Escuela Técnica Superior de Ingeniería Agronómica y de Montes
Departamento de Agronomía

INTERACCIONES MULTITRÓFICAS REGULADAS POR HONGOS ENTOMOPATÓGENOS PARA LA PROTECCIÓN SOSTENIBLE DE CULTIVOS

ENTOMOPATHOGENIC FUNGAL-MEDIATED MULTITROPHIC INTERACTIONS FOR SUSTAINABLE CROP PROTECTION

Tesis doctoral presentada por D. **Pedro Miranda Fuentes** para optar al grado de Doctor por la Universidad de Córdoba

Directores:

Prof. Dr. D. Enrique Quesada Moraga

Dra. Dña. Leire Molinero Ruiz

Córdoba, a 28 de enero de 2021

TITULO: *Interacciones multitróficas reguladas por hongos entomopatógenos para la protección sostenible de cultivos*

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Programa de Doctorado en Ingeniería Agraria, Alimentaria, Forestal y del Desarrollo Rural Sostenible

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TÍTULO DE LA TESIS: Interacciones multitróficas reguladas por hongos entomopatógenos para la protección sostenible de cultivos / Entomopathogenic fungal-mediated multitrophic interactions for sustainable crop protection

DOCTORANDO: Pedro Miranda Fuentes

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS:

El Prof. **D. Enrique Quesada Moraga**, Doctor Ingeniero Agrónomo, Profesor Catedrático de Producción Vegetal de la Universidad de Córdoba, y **Dña. Leire Molinero Ruiz**, Doctor Ingeniero Agrónomo, Científico Titular del Departamento de Protección de Cultivos del Instituto de Agricultura Sostenible (CSIC),

INFORMAN: que el trabajo titulado "Interacciones multitróficas reguladas por hongos entomopatógenos para la protección sostenible de cultivos / Entomopathogenic fungal-mediated multitrophic interactions for sustainable crop protection" realizado bajo su dirección por **D. Pedro Miranda Fuentes**, se encuentra finalizado y cumple los requisitos para su presentación como Tesis Doctoral por compendio de publicaciones, las cuales se indican a continuación:

- Miranda-Fuentes, P., Quesada-Moraga, E., Aldebis, H.K. and Yousef-Naef, M. (2020) Compatibility between the endoparasitoid *Hyposoter didymator* and the entomopathogenic fungus *Metarhizium brunneum*: a laboratory simulation for the simultaneous use to control *Spodoptera littoralis*. *Pest Management Science*, 76, 1060–1070. doi: 10.1002/ps.5616. Esta revista es D1: 7/101 en "Entomology" con un factor de impacto de 3.750.

- Miranda-Fuentes, P., Yousef-Yousef, M., Valverde-García, P., Rodríguez-Gómez, I.M., Garrido-Jurado, I. and Quesada-Moraga, E. (2021) Entomopathogenic fungal endophyte-mediated tritrophic interactions between *Spodoptera littoralis* and its parasitoid *Hyposoter didymator*. *Journal of Pest Science*. doi: 10.1007/s10340-020-01306-7. Esta revista es D1: 2/101 en "Entomology" con un factor de impacto de 4.578.

- Miranda-Fuentes, P., García-Carneros, A.B., Montilla-Carmona, A.M. and Molinero-Ruiz, L. (2020) Evidence of soil-located competition as the cause of the reduction of sunflower verticillium wilt by entomopathogenic fungi. *Plant Pathology*, 69, 1492–1503. doi: 10.1111/ppa.13230. Esta revista es Q1: 21/91 en "Agronomy" con un factor de impacto de 2.169.

- Miranda-Fuentes, P., García-Carneros, A.B. and Molinero-Ruiz, L. (2021) Updated characterization of races of *Plasmopara halstedii* and entomopathogenic fungi as endophytes of sunflower plants in axenic culture. *Agronomy* (accepted with minor revision). Esta revista es Q1: 18/91 en "Agronomy" con un factor de impacto de 2.603.

Y, por ello, puede ser presentado para su exposición y defensa como Tesis Doctoral por compendio de publicaciones en la Universidad de Córdoba.

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TESIS POR COMPENDIO DE ARTÍCULOS

Esta tesis cumple el requisito establecido por la Universidad de Córdoba para su presentación por compendio de artículos, consistente en un mínimo de tres artículos publicados o aceptados en revistas incluidas en los tres primeros cuartiles de la relación de revistas del ámbito de la especialidad y referenciadas en la última relación publicada por Journal Citations Report (SCI);

- Miranda-Fuentes, P., Quesada-Moraga, E., Aldebis, H.K. and Yousef-Naef, M. (2020) Compatibility between the endoparasitoid *Hyposoter didymator* and the entomopathogenic fungus *Metarhizium brunneum*: a laboratory simulation for the simultaneous use to control *Spodoptera littoralis*. *Pest Management Science*, 76, 1060–1070. doi: 10.1002/ps.5616. Esta revista es D1: 7/101 en "Entomology" con un factor de impacto de 3.750.

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RESUMEN

El empleo de ascomicetos mitospóricos entomopatógenos (AME) para el control de plagas de insectos es objeto de un gran interés en tiempos recientes. El principal modo de acción de los AME es el del contacto directo a través del tegumento del insecto, lo que ha propiciado el desarrollo de un amplio abanico de bioinsecticidas (micoinsecticidas) que actúan por contacto. Sin embargo, estos hongos cuentan con un modo de acción secundario asociado a una función ecológica descrita en el siglo XXI que alberga un notorio potencial en protección de cultivos, su condición endófita, que consiste en la ingestión por parte del insecto de tejidos de la planta colonizados por el agente fúngico de control biológico, que complementa al anterior y brinda un grado de control adicional. Asimismo, se están explorando nuevos horizontes de aplicación de estos hongos (ya sea por contacto o por vía endofítica), lo que incluye su uso conjunto con enemigos naturales, depredadores y parasitoides, con objeto de definir estrategias innovadoras de manejo integrado. Por otro lado, algunos AME pueden desempeñar papeles relacionados con la protección de cultivos más allá del control de insectos fitófagos, donde destaca el antagonismo frente a organismos fitopatógenos. De esta forma, la aplicación de AME en campo podría no solo controlar eficazmente las plagas de insectos, sino proporcionar, además, cierta protección frente a enfermedades de cultivos.

La presente tesis explora el potencial de cepas endófitas de hongos entomopatógenos para el control de plagas, con énfasis en el impacto de este nuevo modo de acción sobre las relaciones tritróficas insecto-planta-enemigo natural entomófago, así como su posible acción dual para proteger a la planta frente a microorganismos fitopatógenos. En el primer caso, se ha evaluado el empleo conjunto de la cepa endófita EAMa 01/58-Su del AME *Metarhizium brunneum* (Petch) (Hypocreales: Clavicipitaceae) y del himenóptero endoparasitoide *Hyposoter didymator* (Thunberg) (Hymenoptera: Ichneumonidae) para el control de la rosquilla negra, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae), de acuerdo con los dos modos de acción descritos. Este noctuido es uno de los insectos fitófagos más nocivos y ubicuos de toda la cuenca mediterránea, donde origina de manera recurrente severas plagas en cultivos de enorme importancia económica. La literatura científica provee evidencias de la aptitud de ambos agentes, la cepa EAMa 01/58-Su y el referido parasitoide, para el control de *S. littoralis*, si bien no se ha incidido en su aplicación simultánea. Por ello, se han diseñado diferentes estrategias de aplicación combinada para el control de "la rosquilla negra" tanto en experimentos de laboratorio como *in planta*, para dilucidar las posibles interacciones multitróficas derivadas de la conjunción de la planta, el entomopatógeno, el fitófago y el enemigo natural entomófago. Los resultados de esta primera línea se presentan en los capítulos II y III. En la segunda línea de la tesis, se ha profundizado en el potencial de cinco cepas de los AME *M. brunneum* (EAMa 01/58-Su, EAMb 01/158-Su y EAMb 09/01-Su) y *Beauveria bassiana* Bals. (Vuill) (Hypocreales: Clavicipitaceae) (EABb 01/33-Su y EABb 04/01-Tip) frente a importantes patógenos de girasol. En concreto, los patógenos objeto de estudio fueron los hongos de suelo *Verticillium dahliae* Kleb. y *Cadophora helianthi* (L. Molinero-Ruiz, A. Martin-Sanz, C. Berlanas and D. Gramaje), cuyo control en campo se halla amenazado por la aparición de nuevas razas en el primer caso y por la ausencia de alternativas para su control en el segundo, y el oomiceto *Plasmopara halstedii* Farl. Berl. & de Toni, agente causal del mildiu de girasol, cuyo manejo está condicionado por la aparición de poblaciones resistentes a las materias activas registradas para uso contra este patógeno y por la identificación de nuevas razas de mayor virulencia. Específicamente, se evaluó el antagonismo de los AME frente a *V. dahliae* y *C. helianthi* mediante la técnica del cultivo dual y la reducción de síntomas de verticilosis en plantas de girasol tratadas con los AME en condiciones de invernadero. El efecto de los AME sobre *P. halstedii* se estudió en cultivo axénico durante 14 días, periodo máximo durante el que las plántulas pudieron crecer en este sistema *in vitro* que limita su desarrollo fenológico completo. Para ello, se evaluaron los síntomas de mildiu expresados por las plántulas y se analizó el efecto que ejercieron las cepas de AME sobre el crecimiento del girasol durante este limitado periodo de tiempo. Adicionalmente, se estudió el perfil racial de las poblaciones de *P. halstedii* presentes en España y otros países europeos en los últimos años. Los resultados concernientes a esta segunda línea se exponen a lo largo de los capítulos IV y V.

En el capítulo II se incidió en la compatibilidad entre *H. didymator* y la cepa EAMa 01/58-Su de *M. brunneum* en condiciones de laboratorio. Se estudiaron los efectos directos e indirectos derivados de la aplicación directa del AME en adultos del parasitoide mediante pulverización de los mismos con suspensiones de conidios. Aunque el tratamiento fúngico por contacto directo a dosis elevadas, escenario

que supera las peores condiciones posibles en pleno campo, redujo de manera significativa la esperanza de vida del parasitoide con mortalidad algo superior al 60%, no tuvo efecto sobre su potencial reproductivo durante los días que siguieron a la inoculación. Asimismo, se evaluaron dos estrategias de aplicación simultánea del hongo y el parasitoide para el control de *S. littoralis*, exposición al hongo de manera previa al parasitoide y viceversa, con diferentes intervalos de aplicación de cada agente. Las dos estrategias resultaron adecuadas para el control del noctuido, evidenciando la compatibilidad de ambos agentes de biocontrol al tener su empleo conjunto efecto aditivo en todas las estrategias y combinaciones. No obstante, se observó una disminución significativa en el potencial reproductivo de los parasitoides de la siguiente generación emergidos a partir de las larvas de *S. littoralis* tratadas con el hongo respecto a los emergidos de larvas no tratadas de la misma generación. Además, se constató que el parasitismo de *H. didymator* ocasiona una reducción en el número de hemocitos presentes en la hemolinfa de las larvas de *S. littoralis*, una depresión del sistema inmune de los individuos parasitados con impacto en una mayor proporción de larvas diagnosticadas con crecimiento fúngico. Por último, las hembras de *H. didymator* evidenciaron una preferencia significativa por aquellas larvas de *S. littoralis* no tratadas con hongo en ensayos de elección junto a larvas inoculadas con el entomopatógeno. Por lo tanto, los resultados obtenidos en este capítulo ponen de manifiesto la elevada compatibilidad entre la cepa EAMa 01/58-Su y el parasitoide *H. didymator*, a pesar de los efectos derivados del contacto directo del hongo con el himenóptero. Se ha demostrado la eficacia de su aplicación combinada para controlar a la rosquilla negra, abriendo puertas a los experimentos desarrollados en el capítulo III.

