10^6$, $1.0x10^7$, $1.0x10^8$, and $1.0\ x10^9$ conidia ml^{-1}) in sterile 0.1% Tween 80 aqueous solution and applied as described in subheading 2.3. Each larva was placed in a 28x13 mm cage and was fed with an alfalfa disc. The application of crude extract consisted of 10 µl placed on the alfalfa disc.

Three replicates of 30 larvae per replicate were used for each treatment (controls, each concentration of fungal suspension, each fungal suspension + crude extract at 0.5 mg protein ml^{-1}, and each fungal suspension + crude extract at 1 mg protein ml^{-1}), and water was used in the controls. The discs were replaced with new, freshly treated alfalfa discs each day for five days. The experiment was conducted under insectarium conditions. Larval mortality was recorded every 24 h for 10 days. Two replications were performed for each treatment and control, and the entire experiment was repeated once more with a new batch of insects, new inoculum and new extracts. The bioassay was conducted under a photoperiod of 16:8 h (L:D) at 50-60% RH and a temperature of 26 ± 2 ºC. The cumulative mortality response across the assessment period and the average survival times (ASTs) were analysed with the Kaplan-Meier survival test using SPSS 19.0 for Windows (IBM Company, 2010). The median lethal concentration (LC_{50}) and median lethal time (LT_{50}) was calculated as in subheading 2.4.

The final mortality data were analysed to determine whether there were synergistic, additive or antagonistic effects between combinations of the fungus *M. brunneum* (EAMb 09/01-Su and EAMa 01/58-Su) and their crude extracts and between the fungus *B. bassiana* (EABb 01/33-Su) and the extracts of both strains of *M. brunneum*.

This analysis was conducted using the χ^2 test (Hernández et al., 2012), and the expected mortality value, M_E, for the combined agents was calculated

using the formula $M_E = M_H + M_{CE} -(M_H*M_{CE}/100)$ (Colby, 1967). M_H represents the observed Abbott (1925) corrected mortality percentage caused by the fungi, whereas M_{CE} represents the Abbott corrected mortality percentage due to the crude extract alone. The results from the χ^2 test were compared to the χ^2 table values for 1 degree of freedom ($\chi^2_{\text{Table value}}$: $p>0.001$, $p>0.05$), using the formula $\chi^2= (M_{HCE} - M_E)^2/M_E$, wherein M_{HCE} is the corrected observed mortality. In the case of $\chi^2_{\text{Calculated}}>\chi^2_{\text{Table value}}$, χ^2 if $M_{HCE} - M_E$ was positive, a significant interaction was considered to be synergistic. However, if $M_{CE} - M_E$ was negative, the interaction was considered to be antagonistic.

2.8. Effect of exposure time on the combined treatments of entomopathogenic fungi and their extracts

The effects of exposure time on the combined treatments of EAMb 09/01-Su and EAMa 01/58-Su isolates of *M. brunneum* and their extracts were studied for 4 exposure times. Three replicates of 10 *S. littoralis* larvae (L_2) each were treated as described above by immersion in 10^8 conidia ml^{-1} suspension and *per os* application of 1 mg protein ml^{-1} of crude extract. Insects were fed for 12, 24, 36, and 48 h, respectively, with a disc (5 mm in diameter) treated with 10 µl of crude extract, and they were then transferred to fresh, untreated leaf discs for the rest of the experiment. Controls were fed in the same manner with culture medium and water. After 24 h, the antifeedant index (AI) was calculated with the following equation: $AI = [(C-T)/(C+T)]\times100$, where C is the leaf area (mm^2) consumed in the control, and T is the leaf area (mm^2) consumed in the treatment, as assessed using Image-Pro Plus 4.5.0.29 software for Windows.

The cumulative mortality response across the assessment period and the average survival times (ASTs) were analysed with the Kaplan-Meier survival test using SPSS 19.0 for Windows (IBM Company, 2010). Digital

images of the alfalfa discs were captured to evaluate the leaf consumption of each larva. The area of each disc was marked using Image Pro-Plus software (Media Cybernetics, Atlanta, GA), and leaf consumption was compared using Tukey's test (HDS) with 5% significance (Mansson and Schlyter, 2004).

3. RESULTS

3.1. Initial pathogenicity assay of entomopathogenic fungal isolates against *Spodoptera littoralis* larvae

The treatments differed significantly from the control, both for mortality with fungal outgrowth ($F_{1,54}$ = 11.67, P = 0.001) and for mortality due to other causes ($F_{1,54}$ = 6.33, P = 0.002). There were no significant differences between the genera *Metarhizium* and *Beauveria* for mortality with fungal outgrowth ($F_{1,54}$ = 0.48, P = 0.49) or for mortality due to other causes ($F_{1,54}$ = 0.22, P = 0.64). Among the *Beauveria* isolates, significant differences were found in mortality with fungal outgrowth between *B. bassiana* as a group and the *B. pseudobassiana* and *B. brongniartii* isolates ($F_{1,54}$ = 11.86, P < 0.001). All other comparison were not significant (P>0.05). However, the *B. bassiana* isolates EABb 01/33-Su and EABb 01/88-Su caused the highest mortality with fungal outgrowth, with values of 78.3% and 75.0%, respectively (Figure 1). These mortality rates were not significantly different from those of the *B. bassiana* isolates EABb 01/103-Su, 3155, EABb 07/06-Su, and EABb 09/07-Fil or from those of the *M. brunneum* isolates EAMb 09/03-Su and EAMb 09/01-Su, which also caused mortality rates with fungal outgrowth of higher than 40% (Figure 1). The average survival times (ASTs) ranged from 8.6 to 17.6 days for EABb 01/103-Su and 1222, respectively. The lowest ASTs were observed for the isolates EABb 01/103-Su, EABb 01/88-Su, and EABb 01/33-Su of *B. bassiana*, at 8.6, 8.7, and 9.7 days, respectively, and for the isolate EAMb 09/03-Su of *M. brunneum*, at 9.0 days (χ^2 = 881.17, P<0.001; Figure 1).

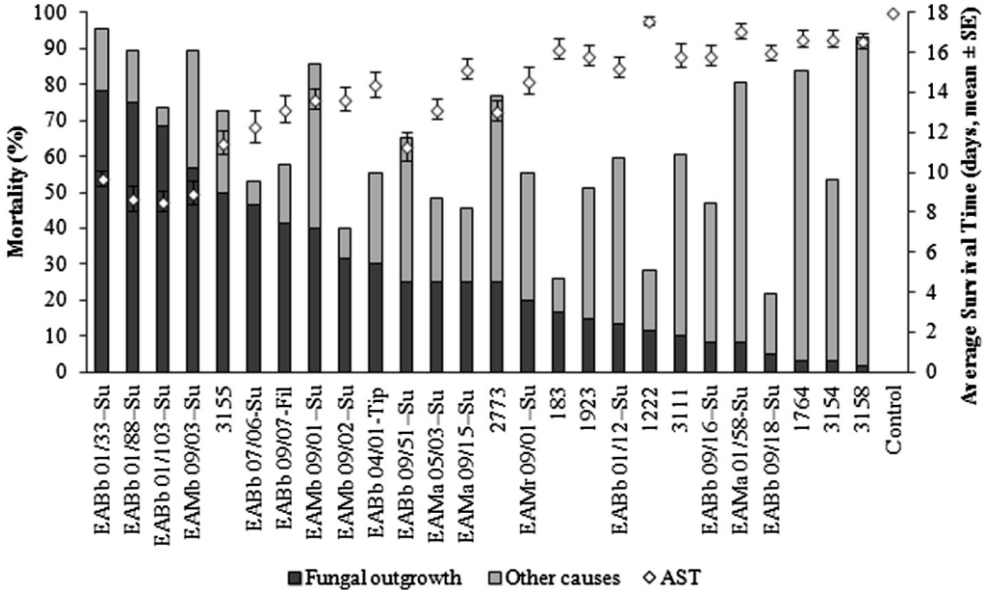

Figure 1. Relative percentages of dead L_2 *Spodoptera littoralis* showing fungal outgrowth and larvae death by other causes after immersion in $1.0x10^8$ conidia ml^{-1} suspensions of entomopathogenic fungi. The dots represent average survival times (ASTs), which were limited to 18 days. The data are presented as the mean ± standard error.

3.2. Virulence assay of entomopathogenic fungal isolates against *Spodoptera littoralis* larvae

Concentration-mortality response regression analysis for each isolate was performed using the five concentrations of conidia (Table 1). All χ^2 values were non-significant, as determined by goodness-of-fit Pearson's test (χ= 0.05), with regression coefficients varying from 0.07 to 0.14. The three isolates did not showed significant differences in their virulence according to the confidence interval of their relative potencies. None of the confidence intervals contained unity. While the isolate EABb 01/33-Su was the most virulent, showing mortality rates of between 43.3% and 93.3%, an LC_{50} value of $5.7x10^6$ and an LT_{50} value of 6.8 days at the dose of $1.0x10^8$ conidia ml^{-1} (Table 1).

The relative potencies of the treatments with the isolates EABb 01/33-Su and EABb 01/88-Su with respect to the treatment with EAMa 01/58-Su were 1.70 and 1.12, respectively.

Table 1. Probit analysis of log-dose mortality and time-mortality response of bioassays with two isolates of *Beauveria bassiana* and *Metarhizium brunneum* against second-instar *Spodoptera littoralis* larvae.

Isolates[a]	Regression Probit	Ep[b]	χ^2 (2 g.d.l)	LC_{50} (conidia ml^{-1})	Confidence limit 95%		Potency relative	χ^2 (g.d.l)	LT_{50}[c]	Confidence limit 95%	
					lower	upper				lower	upper
EABb 01/88-Su	y= 0.55x+1.15	0.12	5.33	$1.05x10^7$	-	-	1.12	13.85(6)	7.02	-	-
EABb 01/33-Su	y= 0.72x-4.85	0.14	3.04	$5.69x10^6$	$2.86x10^6$	$1.00x10^7$	1.70	13.85(6)	6.76	6.46	7.07
EAMa 01/58-Su	y=0.35x+3.63	0.07	0.79	$2.50x10^7$	$8.20x10^6$	$1.0x10^8$	1.00	13.85(6)	13.0	-	-

[a] Regression equation of EAMb 09/01-Su did not performed since any mortality data was higher than 50%.
[b] Slope error
[c] LT_{50} measures in days.

3.3. *Per os* insecticidal activity of the crude extracts against *Spodoptera littoralis* larvae

The insecticidal activities of all of the treatments were significantly different from that of the control ($F_{1,56}$ = 10.16, P = 0.002). However, there were no significant differences between the extracts produced by *Metarhizium* spp. and those produced by *Beauveria* spp. ($F_{1,56}$ = 0.26, P = 0.62). Further, no significant differences were found between the *Beauveria* and *Metarhizium* isolates ($F_{1,56}$ = 1.11, P=0.29 and $F_{1,56}$ = 0.02, P=0.89, respectively). All other comparison were not significant (P>0.05) and AST showed significant differences (χ^2=136.01, P<0.001). However, the crude extract of the *M. brunneum* isolate EAMb 09/01-Su showed the highest mortality rate of 83.3% and the lowest AST of 5.1 days (Figure 2). Further, extracts from the isolates EAMa 01/58-Su and EAMa 05/03-Su of *Metarhizium*, EABb 09/16-Su of *B. pseudobassiana*, EABb 04/01-Tip, and 3158, 3155, and 3154 of *B. bassiana* also caused high mortality rates, ranging between 50.0% and 66.7%. Only the *B. bassiana* isolate EABb 01/33-Su did not result in mortality (Figure 2).

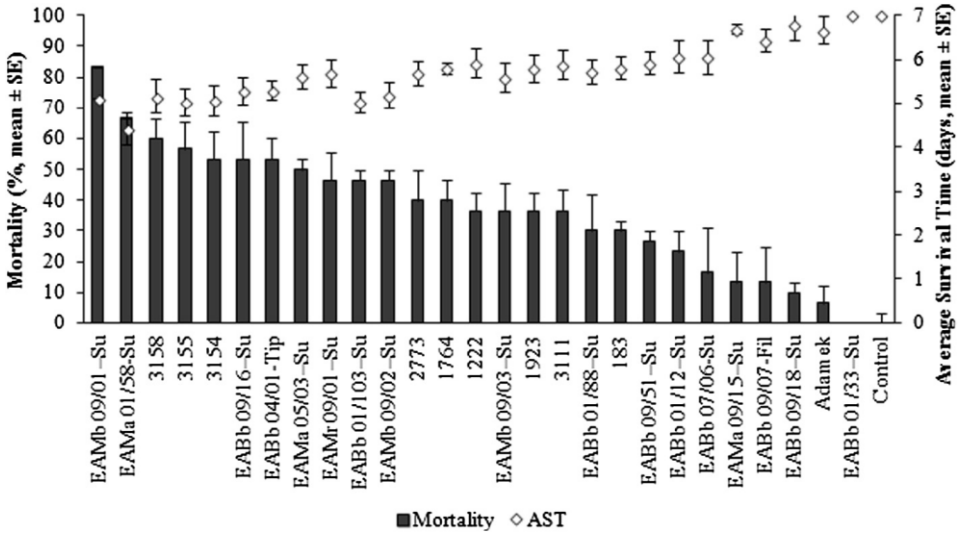

Figure 2. *Per os* insecticidal activities of the crude extracts of entomopathogenic fungi against L_2 *Spodoptera littoralis* larvae after 7 days of treatment with 10 µl of extract (1 mg of protein ml^{-1}) on alfalfa leaf discs. The dots represent average survival times (ASTs), which were limited to 18 days. The data are presented as the mean ± standard error.

3.4. Evaluation of the insecticidal activities of spray applications of the crude extracts against *Spodoptera littoralis*

The crude extracts of the isolates that showed high per os mortality were also applied by contact. None of the crude extracts caused mortality. Furthermore, even after the treatments, the larvae continued to feed until pupation.

3.5. Combined treatments with entomopathogenic fungal conidia and their extracts

The combination of the isolate EAMb 09/01-Su and its extract showed the highest mortality when larvae were treated with $1.0x10^8$ conidia ml^{-1} and extracts of 1 mg protein ml^{-1}, with 100.0% mortality; the isolate EAMa 01/58-Su also induced high mortality (78.3%) at the highest dose tested

(Table 2). The ASTs ranged from 3.6 to 4.7 days for EAMb 09/01-Su and from 5.1 to 6.9 days for the EAMa 01/58-Su isolates.

However, the combined use of the crude extract of EAMb 09/01-Su and suspensions of EABb 01/33-Su resulted in the highest possible mortality rates with the use of $1.0x10^8$ conidia ml^{-1}, with rates of 100.0% and 96.7% for extracts containing 1 and 0.5 mg protein ml^{-1}, respectively. Further, the highest dose of the isolate EAMa 01/58-Su caused significantly high mortality rates of 76.7% and 70.0% at 1 and 0.5 mg protein ml^{-1}, respectively (Table 2). The ASTs ranged from 6.9 to 9.7 days for the combination of the fungal suspension of EABb 01/33-Su and the crude extract of EABb 09/01-Su isolate and from 5.3 to 9.1 days for the combination of the fungal suspension of EABb 01/33-Su and the crude extract of the EAMa 01/58-Su isolate. Meanwhile, the antifeedant indexes (AIs) of the alfalfa leaf discs were calculated at 24 h after treatment and showed significant differences between the treatments and isolates (Figure 3).

In general, the larvae fed with the crude extract of the EAMb 09/01-Su isolate showed AIs that were higher than those of the larvae fed with the crude extract of the EAMa 01/58-Su isolate. The differences were the highest for the treatment combining a conidial suspension with crude extracts. In general, the two higher conidial concentrations ($1.0x10^7$ and $1.0x10^8$ conidia ml^{-1}) showed additive effects, except for the EAMa 01/58-Su isolate and its extract at 0.5 mg protein ml^{-1}, which showed an antagonistic effect (Table 2). Usually, antagonistic interactions were detected with the lower conidial concentrations ($1.0x10^5$ conidia ml^{-1}) and 0.5 and 1.0 mg protein ml^{-1} of crude extract, with the exception of the EAMb 09/01-Su isolate and its crude extract, which showed an additive effect. Nearly all of the combinations of the crude extract of EAMa 01/58-Su isolate and its crude extract had antagonistic effects when applied to *S. littoralis* larvae (Table 2).

Table 2. Susceptibility of second-instar larvae of *Spodoptera littoralis* to combined treatments with serial suspensions of conidia of the isolates EAMb 09/01-Su or EAMa 01/58-Su and their crude extracts, as well as fungal suspensions of EAMb 01/33-Su and the crude extracts of the isolates EAMb 09/01-Su and EAMa 01/58-Su.