En el capítulo III se ha explorado la vía endofítica de la cepa EAMa 01/58-Su, así como la del contacto, en sistemas tritróficos con plantas de melón pulverizadas con suspensiones de conidios del entomopatógeno. Las plantas tratadas, que fueron colonizadas endofíticamente por el AME, se ofrecieron como única fuente de alimento a larvas *S. littoralis* en experimentos tanto *in vitro* como *in vivo*. Asimismo, las larvas se expusieron a adultos de *H. didymator* en diferentes intervalos de tiempo. Se evaluó la colonización endofítica de las plantas 48 h después del tratamiento, que alcanzó porcentajes muy altos (≈90%) en todos los bioensayos, lo que pone de manifiesto la destacada propiedad endófita de la cepa EAMa 01/58-Su. La inclusión del parasitoide afectó significativamente la mortalidad de *S. littoralis*; de hecho, aquellos tratamientos en los que las larvas del noctuido se expusieron al parasitoide registraron los mayores porcentajes de mortalidad en los ensayos *in vitro* e *in planta*. Sin embargo, la exposición simultánea al hongo y el parasitoide en los diferentes sistemas multitróficos no aumentó la mortalidad de *S. littoralis* respecto a los tratamientos en los que las larvas únicamente se ofrecieron al parasitoide. Por otro lado, el tiempo de exposición al hongo y al parasitoide no tuvo ningún efecto sobre la mortalidad de *S. littoralis*, sobre los ratios de parasitismo ni tampoco sobre la proporción de larvas con micosis. Al igual que en el capítulo II, se evaluó la preferencia del parasitoide por larvas expuestas al AME, si bien en el capítulo III se alimentó a las larvas del noctuido con hoja de melón colonizada endofíticamente, en lugar de efectuar un tratamiento directo de las larvas con el entomopatógeno. Como en el capítulo II, las hembras del parasitoide evidenciaron una inequívoca predilección por las larvas que no habían sido expuestas al hongo. Finalmente, se llevó a cabo un estudio histológico en el que se examinaron larvas de *S. littoralis* expuestas conjuntamente a *H. didymator* y el entomopatógeno a fin de determinar la posible existencia de relaciones intrahospedante y observar el desarrollo de ambos agentes de biocontrol en el interior del noctuido. Las micrografías presentadas muestran, por primera vez, la coexistencia de los dos agentes dentro del hospedante y el desarrollo de la larva del parasitoide a pesar de la infección fúngica. Los resultados presentados ponen de manifiesto la compatibilidad entre *H. didymator* y *M. brunneum* en sistemas *in planta*, sentando las bases de futuras estrategias de aplicación simultánea para controlar a la rosquilla negra en el campo o en invernadero.

En el capítulo IV se investigó el efecto de dos cepas de *B. bassiana* (EABb 01/33-Su y EABb 04/01-Tip) y tres de *M. brunneum* (EAMa 01/58-Su, EAMb 09/01-Su y EAMb 01/158-Su) frente a los hongos de suelo causantes de marchiteces de girasol *V. dahliae* y *C. helianthi*. Los cinco AME inhibieron significativamente el crecimiento miceliar de los fitopatógenos en cultivo dual en medio de cultivo agar extracto de malta. Los porcentajes de inhibición oscilaron entre el 8.3 y el 63.5% en el caso de *V. dahliae*, y entre el 19.6 y el 37.4% en el de *C. helianthi*. Esta inhibición dependió tanto de la cepa del AME como del aislado del patógeno en los cultivos duales con *V. dahliae*, pero solo de la cepa del AME en los cultivos con *C. helianthi*. La cepa EABb 01/33-Su de *B. bassiana* presentó la mayor capacidad inhibitoria frente ambos patógenos. De acuerdo a los cultivos duales y a observaciones al microscopio, los AME ejercieron dos tipos de antagonismo: competición o antibiosis. Sin embargo, mientras que cuatro de los

cinco entomopatógenos causaron únicamente uno de los dos antagonismos, la cepa EAMa 01/58-Su (incluida en los capítulos II y III), fue capaz de ejercer ambos, como ya se había descrito con anterioridad. Por otro lado, dos de los AME (EABb 01/33-Su y EAMb 09/01-Su) redujeron significativamente la severidad de los síntomas de verticilosis en plantas de girasol cultivadas en invernadero (severidades de 31 y 53%, respectivamente, en comparación con 95% en las plantas control inoculadas con *V. dahliae* pero no tratadas con AME). Los AME persistieron en el sustrato durante al menos dos meses tras el tratamiento, a excepción de la cepa EABb 04/01-Tip, que no pudo recuperarse del sustrato más allá de la séptima semana. Por último, los cinco AME se detectaron molecularmente en las plantas al final del experimento (dos meses después de haber sido aplicados al suelo). Curiosamente, solo se detectaron molecularmente en aquellas plantas tratadas con AME pero no inoculadas con *V. dahliae*, lo que sugiere que la inhibición del hongo fitopatógeno por parte del AME tiene lugar en el suelo y, como consecuencia, la subsiguiente colonización de la planta por *V. dahliae* se reduce.

En el capítulo V se ha abordado el manejo del mildiu de girasol desde una doble perspectiva, esto es, resistencia genética y control biológico. En la primera aproximación se han obtenido datos actualizados acerca del perfil racial de las poblaciones de *P. halstedii* presentes en España y, en menor grado, Francia, Italia, Portugal y Rumanía, en los últimos años. El método empleado para ello, basado en la reacción de nueve líneas diferenciales de girasol a la inoculación con el patógeno, permitió la identificación de 23 razas diferentes en Europa, de las cuales 22 se confirmaron en España, siendo las razas 310, 304, 705 y 715 más frecuentes que el resto. No solo la diversidad racial de *P. halstedii* fue mayor que en años anteriores, sino también la presencia de razas altamente virulentas. Por otro lado, se evaluó el efecto de las cinco cepas de AME sobre la severidad de la enfermedad en plántulas de girasol y sobre el desarrollo de estas en cultivo axénico solo durante los primeros 14 días, dado el carácter limitante de este cultivo *in vitro* para el desarrollo vegetal. Ninguna de las cepas de AME redujo de manera significativa la severidad ni afectó al crecimiento de las plantas durante las dos semanas estudiadas, cuando estas se trataron e inocularon, respectivamente, con ambos organismos. Cuando las plantas se trataron solo con los AME, se observaron para la cepa EABb 01/33-Su y, en menor medida, otras dos, las pautas características de evolución de su desarrollo asociadas al establecimiento del endófito. Finalmente, se obtuvieron porcentajes variables de aislamiento de los AME a partir de las plántulas, si bien la detección molecular de los entomopatógenos solo resultó posible en algunas cepas y únicamente en ausencia de *P. halstedii*, lo que revela que el método empleado no favorece la acción de los entomopatógenos ni es el más adecuado para la colonización endofítica.

En conclusión, la presente tesis doctoral incide en los mecanismos subyacentes tras las interacciones que tienen lugar en sistemas multitróficos que incluyen plantas, agentes de control biológico macrobianos y microbianos y organismos fitófagos o fitopatógenos. El AME *M. brunneum* posee un destacable potencial para su aplicación conjunta con el parasitoide *H. didymator* en distintas condiciones. Adicionalmente, las cinco cepas de *M. brunneum* y *B. bassiana*, y en particular EABb 01/33-Su y EAMb 09/01-Tip, han resultado ser candidatos muy prometedores para el manejo de algunos hongos patógenos de girasol a la luz del antagonismo *in vitro* frente a *V. dahliae* y *C. helianthi* y, sobre todo, de la reducción de la severidad de la verticilosis en girasol. No obstante, debe profundizarse en el empleo de los AME para el control del mildiu, puesto que no se ha evidenciado un efecto positivo sobre la severidad de dicha enfermedad en cultivo axénico en las condiciones experimentales utilizadas. Finalmente, los cinco entomopatógenos mostraron una aptitud endofítica muy marcada en girasol (en el caso de la cepa EAMa 01/58-Su, también en melón). La profundización en estas líneas de investigación contribuirá a definir estrategias de aplicación de los AME en el campo como parte de sistemas de gestión integrada de plagas.

Palabras clave: control biológico, hongos entomopatógenos, interacciones multitróficas, relaciones intrahospedante, rosquilla negra, parasitoides, gestión integrada de plagas, patógenos de suelo, enfermedades de girasol, patógenos de plantas, compatibilidad, control integrado de plagas y enfermedades

ABSTRACT

The use of entomopathogenic mitosporic ascomycetes (EMA) for pest control has received increased attention in the last years. The main mode of action of EMA is via direct contact through the insect integument, which has led to the development of a wide variety of contact bioinsecticides (mycoinsecticides) around the world. However, an alternative mode of action of these EMA related to their new ecological role as endophytes has been described in the XXI century. Indeed, feeding of insect pest on endophytically colonised plant tissues may cause an additional insect mortality that improves the outcome of foliar applications of EMA against chewing. Moreover, strategies including joint use of EMA (either by direct contact or via endophytism) and macrobial biocontrol agents (i.e., predators and parasitoids) are being considered as promising alternatives within IPM programs. On the other hand, EMA may play other roles linked to plant protection in the frame of these new ecological roles, such as the protection of the plant against plant pathogens. Thus, these fungi may provide certain levels of disease control while effectively controlling insect pests.

In this way, the present thesis explores the potential of endophytic strains of entomopathogenic fungi for pest control, with emphasis on the impact of this new mode of action on the tritrophic insect-plant-entomophagous natural enemy relationships, as well as its possible dual action to protect the plant against phytopathogenic microorganisms. Regarding the former, simultanous use of an endophytic strain of the EMA *Metarhizium brunneum* (Petch) (Hypocreales: Clavicipitaceae) (EAMa 01/58-Su) and the hymenopteran endoparasitoid *Hyposoter didymator* (Thunberg) (Hymenoptera: Ichneumonidae) to control the cotton leafworm, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) under different conditions has been explored. This noctuid is one of the most noxious polyphagous widespread insect pests in the Mediterranean basin, causing economic losses to several crops of great economic importance. Both EAMa 01/58-Su strain and the parasitoid have been proved to be suitable candidates for controlling *S. littoralis* in previous research, although their combined use has not yet been investigated. Thus, we assessed different strategies to control the cotton leafworm both in laboratory and *in planta* conditions, delving into multitrophic interactions between plant, pest and natural enemy. The results concerning this line are presented in chapters II and III. In the second line of the thesis, it has been investigated the potential of five strains of the EMA *M. brunneum* (EAMa 01/58-Su, EAMb 01/158-Su and EAMb 09/01-Su) and *Beauveria bassiana* Bals. (Vuill) (Hypocreales: Clavicipitaceae) (EABb 01/33-Su and EABb 04/01-Tip) against important sunflower pathogens. Specifically, the pathogens were *Verticillium dahliae* Kleb. and *Cadophora helianthi* (L. Molinero-Ruiz, A. Martin-Sanz, C. Berlanas and D. Gramaje), two soilborne fungi causing sunflower wilts whose management is threatened by the appearance of populations of increased pathogenicity and by the total absence of control alternatives, respectively. Also the effect of EMA against *Plasmopara halstedii* Farl. Berl. & de Toni, causal agent of downy mildew of sunflower, was assessed. In this case, the management of the disease is hampered by either fungicide-resistant or highly virulent pathogen populations. The antagonism of the EMA against *V. dahliae* and *C. helianthi* was tested *in vitro* by dual plating, whereas the reduction of verticillium wilt was evaluated in sunflowers treated with the EMA and grown under greenhouse conditions. The effect of AME on *P. halstedii* was studied in axenic culture for 14 days, the maximum period during which the seedlings could grow in this *in vitro* system that limits their complete phenological development. Hence, the symptoms of mildew expressed by the seedlings were evaluated and the effect exerted by the AME strains on the growth of sunflower during this limited period of time was analysed. Moreover, we present an updated racial characterization of *P. halstedii* populations from Spain and other European countries. The results obtained in this line are presented in chapters IV and V.

In chapter II, compatibility between *H. didymator* and *M. brunneum* EAMa 01/58-Su strain was investigated in laboratory conditions. We studied both direct and indirect effects of the EMA toward parasitoid adults when directly applied by spraying them with conidial suspensions. Although the direct contact fungal treatments at the higher dose significantly reduced the parasitoids' life expectancy and caused a mortality slightly above 60%, their reproductive potential during the days after treatment was not affected. Furthermore, we evaluated two strategies for simultaneous application of fungus and parasitoid to control *S. littoralis* (exposure to fungus before parasitoid and *vice versa* at different releasing times). Both strategies were suitable for controlling this noctuid and showed compatibility between the two biocontrol agents, with additive effect in all combinations. However, we observed a significant reduction on the parasitization capability of the F1 parasitoid generation emerged from fungus-treated *S.*

littoralis larvae compared with parasitoids emerged from non-treated larvae of the same generation. Moreover, we observed that parasitization significantly reduced the number of haemocytes in the haemolymph of *S. littoralis* larvae, which caused a depletion on the immune system of the parasitized larvae, therefore promoting fungal pathogenesis and fungal outgrowth from the cadavers. Finally, parasitoid females showed a significant preference for non-treated *S. littoralis* larvae compared with fungus-treated larvae. Therefore, the results obtained in this chapter demonstrated high compatibility between the fungal strain and the parasitoid, despite direct and indirect effects of the fungus on parasitoids via direct contact, as well as a high efficacy of the simultaneous application of both biocontrol agents to control the cotton leafworm. This work opens doors to the experiments performed in chapter III.

In chapter III, we explored the impact of the endophytic behaviour of the EAMa 01/58-Su strain on the abovementioned multitrophic interactions. Hence, we sprayed melon plants with conidial suspensions, being subsequently colonized endophytically, and fed *S. littoralis* larvae on colonized plants in different *in vitro* and *in vivo* experiments. In addition, larvae were exposed to *H. didymator* adults at different releasing times. The sprayed plants were successfully colonized by the EMA within the first hours after fungal treatment in all the experiments, revealing the high endophytic behaviour of this strain. Total mortality of *S. littoralis* larvae was significantly affected by the presence of the parasitoid. Moreover, all treatments including the parasitoid achieved the highest mortality rates both *in vitro* and *in planta*. However, simultaneous exposure to the fungus and the parasitoid did not significantly affect the total mortality of *S. littoralis* larvae compared with when the parasitoid was used alone. The time between exposure to fungus and parasitoid did not affect *S. littoralis* mortality, nor parasitism or infection rates in any experiment. As in chapter II, we evaluated the parasitoid's preference for larvae exposed to the EMA, although in this case larvae were not directly treated, but fed on fungal-colonized plant. As in the previous chapter, parasitoids showed a significant preference for those larvae that had not been exposed to the EMA. Finally, we conducted a histological study of *S. littoralis* larvae simultaneously parasitized by *H. didymator* and infected by *M. brunneum* in order to examine intra-host relationships and observe the development of both biocontrol agents inside *S. littoralis*. Our micrographs showed, for the first time, the coexistence of both agents within the same host and the development of *H. didymator* larvae despite fungal colonization of *S. littoralis*. Our findings provide key information on compatibility between *H. didymator* and *M. brunneum* in *in planta* systems, what is a first step to deploy sustainable IPM programs for controlling the cotton leafworm at field and greenhouse conditions.

In chapter IV, the effect of two *B. bassiana* strains (EABb 01/33-Su and EABb 04/01-Tip) and three strains of *M. brunneum* (EAMa 01/58-Su, EAMb 09/01-Su and EAMb 01/158-Su) against the soilborne pathogens causing sunflower wilt *V. dahliae* y *C. helianthi*, was investigated. The five EMA were able to significantly inhibit the mycelial growth of both *V. dahliae* and *C. helianthi* when dually plated onto malt extract agar medium. The inhibition ranged from 8.3 to 63.5% in the case of *V. dahliae*, and from 19.6 to 37.4% in *C. helianthi*. Percentages of inhibition were dependent on both the EMA and the pathogen isolate in dual cultures with *V. dahliae*, whereas they were only dependent on the EMA in the case of *C. helianthi*. EABb 01/33-Su strain was the most effective antagonist against both pathogens. According to the dual cultures and microscopic examinations, two types of antagonism were exerted by the EMA: competition or antibiosis. Whereas most of the EMA were associated to only one type of antagonism, EAMa 01/58-Su strain (included in chapters II and III) was able of exert both, which had been reported in previous research. On the other hand, two EMA (EABb 01/33-Su and EAMb 09/01-Su) significantly inhibited the severity of verticillium wilt symptoms in sunflowers grown in the greenhouse (severities of 31 y 53%, respectively, as compared to 95% in control plants only inoculated with *V. dahliae*). The EMA were able to persist in the soil for at least two months after treatments, with the exception of EABb 04/01-Tip strain, which persistence reached up to the seventh week. Finally, the five EMA were molecularly detected inside the sunflowers at end of experiments (two months after application to soil), showing that their endophytic ability can exceed the transient period of plant colonization that has been reported for these fungi. Interestingly, the EMA only were molecularly detected inside the sunflowers in treatments with EMA not inoculated with *V. dahliae*. This finding suggests that the inhibition of *V. dahliae* by EMA occurs in the soil and, as a consequence, subsequent colonization of sunflower by the pathogen is reduced.

In chapter V, the management of downy mildew of sunflower was explored from two perspectives: genetic resistance and biological control. On the one hand, we present an updated racial

characterization of *P. halstedii* populations from Spain, France, Italy, Portugal and Romania. The races were assigned by means of the response of nine sunflower differential lines to inoculation with each of the pathogen populations. Twenty-three races were identified in Europe, whereas twenty-two of them were detected in Spain. The most frequent races were 310, 304, 705 and 715. The variety of races of *P. halstedii* was wider than in previous years, whereas we identified a high number of highly virulent ones. On the other hand, the effect of the five EMA strains on the severity of the disease in sunflower seedlings and on their development in axenic culture was evaluated only for 14 days, given the limitations of this *in vitro* culture for plant development. None of the EMA strains significantly reduced the severity or affected the growth of the plants during the two weeks studied, when they were treated and inoculated, respectively, with both organisms. When the plants were inoculated only with EMA strains, the characteristic growth developmental patterns associated with the establishment of the endophyte were detected in plants treated with EABb 01/33-Su and, to a lesser extent, other two fungal strains. Although the EMA were isolated from plants in different percentages, they were molecularly detected only when they were applied without *P. halstedii*. Our results suggest that the assayed conditions were not suitable for the endophytic colonization of plants by EMA nor for the infection by *P. halstedii*.

In conclusion, the present thesis delves into the mechanisms underlying multitrophic interactions in systems including plants, macro- and/or microbial biological control agents and plant pests or pathogens. The EMA *M. brunneum* has shown a remarkable potential to be simultaneously applied with *H. didymator* under different conditions. Furthermore, the five strains of the EMA *M. brunneum* and *B. bassiana*, particularly EABb 01/33-Su and EAMb 09/01-Tip, are promising candidates to control some sunflower pathogens due to their *in vitro* antagonism against *V. dahliae* and *C. helianthi* and, above all, to the reduced severity of verticillium wilt when they were applied to sunflowers. However, the potential of EMA against downy mildew of sunflower must be thoroughly studied. In axenic culture, none of the tested EMA showed any effect on the severity of symptoms by *P. halstedii*. Finally, the five EMA showed a lasting endophytic property in sunflower and EAMa 01/58-Su also in melon. However, further research will elucidate possible strategies for application of these EMA in the field as part of IPM programs.