Treatment	Doses (conidia ml^{-1}/mg; proteinml^{-1})	Mortality Mean (%)±SE	AST (mean ± SE; d)	IC (95%)	Expected mortality	χ^2 calculate((*gf*=l))	χ^2 Table valu	D	Effect on larvae
EAMb 09/01-Su + its extract	Adamek's control	0±0	10.0±0	(10.0; 0)	-	-	-	-	-
	Water control	0±0	18.0±0	(18.0; 0)	-	-	-	-	-
	0.5	78.3±8.8	4.2±0.2	(3.8±4.6)	-	-	-	-	-
	1.0	96.0±5.2	4.3±0.2	(4.0±4.6)	-	-	-	-	-
	$1.0x10^5$	8.3±5.4	13.9±0	(13.9±14.0)	-	-	-	-	-
	$1.0x10^6$	16.7±10.3	13.5±0.2	(13.1±13.8)	-	-	-	-	-
	$1.0x10^7$	26.7±6.1	13.2±0.3	(13.1±13.8)	-	-	-	-	-
	$1.0x10^8$	56.7±12.3	12.2±0.4	(11.5±12.9)	-	-	-	-	-
	$1.0x10^5$ +0.5	55.0±13.1	4.3±0.1	(4.1; 4.5)	80.1	7.9	10.8*	-	additive
	$1.0x10^6$ +0.5	55.0±13.1	4.2±0.1	(4.0; 4.4)	81.9	8.9	10.8*	-	additive
	$1.0x10^7$ +0.5	63.3±10.3	4.2±0.1	(4.0; 4,4)	84.1	5.1	10.8*	-	additive
	$1.0x10^8$ +0.5	83.3±13.3	4.1±0.9	(4.1; 4.5)	90.6	0.6	3.8**	-	additive
	$1.0x10^5$ +1	55.0±16.4	4.7±0.2	(4.2; 4.5)	96.3	17.7	10.8*	-41.3	antagonistic
	$1.0x10^6$ +1	61.7±7.2	4.4±0.1	(4.2; 5.2)	96.7	12.7	10.8*	-35.0	antagonistic
	$1.0x10^7$ +1	86.7±8.2	4.3±0.1	(4.0; 4.3)	97.1	1.1	3.8**	-	additive
	$1.0x10^8$ +1	100.0±0	3.6±0.1	(3.5; 3.7)	98.3	0.1	3.8**		additive
EAMa 01/58-Su + its extract	Adamek's control	0±0	10.0±0	(10.0; 0)	-	-	-	-	-
	Water control	0±0	18.0±0	(18.0; 0)	-	-	-	-	-
	0.5	65.0±10.5	4.9±0.2	(4.5±5.4)	-	-	-	-	-
	1.0	75.0±8.9	4.8±0.2	(4.3±5.2)	-	-	-	-	-
	$1.0x10^5$	30.0±4.5	15.5±0,2	(15.1±15.9)	-	-	-	-	-
	$1.0x10^6$	33.3±3.3	13.4±0.2	(13.1±13.8)	-	-	-	-	-
	$1.0x10^7$	56.7±4.2	13.7±0.4	(12.9±14.6)	-	-	-	-	-
	$1.0x10^8$	75.0±6.2	12.8±0.2	(11.8±13.7)	-	-	-	-	-
	$1.0x10^5$ +0.5	10.0±16.7	6.9±0.1	(6.7; 7.0)	75.5	56.8	10.8*	-65.5	antagonistic
	$1.0x10^6$ +0.5	20.0±12.6	6.5±0.1	(6.7; 6.8)	76.7	41.9	10.8*	-56.6	antagonistic
	$1.0x10^7$ +0.5	30.0±12.6	6.1±0.1	(5.7; 5.9)	84.8	35.4	10.8*	-54.8	antagonistic
	$1.0x10^8$ +0.5	46.7±17.5	5.8±0.2	(5.8; 6.4)	91.3	21.8	10.8*	-44.6	antagonistic
	$1.0x10^5$ +1	28.3±13.3	5.9±0.3	(5.9; 5.9)	82.5	35.6	10.8*	-44.3	antagonistic
	$1.0x10^6$ +1	35.0±9.7	5.6±0.9	(5.4; 5,8)	83.3	28.0	10.8*	-44.2	antagonistic
	$1.0x10^7$ +1	45.0±15.2	5.4±0.1	(5.2; 5.6)	89.2	21.8	3.8**	-15.4	antagonistic
	$1.0x10^8$ +1	78.3±14.7	5.1±0.2	(4.8; 5.5)	93.8	2.5	3.8**	-	adiditive
EABb 01/33-Su + EAMb 09/01-Su extract	Adamek's control	0±0	10.0±0	(10.0; 0)	-	-	-	-	-
	Water control	0±0	18.0±0	(18.0; 0)	-	-	-	-	-
	0.5	80.0±10.9	5.9±0.15	(5.6±6.2)	-	-	-	-	-
	1.0	90.0±8.9	5.2±0.2	(4.8±5.6)	-	-	-	-	-
	$1.0x10^5$	43.3±8.8	6.7±0.2	(6.1±7.2)	-	-	-	-	-
	$1.0x10^6$	53.3±5.8	15.6±1.2	(13.2±17.9)	-	-	-	-	-
	$1.0x10^7$	66.7±13.3	11.6±1.2	(9.2±13.9)	-	-	-	-	-
	$1.0x10^8$	93.3±6.7	10.6±1.3	(8.7±14.6)	-	-	-	-	-
	$1.0x10^5$ +0.5	30.0±10.0	9.0±0.4	(8.4; 9.7)	88.7	38.8	10.8*	-58.6	antagonistic
	$1.0x10^6$ +0.5	66.7±15.3	8.4±0.4	(7.6; 9.2)	90.7	6.4	10.8*	-	additive
	$1.0x10^7$ +0.5	76.7±18.1	8.1±0.4	(7.3; 8.8)	85.3	0.9	3.8**	-	additive
	$1.0x10^8$ +0.5	96.7±5.8	8.0±0.4	(7.2; 8.8)	98.7	0.04	3.8**	-	additive
	$1.0x10^5$ +1	33.3±11.5	9.7±0.1	(9.5; 9.9)	94.3	39.5	10.8*	-61.0	antagonistic
	$1.0x10^6$ +1	70.0±10.0	8.4±0.4	(7.7; 9.1)	95.3	6.7	10.8*	-	additive
	$1.0x10^7$ +1	93.3±11.5	7.6±0.4	(6.8; 8.4)	83.7	1.1	3.8**	-	additive
	$1.0x10^8$ +1	100.0±0	6.9±0.4	(6.1; 7.8)	98.9	0.01	3.8**	-	additive
EABb 01/33-Su + EAMa 01/58-Su extract	Adamek's control	0±0	10.0±0	(10.0; 0)	-	-	-	-	-
	Water control	0±0	18.0±0	(18.0; 0)	-	-	-	-	-
	0.5	60.0±10.9	7.1±0.3	(6.6±7.7)	-	-	-	-	-
	1.0	75.0±12.6	6.7±0.2	(6.1±7.2)	-	-	-	-	-
	$1.0x10^5$	43.3±8.8	15.6±0.2	(13.2±17.9)	-	-	-	-	-
	$1.0x10^6$	53.3±5.8	11.6±0.2	(9.2±13.9)	-	-	-	-	-

Table 2 (continued). Susceptibility of second-instar larvae of *Spodoptera littoralis* to combined treatments with serial suspensions of conidia of the isolates EAMb 09/01-Su or EAMa 01/58-Su and their crude extracts, as well as fungal suspensions of EAMb 01/33-Su and the crude extracts of the isolates EAMb 09/01-Su and EAMa 01/58-Su.

Treatment	Doses (conidia ml^{-1}/mg; proteinml^{-1})	Mortality Mean (%)± SE	AST (mean ± SE; d)	IC (95%)	Expected mortality	χ^2 calculated (gf=l))	χ^2 table value	D	Effect on larvae
EABb01/33-Su + EAMa01/58-Su extract	$1.0x10^7$	66.7±13.3	10.6±0.3	(8.7±14.6)	-	-	-	-	-
	$1.0x10^8$	93.3±6.7	11.7±0.4	(7.9±13.3)	-	-	-	-	-
	$1.0x10^5$ +0.5	30.0±0	7.7±0.1	(7.5; 8.0)	77.3	28.9	10.8*	-47.3	antagonistic
	$1.0x10^6$ +0.5	53.3±5.8	6.3±0.3	(5.6; 6.9)	81.3	9.6	10.8*	-	additive
	$1.0x10^7$ +0.5	63.3±5.8	6.2±0.2	(5.6; 6.8)	86.6	6.2	10.8*	-	additive
	$1.0x10^8$ +0.5	70.0±10.0	5.3±0.2	(4.9; 5.7)	97.3	7.7	10.8*	-	additive
	$1.0x10^5$ +1	20.0±12.6	9.1±0.3	(8.6; 9.7)	85.8	50.5	10.8*	-65.8	antagonistic
	$1.0x10^6$ +1	60.0±10.0	8.0±0.4	(7.2; 8.9)	88.3	9.1	10.8*	-	additive
	$1.0x10^7$ +1	63.3±15.3	8.3±0.4	(7.4; 9.1)	91.6	8.8	10.8*	-	additive
	$1.0x10^8$ +1	76.7±18.5	7.1±0.4	(6.4; 7.1)	98.3	4.8	10.8*	-	additive

Mean (%) ± SE: means of mortality rates.

ME: expected mortality ME = MH + MCE (1-MH/100). MH represents the observed Abbott (1925) corrected mortality percentage caused by the fungi whereas MCE represents

the Abbott corrected mortality percentage due to the crude extract alone. Results from χ^2 test were compared to the χ^2 Table value for 1 degree of freedom (χ^2Table value: P > 0.001, P > 0.05), using the formula χ^2= (MHCE -ME)^2/ME, wherein MHCE is the corrected observed mortality. In the case of χ^2

calculated > χ2 Table value, χ^2 if MHCE-ME was positive a significant interaction was considered to be synergistic. However, if MCE-ME was negative, the interaction was considered to be antagonistic.

χ^2Table value: *P > 0.001, **P > 0.05.

D = (MCE-ME) > 0 a significant synergistic interact.

Mean (%) ± SE: means of mortality rates.

χ^2Table value: *P > 0.001, **P > 0.05.

D = (MHCE-ME) > 0 a significant synergistic interact.

ME = MH + Me [1- (MH/100)].

3.6. Effect of duration of exposure to the combined treatments of the fungal suspension and extract of EAMb 09/01-Su

There were significant differences in mortality between the treatments ($F_{8,26}$= 16.6, P<0.001) (Table 3). The mortality increased over time, and significant differences were observed at 48 h of per os administration of the crude extract (Table 3). The application of fungal suspension and crude extract at 1mg protein ml^{-1} for 48 h yielded higher mortality rates (96.7%) and lower ASTs (6.0 days) than the treatments with either fungal suspension or crude extract alone.

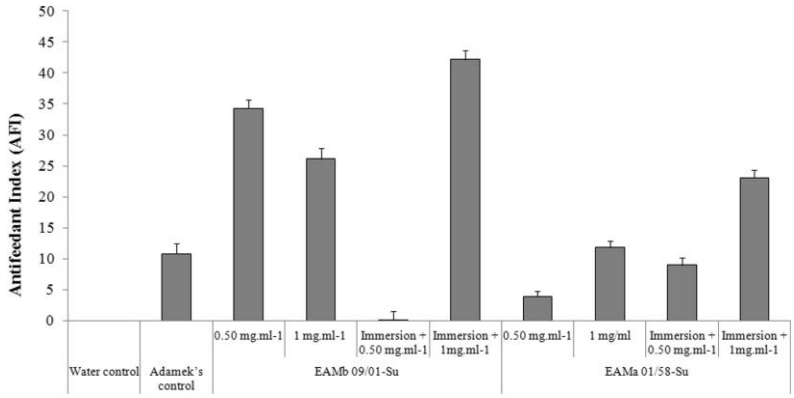

Figure 3. Antifeedant indexes of *Spodoptera littoralis* second-instar larvae exposed to various combined treatments of fungal suspensions and crude extracts at 24 h after treatment. The data are presented as the mean ± standard error.

Table 3. Effect of exposure time on the combined treatment with the conidia and extract of the isolate EAMb 09/01-Su against second-instar *Spodoptera littoralis* larvae at 24, 48, 72 and 96 h.

Treatments	Mortality (%mean ± SE)*	Survival Analysis- Kaplan Meier**	
		AST (mean ± SE; d)	IC (95%)
Control absolute	0±0a	18.0±0a	-
Adamek's control	6.7± 3.3a	17.2±0.5a	-
$1.0x10^8$ conidia ml^{-1}	36.7±8.8b	14.8±0.8b	(13.2; 16.4)
0.5 mg protein ml^{-1}	70.0±5.7c	8.1±1.2c	(5.8; 10.4)
1 mg protein ml^{-1}	70.0±5.7c	8.2±1.2c	(5.8; 10.6)
$1.0x10^8$ + 1 mg protein ml^{-1} (12h)	36.7±6.7b	13.0±1.2b	-
$1.0x10^8$ + 1 mg protein ml^{-1} (24h)	60.0±5.7bc	5.0±0.4c	(4.2; 5.9)
$1.0x10^8$ + 1 mg protein ml^{-1} (36h)	60.0±0bc	10.3±1.1b	(8.1; 12.6)
$1.0x10^8$ + 1 mg protein ml^{-1} (48h)	96.7±3.3d	6.0±0.4c	(5.1; 7.0)

* Means within columns with the same letter are not significantly different according to the Tukey test ($P < 0.001$).
** Means within columns with the same letter are not significantly different according to the log rank test (Analysis Survival - Kaplan Meier) AST: average survival time measured at 18 days.

4. DISCUSSION

The results of this work indicate that *S. littoralis* larvae are susceptible to the studied entomopathogenic fungi (EF) and their extracts. Most of the 26 assayed isolates were pathogenic, but eight of them, namely EABb 01/33-Su, EABb 01/88-Su, EABb 01/103-Su, EAMb 09/03-Su, 3155, EABb 07/06-Su, EABb 09/07-Su and EAMb 09/01-Su, caused markedly higher mortality rates. The isolates 3158, 3154, 1764, and EAMa 01/58-Su, which caused high mortality rates, were not frequently isolated from the dead treated larvae, likely in accordance with the ‘‘toxin strategy” proposed by Kershaw et al. (1999). Although EF have shown high potential in soil applications against soil-dwelling insect pests (Ekesi et al., 2003; Quesada-Moraga et al., 2006; Garrido-Jurado et al., 2011), there is little information available on the effects of soil treatments with EF on soil-dwelling lepidopteran pests. Our results showed that several of the assayed isolates, especially the abovementioned isolates EABb 01/33-Su and EABb 01/88-Su, could also lead to significant reductions in the survival rates of treated larvae. The isolate EABb 01/33-Su was the most virulent against *S. littoralis* larvae, with an LC_{50} value of $5.69x10^6$ conidia ml^{-1}. Working with *S. littoralis*, Sahab and Sabbour (2011) reported LC_{50} values of $1.63x10^6$, $1.75x10^6$, $1.99x10^6$ and $1.87x10^6$ conidia ml^{-1} for *B. bassiana*, *M. anisopliae*, *Paecilomyces fumosoroseus* and *Verticillium lecanii* isolates, which are similar to the values obtained in our study. However, El-Khawas and Abed El-Gward (2002) evaluated a commercial product of *B. bassiana* and reported that the LC_{50} for *S. littoralis* was $3.07x10^8$ conidia ml^{-1}. This study has shown that extracts from EF have oral toxicity against *S. littoralis* larvae, as previously reported by Quesada-Moraga et al. (2006). However, fungal extracts or, more precisely, their secondary metabolites have also shown insecticidal activity by injection or contact in other lepidopteran pests (Hu et al., 2007; Fan et al., 2013).

However, in our work, spray application of the extracts did not result in mortality of *S. littoralis* larvae, indicating the ineffectiveness of their use by contact. Among all of the isolates tested in bait, the crude extracts from EAMb 09/01-Su and EAMa 01/58-Su strains showed the higher insecticidal activity against *S. littoralis*. However, the activities of the crude extracts of the isolates 3158, 3155, 3154 and EABb 09/16-Su should not be underestimated.

Continuous application of the EAMb 09/01-Su and EAMa 01/58-Su extracts resulted in high mortality values. The crude extract of the EAMa 01/58-Su strain also showed *per os* insecticidal activity against medfly *Ceratitis capitata* (Wied.) adults. It is secreted in vivo during the infection process (virulence factor) (Ortiz-Urquiza et al., 2009), and its active fraction has been recently purified and characterised as a 15 kDa protein that has been renamed secreted insect toxin^{-1} (SIT1) and has shown reported similarity with a family of phytotoxins secreted by plant pathogenic fungi (Ortiz-Urquiza et al., 2013). Our research has demonstrated that the partially purified active fraction of the EAMb 09/01-Su crude extract is composed of a mix of destruxins (Lozano-Tovar et al., 2015). The values obtained with the crude extracts of the isolates EAMb 09/01-Su and EAMa 01/58-Su, containing destruxins and insecticidal proteins, respectively, are similar to those produced in lepidopterans by secondary metabolites of entomopathogenic fungi, such as destruxins, efrapeptins and cordycepin (Amiri et al., 1999; Bandani and Butt, 1999; Kim et al., 2002), by Photorhabdus toxins (Blackburn et al., 1998; Gerritsen et al., 2005) and by certain plant-derived compounds (Wheeler and Isman, 2001; El-Aswad et al., 2003; Sadek, 2003).