Keywords: biological control, entomopathogenic fungi, multitrophic interactions, intra-host relationships, cotton leafworm, parasitoids, integrated pest management, soilborne pathogens, sunflower diseases, plant pathogens, compatibility, integrated pest and disease management

ÍNDICE GENERAL

ÍNDICE DE FIGURAS

CAPÍTULO I. INTRODUCCIÓN

Figure V.2. Bands from PCR amplification of the ribosomal DNA region (5.8S rDNA and internal transcribed spacers 1 and 2) with the universal primer set ITS5 and ITS4 from individual sunflower plants only treated with entomopathogenic fungi (EF). The higher molecular weight band is sunflower-specific, and the lower molecular weight band is EF-specific. M, 100–2000 bp BrightMAX^{TM} DNA ladder (Canvax Biotech); Lanes 1–4, DNA from four individual plants of sunflower breeding line HA-304 treated with *Beauveria bassiana* strain EABb 04/01-Tip; Lane 5, DNA of EABb 04/01-Tip; Lane 6, DNA of sunflower HA-304; Lanes 7–10, DNA from four individual plants of sunflower breeding line HA-304 treated with *Metarhizium brunneum* strain EAMa 01/58-Su; Lane 11, DNA of EAMa 01/58-Su; Lane 12, DNA of sunflower HA-304; Lanes 13–16, DNA from four individual plants of sunflower breeding line HA-304 treated with *M. brunneum* strain EAMb 09/01-Su; Lane 17, DNA of EAMb 09/01-Su; Lane 18, DNA of sunflower HA-304; Lanes 19–22, DNA from four individual plants of sunflower breeding line HA-304 treated with *M. brunneum* strain EAMb 01/158-Su; Lane 23, DNA of EAMb 01/158-Su; Lane 24, DNA of sunflower HA-304; Lane 25, negative control of PCR amplification. 155

ÍNDICE DE TABLAS

CAPÍTULO I. INTRODUCCIÓN

CAPÍTULO II. COMPATIBILITY BETWEEN THE ENDOPARASITOID *Hyposoter didymator* AND THE ENTOMOPATHOGENIC FUNGUS *Metarhizium brunneum*: A LABORATORY SIMULATION FOR THE SIMULTANEOUS USE TO CONTROL *Spodoptera littoralis*

CAPÍTULO IV. EVIDENCE OF SOIL-LOCATED COMPETITION AS THE CAUSE OF THE REDUCTION OF SUNFLOWER VERTICILLIUM WILT BY ENTOMOPATHOGENIC FUNGI

CAPÍTULO V. UPDATED CHARACTERIZATION OF RACES OF *Plasmopara halstedii* AND ENTOMOPATHOGENIC FUNGI AS ENDOPHYTES OF SUNFLOWER PLANTS IN AXENIC CULTURE

CAPÍTULO I. INTRODUCCIÓN

I.1. Introducción

La agricultura (vocablo que deriva del latín *agri*, "campo", y *cultūra*, "cultivo o crianza") es definida por el Diccionario de la Lengua Española como el arte de cultivar la tierra, o la labranza o cultivo de la tierra, y comprende el empleo de diversas técnicas, destrezas y conocimientos encauzados a la producción primaria de bienes de naturaleza agraria para su uso, consumo, transformación, comercialización o incorporación a otros sistemas productivos (Manley et al., 2019). Desde un punto de vista práctico, y excluyendo tanto la componente animal de la rama agraria como los modernos sistemas de cultivo forzado que prescinden en gran medida de activos fijos tan comunes en el balance de cualquier explotación agraria como lo es el suelo, la agricultura conlleva la roturación de tierras para el cultivo de especies vegetales de las cuales se pretende obtener un producto con una función o destino comercial predeterminados (Manley et al., 2019).

La práctica agrícola se encuentra sometida a diferentes factores o agentes que condicionan el rendimiento de los cultivos. Son tan numerosos y variados que no podrían abarcarse en su totalidad sino en una exhaustiva revisión *ad hoc*, que no supone el objeto del presente documento. Baste decir que, amén de la climatología, la edafología, la calidad de perecederos de los productos agrarios, los desastres naturales, los complejos mecanismos que rigen los mercados y las políticas nacionales e internacionales, los cultivos y productos agrarios se hallan expuestos al efecto de distintos agentes bióticos, que pueden ocasionar problemas fitosanitarios de impacto disímil. A grandes rasgos, los principales organismos nocivos de interés agrícola son los animales fitófagos, los microorganismos fitopatógenos y otras plantas que compiten por recursos con la cultivada o bien pueden parasitarla (Tremblay, 2000).

Los estudios geológicos y paleontológicos constatan que los seres autótrofos, las plantas, aparecen primero que los heterótrofos, con énfasis en los animales, y durante millones de años los dos grupos han estado interaccionando sin que ello supusiera peligro para la subsistencia de unos y otros. Ni siquiera la aparición del hombre, hace aproximadamente 315000 años (Callaway, 2017), consigue interferir negativamente en el equilibrio existente entre autótrofos y heterótrofos. Sin embargo, en algún momento de la historia, entre 20000 y 10000 años a.C., se comenzó a domesticar a algunas especies vegetales comestibles por parte de comunidades humanas que dejan de ser nómadas para hacerse sedentarias: había surgido la agricultura (Maroto, 1998; Brown et al., 2009; Cubero, 2018). La acción del hombre altera el equilibrio de estos primitivos ecosistemas agrícolas, que pierden complejidad y diversidad respecto a los ecosistemas naturales, para favorecer la acción de los insectos fitófagos, que comienzan a competir con los intereses del hombre; son las primeras plagas de insectos, cuyo azote ha llegado a nuestros días (Rajendran y Sing, 2016).

Existen numerosas referencias a este azote, muy conocidas las bíblicas sobre las plagas de langosta que azotaban los cultivos en tiempos de los faraones. Pero probablemente, la primera referencia documentada acerca de la importancia de los agentes fitoparásitos en las plantas cultivadas pueda encontrarse en el tratado *De*

historia plantarum (“Historia de las plantas” en castellano) del filósofo griego Teofrasto, discípulo de Aristóteles, que ya había descrito a los insectos. En dicha obra, el autor mencionaba que las plantas cultivadas resultaban más propensas a sufrir el ataque de enfermedades que las silvestres, y que su incidencia era a menudo más grave. Como enfermedades más características, se describían “roñas”, “podredumbres”, “tumores” y “hongos”, entre otras, así como la incidencia de invertebrados varios como “gusanos” y “mosquitos” (Teofrasto, siglo IV a.C.). Autores posteriores de la talla de Columela (siglo I), Plinio el Viejo (siglo I), San Isidoro (siglo VII), Abú Zacaría (siglo XII), Olivier de Serres (siglo XVI), Alonso de Herrera (siglo XVI) y Fray Benito Feijóo (siglo XVIII) han incidido en esta problemática, sugiriendo el empleo de remedios para paliar los efectos nocivos de los agentes bióticos sobre los cultivos.

Actualmente, las disminuciones de rendimiento ocasionadas por problemas fitosanitarios a nivel mundial oscilan, habitualmente, entre el 26 y el 50% dependiendo del cultivo y su sistema de manejo (Oerke, 2006; Savary et al., 2019). En consecuencia, una de las principales preocupaciones del ser humano ha sido, desde hace siglos, reducir el perjuicio ocasionado por los agentes bióticos responsables de la minoración del producto cosechado. A lo largo del tiempo, han aparecido diferentes métodos, remedios y soluciones tecnológicas con las que los agricultores han podido controlar, en mayor o menor grado, a los organismos parasitarios que amenazan sus cultivos, aliviando los estreses producidos por ellos.

I.1.1. Control integrado de plagas y agricultura sostenible

La expresión “gestión integrada de plagas” (GIP), sinónimo de manejo integrado, control integrado o lucha integrada, es una traducción del inglés “Integrated Pest Management”. Las primeras definiciones de este sistema de manejo fueron acuñadas en los años cincuenta (Smith y Allen, 1954; Stern et al., 1959), cuyas líneas principales quedan plasmadas en la definición que acuñasen Smith y Reynolds en un simposio de la FAO en los años sesenta, en pleno ocaso de la revolución verde: “*a pest management system that, in the context of the associated environment and the population dynamics of the pest species, utilizes all suitable techniques and methods in as compatible a manner as posible and maintains the pest population at levels below those causing economic injury*” (Smith y Reynolds, 1966).

La concepción actual no dista mucho de la descrita, pues la Directiva 2009/128/EC del Parlamento Europeo y del Consejo define “Gestión Integrada de Plagas” de la siguiente manera: “*El examen cuidadoso de todos los métodos de protección vegetal disponibles y posterior integración de medidas adecuadas para evitar el desarrollo de poblaciones de organismos nocivos y mantener el uso de productos fitosanitarios y otras formas de intervención en niveles que estén económica y ecológicamente justificados y que reduzcan o minimicen los riesgos para la salud humana y el medio ambiente*”.

La GIP, tal como se concibe hoy día, conlleva la racionalización del empleo de productos fitosanitarios; aunque no proscribe su utilización, prioriza los métodos de control no químicos, más respetuosos con el medioambiente, tales como: las disposiciones legales, esto es, todas aquellas actuaciones de carácter normativo llevadas

a cabo por los organismos internacionales y los gobiernos de las naciones que pretenden evitar la llegada a un territorio de un organismo nocivo o limitar su dispersión si ya se encuentra presente; las medidas físicas, agronómicas y culturales, como la rotación de cultivos, racionalización de las labores agrícolas, y otras de diferente naturaleza; la obtención de variedades resistentes, donde la mejora vegetal y la ingeniería genética permiten la obtención de cultivares resistentes que incorporen, al mismo tiempo, los caracteres agronómicos de interés; el control químico, que trata de minimizarse en la normativa vigente por su efecto negativo sobre el medioambiente, la fauna auxiliar y los seres vivos; y el control biológico de plagas.

La importancia del estudio y la aplicación de la GIP no se limita a su interés científico, agronómico y medioambiental, puesto que este método de manejo es una obligación para muchos productores agrarios en todo el mundo. Desde la Conferencia de las Naciones Unidas sobre el Medio Ambiente y el Desarrollo de Río de Janeiro, en 1992, la GIP ha comenzado a integrarse en las políticas agrarias de los diferentes Estados que componen la ONU (Coll y Wajnberg, 2017). En la Unión Europea (UE), la Directiva 2009/128/EC es la primera norma jurídica de carácter vinculante que obliga a los Estados miembros a su aplicación (Artículo 19: "*En virtud del Reglamento (CE) no 1107/2009 y de la presente Directiva, la aplicación de los principios de la gestión integrada de plagas es obligatoria*").

En España, la transposición de su contenido fue llevada a cabo mediante el Real Decreto 1311/2012, que, entre otras cuestiones, asienta el marco legal por el que la GIP es una obligación para los productores españoles desde 2013, debiendo registrar los tratamientos fitosanitarios que lleven a cabo en su cuaderno de explotación. Esta normativa prioriza los métodos no químicos de control de plagas, donde destaca el control biológico, que contribuye a garantizar la seguridad e inocuidad alimentaria, así como a proteger la biodiversidad, en una clara promoción de los principios de la agricultura sostenible.

I.1.2. Control biológico de plagas

El control biológico o biocontrol consiste en el empleo de organismos vivos, ya sean de naturaleza macro (entomófagos) o microscópica (entomopatógenos), para reducir la presencia o la incidencia de los agentes nocivos bióticos en las explotaciones agrarias (Eilenberg et al., 2001; Barratt y Ehlers, 2017). Esta definición excluye todas aquellas praxis que no conlleven la utilización de "organismos vivos" *sensu stricto*, e.g. la aplicación de formulados inertes a partir de metabolitos (Eilenberg et al., 2001), aunque la Organización Internacional de la Lucha Biológica e Integrada contiene en su concepto de control biológico no solo a los seres vivos, sino también a sus productos ("*la utilización de organismos vivos, o de sus productos, para evitar o reducir las pérdidas o daños causados por los organismos nocivos*"), definición de gran impacto en la concepción moderna de bioinsecticida, que incluiría no solo agentes entomófagos y microorganismos entomopatógenos, sino además feromonas, extractos vegetales y extractos de microorganismos.

Tradicionalmente, se han considerado tres variantes del control biológico: en primer lugar, **el control biológico clásico**, consistente en la importación de enemigos

naturales foráneos (habitualmente, del área geográfica de origen del organismo nocivo) con el objetivo de que se diseminen por el medio y den lugar a poblaciones estables; el segundo tipo, **el control biológico por conservación**, conlleva aquellas actuaciones y modificaciones del entorno que propician la potenciación de los herbívoros, depredadores, parasitoides y patógenos naturalmente presentes; y, por último, **el control biológico aumentativo** se basa en la liberación de enemigos naturales durante estaciones favorables o en ambientes confinados para que efectúen un menoscabo en las poblaciones del organismo diana, sin vocación de su establecimiento duradero en el sistema (Barratt y Ehlers, 2017). Empero, ciertos autores discrepan de esta sistemática y proponen modificaciones parciales a la misma. Eilenberg et al. (2001) subdividió el control aumentativo en dos prácticas diferentes: el **control biológico por inoculación**, entendido este como la liberación de un agente de biocontrol con la intención de que se multiplique y efectúe un control de organismos nocivos prolongado en el tiempo, pero no permanente; y el **control biológico por inundación**, en el que se suelta un agente de biocontrol, al igual que en el caso anterior, pero sin la intención de su reproducción, limitándose el control ejercido a los propios organismos liberados y no a sus descendientes. Otras clasificaciones mantienen gran parte de esta nomenclatura, enumerando cuatro vertientes de este método de manejo: control biológico clásico o inoculativo, aumentativo o inoculativo estacional, inundativo y por conservación (Urbaneja y Jacas, 2008); si bien, las bases y las técnicas comprendidas son análogas, en cualquier caso. Los agentes de control biológico pueden dividirse en agentes **microbianos**, a los que nos referiremos más adelante, y agentes **macrobianos.**

I.1.2.1. Control macrobiano de plagas

Los agentes macrobianos de control de plagas son aquellos que pueden integrarse en una escala macroscópica, donde los más recurrentes en el ámbito agrícola son los animales **entomófagos**, que a su vez se dividen en **depredadores y parasitoides** (Nazir et al., 2019). Los depredadores son animales que se alimentan a expensas de otros insectos, bien en su estado preimaginal, bien en su estado imaginal, bien durante ambos (Jacas et al., 2008). Los depredadores pueden devorar a su presa o parte de ella, ingerir sus huevos o nutrirse de su hemolinfa. Los parasitoides, por otro lado, son animales que parasitan a otros durante sus estadios inmaduros, teniendo el imago por lo general una vida libre (Ahmed et al., 2016). Para ello, efectúan su oviposición junto al insecto al que se dirige su ataque, o bien directamente en su interior (Pina, 2008). Este actuará como hospedante de su prole, que permanecerá gran parte de su estado larvario en un íntimo contacto con aquel (pudiendo vivir dentro o fuera de su cuerpo, en cuyo caso es calificado de endoparasitoide o ectoparasitoide, respectivamente) y se alimentará de su hemolinfa u órganos hasta que lo haya consumido por completo o haya ingerido el suficiente alimento para completar su desarrollo larvario, causando un perjuicio al insecto hospedante que desembocará inequívocamente en la muerte del mismo o su esterilidad (Clarke et al., 2019). Esta última particularidad es la que distingue a los parasitoides de los parásitos convencionales.

El empleo de agentes de control biológico por el hombre es tan antiguo, probablemente, como la propia civilización: desde el uso de felinos en el antiguo Egipto

para hacer frente a plagas urbanas como las ratas (Jacas y Urbaneja, 2008) hasta los agricultores chinos que, en el siglo IV, reubicaban hormigueros de *Oecophylla smaragdina* (F.) (Hymenoptera: Formicidae) en sus plantaciones para garantizar el trasiego de individuos que depredasen a la chinche *Tessaratoma papillosa* Drury (Hemiptera: Pentatomidae) (DeBach, 1974), práctica que ha pervivido hasta nuestros días (Jacas y Urbaneja, 2008). En la actualidad, diversos autores consideran al control biológico como una pieza clave de la protección de cultivos moderna, así como la piedra angular de la GIP y la agricultura sostenible (Jacas y Urbaneja, 2008).

I.1.2.2. Control microbiano de plagas

Los agentes microbianos de control de plagas incluyen a todos los microorganismos entomopatógenos, principalmente hongos, bacterias y virus, que han tenido un pronunciado desarrollo comercial (Hatting et al., 2019; Nazir et al., 2019), así como otros organismos tales como protozoos y nematodos (Lacey, 2008). El modo de actuación de cada uno es dependiente del taxón al que pertenecen, desde el Reino hasta la especie, si bien, en general, coinciden en causar la infección del insecto —pudiendo ocasionar su muerte— y, en ocasiones, en diseminarse hacia otros individuos de esta o distinta especie.

La vía de entrada más común en gran parte de ellos es la ingestión, tal es el caso de virus, bacterias y protozoos entomopatógenos; mientras tanto, otros penetran directamente a través de aperturas naturales, como hacen los nematodos entomopatógenos (Gozel y Gozel, 2016). Por último, los hongos entomopatógenos (HE) hallan en el tegumento su principal ruta de infección, sin que ello ocasione detrimento alguno en la otra vía de entrada referida (Quesada-Moraga et al., 2020).

En la definición *sensu latto* de control biológico, que incluye los productos derivados de organismos vivos, el modo de acción dependerá completamente de la naturaleza de los formulados obtenidos, donde resulta frecuente la acción tópica en los extractos (Akhtar et al., 2009) y la ingestión en los bioinsecticidas a partir de la proteína Cry de *Bacillius thuringiensis* Berliner (Bacillales: Bacillaceae) (Bravo et al., 2011).

Los productos de control biológico gozan de una creciente popularidad que se manifiesta en el incremento de su presencia en los mercados, ostentando una tasa de crecimiento sostenida en el último decenio que dobla a la de los fitosanitarios de origen químico (Dunham y Trimmer, 2017). No obstante su rápida penetración comercial, los formulados y agentes de biocontrol aún no alcanzan una magnitud equiparable a la de aquellos (Rubin, 2010; Quesada-Moraga et al., 2020). La equiparación de ambos acontecería, de manera presumible, en 2050 (Orson, 2015). Sin duda, la normativa vigente promueve intensamente el biocontrol, y existe una necesidad urgente en el mercado de nuevos productos *ad hoc*, no solo para ajustarse a los principios de sostenibilidad en la agricultura, sino por la reducción vertiginosa del número de materias activas de origen químico autorizadas en el registro de productos fitosanitarios.

I.2. Los hongos entomopatógenos

I.2.1. Clasificación y diversidad de los hongos entomopatógenos

Los HE se engloban en cuatro divisiones: dos de ellas, Entomophthoromycota y Ascomycota, mayoritarias en cuanto a número de especies de carácter entomopatogénico (Tabla I.1); y las otras dos, Blastocladiomycota y Basidiomycota, que abarcan una menor cantidad. Fruto de su diversidad, se ha descrito toda una plétora de especies de HE, estimándose su número total en 700-750, los órdenes más representativos en términos cuantitativos siendo Entomophtorales e Hypocreales, que pertenecen, respectivamente, a la primera y la segunda de las divisiones citadas (Hibbett et al., 2007; Humber, 2012; Gryganskyi et al., 2013). Los entomoftoromicetos son biotrofos obligados, resultando su cultivo difícil o imposible, según el caso; en consecuencia, las perspectivas de su explotación comercial son, cuanto menos, limitadas (Keller, 2007; Pell et al., 2010). Distinta es la situación de los ascomicetos mitospóricos entomopatógenos (AME), clasificación que engloba a más de seiscientas de las especies de HE, que poseen ciclos complejos que habitualmente alternan fases de infección en que residen en sus hospedantes invertebrados con otras en las que habitan el medio edáfico, o bien se mantienen en estrecha asociación de epifitismo o endofitismo con los individuos vegetales que en él hallan soporte (Quesada-Moraga y Santiago-Alvarez, 2008; Chandler, 2017; Quesada-Moraga et al., 2019; Quesada-Moraga et al., 2020; Quesada-Moraga, 2020). En contraposición a los anteriores, el manejo y cultivo en laboratorio de estos hongos entraña una menor complejidad, lo que facilita su multiplicación masiva, propiciando de este modo la producción de inóculo para el desarrollo de bioinsecticidas o cuantas soluciones comerciales resulten factibles (Lacey et al., 2015; Lacey, 2017). Resultan de obligada mención los órdenes Hypocreales y Eurotiales, dentro de los cuales hay familias, y en concreto Clavicipitaceae, Cordycipitaceae, Ophiocordycipitaceae y Trichocomaceae, que han suscitado un especial interés en la comunidad científica (Lacey et al., 2015; Lacey, 2017).

I.2.2. Patogénesis de los ascomicetos mitospóricos entomopatógenos

Los AME presentan, de manera natural, un efecto bioinsecticida frente a insectos y otros artrópodos (Litwin et al., 2020), facilitado por la adaptación del hongo al ciclo vital de su hospedante animal, así como a las condiciones ambientales, al entorno e, incluso, a las modificaciones antrópicas (Quesada-Moraga y Santiago-Alvarez, 2008; Nowak et al., 2019; Litwin et al., 2020). El ciclo vital de los AME consta de dos fases subsecuentes, una patogénica y la otra saprofítica, que van a describirse a continuación:

-La fase patogénica conlleva la sucesión de una serie de eventos que posibilitan la infección del insecto: una vez los conidios, esporas asexuales de carácter infectivo que producen estos hongos, alcanzan el tegumento del insecto, pues es esta su vía de entrada más frecuente, se adhieren al mismo (Senthil-Nathan, 2015; Quesada-Moraga et al., 2020). La adhesión se logra gracias a la interacción de fuerzas hidrofóbicas de la pared celular (Ortiz-Urquiza y Keyhani, 2013). Acontece en ese momento la germinación de las referidas esporas, resultando comunes la subsiguiente formación de

Tabla I.1. Clasificación sistemática de las principales divisiones de hongos entomopatógenos. Fuente: Adaptado de Hibbett et al., 2007, Humber, 2012 y Gryganskyi et al., 2013