The current study reports on the novel control of *S. littoralis* with EF and their extracts, either as single applications or using both in combination. The highly toxic extracts of the isolates EAMb 09/01-Su and EAMa 01/58-Su, when applied in combination with fungal suspensions of the same isolates

or with a fungal suspension of the isolate EABb 01/33-Su, showed strong insecticidal effects against L_3 *S. littoralis* larvae. In general, additive effects were observed for almost all of the treatments, although antagonistic interactions were found with the isolate EAMa 01/58-Su and its extract and at the lower doses of the fungal suspensions for all the isolates. These findings highlight the needs for strain selection in strategies of combined applications of EF and their extracts. Similarly, Yousef et al. (2014) have found additive effects for all fungal concentrations using the same concentrations of extracts and various exposure times. However, in the present work, 48 h of exposure produced significantly higher mortality than the other exposure times assessed. The mortality of the combination treatment might have been due to fungal disease acquired through infection and the toxicity of the secreted compounds. The compatibility of entomopathogens with synthetic insecticides or other biological control agents has been proposed as a strategy to improve the efficacy of microbial control agents. Such is the case for the interaction between *M. anisopliae* and spinosad, which shows a synergic effect and increased mortality in the house fly (*Musca domestica*) while reducing the lethal time (Sharififard et al., 2011), that of *B. bassiana* and a nereistoxin analogue insecticide for the control of *Plutella xylostella* larvae (Tian and Feng, 2006), and the antagonism between *B. bassiana* and imidacloprid for the control of *Bemisia argentifolii* (James and Elzen, 2001). Moreover, synergistic interactions have been observed previously for certain combinations of *B. bassiana* and *B. thuringiensis* var. *israelensis* for the control of house fly larvae (Mwamburi et al., 2009). Despite the success of those combined applications, only Yousef et al. (2014) have addressed interactions between EF and their extracts against the dipteran *C. capitata*, offering a very promising tool for integrated pest control.

In conclusion, this study reveals the potential of certain EF strains for the microbial control of *S. littoralis* larvae in foliar applications. In addition,

the crude extracts of some of these isolates also show potential for use against *S. littoralis* as new insecticidal compounds of natural origin. Furthermore, the current study indicates that selection of a suitable combination of strain and extract it is important in order to develop control strategies based on EF and their own extracts, in the same way as the combination currently use with EF and extracts from other microorganisms or chemical insecticides.

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CAPÍTULO III

Este capítulo es una versión adaptada del artículo: Transient endophytic colonizations of plants improve the outcome of foliar applications of mycoinsecticides against chewing insects, que se encuentra publicado en Journal of Invertebrate Pathology en el quartil Q1 del área de Zoología (posición 27 de 154) del JCR

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Transient endophytic colonizations of plants improve the outcome of foliar applications of mycoinsecticides against chewing insects

G. Resquín-Romero [a,b,1], I. Garrido-Jurado [a,1], C. Delso [a], A. Ríos-Moreno [a], E. Quesada-Moraga [a,*]

[a] Department of Agricultural and Forestry Sciences, ETSIAM, University of Cordoba, Campus de Rabanales, Building C4 "Celestino Mutis", Cordoba 14071, Spain
[b] Faculty of Agrarian Sciences of the National University of Asunción, Paraguay

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ABSTRACT

The current work reports how spray application of entomopathogenic fungi on alfalfa, tomato and melon plants may cause an additional *Spodoptera littoralis* larvae mortality due to a temporal colonization of the leaves and subsequent ingestion of those leaves by the larvae. Most entomopathogenic fungi (EF) (Ascomycota: Hypocreales) endophytes seem to colonize their host plants in a non-systemic pattern, in which case at least a transient endophytic establishment of the fungus should be expected in treated areas after spray application. In this work, all strains were able to endophytically colonize roots, stems and leaves during the first 96 h after inoculation. Whilst the treatment of *S. littoralis* larvae with a 10^8 ml^{-1} conidial suspension resulted in moderate to high mortality rates for the *Metarhizium brunneum* EAMb 09/01-Su (41.7–50.0%) and *Beauveria bassiana* EABb 01/33-Su (66.7–76.6%) strains, respectively, an additive effect was detected when these larvae were also fed endophytically colonized alfalfa, tomato, and melon leaves, with mortality rates varying from 25.0% to 46.7% as a function of the host plant and total mortality rates in the combined treatment of 75–80% and 33–60% for *B. bassiana* and *M. brunneum*

Transient endophytic colonizations of plants improve the outcome of foliar applications of mycoinsecticides against chewing insects

Resquín-Romero, G.[1,2†], Garrido-Jurado, I.[1†], Delso C.[1], Ríos-Moreno, A.[1], Quesada-Moraga, E[1]*

[a] Department of Agricultural and Forestry Sciences, ETSIAM, University of Cordoba, Campus de Rabanales, Edificio C4 Celestino Mutis, 14071 Cordoba, Spain

[b] School of Agrarian Sciences of the National University of Asunción, Paraguay

[†] Both authors contributed equally to this work

* Corresponding author: tel.: + 34-957218475; fax: +34-957218440. E-mail address: equesada@uco.es

ABSTRACT

The current work reports how spray application of entomopathogenic fungi on alfalfa, tomato and melon plants may cause an additional *Spodoptera littoralis* larvae mortality due to a temporal colonization of the leaves and subsequent ingestion of those leaves by the larvae. Most entomopathogenic fungi (Ascomycota: Hypocreales) endophytes seem to colonize their host plants in a non-systemic pattern, in which case at least a transient endophytic establishment of the fungus should be expected in treated areas after spray application. In this work, all strains were able to endophytically colonize roots, stems and leaves during the first 96 h after inoculation. Whilst the treatment of *Spodoptera littoralis* larvae with a 10^8 ml^{-1} conidial suspension resulted in moderate to high mortality rates for the *M. brunneum* EAMb 09/01-Su (41.7-50.0%) and *B. bassiana* EABb 01/33-Su (66.7-76.6%) strains, respectively, an additive effect was detected when these larvae were also fed endophytically colonized alfalfa, tomato, and melon leaves, with mortality rates varying from 25.0% to 46.7% as a function of the host plant and total mortality rates in the combined treatment of 75-80% and 33-60% for *B. bassiana* and *M. brunneum*, respectively. Fungal outgrowth was not detected in any of the dead larvae feeding on colonized leaves, whereas traces of destruxin A were detected in 11% of the insects fed tomato discs endophytically colonized by *M. brunneum*. The combined effects of the fungal spray with the mortality caused by the feeding of insects on transient EF-colonized leaves have to be considered to estimate the real acute impact of field sprays with entomopathogenic fungi on chewing insects.

1. INTRODUCTION

In the last century, entomopathogenic fungi (EF) have been viewed as arthropod pathogens, and research has been focused on all aspects related to their possible development as alternatives to chemicals. Indeed, several hypocrealean ascomycete-based mycoinsecticide products are commercially available (de Faria and Wraight, 2007; Nollet and Rathore, 2015).

Recent studies have revealed that entomopathogenic Hypocreales may play additional unusual roles in the ecosystem such as plant endophytes, rhizosphere-competent fungi, or antagonists of plant pathogens (Vega, 2008; Vega et al., 2009; Quesada-Moraga et al., 2014a). Research on these new lifestyles, particularly plant endophytes, has come of age within the last decade, and the newly recognized importance of these roles is a consequence of the increasing recognition of the potential agronomic benefits that these fungal species may have and their enormous potential in the development of novel integrated crop protection tools (Quesada-Moraga et al., 2014a).

The pioneer studies of Bing and Lewis (1992) in corn first revealed that the establishment of *Beauveria* sp. in planta can confer systemic protection from herbivorous pests. Late in the XXI century, some fungal entomopathogens were identified as naturally occurring endophytes, whereas others have been artificially inoculated into plants using various techniques (Quesada-Moraga, 2014b). Such studies aimed at using these fungi as biological control agents against specific pests whose life cycles (feeding internally and tunnelling extensively in stems, pseudostems, rhizomes, roots, and seeds) seriously limit the effectiveness of chemical insecticides and other control methods.

The techniques used for the artificial inoculation of EF into different crops include leaf spraying, injection into stems, soil drenching, and seed dressing with conidial suspensions, although differences have been noted in the rates of success, the extension of colonization and the occurrences of systemic colonization (Bing and Lewis, 1992; Tefera and Vidal, 2009; Quesada-Moraga et al., 2006; Posada et al., 2007). Despite the occurrence of natural or artificial EF endophytic colonization, much remains unknown about endophyte penetration and colonization processes. Important issues central to the development of this fungus-plant association include the primary portals of fungal entry into plant tissues, the extent of fungal dissemination (especially ascending migration) within the plant host after primary root infection, and fungal population dynamics in plant. The first clue suggesting the ascending migration of an endophytic strain of *B. bassiana* within the opium poppy came from the studies of Landa et al. (2013), who used seed dressing with a conidial suspension to demonstrate, for the first time, the vertical transmission of an entomopathogenic fungus from endophytically colonized maternal plants (Quesada-Moraga et al., 2014a). Interestingly, when the inoculation technique was not seed dressing but leaf spray with the same GFP-tagged *B. bassiana* strain, only a temporal endophytic establishment was detected in the treated area, which gradually decreased until disappearing completely (Landa et al., 2013); these findings were also detected in several crops by different researchers (Bing and Lewis, 1992; Posada et al., 2007; Gurulingappa et al., 2010; Herrero et al., 2012; Biswas et al., 2012; Batta, 2013).

One current drawback to the use of EF endophytes as biocontrol agents is the variability observed in the endophytic persistence of fungi after inoculation. The inoculation method, the fungal strain used, and the host plant genotype are key factors determining the persistence of EF endophytes and the compatibility of plant-endophyte associations (Quesada-Moraga,

2014a). As a result, most known fungal endophytes seem to colonize their host plants in a non-systemic pattern (Rodriguez et al., 2009; Marquez et al., 2012), probably due to a "balanced antagonism" in which the host plant can restrain the growth of the fungus, and the fungus can modulate the effectiveness of plant defence mechanisms (Schulz and Boyle, 2005). As a result, it could be expected that spraying plant tissues with an EF fungal suspension could produce the temporary endophytic establishment of the EF fungus in the treated area in many cases, both in systemic and non-systemic colonization patterns.

In such a scenario, it remains unknown whether spray applications with conidial EF suspensions targeting chewing insect pests can be accompanied by such transient endophytic colonizations of plant tissues or whether this temporary establishment may cause any level of pest control mortality, thereby improving the overall efficacy of fungal treatment. Even if one expects that the bio-controlling abilities of endophytic EF are due to the infection of the insect upon feeding on endophytically colonized plants, very few fungal-infected insects have been observed in the aforementioned studies; thus, apart from antibiosis and feeding deterrence, it could be argued that endophytes kill insects during the first stages of development by secreting toxic compounds in planta. Likewise, various species of endophytes are known to produce metabolites that deter insect feeding (Daisy et al., 2002); this finding suggests that the production of such compounds in planta may inhibit insects from foraging on the plants (Vega et al., 2009). In the current work, we monitored the extent of endophytic colonization of alfalfa, tomato, and melon plants when fungal suspensions of *Beauveria bassiana* and *Metarhizium brunneum* are sprayed onto plant leaves, in order to ascertain whether a spray application of a fungal suspension could produce a temporal colonization of the plants that could improve the overall effect of the treatment. We evaluated whether the feeding of larvae of the beet

armyworm *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) on the leaves of three crops temporally colonized by the fungi caused any level of pest mortality, investigated possible causes of mortality, and assessed whether mortality may improve the efficacy of spray applications with fungal suspensions.

2. MATERIAL AND METHODS

2.1 *Spodoptera littoralis* (Boisduval) (Noctuidae: Lepidoptera) culture

The *Spodoptera littoralis* larvae used in this study were obtained from a stock colony from the Department of Agricultural and Forestry Sciences of the University of Córdoba (Spain). These insects were originally collected in the field from different crops. They were maintained in an environmental chamber set at 26±2ºC, 70±5% RH, and a photoperiod of 16:8 (L:D) h (Poitout and Bues, 1974). The adults were placed in oviposition chambers containing cylindrical filter paper (150×120 mm) that were closed at both ends with a layer of filter paper. Cotton moistened with a 10% honey solution was placed inside a small container. This cotton was replaced every two days to prevent possible contamination and fermentation. Each chamber contained 5 pupae of each sex. The chambers were observed daily, and clusters of collected eggs were externally disinfected by immersion in 10% formalin for 10 minutes and three washes with sterile water to remove the formalin residues. Finally, the disinfected egg clusters were laid on pieces of filter paper to remove the water using the methodology developed by Santiago-Álvarez (1977). The larvae were fed on artificial diet consisting of 85g of alfalfa meal, 34g of brewer's yeast, 32g of wheat germ, 18g of agar-agar (Industrias ROKO, S.A., Spain), 14g of casein (Merck KGaA, Germany); 4.50g of ascorbic acid (Scharlab, Spain), 1.30g of benzoic acid (Scharlab, Spain), 1.10g of nipagin (Guinama S.L.U., Spain), 5ml formaline 10% (formaldehyde 37-38% w/w stabilized with methanol) (Panreac, Spain) and

800ml of distilled water (Santiago-Álvarez, 1977). A 50 mm layer of sterilized peat was added when the larvae reached the last larval instar and kept under controlled conditions of 26±2ºC, 70±5% RH, and a photoperiod of 16:8 (L:D) h. Then the pupae were removed and separated by sex using a stereomicroscope (Nikon SMZ800, Japan). Finally the pupae were classified and transferred to the oviposition chambers under control conditions.

2.2 Fungal strains and suspensions

The four fungal strains, which included three *Beauveria* strains and one *Metarhizium* strain, used in this study came from the culture collection in the Department of Agricultural and Forestry Sciences (AFS) of the University of Córdoba (Table 1). They were sub-cultured on malt agar (MA) supplied with 500 mg l^{-1} streptomycin sulphate salt (product of China) for 15 d at 25°C in darkness. The Petri plates were sealed with Parafilm® (Pechiney Plastic Packaging Co, Chicago, IL). Conidia suspensions were prepared by scraping conidia from the Petri plates into a sterile aqueous solution of 0.1% Tween 80 and filtered through a piece of cheesecloth. The conidia suspensions used for the inoculation bioassays were adjusted by diluting the conidia with 0.1% Tween 80 to a final concentration of 1.0×10^{8} conidia ml^{-1} using a Malassez chamber.

Table 1: Fungal isolates used this study

Isolates	Fungal species	Origin	Ecology	Habitat	Gen Bank accession number
EABb 04/01-Tip	*B. bassiana*	Ecija (Sevilla, Spain)	*Iraella luteipes*	Insect	FJ972963
EABb 01/33-Su	*B. bassiana*	El Bosque (Cádiz, Spain)	Traditional olive Orchard	Soil	FJ972969
EAMb 09/01-Su	*M. brunneum*	Hinojosa del Duque (Córdoba, Spain)	Wheat crop	Soil	KJ158746
Bb04*	*B. bassiana*	Ethiopia	*Busseola fusca*	Insect	

* Isolate assigned by Prof. Stefan Vidal of the Georg-August-Universität Göttingen in Germany.

2.3 Substrate and preparation of plant material

Alfalfa (*Medicago sativa* L.), tomato (*Lycopersicon esculentum* Mill cv Tres Cantos) and melon var. Galia (*Cucumis melo* L. var. *reticulatus* Naud) seeds were surface disinfected. To accomplish this, the seeds were immersed in 70% (v/v) ethanol for 2 min followed by 2% NaOCl for 5 min and rinsed with sterilized water three times. Lastly, they were again immersed in 70% ethanol (v/v) for 1 min and dried under sterile air flow. The last rinse water was plated out to assess the effectiveness of the surface-disinfecting procedure. The seeds were transferred into plastic pots of 110 wells (25x25x25 mm) containing a planting substrate based on washed sand. The substrate was sterilized in an autoclave for 45 min at 121°C for three times with an intervals of 24 h allotted between each sterilization process and allowed to cool for 24 h prior to planting. One seed was sown per pot (110 wells) and maintained at a temperature of 27±3°C, 13 h of daylight (light intensity of 700 lux) and 11 h of darkness. The seeds were planted in compost enriched with sterilized coconut fibre. The plants were transferred immediately to individual pots (70x70x60 mm) containing 300 g of sterilized substrate. The plants were produced and kept under greenhouse conditions (26±5°C, 14:10 h photoperiod) until used. Fertilization was performed weekly by adding 20 ml of Hoagland solution (Hoagland and Arnon, 1950).

2.4. Colonization of plant tissues by entomopathogenic fungi

Three fungal strains belonging to the genus *Beauveria* (Bb04, EABb 04/01-Tip, and EABb 01/33-Su) and one strain belonging to *Metarhizium* (EAMb 09/01-Su) were used to determine the colonization of the plant tissues by foliar spraying. The adaxial and abaxial leaf surfaces were sprayed with 2 ml of fungal suspensions using an aerograph 27085 (piston compressor of 23 l/min, 15-50PSI and 0.3 mm nozzle diameter, China). During treatment, the non-inoculated parts (i.e., soil) were covered with

aluminium foil to avoid run-off of the suspension. Next, the foliar area was coated with a transparent plastic sheet for 24 h to promote fungal growth.

Control plants were sprayed with sterile water with 0.1% Tween 80. To determine the temporary colonizations of the plants, 10-true leaf plants were carefully removed from the pots at 24, 48, 72, and 96 h. They were washed with water for 10 min to remove attached debris, followed by a rinse of 70% ethanol (v/v) for 2 min, a wash with sterilized water and another wash with a 2% aqueous solution of NaOCl for 5 min for the tomato and melon plants or 2 min for the alfalfa plants. They were then washed three times with sterile water and finally with 70% ethanol (v/v) for 1 min under sterile air flow. To ensure total disinfection of the leaves, 100 µl of the last rinse of each sample was cultured on plates containing selective culture medium [20.0 g of Agar Sabouraud Glucose Chloramphenicol (Cultimed Panreac, Spain), 500 mg l^{-1} streptomycin sulphate (Sigma-Aldrich Chemie, China), 500 mg l^{-1} ampicillin (Intron biotechnology, China) and 500 mg l^{-1} dodine 65 WP (Barcelona, Spain)]. Subsequently, one fragment of leaves (5x5 mm) and stems (7-8 mm diameter) per plant were randomly taken. The fragments were then placed on the selective medium and gently pressed down to ensure full contact.

The colonization of the different plant parts was determined by counting the number of fragments that showed fungal outgrowth. The samples were kept in an incubator at 25°C. The results of the inoculated and non-inoculated fragments and control plants were obtained after 12 days.

2.5. Pathogenicity of entomopathogenic fungi against *Spodoptera littoralis* larvae by topical application

Newly molted third instar larvae 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 10^8-conidial suspension was used for each replicate resulting approximately 731 conidia mm^2 (James at al., 2003), and then the larvae were individually placed in a bioassay cage (28×13 mm) and fed with alfalfa discs. Control larvae were treated with sterile water with 0.10% Tween 80. Larval mortality was recorded every two days for each treatment for 18 days in an environmental chamber set at 26±2ºC, 70±5% RH, and a photoperiod of 16:8 (L:D) h. The dead larvae were immediately surface sterilized with 1% NaOCl for 2 min, followed by three rinses with sterile water. They were then placed on sterile wet filter paper in Petri plates sealed with Parafilm® and kept at room temperature (25±2°C) until inspection of the cadavres. After five days, observation of fungal outgrowth on the surface of the insect cuticle was performed with a light microscope (Nikon, Japan). The whole experiment was repeated twice using a new batch of *S. littoralis* larvae and a new fungal inoculum.

2.6. Application of entomopathogenic fungi together with the ingestion of colonized plants against *Spodoptera littoralis* larvae

2.6.1 Combined treatments of EF suspensions and the ingestion of endophytically colonized plants

To study the joint action of the EF application and the ingestion of endophytically colonized plants, third instar larvae of *S. littoralis* were treated with a Potter tower like in the subheading 2.5 with 10^8 conidia ml^{-1} suspension of EABb 01/33-Su and EAMb 09/01-Su strains and were fed with 15 mm colonized discs of alfalfa, melon, and tomato in joint or separate treatments (Figure 1). The controls were treated with sterile water with 0.10% Tween 80 and were fed with non-colonized discs. Those discs were surface disinfected using the procedure described in subheading 2.4. Two replicates of five experimental plants were used per treatment, each of which was repeated twice.

Collected larvae were surface sterilized as previously described and placed in Petri dishes under optimal conditions to promote fungal sporulation. The Petri dishes were maintained in an incubator at 25°C for 8 days prior to evaluation. Larval mortality was recorded every 24 h for 10 days. Larvae that did not show fungal outgrowth were subjected to destruxin extraction (Skrobek et al., 2008), as were the plants used for feeding following the methodology proposed by Liu et al. (2002) with modifications and quantification according to Carpio et al. (2016). In addition, the treated larvae were weighed 120 h after treatment and compared with the control larvae.

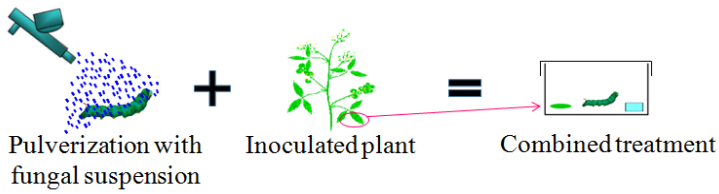

Figure 1. Illustration of the treatments performed to study the combined effect of topical and oral infection with the *Beauveria bassiana* EABb 01/33-Su and *Metarhizium brunneum* EAMb 09/01-Su isolates.

2.6.2 Effect of dose in combined treatments of EF suspensions and ingestion of colonized plants

Larvae were treated as the subheading 2.5 with the appropriate conidial concentration (four tenfold concentrations from $1.0x10^5$ to $1.0x10^8$ conidia/ml) of EABb 01/33-Su and EAMb 09/01-Su strains and were fed with inoculated discs of alfalfa, melon, and tomato in combined and non-combined treatments as described in subheading 2.6.1. The controls were treated with sterile water with 0.10% Tween 80. Larval mortality was recorded every day for each treatment for 12 days. Dead larvae were surface sterilized as previously described and inspected for fungal outgrowth.

2.8 Statistical Analysis

Mortality data and larval weights were analysed using analysis of variance (ANOVA, Statistix 9.0 [Analytical Software 2008]), and the LSD test was used to compare the means. An arcsin transformation was used if the data did not conform to a normal distribution and homogeneity of variance. 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 median lethal concentrations (CL_{50}) values were estimated by probit analysis (Finney, 1971). Probit parallelism test regression lines for all assayed strains were drawn using the χ^2 goodness-of-fit tests. Relative median potencies and their 95% confidence intervals were calculated for different treatments when their slopes did not differ significantly (Finney, 1971). Finally, the synergic, additive, or antagonistic effects between the strains and the inoculated discs were performed according to Resquín-Romero et al. (2015).

3. RESULTS

3.1 Colonization of plant tissues by entomopathogenic fungi

The four strains colonized more than 40% of the leaves and stems of each plant species (Figure 2A and B). Although all strains showed the capacity to colonize plants, differences among the strains were observed. The three plant species were colonized equally by the fungal strains except for Bb04, which exhibited higher colonization percentages in alfalfa, melon, and tomato plants. The four strains produced the highest colonization percentages at different times after inoculation. Bb04 and EABb 01/33-Su produced higher colonization levels after 96 h, but EABb 04/01-Tip and EAMb 09/01-Su produced the highest levels after 48 and 24 h, respectively. Nevertheless, the colonization values remained fairly constant throughout the time (Figure

2). Colonization of the roots was observed 72 h after inoculation in alfalfa and tomato plants and 96 h after inoculation in melon plants (Figure 2C).

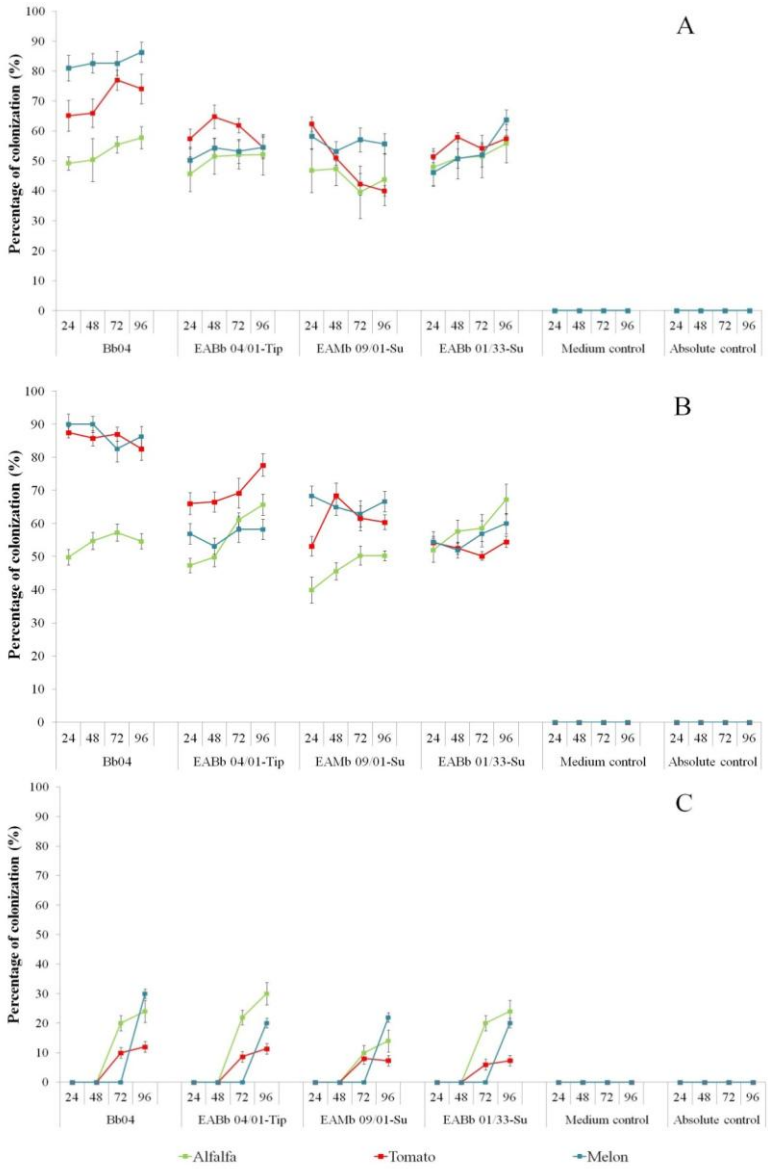

Figure 2. Evolution of endophytic colonization of tomato, melon, and alfalfa plants after foliar spray with $1.0x10^8$ conidia/ml suspensions of three *Beauveria* isolates (Bb04, EABb 04/01-Tip, EABb 01/33-Su) and one *Metarhizium* isolate (EAMb 09/01-Su). Colonization in A) leaves, B) stems, C) roots. The data indicate the percentage of colonization (mean ±EE) of the inoculated fragments collected after 24h, 48h, 72h and 96h of the inoculation.

3.2 Pathogenicity of entomopathogenic fungi against *Spodoptera littoralis* larvae by topical application

The four strains were pathogenic against third instar *S. littoralis* larvae treated with 10^8 conidia ml^{-1} suspensions (Table 2). Both total mortality ($F_{4, 14}$ = 20.17, P<0.001) and mortality with fungal outgrowth ($F_{4, 14}$ = 15.9, P<0.001) showed significant differences between the strains, but no significant differences were detected in mortality due to unknown causes ($F_{4, 14}$ = 2.0, P=0.1460). The highest mortality, either total (95.0%) or with fungal outgrowth (78.3%), was caused by the EABb 01/33-Su strain, together with lowest average survival time (AST), 9.7 days. The AST of larvae treated with the EAMb 09/01-Su, EABb 04/01-Tip, and Bb04 strains showed significant differences compared with the control group, but the mortalities with fungal outgrowth caused by those strains did not show significant differences (Table 2).

Table 2. Pathogenicity of EABb 01/33-Su, EABb 04/01-Tip, and Bb04 *Beauveria bassiana* and EAMb 09/01-Su *Metarhizium brunneum* strains to third instar *S. littoralis* larvae after treatment with a 10^8 conidia ml^{-1} suspension.

Isolate	Mortality (mean ± SE) (%)*			Survival analyses- Kaplan Meier	
	Total mortality	Fungal outgrowth	Other causes	AST** (mean ± SE;d)	CI (95%)
EABb 01/33-Su	95.0±5.0A	78.3±3.3A	16.7±4.2	9.7 ± 0.4 A	(8.8; 10.5)
EAMb 09/01-Su	65.0± 16.1AB	40.0± 17.6AB	25.0±9.3	11.6 ± 0.7 B	(9.9; 12.6)
EABb 04/01-Tip	56.7±3.3AB	46.7± 3.3AB	10.0±0.0	14.4 ± 0.6 B	(13.2; 15.5)
Bb 04	40.0±2.9BC	33.3± 4.4AB	6.7±4.4	13.2 ± 0.5 B	(12.3; 14.2)
Control	0.0 C	0±0 B	0±0	18.0 ± 0 C	(0; 0)

* Means within columns with the same letter are not significantly different according to the test LSD (P<0.001).
**AST: Average survival time. Means within columns with the same letter are not significantly different according log rank test (P≤0.05). Average survival time measured at 18 days.

3.3 Application of entomopathogenic fungi together with the ingestion of colonized plants against *Spodoptera littoralis* larvae

3.3.1 Combined treatments of EF suspensions and ingestion of endophytically colonized plants

Strains EABb 01/33-Su and EAMb 09/01-Su, which were previously shown to endophytically colonize the three crops and to be pathogenic towards *S. littoralis* larvae, were sprayed on L_3 larvae, which were simultaneously fed with inoculated plant discs. Colonized discs with the EABb 01/33-Su strain caused total larval mortality rates ranging between 25.0% and 46.7%, and the larval mortality rates of the insects fed leaves colonized with the EAMb 09/01-Su strain ranged between 10.0% and 38.3%. In addition, none of the larvae fed with colonized discs showed fungal outgrowth, whereas destruxin A (dtx A), with a value of 0.004 ppb dtx A as the mean value of all samples, was found in *S. littoralis* larvae after 72 h of ingestion of endophytically colonized tomato leaves with the strain EAMb 09/01-Su; dtx A was also detected in tomato and melon leaves at 0.007 and 0.0011 ppb dtx A, respectively.

Both fungal strains and host plants significantly influenced larval mortality ($F_{20,125} = 36.75$; $P \leq 0.001$), and significant differences were noted among the treatments for each strain and host plant (Table 3). However, no significant differences were detected among the spray application and the combined treatments (spray application + feeding on colonized plant), which were additive for the two strains and three host plants (Table 3). When a parallelism test was performed, the mortality data caused by the EABb 01/33-Su strain and each plant species were represented by two parallel probit regression lines, which allowed the detection of significant differences in the relative potencies of the six treatments with both methods (individual and combined treatment). The relative potencies of the combined treatments were calculated with respect to the LC_{50} values of the spray application treatments,

which were 31.8 and 3.2 for the treatments in which larvae were fed with alfalfa and tomato plants, respectively. However, the relative potency of the spray application treatment when larvae were fed with melon plants was 1.1 with respect to the LC_{50} value of the combined treatment. The parallelism test could not be performed with the mortality data produced by the EAMb 09/01-Su because these data were lower than 50% in the spray application treatment. The highest mortality values and lowest AST combinations were observed in larvae simultaneously treated with the EABb 01/33-Su strain and fed with colonized alfalfa (80.0% mortality and 7.2 days), tomato (76.7% mortality and 6.1 days), and melon (75.0% mortality and 7.7 days) plants (Table 3). For all plant species, the larvae exposed to all fungal treatments showed decreases in their weights (mg) compared with the controls (Figure 3) but did not show significant differences.

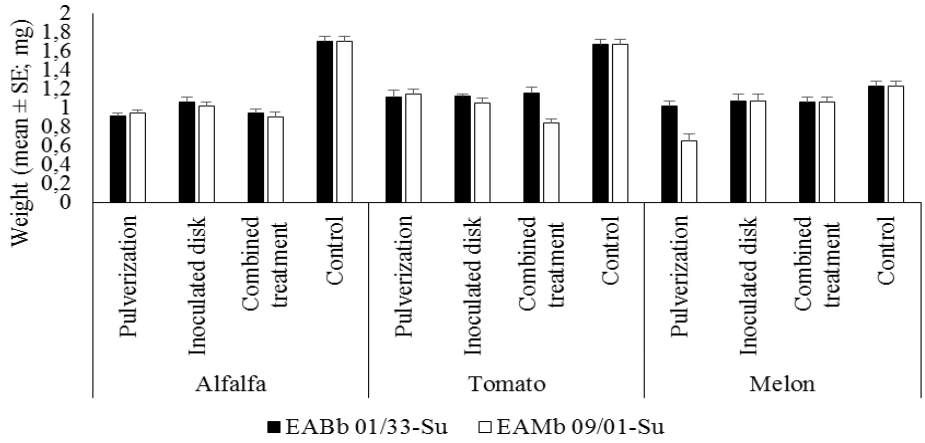

Figure 3. Weight (mg) of *Spodoptera littoralis* larvae treated with EABb 01/33-Su *Beauveria bassiana* and EAMb 09/01-Su *Metarhizium brunneum* strains at $1.0x10^8$ conidia ml^{-1}, after ingestion of colonized plants, and applying the combined treatment of both after 10 days of treatment.

Table 3. Susceptibility of third instars larvae *Spodoptera littoralis* to EABb 01/33-Su and EAMb 09/01-Su strains after treatment with $1.0x10^8$ conidia ml^{-1} suspensions, offering disks of alfalfa, tomato and melon endophytically colonized for 24h and the combination of both.