División	**Clase**	**Orden**	**Familia**	**Género**
Entomophthoromycota	Entomophthoromycetes	Entomophthorales	Entomophthoraceae	*Batkoa, Entomophaga, Entomophthora, Erynia, Eryniopsis, Furia, Massospora, Orthomyces, Pandora, Strongwellsea, Zoophthora*
			Ancylistaceae	*Ancylistes, Conidiobulus, Macrobiotophthora*
			Completoriaceae	*Completoria*
			Meristacraceae	*Meristacrum, Tabanomyces*
	Basidiobolomycetes	Basidiobulus	Basidiobolaceae	*Basidiobolus*
	Neozygitomycetes	Neozygitales	Neozygitaceae	*Apterivorax, Neozygites, Thaxterosporium*
Ascomycota	Sordariomycetes	Hypocreales	Clavicipitaceae	*Hypocrella, Metacordyceps, Regiocrella, Archersonia, Metarhizium, Tipo–Paecilomyces, Pochonia Normurea, Tipo–Verticillium*
			Cordycipitaceae	*Cordyceps,Torrubiella, Isaria, Beauveria, Engyodontium, Akanthomyces, Lecanicillium, Simplicillium, Microhilum,Tipo–Mariannaea*
			Ophicordycipitaceae	*Elaphocordyceps, Hirsutella, Syngliocardium, Paraisaria Ophiocordiceps, Sorosporella, Topypocladium, Hymenostibe Culicinomyces, Metarhiziopsis, Tipo–paecilomyces, Tipo–verticillum*
	Eurotiomycetes	Eurotiales	Trichocomaceae	*Paecilomyces*

una estructura de anclaje que recibe el nombre de apresorio y la emersión de las hifas infectivas que proceden a la penetración de la cutícula del insecto mediante una combinación de acciones mecánicas y alteraciones bioquímicas derivadas fundamentalmente de la producción de enzimas hidrolíticas de tipo proteasa, quitinasa y lipasa (Quesada-Moraga y Santiago-Alvarez, 2008; Vega et al., 2012). Conforme el hongo atraviesa la cutícula, la epidermis y la membrana basal de su víctima, se abre camino hacia el hemocele, donde, hemolinfa mediante, podrá desarrollarse y dispersarse en forma de hifas y cuerpos hifales a lo largo de la anatomía del insecto, si bien deberá hacer frente a la respuesta defensiva de su hospedante, que será tanto de tipo celular (en concreto, fagocitosis y encapsulación) (Han et al., 2013; Tseng et al., 2014) como humoral (producción de péptidos defensivos y proteínas como lectinas o fenoloxidasa) (Vey et al., 2001; Volkoff et al., 2003). Por último, deviene la muerte del insecto, resultado de la combinación de diversos factores entre los que destacan la asfixia por la localización del hongo en el sistema respiratorio, la sustracción de nutrientes, la invasión de órganos y tejidos, el desgarro ocasionado por las hifas durante su trasiego y la producción de metabolitos entomotóxicos (Rios-Moreno et al., 2016; Quesada-Moraga et al., 2020).

-Fase saprofítica, consecuencia de la anterior. Tras la muerte del artrópodo, y únicamente si las condiciones ambientales lo permiten, las hifas del hongo emergen al exterior del cadáver, esporulando y produciendo conidióforos y conidios, los últimos dispersándose entonces por medio del viento, el agua libre o los propios insectos (Quesada-Moraga et al., 2020). Al establecer contacto con el tegumento de nuevos individuos fruto de esta transmisión horizontal, tiene lugar el reinicio del ciclo completo (Goettel et al., 2005; Charnley y Collins, 2007; Ortiz-Urquiza y Keyhani, 2016) (Figura I.1).

I.2.2.1. Nuevos modos de acción de los ascomicetos mitospóricos entomopatógenos

Estudios recientes ponen de manifiesto la existencia de diferentes actividades de los AME por la vía de la ingestión, bien de conidios, bien de material vegetal colonizado endofíticamente por estos hongos. Respecto a la primera ruta, se ha descrito cómo los AME pueden matar al insecto sin necesidad de que se desarrolle el proceso de infección: en este sentido, se ha comprobado que la ingestión de conidios de *Metarhizium* sp. produce elevados niveles de expresión de genes relacionados con el estrés, como las proteínas de choque térmico (HSPs), que a su vez pueden regular la actividad caspasa y conducir a la muerte celular y en último término a la del insecto (Butt et al., 2013).

Respecto a la segunda ruta, una vez los insectos han ingerido tejido vegetal colonizado por los AME, puede ocurrir una elevada mortalidad entre los fitófagos, la cual parece relacionada con la secreción y liberación de compuestos entomotóxicos por el hongo, o de radicales libres por la planta tras una posible reacción sistémica inducida por el entomopatógeno (Quesada-Moraga et al., 2014b; Garrido-Jurado et al., 2015a). Curiosamente, los insectos que han fenecido de esta manera rara vez desarrollan crecimiento fúngico a partir de su cadáver, resultando infrecuente (Garrido-Jurado et al., 2017), si no imposible (Resquin-Romero et al., 2016a) su aislamiento. Tal circunstancia

es atribuida a una interferencia del entomopatógeno con su propio ciclo de patogénesis ocasionando una muerte prematura del hospedante por intoxicación que imposibilita su crecimiento saprofítico, que requiere de más tiempo y humedad (Resquin-Romero et al., 2016a).

Este modo de acción doble, esto es, tanto por ingestión como por vía tegumentaria, sitúa a los HE a la vanguardia de los microorganismos entomopatógenos, lo que los hace idóneos para su inclusión en estrategias de GIP (Quesada-Moraga y Santiago-Alvarez, 2008).

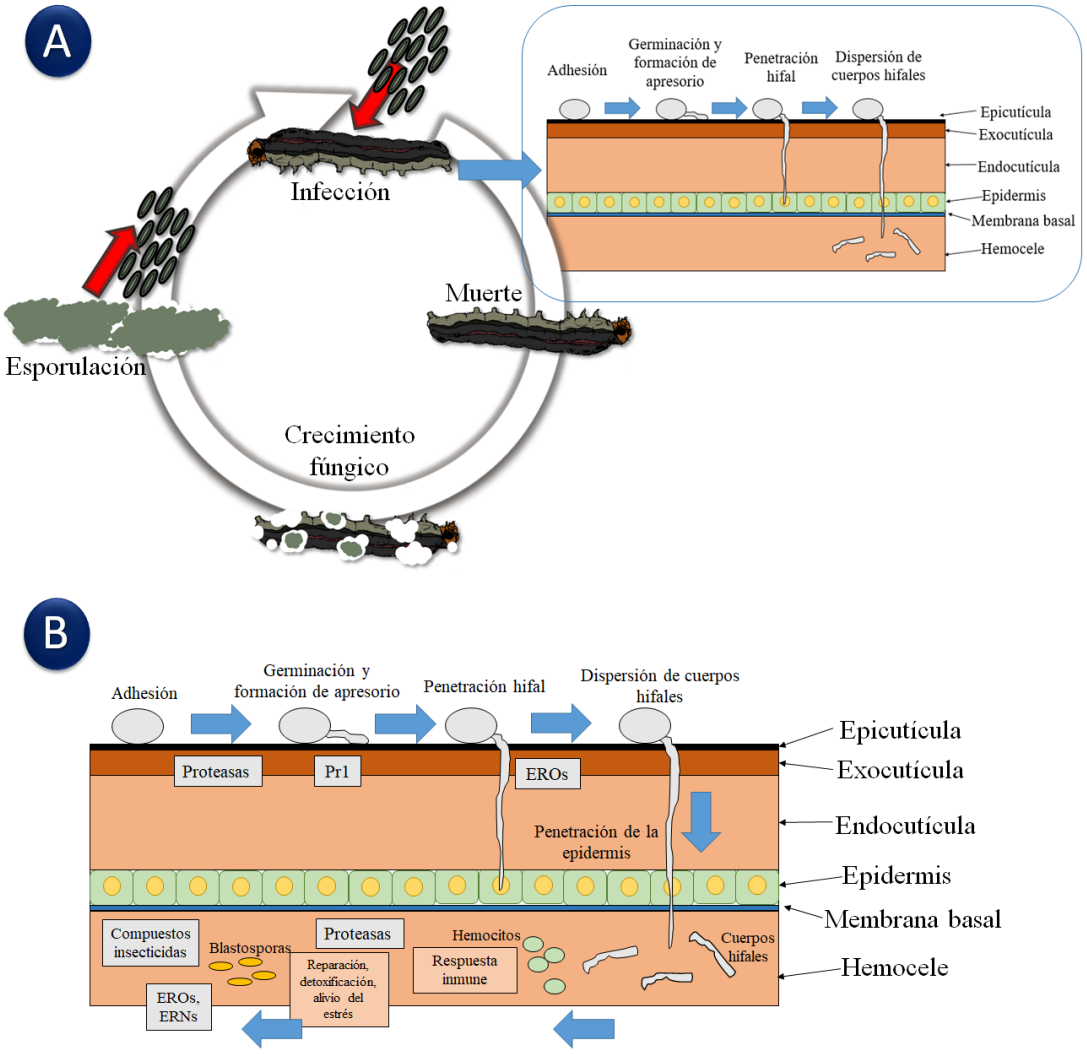

Figura I.1. Ciclo de patogénesis de un ascomiceto mitospórico entomopatógeno. A. Representación esquemática de las cuatro fases de la patogénesis: infección del hospedante, muerte del hospedante, crecimiento fúngico saprofítico desde el cadáver y esporulación y dispersión de conidios. B. Esquema de las principales interacciones que acontecen durante la infección del hospedante. Los compuestos producidos por el entomopatógeno se representan en gris, mientras que las respuestas del hospedante aparecen en naranja. Fuente: Adaptado de Quesada-Moraga et al., 2020.

1.2.3. Empleo comercial de ascomicetos mitospóricos entomopatógenos

El potencial de los AME para el control de insectos se ha investigado desde hace siglos. Se tiene constancia de que en Oriente se conocían asociaciones entre hongos e insectos ya en el año 900 (Steinhaus, 1975). Sin embargo, el primer trabajo científico que demostró el poder entomopatógeno de uno de estos organismos data del siglo XIX, cuando Bassi (1835) aisló al hongo *Beauveria bassiana* Bals. (Vuill) (Hypocreales: Clavicipitaceae) a partir de larvas de *Bombyx mori* L. (Lepidoptera: Bombycidae), el gusano de la seda (Figura I.2). Entre finales del siglo XIX y principios del XX, el interés de la comunidad científica en los AME creció al ser percibidos como una alternativa muy prometedora para el control de plagas de insectos, solo para desvanecerse súbitamente décadas después conforme el empleo de insecticidas químicos se convertía en la norma (Roberts y Hajek, 1992). Paradójicamente, los múltiples obstáculos interpuestos en el recorrido comercial de los fitosanitarios han propiciado el reflorecimiento paulatino de la investigación en los AME (Roberts y Hajek, 1992).


DEL MAL DEL SEGNO
CALCINACCIO O MOSCARDINO
MALATTIA CHE AFFLIGGE
I BACHI DA SETA
E SUL MODO
DI LIBERARNE LE BIGATTAJE
ANCHE LE PIU INFESTATE
TEORIA
LODI
DALLA TIPOGRAFIA ORCESI
1835

Figura I.2. Frontispicio de "*Del mal del segno, calcinaccio o moscardino, malattia che affligge i bachi da seta, e sul modo di liberarne le bigattaje, anche le più infestate*" (Bassi, 1835).