Host plant	Isolate	Treatment	Mortality (mean ± SE) (%)[1]			Survival Analysis– Kaplan Meier		Response to combined treatment***			
			Mean ± SE	Fungal outgrowth	Other causes[2]	AST[3] (mean ± SE; d)	CI (95%)	Expected mortality	$\chi^2_{calculated}$ (gl^c=1))	$\chi^2_{table\ value}$	Effect on larvae
Alfalfa	EABb 01/33-Su	Pulverization	76.6 ± 4.2A	61.6 ±4.2	15.0±5.8	8.3 ± 0.4 B	(7.5;9.1)				
		Inoculated disk	25.0±5.0B	0±0	25.0±5.0	10.9 ± 0.2 C	(10.3;11.4)				
		Combined treatment	80.0 ± 5.8A	64.8±5.8	15.2±5.8	7.2 ± 0.6 A	(5.9;8.5)				
		Control	0±0C	0±0	0±0	12.0 ± 0 D	(0;0)	82.5	0.1	3.84**	Additive
	EAMb 09/01-Su	Pulverization	41.7 ± 4.8b	41.7±4.8	0±0	9.8 ± 0.4 b	(9.0;10.5)				
		Inoculated disk	31.7 ± 7.5c	0±0	31.7±7.5	11.1 ± 0.3 c	(10.5;11.7)				
		Combined treatment	60.0 ± 0.0a	60.0±0	0±0	7.1 ± 0.7 a	(5.7;8.5)				
		Control	0 ± 0d	0±0	0±0	12.0±0 d	(0;0)	60.1	0.001	3.84**	Additive
Tomato	EABb 01/33-Su	Pulverization	66.7±5.8A'	56.7±5.8	10.0±0	6.3±0.3 B'	(5.9;6.8)				
		Inoculated disk	46.7±5.8B'	0±0	46.7±5.8	9.1±0.3 C'	(8.6;9.6)				
		Combined treatment	76.7±5.8A'	66.7±7.2	10.0±0	6.1±0.2 A'	(5.6;6.5)				
		Control	0±0C'	0±0	0±0	12.0±0 D'	(0;0)	76.8	1.4	3.84**	Additive
	EAMb 09/01-Su	Pulverization	33.3±5.8a'	23.4±5.8	10.0±0	8.6±0.3 b'	(8.0;9.2)				
		Inoculated disk	10.0±4.3b'	0±0	10.0±10	9.4±0.3 c'	(8.9;9.9)				
		Combined treatment	50.0±4.3a'	36.7±7.2	13.3±5.8	7.0±0.3 a'	(6.6;7.4)				
		Control	0±0b'	0±0	0±0	12.0±0 d'	(0;0.0)	40.0	2.5	3.84**	Additive
Melon	EABb 01/33-Su	Pulverization	71.7±4.0A''	66.7±3.3	5.0±2.2	7.8±0.2 B''	(7.4;8.1)				
		Inoculated disk	35.0±4.3B''	0±0	35.0±4.3	8.3±0.2 C''	(7.9;8.6)				
		Combined treatment	75.0±5.8A''	68.3±4.0	6.7±3.3	7.7±0.2 A''	(7.4;8.0)				
		Control	0±0C''	0±0	0±0	12.0±0 D''	(0;0)	81.5	0.5	3.84**	Additive
	EAMb 09/01-Su	Pulverization	48.3±6.0a''	31.6±7.0	16.7±4.9	8.1±0.4 b''	(7.3;8.8)				
		Inoculated disk	38.3±4.3a''	0±0	38.3±4.3	8.4±0.2 c''	(7.9;8.8)				
		Combined treatment	55.0±8.1a''	48.3±4.0	6.7±4.2	6.6±4.2 a''	(7.1;8.0)				
		Control	0±0b''	0±0	0±0	12.0±0 d''	(0;0)	78.1	2.5	3.84**	Additive

[1] Mean ± SE: means of mortality rates followed by the same letter (upper-case, upper-case with the quotation marks, upper-case with the double quotation marks, lower-case, lower-case with the quotation marks, and lower-case with the double quotation marks for each plant species and strains EABb 01/33-Su and EAMb 09/01-Su) do not differ significantly according to the test LSD ($P<0.001$). [2] Destruxin A was detected in larvae fed with tomato plants inoculated with the EAMb 09/01-Su isolate. [3]AST: Average survival time. Mean time limited is survival 12 days.

$\chi^2_{table\ value}$: * p>0.001, ** p>0.05

$D = (M_{HCE} - M_E) > 0$ a significant synergistic interact and $D < 0$ was negative, the interaction was considered to be antagonistic

4. DISCUSSION

Most of the fungal products on the market are recommended for use in spraying onto crops to control pests. On the other hand, the aerial applications may produce a temporary endophytic establishment in the plant that could gradually decrease until the endophyte disappears completely (Gurulingappa et al., 2010). This behaviour could be an important issue for registration purposes. The combined actions of topical application and ingestion of EF would improve the overall efficacy of the mycoinsecticide. And therefore, partially overcome the two main constraints for the commercial development of mycoinsecticides, dosage selection and effects on non-target organisms (Butt et al., 2001).

All fungal strains used in this work, including three *B. bassiana* strains and one *M. brunneum* strain, were able to endophytically colonize the leaves, stems, and roots of alfalfa, tomato, and melon plants temporarily when fungal suspensions were sprayed onto the leaves. Foliar inoculation yielded a percentage of colonization in the leaves of approximately 40%, which was constant for the evaluation period in the three crops, and up to 30% in the colonization of the roots 96 h after foliar inoculation. Previous studies have reported the successful endophytic colonization of several plants using different methods of inoculation such as foliar spraying, soil drenching, or seed dressing (Quesada-Moraga et al., 2009; Tefera and Vidal, 2009; Gurulingappa et al., 2010; Biswas et al., 2012). However, in none of these cases did foliar application of the inoculum result in the colonization of the entire plant, including the roots, as in the current work. Transient colonization of rice tissues after foliar application of *B. bassiana* has been reported, attributing this transience to some type of antagonism between the

fungus and the plant or the content of the aerenchyma tissue inside the plant (Jia et al., 2013).

In recent decades, research on the endophytic behaviour of EF when EF is sprayed onto leaves has focused on *Beauveria*, *Lecanicillium*, and *Fusarium* (Tefera and Vidal, 2009; Gurulingappa et al., 2010), with only Batta (2013) reporting the ability of *Metarhizium* to endophytically colonize plants through this inoculation method. Meyling et al. (2011), reported that fungal genera show differences regarding above- or below-ground infection, as well as when they behave as endophytes, in which case *Beauveria* and *Metarhizium* genera were able to control herbivorous insects in the aerial parts of plants and phytophagous pests in the below-ground part of plants, respectively (Cherry et al., 2004; Akello et al., 2008; Quesada-Moraga et al., 2009; Behie et al., 2015). In our work, the *Metarhizium* strain was able to colonize all plant tissues (leaves, stems, and roots) with foliar inoculation, whereas Behie et al. (2015) found the fungus colonizing stems and roots after the inoculation of soil but not the leaves. *Metarhizium* is considered a naturally rhizosphere-competent fungus, and the soil exploits its maximum potential for pest control (Meyling et al., 2011). Indeed, *Metarhizium* has been reported colonizing cortical cells of roots but does not move throughout the plant (Gurulingappa et al., 2010; Sasan and Bidochka, 2012; Jaber, 2015). Nonetheless, our work agreed with the work of Batta (2013), who reported the ability of this fungus to colonize the aerial parts of plants after spraying with a fungal suspension, conferring protection against chewing insects. On the other hand, *Beauveria* is able to colonize plants in an apoplastic way, using apoplastic fluid as a growth medium (Schulz et al., 2002; Landa et al., 2013); therefore *Beauveria* is more commonly found above ground (Meyling et al., 2011).

Of the four tested strains, EABb 01/33-Su caused higher beet armyworm larvae mortality (95.0%) and reduced the average survival time

(7.2 days) of these larvae after spray application. This strain also behaved as a good endophyte, with the mortality of the larvae feeding on the colonized disc of the three crops ranging between 25.0% and 46.7%, whereas the mortality caused by the ingestion of EAMb 09/01-Su colonized discs of the three crops ranged between 10.0% and 38.3%. In previous investigations, the ingestion of endophytically colonized plants has shown varying results in pest control, even if the use of these plants is not yet widespread. Sometimes, the ingestion did not cause insect death but reduced insect fitness (Gurulingappa et al., 2010), whereas high mortality percentages were reported in other experiments (Arab and El-Deeb, 2012; Batta, 2013). For the two strains used in our work to inoculate the leaf discs, none of the larvae that fed on them and were killed showed fungal outgrowth, although fungal metabolites were detected in 11% of the *S. littoralis* cadavers of the larvae fed with plants treated with *M. brunneum* and implicated in their deaths. Metabolites of larvae fed plants treated with *B. bassiana* were not considered due to the absence of similar methods to extract and detect the metabolites secreted by this genus in insects (beauvericin, oosporein, and tenellin). This percentage could have been even greater during the first hours of the experiment before the conversions of dtx A into dtx E and dtx E into dtx E-diol; the activity of the insect enzyme may have reduced the destruxin content up to 10 times within the three hours following acquisition (Skrobek et al., 2008). The destruxin secretion profile is influenced by the host and enables the fungi to kill the insect before body colonization (Amiri-Besheli et al., 2000). Many studies have been published in which destruxin was detected either in insects or in plants (Sulyok et al., 2006; Sree and Padmaja, 2008; Carpio et al., 2016; Taibon et al., 2015), but this study was the first to report destruxin traces in insects fed EF-colonized plants; further investigations are needed to determine the persistence of this compound in the environment.

Combined treatments of fungal suspensions and colonized discs produced an additive effect as shown by the χ^2 test and the potency ratios of the parallelism tests of the PROBIT regression lines. Considering the weight reductions detected in the insects feeding on EF- colonized disc, it could be argued that the insects were weakened by the endophyte and its metabolites, making them more susceptible to fungal infection, which also could reduce their mobility and hinder feeding (Schrank and Vainstein, 2010; Kaur et al., 2015). These steady decreases in the weights of larvae feeding on EF-colonized discs has been previously reported for *Helicoverpa armigera* larvae fed diets supplemented with *B. bassiana* and its crude extract (Leckie et al., 2008). Finally, for all treatments, the larvae fed with alfalfa leaves tended to show lower weights, although no significant differences were detected at the 5% significance level. Previous studies that addressed the influence of the host plants on the susceptibility of insects to fungal infection showed that *S. littoralis* larvae exhibited the same susceptibility to *Nomurea rileyi* fed with cotton, cabbage, eggplant, or beans (Fargues and Maniania, 1992) or *Bemisia tabaci* nymphs fed with 10 different crops (Santiago-Álvarez et al., 2006). In these cases, it was hypothesized that secondary compounds of plant origin found in several botanical genera may possess fungicidal or fungistatic properties and are therefore more or less adapted for fungal virulence. Besides, those secondary compounds are produced by the fungus inside the plant or by the plant in response to the endophytic colonization with effects on the insect fitness or survival (Thakur et al., 2013). On the other hand, a nutritional stress of the plant enhances the efficacy of the entomopathogenic fungi against the insect (Zibaee et al., 2013).

This work shows that spray applications of conidial suspensions of *B. bassiana* and *M. brunneum* targeting the chewing insect pest *S. littoralis* in alfalfa, tomato and melon plants can be accompanied by the transient

endophytic colonization of plant tissues. In the current work is show how temporal colonization may cause an additional larval mortality rate, although not higher that 40%, and also produce additivity to the mortality caused by spray application. The combined effects of the fungal spray and the mortality caused by the feeding of insects on transiently EF-colonized tissues should be considered for estimating the real acute impact of EF field sprays.

5. ACKNOWLEDGEMENTS

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CAPÍTULO IV

Este capítulo es una versión adaptada del artículo: Transient endophytic colonization of melon plants by entomopathogenic fungi after foliar application for the control of *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), que se encuentra aceptada en la revista Journal of Pest Science en el quartil Q1 del área de Entomología (posición 8 de 92) del JCR

Date: 09 Apr 2016
To: "Enrique Quesada-Moraga" equesada@uco.es
From: "Journal of Pest Science (PEST)" Kiruthiga.Jagadeesan@springer.com
Subject: Your Submission PEST-D-15-00509R2

Dear Dr. Quesada-Moraga,

We are pleased to inform you that your manuscript, "Transient endophytic colonization of melon plants by entomopathogenic fungi after foliar application for the control of Bemisia tabaci Gennadius (Hemiptera: Aleyrodidae)", has been accepted for publication in
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Transient endophytic colonization of melon plants by entomopathogenic fungi after foliar application for the control of *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae)

Garrido-Jurado, I.[1†], Resquín-Romero, G.[1,2†], Amarilla, S.P.[3], Ríos-Moreno, A.[1], Carrasco, L.[3], Quesada-Moraga, E[1]*

[1]Department of Agricultural and Forestry Sciences, ETSIAM, University of Cordoba. Campus de Rabanales. Edificio C4 Celestino Mutis. 14071 Cordoba, Spain.

[2]Faculty of Agrarian Sciences of the National University of Asunción, Paraguay.

[3]Department of Anatomy and Comparative Pathology, Faculty of Veterinary Medicine, Campus Universitario de Rabanales, International Excellence Agrifood Campus, CeiA3, 14071 Cordoba, Spain.

†Both authors contributed equally to this work.

*corresponding author: tel.: + 34-957218475; fax: +34-957218440. E-mail address: equesada@uco.es

ABSTRACT

Three *Beauveria bassiana* and two *Metarhizium brunneum* strains, two of which GFP-transformed, were evaluated for virulence against fourth-instar nymphs of the sweet potato whitefly *Bemisia tabaci* (*B. tabaci* nymphs,BTN), with the following results. (1) Immersion of infested melon leaves in suspensions ranging from 10^5 to 10^8conidia ml^{-1} yielded an LC_{50} ranging between $3.4x10^4$ and $2.2x10^7$conidia ml^{-1}. (2)Temporal colonization of the leaf tissues infested with BTN 96 h after inoculation was observed in both sprayed leaves (SL) (ranging between 98.0–40.0%) and leaves of the same plant that were not directly exposed to the fungal treatment (i.e.,leaves that were not sprayed (LNS)) (62.0–12.0%), Total nymphal mortality ranged between 83.9 and 100.0% and between 66.3 and 87.9% in SL and LNS, respectively. Interestingly, from 0 to 16% nymph cadavers from LNS showed fungal outgrowth. (3)The adaxial surfaces of infested melon leaves were brushed with 10^8conidia ml^{-1} suspensions to ascertain the translaminar fate of the fungi in the leaves that resulted in nymphal mortality, which ranged from 53.4 to 96.0%. As before, mortality with fungal outgrowth was detected only in the *B. bassiana* treatments as a result of the different leaf colonization patterns of the two fungal species revealed by histological examination. Destruxin A was present in 43.0% of the melanized nymphs on the leaves treated on their adaxial surfaces with the *M. brunneum* isolate. The effect of transiently colonized leaves must be considered to estimate the true acute impact of field sprays containing entomopathogenic fungi on *B. tabaci* and other sucking insect pests.

1. INTRODUCTION

More than 700 species of entomopathogenic fungi from the fungal taxons Entomophthoromycota and Ascomycota has been described as pathogens of insects (Hibbett, 2007; Humber, 2012; Gryganskyi et al., 2012), which infection occurs through the tegument and have distinct advantages in controlling certain pests. The infection process begins with adhesion of the conidia to the insect cuticle. This process does not require any ingestion by insects; thus, these fungi serve as an alternative for controlling insects with cryptic lifestyles or feeding habits (Quesada-Moraga et al., 2014a).

Over the past few decades, European authorities have considerably restricted allowable residue levels and active ingredients of pesticides because of increasing concerns over their impacts on health and the environment (Ravensberg, 2015). This restriction has promoted an increase in the number of microbial insecticides available commercially, although they currently comprise only 3% of all commercialized pesticides (Ravensberg, 2015). Most of the commercial mycoinsecticides are based on *Beauveria bassiana* Bals. (Vuill), *Lecanicillium muscarium* (Petch) Zare & W. Gams and *Isaria fumosorosea* (Wize) and are primarily recommended to control whiteflies, thrips and certain other soft-bodied insects in greenhouse crops (Ravensberg, 2015), as conditions inside greenhouses are favorable for infection. However, many of these products have shown only moderate efficacy against immature sucking insect pests due to their short intermolt periods, and it is estimated that only 0.02–0.03% of foliar-applied insecticides target sucking insects (Bateman and Chapple, 2001; Jandricic et al., 2014). On the other hand, when using these mycoinsecticides against aphids or whiteflies, the presence of a pest complex that leads to outbreaks of more than one species must be considered (Jandricic et al., 2014). Therefore,

the development of a mycoinsecticide that can persist on plants for several days after a spray application would be beneficial for controlling pest complexes.

Entomopathogenic fungi are usually applied using an inundative approach, although their role as endophytes has also been exploited to control insect pests in recent years (Vega et al., 2009). Endophytes are useful control agents because they exhibit enhanced persistence in the plant, and use of endophytes allows the total quantity of applied fungi to be reduced (Quesada-Moraga et al., 2014b; Vega, 2008; Vega et al., 2008). In particular, endophytic colonization with EF confers systemic protection against whiteflies and can have negative effects on their development because of the chemical changes induced in the plant by the endophyte or the secondary metabolites secreted by the fungus (Akello and Sikora, 2012; Castillo Lopez et al., 2014; Gurulingappa et al., 2010). However, not all fungi are able to colonize plants—at least, not permanently—because they first need to adapt to the nutrient content inside the plant (Mercado-Blanco and Lugtenberg, 2014). Nevertheless, fungal/plant associations can persist for a few days after a spray application because of the propagule abundance on the leaves, resulting in a transient relationship between the plant host and the fungi (Arnold and Herre, 2003). The current work aims to ascertain whether temporal colonization can occur as result of foliar application of EF and whether this colonization can successfully improve foliar applications used for the control of whiteflies. The sweet potato whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), one of the world's most prevalent pests, was used in the current study, and the infection process was documented.

2. MATERIALS AND METHODS

2.1 Insects

2.1.1 *Bemisia tabaci* (Hemiptera: Aleyrodidae) culture.

A *Bemisia tabaci* Q biotype population was obtained from the Institute of Mediterranean and Subtropical Horticulture (IHS) "La Mayora" in Malaga (Spain). Whiteflies were mass-reared using the methodology of Quesada-Moraga et al. (2006). Briefly, whiteflies were reared on melon plants (*Cucumis melo* L., cv. Galia) in screened cages (40x40x30 cm). Plants at the five-leaf stage were selected and placed individually into cylindrical cages (diameter: 14 cm, height: 24 cm) with an organdy sleeve (300-µm mesh) and infested with 100 newly molted-adult whiteflies (sex ratio 1:1). Whitefly adults were allowed to lay eggs in 48 h, resulting in at least 50–100 eggs/leaf. They were maintained in an environmental chamber set at 26±2ºC, 70±5% RH, with a photoperiod of 16:8 (L:D) h until their use in bioassays.

2.2 Strains and fungal suspensions

Three *Beauveria bassiana* strains (Bb04, EABb 01/33-Su, and EABb 04/01-Tip) and two *Metarhizium brunneum* strains (EAMb 09/01-Su and EAMa 01/58-Su) belonging to the culture collection from the Department of Agricultural and Forestry Sciences (AFS) of the University of Córdoba (Table 1) were used in this study. Two (EABb 01/33-Su and EAMa 01/58-Su) were transformed with green fluorescent protein (GFP) and subcultured on malt agar (MA) supplied with 500 mg l^{-1} streptomycin sulfate salt (Sigma-Aldrich Chemie, China) for 15 d at 25°C in darkness. The Petri plates were sealed with Parafilm® (Pechiney Plastic Packaging Co, Chicago, IL).

The two strains were transformed with GFP following the efficient Agrobacterium-mediated transformation method described by Fang et al.

(2004). To check for the successful expression of GFP, single-spore cultures of transformants were examined with a confocal laser microscope (Nikon TE 2000-S, Melville, NY, USA). One out of nine transformants showing the appropriate fluorescence emission and stability was selected to carry out the bioassay. Monosporic cultures of the wild-type and GFP-transformed strains were grown on slants of malt agar (MA; Biocult, Madrid, Spain) at 25ºC in the dark and stored at 4ºC.

Table 1: Fungal strains used in this study

Strains	Fungal species	Origin	Ecology	Habitat
EABb 04/01-Tip	*B. bassiana*	Ecija (Sevilla, Spain)	*Iraella luteipes*	Insect
EABb 01/33-Su*	*B. bassiana*	El Bosque (Cádiz, Spain)	Traditional olive orchard	Soil
Bb04**	*B. bassiana*	Ethiopia	*Brusseola fusca*	Insect
EAMb 09/01-Su	*M. brunneum*	Hinojosa del Duque (Córdoba, Spain)	Wheat crop	Soil
EAMa 01/58-Su*	*M. brunneum*	Hinojosa del Duque (Córdoba, Spain)	Wheat crop	Soil

* GFP-transformed strain
** Strain assigned by Prof. Stefan Vidal of the Georg-August-Universität Göttingen in Germany

Conidial suspensions were prepared by scraping conidia from the Petri plates into a sterile aqueous solution of 0.1% Tween 80, which was then filtered through a piece of cheesecloth. The viability of the conidia was validated by germination on MA supplied with 500 mg l^{-1} streptomycin sulfate salt (Sigma-Aldrich Chemie, China). The conidial suspensions used for the inoculation bioassays were adjusted by diluting them with 0.1% (v/v) Tween 80 to a final concentration of 1.0×10^{8} conidia ml^{-1} using a Malassez chamber (Quesada-Moraga et al., 2006).

2.3 Substrate and preparation of plant material

Ten grams of melon seeds (Galia variety) were disinfected by immersion in 70% (v/v) ethanol for 2 min, followed by 2% NaOCl (Sigma-Aldrich, MO, USA) for 5 min. The samples were then rinsed with sterilized water three times. Lastly, they were immersed in 70% ethanol (v/v) for 1 min

and dried under a sterile airflow. Water used for the last rinse was plated to assess the effectiveness of the described surface disinfection procedure. Seeds were kept overnight at 4°C and were transferred into plastic pots with 110 wells (25x25x25 mm) containing a substrate based on washed sand. The substrate was sterilized in an autoclave for 45 min at 121°C three times with a 24 h interval between each sterilization process and allowed to cool down before planting for 24 h. One seed per pot was sowed and maintained at a temperature of 27 ± 3°C with 13 h of daylight (light intensity of 700 lux) and 11 h of darkness. Plants were transferred to individual pots (70x70x60 mm) containing 300 g of sterilized substrate and kept under greenhouse conditions (26±5°C, 14:10 h photoperiod) until use. Fertilization was performed weekly using 20 ml of nutrient solution (Hoagland and Arnon, 1950).

2.4 Virulence of entomopathogenic fungi on nymphs of *Bemisia tabaci*

Melon leaf discs (30 mm diameter) infested with 30–50 fourth instar nymphs were selected per replicate, immersed in 5 ml of 10^8 conidia ml^{-1} fungal suspension for 30 seconds and then individually placed in a Petri plate with 5% water agar as a culture medium to provide moisture under insectarium conditions (26±2ºC, 70±5% RH) for a week. For the control, nymphs were immersed in sterile water with 0.10% Tween 80. The nymphs' mortality was recorded every 24 hours for 12 days. The surfaces of the discs with dead nymphs were immediately sterilized at the end of the experiment with 1% NaOCl for 1 min, followed by three rinses with sterile water. The discs were placed on sterile wet filter paper in sterile Petri plates with 5% water agar, sealed with Parafilm® and kept at room temperature (25 ± 2°C) prior to inspection for fungal outgrowth on cadavers. After five days, the observation of fungal outgrowth on the surface of the insect cuticle was performed with a light microscope (Nikon, Japan). The entire experiment was

repeated twice using a new batch of whitefly nymphs and new fungal inocula each time.

The same *B. bassiana* strains (Bb04, EABb 01/33-Su, and EABb 04/01-Tip) and the two *M. brunneum* strains (EAMb 09/01-Su and EAMa 01/58-Su) were used for the virulence studies against third instar whitefly nymphs. The conidial suspension was prepared by mixing the conidia with a sterile aqueous solution of 0.1% (v/v) Tween 80, and the conidial concentration was adjusted from 10^5 to 10^8 conidia ml^{-1}, as determined using a Malassez hemocytometer.

The leaf discs with nymphs were directly immersed in groups into 5 ml of the various conidial suspensions for 30 seconds. A control was used consisting of sterile 0.1% (v/v) Tween 80 aqueous solution. Five replicates with 30–50 nymphs were used for each concentration. Nymph mortality was recorded for each treatment at 24, 48, 72, and 96 hours. Dead nymphs were inspected as described above.

2.5. Effects of spray application of fungal suspensions on *Bemisia tabaci* in plant

Five melon plants infested with 30–50 BTN per leaf were selected to evaluate BTN mortality in leaves sprayed with fungal suspensions (10^8 conidia ml^{-1}) of the five isolates and in leaves of the same plant that were not exposed to the fungal treatment. Leaves were sprayed with 1 ml of the fungal suspensions with an aerograph 27085 (piston compressor of 23 l/min, 15–50 PSI, nozzle diameter of 0.3 mm, China). During the application, some infested leaves were not inoculated; these leaves were separated from the plant during the treatment by placing on polystyrene plates covered with aluminum foil (Online Resource 1). Moreover, the soil was covered with aluminum foil to prevent run-off of the suspension. Then, the foliar area was covered with a transparent plastic sheet to promote fungal growth for 24 h.

Control plants were sprayed with sterile water with 0.10% Tween 80. Five infested plants at the 10-true-leaf stage were used per strain. Five leaves were sprayed, and five leaves were not sprayed. The experiment was repeated twice. Nymphal mortality was recorded daily over a 96-hour period. Dead nymphs were inspected as described in section 2.4. Fungal outgrowth of cadavers on sprayed leaves and leaves of the same plant that were not exposed to the fungal treatment was observed using a Zeiss fluorescence microscope.

Online Resource 1. Treated leaves with fungal suspension separated from the plant during the treatment by placing on polystyrene plates covered with aluminum foil

2.5.1 Short-term colonization of the plant by entomopathogenic fungi

The five fungal strains were sprayed onto five new melon plants per evaluated period to determine whether they were able to temporally colonize the plants for at least 96 h. Evaluations were carried out at 24, 48, 72, and 96 h after the inoculation. To determine the colonization percentage, the

inoculated plants were carefully removed from the pots and then washed with water for 10 min to remove any attached soil. This was followed by a wash with 70% ethanol (v/v) for 2 min, a wash with sterilized water, a wash with an aqueous solution of 2% NaOCl for 5 min, three washes with sterilized water, and a wash with 70% ethanol (v/v) for 1 min, all of which were performed under sterile air-flow. The water used for the last rinse was plated out on plates containing selective culture medium [20 g of Agar Sabouraud Glucose Chloramphenicol (Cultimed Panreac, Spain), 500 mg l-1 streptomycin sulfate (Sigma-Aldrich Chemie, China), 500 mg l^{-1} ampicillin (Intron Biotechnology, China) and 500 mg l^{-1} dodine 65 WP (Barcelona, Spain)] to assess the effectiveness of the surface disinfection procedure.

Subsequently, 10 fragments of 5x5 mm and 7–8 mm diameter were randomly taken from the leaves, placed on the selective medium and gently pressed down to ensure full contact. Leaf colonization was determined by counting the number of fragments that showed fungal outgrowth in the 5 replicates per inoculated fungal strain. The samples were kept in an incubator at 25°C. The results from the inoculated and non-inoculated fragments and control plants were obtained after 12 days.

2.6 Effects of the translaminar growth of entomopathogenic fungi of *Bemisia tabaci* across melon leaves

In order to elucidate if non sprayed nymphs can be infected by the fungi during the temporal colonization of the leaves after spraying, five infested plants were brushed onto the adaxial leaf surfaces with fungal suspensions (10^8 conidia ml^{-1}) of the EABb 01/33-Su and EAMb 09/01-Su isolates while the abaxial surfaces were covered with aluminum foil to protect them against any treatment overflow. A similar set of control plants were sprayed with sterile water with Tween 80%. The experiment was repeated twice. The spore germination and mycelial growth of *Beauveria* and

Metarhizium from each part of the plant were determined in vitro using the culture medium described in section 2.5.1. and evaluated every 24 h. In addition, nymphal mortality was recorded daily over 96 hours. Dead nymphs were inspected as described in section 2.4.

2.6.1 Histological studies of plant and insect penetration and colonization by endophytic fungi

Fungal suspensions of the strains EAMb 09/01-Su of *M. brunneum* and EABb 01/33-Su of *B. bassiana* were brushed onto the adaxial surfaces of melon leaves. Treated and non-treated leaves containing whitefly populations were selected after 24 hours, and their surfaces were disinfested as described in section 2.4. Three small sections of leaf per treatment were randomly selected and fixed by immersion in 10% neutral buffered formalin (Fisher Scientific Ltd., Leicestershire, UK) for 24 h and then processed for histological analysis by embedding in paraffin. Samples were cut at a thickness of 3 µm with a microtome, stained with Periodic Acid-Schiff (PAS), and examined under a light microscope. Identification of the different tissues was performed according to Landa et al. (2013).

2.6.2 Extraction and identification of metabolites in infected nymphs and colonized plants

The melon leaves brushed with the strain EAMb 09/01-Su of *M. brunneum* were processed following Carpio et al. (2015) with modifications to determine the destruxin A (dtx A) content. Briefly, 1.5 g of the lyophilized leaves of the plants treated with the EAMb 09/01-Su isolate (described in section 2.6) were added to 50 ml of sterilized water and centrifuged at 27000 g for 20 min. The supernatant was discarded. Then, the same volume of ethyl acetate was added and evaporated. Finally, 0.5 ml of acetonitrile: water (1:1) was added. The samples were stored at -4ºC.

Moreover, we investigated the presence of dtx A in whitefly nymphs placed on the treated leaves using the methodology proposed by Skrobek et al. (2008) and Carpio et al. (2015). Briefly, dead nymphs were triturated and immersed for 2.5 h in 150 ml of ethyl acetate (EtOAc): dichloromethane (CH_2Cl_2) (1:1, v/v) on an orbital shaker (100 rpm) at room temperature and for a further 30 min in an ultrasonic bath.

The solvent extract was filtered through Whatman No. 1 filter paper and evaporated in a flow chamber. Crude extracts were re-dissolved in methanol (MeOH): acetonitrile (MeCN) (1:1, v/v) and then stored at –20ºC until analysis.

2.7 Statistical Analysis

All mortality data obtained from the experiments were subjected to analysis of variance, and the LSD test was used to compare means. The total percentage of colonization was transformed as follows:

$$\text{Arcsin} = 180 * \left[\text{Arcsin}\left(\frac{\text{mortality}}{100}\right)^{0.5}\right] / \text{Pi}$$

The median lethal concentration (CL_{50}) values were estimated by probit analysis (Finney, 1971). Probit parallelism test regression lines for all assayed strains were constructed using $\chi 2$ goodness-of-fit tests. Relative median potencies and their 95% confidence intervals were calculated for different treatments when their slopes did not differ significantly (Finney, 1971). Relative potency is a statistical parameter generated from probit analysis based on a comparison of mortality responses at range of fungal suspension doses between the fungal isolates. Statistical analyses were

performed using Statistix 9.0 (Analytical Software, 2008) and SPSS 19.0 for Windows (IBM Company, 2010).

3. RESULTS

3.1 Virulence of entomopathogenic fungi on nymphs of *Bemisia tabaci*

The fungal treatment had a significant effect on nymph mortality ($F_{(5,47)}$=114.3, P<0.001). Mortality varied from 70 to 92% when nymphs were treated with the fungal suspensions compared with the control nymphs, which exhibited a mortality rate of 0% (Figure 1). The most virulent strain was EABb 01/33-Su, which caused 92.25% mortality and was significantly different from the other strains.

Concentration-mortality response regression analysis for each strain was performed using the four concentrations of conidia (Table 2). The five strains showed significant differences in virulence according to the confidence interval of their relative potencies.

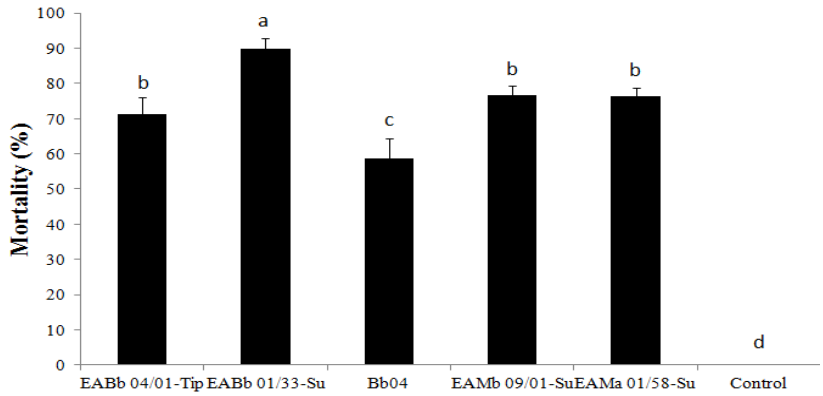


Figure 1. Relative percentages of dead *B. tabaci* nymphs showing fungal outgrowth after immersion in suspensions ($1.0x10^8$ conidia ml^{-1}) of three *B. bassiana* (Bb04, EABb 04/01-Tip, EABb 01/33-Su) and two *M. brunneum* (EAMb 09/01-Su and EAMa 01/58-Su) strains. Data are presented as the mean ± standard error.

The strain EABb 01/33-Su was the most virulent, with an LC_{50} value of $3.4x10^4$ conidia ml^{-1} (Table 2). The relative potencies of the treatments with the strains EABb 01/33-Su, EAMb 09/01-Su, EAMa 01/58-Su and EABb 04/01-Su with respect to the treatment with Bb04 were 9391, 233, 134 and 84, respectively.

Table 2. Probit analysis of log-dose mortality response of bioassay by immersion with two isolates of *Beauveria bassiana* and *Metarhizium brunneum* against nymphs of *Bemisia tabaci*

Strains	Regression equation	Se[1]	χ^2 (2g.d.l)	LC_{50} (conidia ml^{-1})	Confidence Interval (CI 95%, conidia ml^{-1})		Relative potency	CI (95%)	
					lower	upper		lower	upper
EABb04/01-Tip	y= 0.4x-2.6	0.05	1.74	$7.9x10^5$	$3.6x10^5$	$1.5x10^5$	84	6	2732
EABb01/33-Su	y= 0.3x+3.9	0.06	3.13	$3.4x10^4$	$1.1x10^2$	$1.9x10^4$	9391	359	1441239
Bb04	y= 0.6x-0.7	0.07	0.77	$2.2x10^7$	$1.3x10^7$	$4.4x10^7$	1	-	-
EAMb09/01-Su	y= 0.3x+3.5	0.04	29.98	$1.4x10^5$	-	-	233	17	9174
EAMa01/58-Su	y= 0.3x+3.5	0.05	6.67	$7.1x10^6$	-	-	134	10	4680

[1]Slope error

3.2. Short-term colonization of the plant by entomopathogenic fungi

The five strains colonized more that 40% of the leaves treated with the fungal suspensions over the 96 h period (Figure 2). Non-treated leaves also showed fungal colonization, ranging from 10 to 60%. The three *Beauveria* strains colonized a higher percentage of non-inoculated leaves than the two *Metarhizium* strains. Leaves inoculated with the strain EAMa 01/58-Su showed the lowest percentages—between 40 and 60%.