El empleo comercial de los AME se encuentra en pleno desarrollo en la actualidad, si bien se ha visto lastrado por las limitaciones relacionadas con las exigencias de registro, aún idénticas a las de los insecticidas químicos, así como los derivados de sus tiempos letales, si se comparan con sustancias químicas con efecto de choque. Sin embargo, la reducción progresiva del número de sustancias químicas disponibles, la promoción creciente de la sostenibilidad agrícola, sin olvidar el mejor conocimiento de la respuesta de estos hongos a los factores ambientales, amén del perfeccionamiento de las tecnologías de producción y aplicación, han permitido un

acceso creciente al mercado de estos microorganismos (Lacey et al., 2015). De hecho, la penetración en el mercado de los formulados de control biológico durante las pasadas dos décadas ha alcanzado nada menos que el 7% de la cuota de mercado, con una tasa de crecimiento estable del 15% que dobla a la de los productos químicos de síntesis (Marketsandmarketc.com, 2016; Dunham y Trimmer, 2017). El volumen de ventas de los formulados de control biológico de origen fúngico fue de 401.6 M$ en 2016, con una tasa de crecimiento superior al 19% (Marketreportsworld.com, 2019). A día de hoy, existen más de 30 formulaciones comerciales de AME en todo el mundo, que incorporan a un total de 13 especies fúngicas diferentes, si bien el 80% de ellas pertenecen a *B. bassiana* (Butt et al., 2016; Quesada-Moraga et al., 2020); destaca por su presencia comercial, asimismo, el género *Metarhizium*, concretamente las especies *M. anisopliae* (Metschn.) y *M. brunneum* (Petch) (Hypocreales: Clavicipitaceae) (Lacey et al., 2015). A pesar de ello, las cifras reales de ventas de estos productos son elusivas y, en gran medida, contradictorias: según un estudio de mercado, las ventas de formulados comerciales de AME superaron en 2019 los 50 M$, de las cuales más del 31% se efectuaron en EEUU, que se posiciona como primer demandante de estos productos, y el 30% en la UE (Reportsmonitor.com, 2019); sin embargo, otras fuentes aluden a Brasil y China como los principales consumidores de micoinsecticidas (Li et al., 2010).

Por último, cabe destacar que el empleo de AME en estrategias de GIP suscita un gran interés por la destacada aptitud insecticida de ciertas cepas (Quesada-Moraga et al., 2014b), su compatibilidad con otros agentes de biocontrol (Gonzalez-Mas et al., 2019a) y su seguridad desde el punto de vista ecológico y medioambiental (Vega, 2018).

I.2.4. Presencia natural de ascomicetos mitospóricos entomopatógenos

Los AME exhiben una distribución cosmopolita, encontrándose naturalmente presentes en una gran variedad de ecosistemas terrestres a lo largo y ancho de los cinco continentes (Aira et al., 2007). Pese a su relevancia en el contexto del control de plagas, no ha habido disponibilidad de información comprehensiva acerca del rol de estos organismos en los ecosistemas sino hasta la pasada década (Garrido-Jurado et al., 2015b).

Tradicionalmente se ha considerado al suelo como el principal reservorio natural de estos hongos, amén de los propios artrópodos infectados (Klingen y Haukeland, 2006; Quesada-Moraga et al., 2007; Jaronski, 2010). No obstante, estudios recientes han puesto de manifiesto la ubicuidad de los AME, los cuales son capaces de persistir con mayor o menor duración y frecuencia en hábitats y entornos como la rizosfera (Hu y St Leger, 2002), la filosfera (Garrido-Jurado et al., 2015b), el interior de las plantas (Vega, 2018) o incluso el aire (Aira et al., 2007).

I.2.4.1. Presencia en el suelo

Los AME pueden encontrarse con frecuencia en el suelo, tanto virgen como roturado (Quesada-Moraga et al., 2007; Jaronski, 2010), donde se ha identificado a gran

parte de las especies de AME descritas (Quesada-Moraga et al., 2007). Estos microorganismos se localizan de manera preferente en el estrato edáfico superior, abundando en los primeros diez centímetros y escaseando conforme aumenta la profundidad, siendo su origen, por lo general, conidios desprendidos de insectos infectados (Jaronski, 2007; Quesada-Moraga et al., 2007). La distribución de los AME a lo largo del perfil del suelo, su dinámica poblacional y su diversidad se ven profundamente afectadas por el manejo antrópico de los agroecosistemas que pueblan (Keller et al., 2003; Meyling y Eilenberg, 2006; Quesada-Moraga et al., 2007).

Resulta el medio terrestre un hábitat muy propicio para la supervivencia y dispersión de los AME, pues proporciona cobijo a los propágulos fúngicos, protegiéndolos de las inclemencias meteorológicas, a la vez que otorga los medios necesarios para extender la infección entre los artrópodos que transitan la hojarasca, el sustrato húmico, la filosfera o sus aledaños (Jaronski, 2007). Así, mientras que la dispersión de los entomopatógenos en el medio epigeo se ve facilitada por el viento, la lluvia, los aerosoles o los insectos (Goettel et al., 2005), la distribución vertical en el medio hipogeo se efectúa gracias a la acción de agentes físicos, como el agua, o a las interacciones con la fauna y flora y con el microbioma, especialmente invertebrados geófilos o geobiontes como ácaros, anélidos o colémbolos (Meyling y Eilenberg, 2007; Jaronski, 2010; Lacey et al., 2015).

Aun con todo, ha de considerarse que la presencia espacial y temporal de los AME en el medio edáfico se halla condicionada por multitud de factores, como las propiedades fisicoquímicas del suelo (temperatura, humedad, pH, capacidad de intercambio catiónico, riqueza en materia orgánica, etc.) o la diversidad de organismos macro y microbiológicos que en él habitan (Jaronski, 2010). Cuando los hospedantes escasean o las condiciones no resultan favorables para la dispersión de los AME, estos producen estructuras de resistencia —como microesclerocios o clamidosporas— que les permiten mantenerse en estado viable en el ambiente hipogeo durante periodos prolongados de tiempo (Quesada-Moraga et al., 2007).

I.2.4.2. Presencia en los artrópodos

Los AME han experimentado un dilatado proceso de coevolución junto a los artrópodos, a expensas de los cuales se nutren, que ha condicionado su biología, ligándola íntimamente a la de aquellos (Roy et al., 2006). Actúan los abundantes artrópodos que pululan por ecosistemas de toda clase y condición —especialmente los insectos y ácaros— como portadores ideales para la (auto) diseminación de estos hongos: en el medio epigeo, el viento facilita la dispersión de los conidios hacia nuevos hospedantes, y estos, a su vez, hacia otros individuos de su misma u otra especie, el suelo, la vegetación o allá donde den a parar (Quesada-Moraga et al., 2007). En general, las esporas procurarán el inicio del ciclo patogénico y la consecuente invasión del artrópodo tan pronto se adhieran a su tegumento, mas no resulta inusual la presencia de propágulos fúngicos en reposo sobre la epicutícula (Greif y Currah, 2007).

La transmisión horizontal de los AME ocasiona epizootias entre la artropodofauna que dependen de factores tanto del propio insecto hospedante (sirvan de ejemplo su gregarismo, su susceptibilidad a los hongos o su capacidad de vuelo) como

de las poblaciones del hongo (como su virulencia, poder de dispersión, la densidad del inóculo o su distribución espacial), en claro contraste unos AME, como *B. bassiana*, muy generalistas y capaces de infectar a una miríada de especies, y otros, como *Metarhizium acridum* (Driver and Milner) (Hypocreales: Clavicipitaceae), altamente específicos y restringidos a un abanico de hospedantes muy estrecho (de Faria y Wraight, 2007; Quesada-Moraga et al., 2007).

Dependiendo de la naturaleza de la asociación trófica HE-insectos hospedantes, los primeros pueden clasificarse de la siguiente forma: biotrofos, si obtienen su sustento exclusivamente de las células vivas de los artrópodos, cesando su nutrición al morir aquellos; necrotrofos, capaces de alimentarse únicamente de tejidos sin vida, por lo que, de manera previa a su alimentación, deben ocasionar la muerte del artrópodo, y hemibiotrofos, que actúan como biotrofos hasta que su hospedante expira, y como necrotrofos a partir de entonces (Quesada-Moraga y Santiago-Alvarez, 2008; Vega et al., 2009).

I.2.4.3. Asociaciones con las plantas

Hasta finales del siglo XX, se daba por hecho que la presencia de los AME se restringía a los artrópodos y el medio terrestre, y que su función en los ecosistemas no era otra que el control natural de poblaciones de artrópodos como consecuencia lógica de su patogenicidad (Vakili, 1990; Bing y Lewis, 1991). En el año 1990 se revelaría un importante sesgo en la concepción de estos hongos por parte de la comunidad científica, al descubrirse al entomopatógeno *B. bassiana* como endófito en una planta de maíz, donde actuaba al mismo tiempo como agente de control de insectos barrenadores del tallo (Vakili, 1990; Bing y Lewis, 1991). Ello abrió un mundo de posibilidades a la investigación de los AME; desde entonces, el interés por dilucidar la naturaleza, magnitud y extensión del rol ecológico de estos microorganismos no ha hecho más que aumentar.

La asociación entre los AME y las plantas puede manifestarse en distintas vías conforme las estructuras fúngicas establecen contacto con la planta, siendo las más comunes las tres situaciones que van a enumerarse: en la rizosfera, donde los exudados de las raíces y otros mecanismos contribuyen a que los AME persistan en el suelo durante periodos de tiempo más prolongados (Hu y St Leger, 2002); en la filosfera, donde permanecen como epífitos (Garrido-Jurado et al., 2015b); y, por último, en el interior de las propias plantas (Vakili, 1990). Por su complejidad y posibles implicaciones, esta última amerita una atención acentuada. Ciertas especies de hongos son, además de entomopatógenos, endófitos (Vega, 2018; Quesada-Moraga et al., 2019; Quesada-Moraga, 2020); esta facultad, común entre los ascomicetos, se manifiesta por la capacidad de estos microorganismos de colonizar los tejidos internos de las plantas, residiendo en el interior de ellas durante su fase endófita sin ocasionarles síntoma adverso alguno (Hyde y Soytong, 2008). La colonización de los AME puede ser a nivel intercelular o intracelular, así como localizada o sistémica (Stone et al., 2000; Arnold y Lutzoni, 2007; Garrido-Jurado et al., 2017); los referidos hongos son capaces de colonizar cualquier tejido de la planta, pudiendo dar lugar a una transmisión vertical a través de las semillas (Bacon y White, 2000; Quesada-Moraga et al., 2014b). Una vez

los AME se han abierto camino al interior del autótrofo, lo cual sucede habitualmente mediante penetración directa gracias a la enzima MAD2, o simplemente a través de aberturas naturales como los estomas (Wang y St Leger, 2007); el movimiento de los entomopatógenos se limita al espacio intercelular, siguiendo la vía del apoplasto (Landa et al., 2013). Pese a su inequívoca relación trófica con los artrópodos, no puede descartarse que exista algún tipo de nutrición por parte del hongo cuando se hallan en el interior de las plantas (Vega et al., 2012).

I.3. Nuevas aplicaciones en protección, producción vegetal y otras áreas derivadas de los ascomicetos mitospóricos entomopatógenos y sus asociaciones con las plantas

Las diversas funciones de los AME pueden tener una gran utilidad desde el punto de vista práctico y, por tanto, comercial, si se desarrollan debidamente. Su principal rol en los agroecosistemas es el control de las poblaciones de insectos, lo que ha condicionado que su utilización en la agricultura se haya encaminado, tradicionalmente, a la lucha biológica (Vega, 2018; Quesada-Moraga et al., 2020); sin embargo, el descubrimiento de nuevas propiedades de estos microorganismos ha propiciado que últimamente se estén explorando horizontes inéditos en los campos de la protección de cultivos, la producción vegetal y otras áreas (Quesada-Moraga et al., 2014a), algunos derivados de sus asociaciones con las plantas (Quesada-Moraga, 2020) y otros no (Litwin et al., 2020).