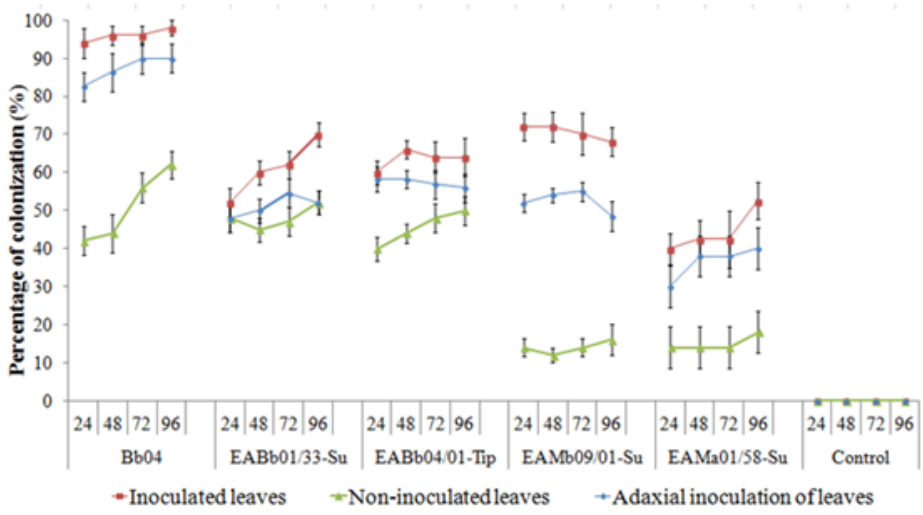


Figure 2. Progression of endophytic colonization in melon plants after foliar application with suspensions ($1.0x10^8$ conidia ml^{-1}) of three *B. bassiana* (Bb04, EABb 04/01-Tip, EABb 01/33-Su) and two *M. brunneum* (EAMb 09/01-Su and EAMa 01/58-Su) strains. The data are presented as the mean ± SE of fragments from sprayed leaves, leaves of the same plant that were not exposed to the fungal treatment and leaves inoculated on the adaxial surface. Leaves were collected 24 h, 48 h, 72 h and 96 h after inoculation.

On the other hand, leaves inoculated on their adaxial surfaces showed colonization percentages ranging between 30 and 90% and followed the same trend as leaves inoculated with the normal foliar application.

3.3. Effects of spray application of fungal suspensions on *Bemisia tabaci* in plant

The fungal strains ($F_{(6,69)}$ = 739, P < 0.001) had a significant effect on total nymph mortality in the sprayed leaves, ranging from 84% (EAMa 01/58-Su) to 100% (EABb 04/01-Tip and EAMb 09/01-Su). The lowest average survival times (ASTs) for strains EABb 01/33-Su and EABb 04/01-Tip of *B. bassiana* were 76 hours. Moreover, the fungal strains ($_{F(6,69)}$ = 151, P < 0.001) had a significant effect on the mortality of nymphs that showed fungal outgrowth. In this case, the mortality ranged from 60% for the nymphs

in leaves sprayed with strain EAMa 01/58-Su to 94% for those inoculated with strain EABb 04/01-Su (Table 3). The ASTs were significantly different between treatments even though all the treatments with fungal strains were different from the controls. Their ASTs ranged between 77 and 82 hours. The mortality of nymphs in leaves of the same plant that were not exposed to the fungal treatment was lower than that in the inoculated leaves, ranging from 66% (EABb 04/01-Tip and EAMa 01/58-Su) to 88% (EABb 01/33-Su), and most of them did not show fungal outgrowth (Table 3). While the ASTs differed significantly from the control, they did not differ significantly between treatments with the fungal strains, ranging from 64 to 68 hours. Mycelia of strain EABb 01/33-Su growing on nymphs on leaves of the same plant not exposed to the fungal treatment were confirmed using the GFP-transformed strain (Figure 3).

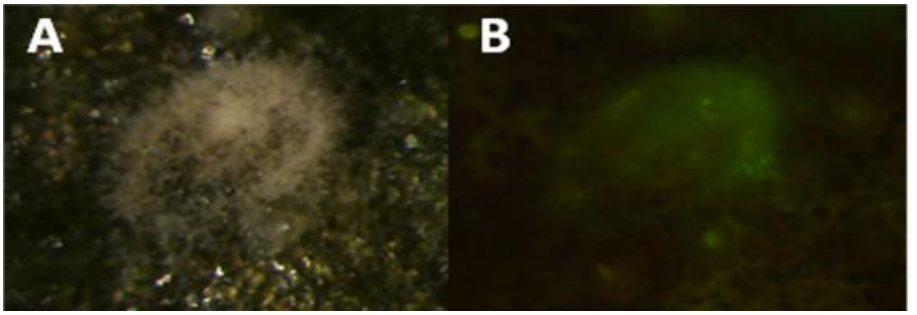


Figure 3. A) Nymphs with external fungal outgrowth on leaves of the same plant not exposed to any fungal treatment; B) *B. bassiana* mycelia expressing GFP in sporulated cadavers from leaves of the same plant not exposed to any fungal treatment.

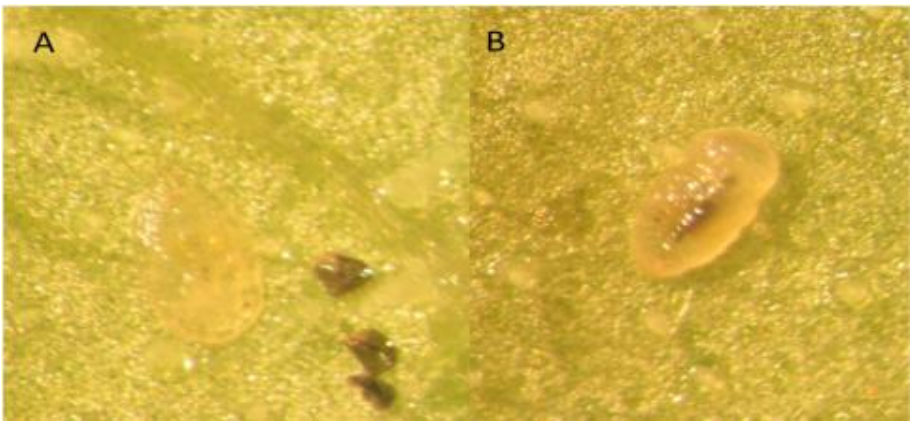
Table 3. Virulence of *B. bassiana* and *M. brunneum* strains on *B. tabaci* nymphs in leaves sprayed, leaves of the same plant not exposed to the fungal treatment, and inoculated leaves in the adaxial surface with 10^8 conidia ml^{-1} fungal suspensions

Treatment	Inoculated leaves					Non-inoculated leaves					Leaves inoculated in the adaxial surface				
	Mortality (mean±SE) (%)*			Survival Analysis–Kaplan Meier**		Mortality (mean±SE) (%)*			Survival Analysis–Kaplan Meier**		Mortality (mean±SE) (%)*			Survival Analysis–Kaplan Meier**	
	Total mortality	Fungal outgrowth	Other causes	AST (mean ± SE ; h)	CI (95%)	Total mortality	Fungal outgrowth	Other causes	AST (mean ± SE ; h)	CI (95%)	Total mortality	Fungal outgrowth	Other causes	AST (mean ± SE ; h)	CI (95%)
EABb 01/33-Su	93.7±3.2b	93.7±3.2a	0.0±0.0d	76.7±0.6 a	75.5; 77.9	87.9±3.3a	10.0±2.6 ab	77.9±0.7 b	79.6±0.5 ab	78.6; 8.7	85.0±3.1a	84.0± 3.1a	1.0±0.0b	64.4±0.5 a	63.4; 65.4
EABb 04/01-Tip	100.0±0.0a	94.1±2.7a	5.9±2.7d	76.7±0.5 a	75.7; 77.7	66.3±2.7b	16.6±2.1 a	49.7±0.6 a	77.9±0.5 a	76.9; 78.9	86.2±4.3a	85.2±3.7a	1.0±0.6b	66.7±0.7 a	65.5; 67.8
Bb04	97.0±1.5ab	81.7±4.8b	15.3±3.8c	80.8±0.7 b	79.5; 82.2	79.2±4.8ab	6.6±2.1 bc	72.6±2.7 b	77.8±0.5 a	76.9; 78,8	53.4±5.5b	52.4±5.5b	1.0±0.0b	64.6±0.6 a	63.7; 65.7
EAMb 09/01-Su	100.0±0.0a	64.6±3.7c	35.4±3.7a	80.1±0.7 b	78.8; 81.4	68.3±4.2b	0.0±0.0c	58.3±4.2 ab	80.6±0.5 b	79.5; 81.6	96.0±2.5a	0.0±0.0c	96.0±2.5a	67.8±0.7 a	66.3; 69.3
EAMa 01/58-Su	83.9±3.2c	60.8±4.5c	23.1±3.4b	82.4±0.7 b	80.9; 83.8	66.6±5.8b	0.0±0.0c	56.6±5.8 ab	79.7±0.5 ab	78.7; 80.8	86.0±3.8a	0.0±0.0c	86.0±3.8a	68.6±0.6 a	67.5; 69.7
Absolute control	0.0±0.0d	0.0±0.0d	0.0±0.0d	96.0±0.0 c	0.0; 0.0	0.0±0.0c	0.0±0.0c	0.0±0.0c	96.0±0.0 c	0.0; 0.0	0.0±0.0c	0.0±0.0c	0.0±0.0b	96.0±0.0 c	0.0; 0.0
Tween 80% control	0.0±0.0d	0.0±0.0d	0.0±0.0d	96.0±0.0 c	0.0; 0.0	0.0±0.0c	0.0±0.0c	0.0±0.0c	96.0±0.0 c	0.0; 0.0	0.0±0.0c	0.0±0.0c	0.0±0.0b	96.0±0.0 c	0.0; 0.0

*Means followed by the same lower case letter in each column do not differ according to LSD test (P<0.001). ** Means within columns with the same letter are not significantly different according to the log rank test. AST: Average survival time. AST measured at 96 hours after inoculation

3.4. Effects of translaminar growth of entomopathogenic fungi of *Bemisia tabaci* across melon leaves

When the inoculation was performed only on the adaxial surface of the leaves, mortality varied between 53 and 96% for treatments with strains Bb04 and EAMb 09/01-Su, respectively (Table 3). Nymphs on leaves treated with the *Beauveria* strains on their adaxial surfaces showed fungal outgrowth, with values of 52, 84, and 85% for the treatments with strains Bb04, EABb 01/33-Su, and EABb 04/01-Tip, respectively. However, nymphs on leaves treated with the *Metarhizium* strains did not show any fungal outgrowth. In contrast, mortalities due to other causes in the *Metarhizium* treatments were higher than those in the *Beauveria* treatments, with rates of 86 and 96% for the EAMa 01/58-Su and EAMb 09/01-Su treatments, respectively (Table 2). In both inoculation types, internal melanization was observed in nymphs treated with the *M. brunneum* strains that did not show fungal outgrowth (Online Resource 2).



Online Resource 2. Fourth instar nymphs of *Bemisia tabaci* on leaves A) control, B) treated with a *Metarhizium* isolate

3.4.1. Histological studies of plant and insect penetration and colonization by endophytic fungi

Histopathological examination of the leaf tissues treated with the strains EAMb 09/01-Su of *M. brunneum* and EABb 01/33-Su of *B. bassiana* revealed fungal structures stained positively by PAS. Control leaves showed a normal structure under microscopy (Figures 4A and 5A). However, leaves infested with nymphs displayed changes in their normal structure (Figure 5B). The leaves showed a high number of hyphae of the *M. brunneum* and *B. bassiana* strains in the intercellular spaces and on leaf surfaces (Figure 4BD and 5B). The hyphae inside the leaf grew through the intercellular spaces between parenchyma cells (Figure 5B). They were restricted to the infected area of the leaves of plants inoculated with *M. brunneum* (Figure 4D), but they colonized the entire leaf area when the plants were inoculated with *B. bassiana* (Figure 5B). The associations between fungi, leaves and nymphs were observed in all leaves infected with nymphs and both fungal species (Figures 4D and 5B). Serial sections of the leaves showed a unique point of contact (stylet) between the nymph and the surface of the leaf. In addition, it was observed that fungal hyphae penetrated the nymphs through either this contact point (Figure 4D) or an adjacent contact point between the leaf and the insect (Figure 5B). Finally, nymphs infected with EF showed a disorganized internal structure due to apparent cellular degeneration of some cells, as evidenced by the presence of pyknotic nuclei. These cells formed a dense necrotic mass that led to the subsequent formation of apoptotic bodies (Figure 4EF).

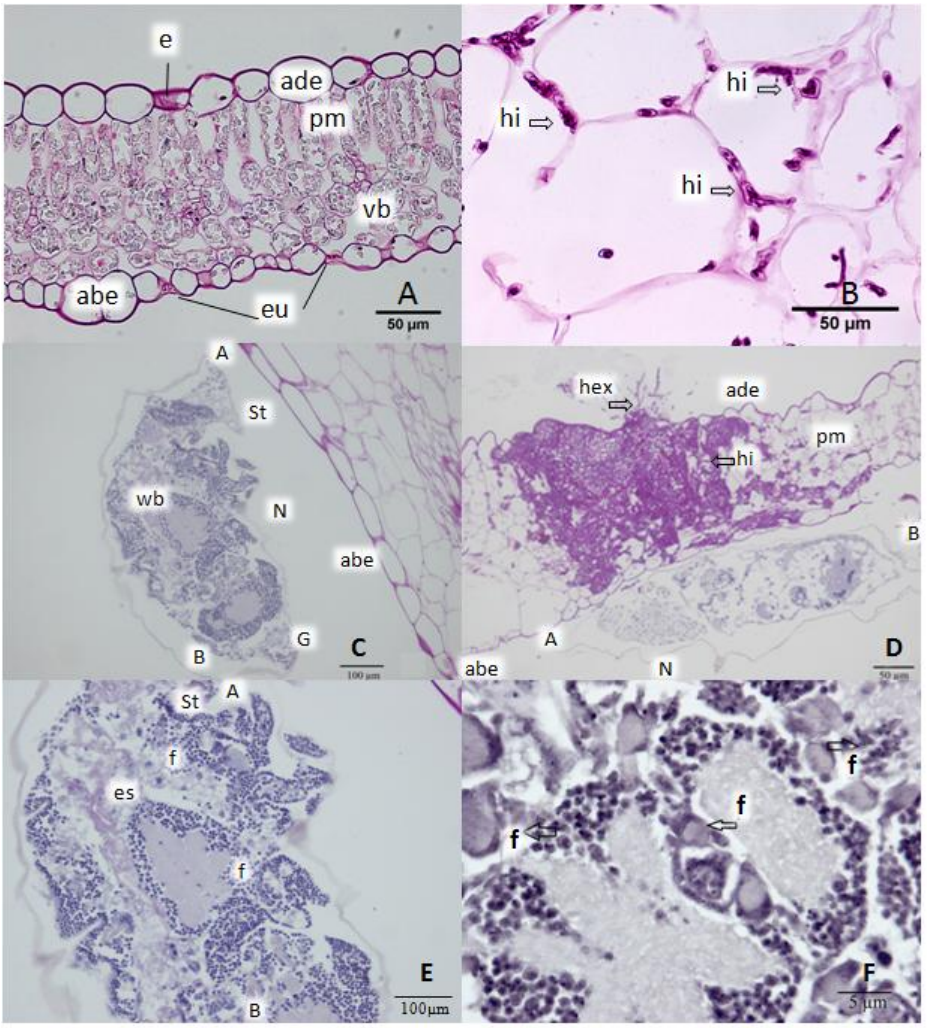


Figure 4 Whitefly nymphs on melon leaves inoculated with *M. brunneum* (EAMb 09/01-Su). A) Control leaf with a normal microscopic structure (Bar, 50 µm). B) Hyphae of *M. brunneum* in the intercellular spaces (Bar, 50 µm); C) Differences in staining between a healthy leaf and a nymph (Bar, 100 µm); D) Association between fungi, leaves and nymphs (Bar, 50 µm); E) *Bemisia tabaci* control nymphs (Bar, 100 µm); F) *Bemisia tabaci* nymphs infected with the EAMb 09/01-Su isolate showing changes in internal structure characterized by a large empty space, showing degeneration of some cells and pyknotic nuclei (Bar, 5 µm). A: thorax; ade: adaxial epidermis; B: hind limb; e: stoma; ebd: abaxial epidermis; es: esophagus; eu: closed stoma; f: phagocytosis; hex: external hyphae; hi: hyphae; N: nymph; pm: palisade mesophyll; st: stylet; vb: beam vascular; wb: wing formation.

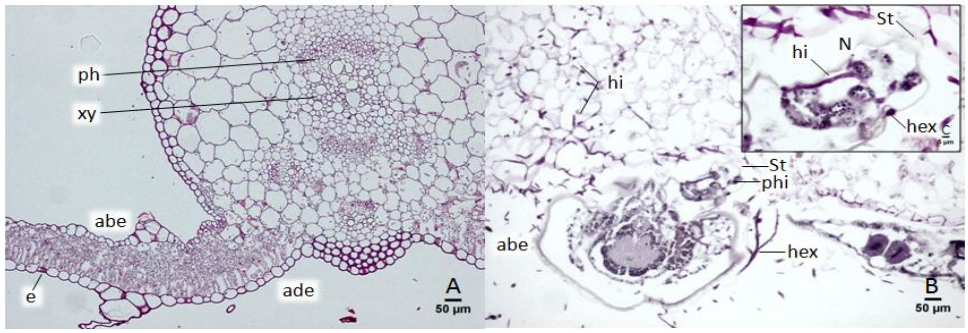


Figure 5 *Bemisia tabaci* nymphs on melon leaves inoculated with EABb 01/33-Su *B. bassiana*. A) Control leaf (Bar, 50 µm); B) Association between fungi, leaves and nymphs. Leaf showing changes in structure and high numbers of *B. bassiana* hyphae in the intercellular spaces (Bar, 50 µm). C) Inset showing details of the association. (Bar, 5 µm). ade: adaxial epidermis; abe: abaxial epidermis; e: stoma; es: esophagus; hex: external hyphae; hi: hyphae; N: nymph; ph: phloem; phi: penetrated hyphae; St: stylet; xy: xylem.

3.4.2. Detection of secondary metabolites

Dtx A was quantified in 43% of the nymphs with internal melanization and on the leaves where they were feeding. At 72 hours after inoculation, dtx A production reached 10.4±0.4 ppb in inoculated leaves and 4.7±0.7 ppb in melanized nymphs of *B. tabaci* fed on leaves inoculated on their adaxial surfaces with the isolate EAMb 09/01-Su.

4. DISCUSSION

This is the first study to report fungal colonization of an insect pest by entomopathogenic fungi via ingestion of hyphae growing as endophytes. All fungal strains from the *Beauveria bassiana* and *Metarhizium brunneum* species used to inoculate the melon plants were able to colonize the leaves within 96 hours, indicating that foliar spray of mycoinsecticides results in at least a transient fungal colonization of the plant tissues. All the evaluated strains in the current work were highly virulent against the sweet potato whitefly *B. tabaci*, causing mortality rates above 60% at 10^8 conidia ml^{-1} of fungal suspension, with the highest nymph mortality produced by strain EABb 01/33-Su. Moreover, EABb 01/33-Su was the most virulent against *B. tabaci* (LC_{50}=3.4x10^4 conidia ml^{-1}) and showed significantly higher virulence compared to the rest of the isolates. This result agrees with that obtained by Quesada-Moraga et al. (2006) with *B. bassiana* strains, who found high variability among strains regarding their virulence against *B. tabaci*, but whose LC_{50} values were higher than the LC_{50} value of EABb 01/33-Su in all cases.

Most of the nymphs on the plant leaves treated with conidial suspensions showed external fungal outgrowth, while mortality was due to other causes in only a few of them. However, the scenario was completely reversed in the non-treated plant leaves, where only a few showed fungal outgrowth. The typical mode of action of entomopathogenic fungi requires direct contact with the insect cuticle, leading to penetration of the insect body, but some fungi are able to produce fungal compounds, both proteins and secondary metabolites, inside the host (Quesada-Moraga et al., 2014a). These secreted compounds can also be produced inside colonized plants; insects contact these compounds during feeding, which results in reduced

survival and fecundity (Gurulingappa et al., 2010). In our work, 43% of the evaluated nymphs contained dtx A, supporting the hypotheses of previous studies, in which secondary metabolites secreted by endophytic strains produced an increase in mortality (Ownley et al., 2008; Gurulingappa et al. 2010). Moreover, destruxins, can upregulate several stress proteins such as caspases in invertebrates, whose activation triggers apoptosis (Garrido-Jurado et al., 2015). This apoptosis can be observed in the histological sections of nymphs in the present study. On the other hand, those nymphs showed internal melanization that likely increased their stress tolerance (Tseng et al., 2014), although the mechanisms by which genes producing those toxins are regulated in insects remain unknown (Vidal and Jaber, 2015). Nevertheless, it has been reported that one stress response strategy employed by *B. tabaci* against other pathogens is to increase its capacity to tolerate infection (Zhang et al., 2014).

Beauveria bassiana strains were able to colonize the plants more extensively than *M. brunneum* strains as a result of both total and adaxial inoculation of the leaves as showed results of subheadings 3.3 and 3.4 of the current work. Indeed, the *B. bassiana* strains showed increased colonization of non-treated tissues. Interestingly, histological sections showed *B. bassiana* hyphae growing profusely through the intercellular spaces between parenchyma cells, which agrees with previous observations using confocal laser scanning microscopy (Landa et al., 2013). In contrast, the growth of *M. brunneum* hyphae was restricted to the infection area of the leaf, a fact that may explain why nymphs showed *Beauveria* outgrowth but not *Metarhizium* outgrowth on non-inoculated leaves and leaves inoculated on the adaxial surface. Behie et al., (2015) reported differential localization of fungal species in the plant 60 days after inoculation, with *Beauveria* preferring aerial parts and *Metarhizium* preferring the roots, and suggested that they adopted this configuration because of environmental conditions; indeed, *Metarhizium*

is considered to be naturally rhizosphere competent (Hu and St. Leger, 2002). In the current work, fungal colonization was evaluated during the first 96 hours after inoculation at 24-hour intervals; therefore, there was not enough time for environmental conditions to influence fungal colonization of the plants. Both *Beauveria* and *Metarhizium* colonized leaves, but they exhibited different patterns. To the best of our knowledge, for the first time, we have confirmed fungal colonization of insects with entomopathogenic fungi in non-treated areas of the plant based on observations of sporulated cadavers via fluorescence microscopy, although only a small percentage of dead nymphs in non-inoculated leaves showed external fungal outgrowth. Additionally, use of the PAS stain allowed detection of translaminar colonization of the leaves and positively stained hyphae inside whiteflies placed on non-treated surfaces. Both *B. bassiana* and *M. brunneum* were able to temporarily colonize the leaf tissues in the adaxial area and control whiteflies deposited in the abaxial area. This translaminar colonization resulted in high mortality of nymphs due to either fungal growth or secreted compounds, depending on the fungal species, and did not influence the AST of the treatments.

In conclusion, 1) foliar applications of mycoinsecticides lead to transient endophytic colonization of crop tissues; 2) both *Beauveria* and *Metarhizium* are able to temporarily colonize leaves but display different colonization patterns; 3) whiteflies can be infected with entomopathogenic fungi via ingestion of hyphae growing as endophytes; and 4) secondary metabolites contribute to the control of whitefly pests.

5. ACKNOWLEDGEMENTS

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CAPÍTULO V

DISCUSIÓN GENERAL

A lo largo de los distintos capítulos de esta Tesis Doctoral se ha puesto de manifiesto que los ascomicetos mitospóricos entomopatógenos (AME), *Beauveria bassiana* y *Metarhizium brunneum* secretan *in vivo* e *in vitro* moléculas insecticidas, y que además pueden comportarse como endófitos, aspectos ambos que pueden potenciar su empleo inundativo para el control de dos fitófagos polífagos de gran importancia a nivel mundial, uno masticador *Spodoptera littoralis* (Boisduval) (Noctuidae: Lepidoptera) y otro picador-suctor *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae).

El capítulo II revela la existencia de variación interespecífica e intraespecífica en la virulencia de los AME evaluados frente a larvas *de S. littoralis*, así como en sus estrategias patogénicas (Kershaw et al.,1999; Ortiz-Urquiza et al., 2010). Mientras que las cepas EABb 01/33-Su y EABb 01/88-Su de *B. bassiana* pudieron seguir la "estrategia de crecimiento", con altos porcentajes de cadáveres con crecimiento fúngico externo, las cepas 3158, 3154 y 1764 de *B. bassiana* y EAMa 01/58-Su de *M. brunneum* mostraron una "estrategia toxicogénica", donde los altos porcentajes de mortalidad no estuvieron asociados a crecimiento fúngico en los cadáveres, sino probablemente a la producción de compuestos insecticidas en el hemocele (Ortiz-Urquiza et al., 2010). Los extractos brutos en medio líquido de estas cepas fueron ofrecidos por ingestión a larvas de *S. littoralis*, con altos porcentajes de mortalidad en los procedentes de EAMb 09/01-Su y EAMa 01/58-Su de *M. brunneum*, lo que pudo deberse tanto a la secreción de compuestos macromoleculares (Quesada-Moraga et al., 2006; Ortiz-Urquiza

et al., 2013), como a la de metabolitos insecticidas como las destruxinas (Parth et al., 2014; Lozano-Tovar et al., 2015).

En este capítulo se explora por primera vez el empleo conjunto de hongos entomopatógenos y sus extractos para el control de plagas. Se seleccionaron dos cepas fúngicas que habían presentado distintas estrategias patogénicas, cepa EABb 01/33-Su con "estrategia de crecimiento" y cepa EAMa 01/58-Su con "estratégica toxicogénica", y se aplicaron a larvas de *S. littoralis* de forma simultanea con sus extractos, y en el caso de la primera cepa, con los extractos de la segunda y de la cepa EABb 09/01-Su, de estrategia patogénica intermedia. Todas las combinaciones mostraron un efecto aditivo, excepto la correspondiente a la cepa EAMa 01/58-Su y su extracto, donde este resultó ser antagónico, por razones aún no aclaradas. A la luz de estos resultados, y siempre que se constate la compatibilidad de los AME con sus extractos, podría recomendarse su aplicación conjunta bien como parte de bioinsecticidas diferentes, bien como resultado del proceso de producción y fermentación.

Las estrategia convencional de aplicación de hongos entomopatógenos es la pulverización dirigida al suelo o al sustrato vegetal, donde los conidios deben iniciar ciclos patogénicos al entrar en contacto con la cutícula de sus insectos hospedantes. Sin embargo, en esta tesis se ha intentando dilucidar en los capítulos III y IV si existe colonización endofítica transitoria después de pulverizar el sustrato vegetal con AME para el control de insectos masticadores (capítulo III) o chupadores (capítulo IV), así como si se inician ciclos patogénicos por esta vía inusual.

En el capítulo III, la pulverización de las cepas fúngicas seleccionadas sobre plantas de alfalfa, tomate y melón estuvo asociada a una colonización de las tres especies vegetales durante las 96 horas posteriores a la inoculación, no soló en las hojas pulverizadas, sino también en el tallo e incluso la raíz de los tres cultivos, con mayores porcentajes de colonización

para las cepas de *B. bassiana*. Cuando hojas de los tres cultivos obtenidas durante este periodo y colonizadas endofíticamente fueron ofrecidas a larvas de *S. littoralis*, se obtuvieron porcentajes significativos de mortalidad en el rango del 10,0-50,0%, pero de forma sorprendente, nunca se observó crecimiento fúngico a partir de estos cadáveres. Esto sugiere que la mortalidad causada por los hongos entomopatógenos en insectos masticadores cuando los ciclos de infección se inician por la vía digestiva al ingerir tejido vegetal colonizado endofíticamente podría estar asociada a la "estrategia toxicogénica", como indica la presencia de destruxina A en el 11,0% de los cadáveres tratados con las cepas de *M. brunneum*. Aún así, no deben descartarse otras causas asociadas a la posible respuesta de la planta frente al fitófago cuando ésta es colonizada por el endófito, que podrían contribuir de forma simultanea al efecto observado. En cualquier caso, resulta muy importante la contribución aditiva de la mortalidad procedente de la colonización endofítica transitoria a la que tiene lugar como consecuencia del tratamiento directo de los insectos con las suspensiones fúngicas, y debe ser considerada para evaluar la eficacia global de estas aplicaciones.

En el capítulo IV, se describe por vez primera como los AME pueden iniciar ciclos de infección en insectos picadores-suctores a través del estilete cuando éstos se alimentan a expensas de sustrato vegetal colonizado de forma endofítica por los mismos. La utilización de técnicas histológicas e incluso el empleo de cepas de *B. bassiana* y *M. brunneum* transformadas con una proteína verde fluorescente (GFP), ha permitido dilucidar estrategias diferentes tanto en la colonización de las hojas, como en los ciclos patogénicos iniciados a través de las mismas. Así, *B. bassiana*, con una gran capacidad de colonización por la vía del apoplasto, incluso con efecto translaminar, pudo iniciar ciclos de infección por contacto directo con el tegumento de las ninfas de *B. tabaci*, cuya mortalidad estuvo asociada a crecimiento fúngico en los cadáveres. Sin embargo, *M. anisopliae*, que tuvo

un menor desarrollo en el apoplasto, sólo fue detectado en el punto de penetración del estilete de las ninfas, cuya mortalidad no estuvo asociada a crecimiento fúngico pero si a la presencia de dextruxina A en el 43,0% de los cadáveres, con evidencias histológicas de muerte celular o apoptosis (Garrido-Jurado et al., 2015). De esta forma, las nuevas funciones ecológicas de los ascomicetos mitospóricos deben ser tenidas en cuenta no solo para el desarrollo de nuevas estrategias de control de plagas, sino para la correcta evaluación de su eficacia.

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CAPÍTULO VI

CONCLUSIONES

Las conclusiones de esta Tesis Doctoral son las siguientes:

1. Las 26 cepas fúngicas evaluadas frente a larvas de segundo estadio de *Spodoptera littoralis*, 20 de *Beauveria* sp. y 6 de *Metarhizium* sp., fueron patogénicas pero presentaron diferencias en su virulencia y en sus estrategias patogénicas, con tres cepas de *Beauveria bassiana* 1764, 3158 y 3154, y dos de *Metarhizium brunneum* EAMb 09/01-Su y EAMa 01/58-Su como representantes de la "estrategia toxicogénica", mientras que el resto presentó una "estrategia de crecimiento.

2. Los extractos crudos en medio líquido de estas 26 cepas no mostraron actividad por contacto frente a larvas de segundo estadio de *S. littoralis*, pero si mostraron porcentajes variables de actividad insecticida por ingestión frente a las mismas aplicados en discos de hoja de alfalfa, a excepción del extracto obtenido de la cepa EABb 01/33-Su de *B. bassiana*, por lo que estos agentes fúngicos de control microbiano ofrecen una nueva fuente de moléculas insecticidas de origen natural para su empleo en programas de control de integrado de plagas.

3. Los tratamientos conjuntos de las cepas EABb 01/33-Su de *B. bassiana*, con "estrategia de crecimiento", y EAMa 01/58-Su de *M. brunneum*, con "estrategia toxicogénica", y sus extractos crudos, y con el extracto de la cepa EAMb 09/01-Su de *M. brunneum* frente a larvas de segundo estadio de *S. littoralis* presentaron efectos aditivos, a excepción de la combinación de la cepa EAMa 01/58-Su y su extracto. En caso de compatibilidad, podrían recomendarse estas aplicaciones bien como parte de bioinsecticidas

diferentes, bien como resultado del proceso de producción y fermentación de los mismos.

4. Aplicaciones foliares de suspensiones fúngicas de las cepas EABb 04/01-Tip, EABb 01/33-Su y Bb04 de *B. bassiana* y EAMb 09/01-Su de *M. brunneum* en plantas de alfalfa, tomate y melón estuvieron asociadas con distintos porcentajes de colonización endofítica en hoja, tallo y raíz durante las 96 horas posteriores a la inoculación. Además, al alimentar larvas de segundo estadio de *S. littoralis* con hojas colonizadas endofíticamente se obtuvieron porcentajes de mortalidad significativos, pero nunca se observó crecimiento fúngico a partir de los cadáveres, lo que sugiere que estos ciclos de infección que se inician por la vía digestiva podrían estar asociados a la "estrategia tóxica", al detectarse destruxina A en el 11.0% de los cadáveres tratados con las cepas de *M. brunneum*.

5. Las cepas EABb 04/01-Tip, EABb 01/33-Su y Bb04 de *B. bassiana* y EAMb 09/01-Su y EAMa 01/58-Su de *M. brunneum* presentaron una gran eficacia insecticida al ser aplicadas a plantas de melón infestadas por ninfas de *Bemisia tabaci*, tanto cuando estas se alimentaban en el envés de hojas que recibieron el impacto del tratamiento en el haz, como cuando lo hacían en hojas distantes de estas y no tratadas, lo que revela la existencia de ciclos de infección en insectos picadores-suctores a través del estilete.

6. Las especies *B. bassiana* y *M. brunneum* presentaron estrategias diferentes tanto para colonizar endofíticamente las hojas de melón, como en sus ciclos patogénicos iniciados por esta vía. *B. bassiana* colonizó de forma profusa las hojas por la vía del apoplasto, con efecto translaminar, para iniciar ciclos de infección por contacto directo con el tegumento de las ninfas de *B. tabaci*, mientras que *M. brunneum* sólo se detectó en el punto de penetración del estilete, con mortalidad de ninfas asociada a la producción de destruxina A.

Las conclusiones 1, 2 y 3 corresponden al objetivo 3.1. (Capítulo II), manuscrito Resquín-Romero, G., Garrido-Jurado, I., Quesada-Moraga, E. 2016. Combined use of entomopathogenic fungi and their extracts improves the control of Spodopteralittoralis (Boisduval) (Lepidoptera:Noctuidae). BIOLOGICAL CONTROL 92: 101-110.

La conclusión 4 corresponde al objetivo 3.2. (Capítulo III),manuscrito Resquín-Romero, G., Garrido-Jurado, I., Delso, C., Ríos-Moreno, A., Quesada-Moraga, E. 2016. Transient endophytic colonizations of plants improve the outcome of foliar applications of mycoinsecticides against chewing insects. JOURNAL OF INVERTEBRATE PATHOLOGY. 136: 23-31.

Las conclusiones 5 y 6 corresponden al objetivo 3.3. (Capítulo IV),manuscrito Garrido-Jurado, I., Resquín-Romero, G., Amarilla, S.P., Ríos-Moreno, A., Carrasco, L., Quesada-Moraga, E. 2016. Unravelling the effects of temporally colonization of plants after foliar application of mycoinsecticides for the control of sucking insects. JOURNAL OF PEST SCIENCE (Aceptado).