Las aplicaciones de los AME pueden dividirse, en una dimensión pragmática, en tres: protección de cultivos, producción vegetal y otros usos (Figura I.3).

-Protección de cultivos: aparte de su virulencia hacia artrópodos fitófagos, los AME pueden brindar cierto grado de protección a la planta frente a estreses, tanto bióticos como abióticos, de diversa clase y condición (Quesada-Moraga, 2020). En referencia a los estreses bióticos, se ha constatado que algunos AME como *B. bassiana*, de mención recurrente en este espectro de la literatura científica, efectúan un marcado antagonismo frente a un amplio rango de microorganismos (Ownley et al., 2008; Jaber, 2015; Jaber y Ownley, 2018; Rondot y Reineke, 2019); entre ellos, figuran especies de bacterias (Griffin et al., 2006; Ownley et al., 2008), virus (Jaber y Salem, 2014), hongos (Ownley et al., 2010; Lozano-Tovar et al., 2013; Keyser et al., 2016) y oomicetos (Lozano-Tovar et al., 2013; Jaber, 2015). Por otro lado, se ha confirmado que los AME pueden aliviar a la planta frente a los efectos de algunos de los estreses abióticos más relevantes desde el punto de vista agrícola, como lo son las deficiencias nutritivas (Sanchez-Rodriguez et al., 2015; Raya-Diaz et al., 2017a) o los estreses salino (Waller et al., 2005), hídrico y térmico (Sanchez-Rodriguez et al., 2015; 2016; Quesada-Moraga, 2020).

-Producción vegetal: en los últimos años se ha descubierto que los AME también pueden contribuir a la promoción del crecimiento de las plantas cultivadas; de esta manera, algunos AME provocan efectos tan diversos como un mayor desarrollo del sistema radical (Sasan y Bidochka, 2012; Sanchez-Rodriguez et al., 2018), una mejor nutrición (Behie et al., 2012; Raya-Diaz et al., 2017a; 2017b), una precocidad en su

ciclo de desarrollo (Raya-Diaz et al., 2017a), un mayor rendimiento (Sanchez-Rodriguez et al., 2018) o, en general, un mayor porte de la planta (Garcia et al., 2011). En consecuencia, la incorporación de los AME a los sistemas agrarios suscita un interés considerable no solo desde la perspectiva de la sanidad vegetal, sino como parte de una visión holística del ciclo de desarrollo del cultivo (Quesada-Moraga, 2020).

-Otros usos: adicionalmente, algunos AME secretan compuestos útiles para las industrias química y farmacéutica, tales como terpenoides, esteroides y fenoles (Schulz et al., 2002), o son capaces de eliminar residuos derivados de la actividad industrial (Nowak et al., 2019). Una de las aplicaciones más recientes de estos hongos es la biotransformación de flavonoides para uso alimentario, lo que es actualmente objeto de estudio (Litwin et al., 2020).

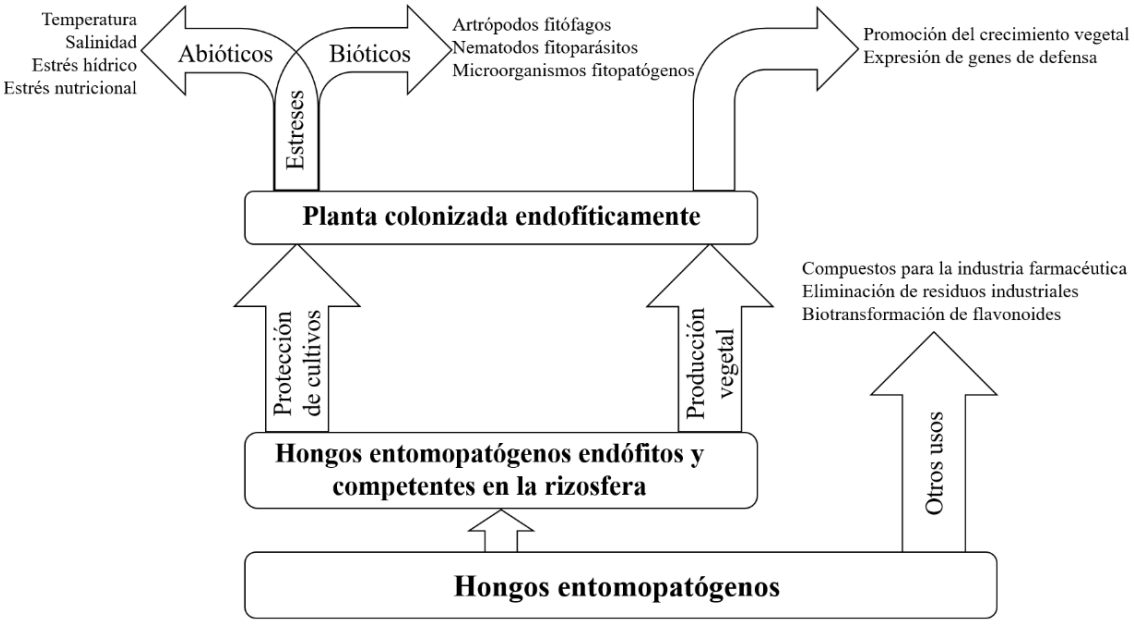

Figura I.3. Principales aplicaciones de los hongos entomopatógenos en agricultura y otras áreas. Fuente: Adaptado de Quesada-Moraga, 2020.

En la presente tesis doctoral se aborda el significado del comportamiento endofítico de los AME tanto para el control de plagas como para el de enfermedades, con implicaciones importantes para el desarrollo de nuevas estrategias sostenibles de protección de cultivos.

I.3.1. Efecto de la asociación de los ascomicetos mitospóricos entomopatógenos con las plantas sobre las cadenas tróficas que incluyen a los insectos fitófagos y sus enemigos naturales entomófagos

Desde los albores de la exploración de la aptitud de los AME para el desarrollo de bioinsecticidas, se han imitado las estrategias de aplicación que tan buenos frutos granjearon en el control de plagas mediante insecticidas químicos, verbigracia la

pulverización foliar (Vega, 2018). A pesar de la reseñable eficacia fitosanitaria de algunas cepas de AME, no es menos cierto que estos métodos han experimentado, en términos generales, severas limitaciones para su aplicación en campo que han ralentizado su desarrollo comercial. Entre ellas, cabe destacar la baja persistencia en la filosfera de algunas cepas, agravada a su vez por los demás factores que van a exponerse; la susceptibilidad de estos hongos a la radiación ultravioleta, las altas temperaturas o a la baja humedad, que influyen negativamente en su supervivencia, germinación o esporulación; la dificultad para que los propágulos infectivos establezcan contacto con el insecto diana, ya sea durante o después de la efectuación del tratamiento; el potencial perjuicio sobre el biotopo (e.g. las aguas superficiales) o la biocenosis (con énfasis en la fauna auxiliar); o, no menos relevante, la ausencia de estudios rigurosos sobre análisis de costes y beneficios que avalen la viabilidad técnica y económica de esta clase de tratamientos (Fernandez-Bravo et al., 2017; Vega, 2018; Quesada-Moraga et al., 2020). Las restricciones para la aplicación de los AME han encaminado a los entomólogos hacia nuevos horizontes, como la investigación de su aptitud endofítica o su empleo combinado con el de agentes de control biológico macrobianos como los artrópodos entomófagos.

En condiciones artificiales, la colonización endofítica puede propiciarse mediante técnicas de inoculación como la pulverización foliar, la inundación, los tratamientos dirigidos a las semillas o las inyecciones (Vega, 2018), y la verificación de la presencia del entomopatógeno en el interior de los tejidos se logra mediante la siembra de fragmentos vegetales en medio de cultivo para recuperarlo de su interior, o bien por medio de técnicas moleculares, de microscopía, de histología, o de otra índole (Garrido-Jurado et al., 2017; Vega, 2018). La benigna invasión del hongo serviría, acaso, para brindar un grado variable de protección de su hospedante vegetal contra los insectos fitófagos gracias a mecanismos ya mencionados en el presente documento, como puedan ser la ingestión del herbívoro de estructuras fúngicas, la secreción fúngica de metabolitos entomotóxicos o la inducción de respuestas defensivas en la planta hospedante (Vega, 2018).

Por otro lado, la liberación conjunta de organismos macro y microscópicos para el control biológico de insectos ha suscitado la atención de una pléyade de investigadores, que han encauzado su trabajo en esta dirección. Las interacciones que ocurren al emplear de manera simultánea más de un agente de biocontrol pueden estar dotadas de una elevada diversidad y complejidad, dificultando así la dilucidación de su resultado, máxime si el entomófago es tan o más susceptible al entomopatógeno que el insecto diana (Ludwig y Oetting, 2001). Por ende, y aunque gran parte de los autores consideran que los tratamientos con AME pueden ser seguros para los enemigos naturales entomófagos si se controlan adecuadamente los parámetros de aplicación (Labbe et al., 2009; Rannback et al., 2015; Mohammed y Hatcher, 2017; Gonzalez-Mas et al. 2019a; Martinez-Barrera et al., 2020), hay estudios donde se ha observado una baja compatibilidad en la aplicación combinada por existir efectos perniciosos sobre el enemigo natural por parte del entomopatógeno (Oreste et al. 2015). En general, y a la vista de los argumentos esgrimidos por la comunidad científica, puede concluirse que la compatibilidad entre agentes de control biológico depende en gran medida de los organismos involucrados y los procedimientos experimentales.

No obstante, la mayoría de la investigación se ha encaminado a la determinación de los efectos de la aplicación directa de AME sobre los enemigos naturales (Shrestha et al., 2017), o indirecta, al exponer a los entomófagos a presas u hospedantes que han recibido un tratamiento directo con hongo (Mesquita y Lacey, 2001), sin que la planta adquiriese un papel relevante en ningún caso; por ello, **apenas se ha investigado acerca de los posibles efectos que ocurrirían sobre los enemigos naturales entomófagos al exponerse a fitófagos que se han alimentado previamente de planta colonizada endofíticamente por AME** (Vega, 2018).

En los trabajos donde se ha evaluado la compatibilidad de la vía endofítica de los AME con los enemigos naturales se señalan algunos aspectos de interés: el empleo de cepas endófitas de AME presenta ventajas destacables, como la necesidad de poco volumen de inóculo para su aplicación, en contraposición a la pulverización foliar —su modo de aplicación más común—, o la protección del hongo frente a factores tanto bióticos como abióticos que limitarían su persistencia en fase epífita (Quesada-Moraga et al., 2020). Adicionalmente, la escasa exposición del entomófago al entomopatógeno mediante este método contribuiría a la seguridad del primero al no afectar significativamente a sus parámetros reproductivos o su esperanza de vida, en detrimento del fitófago, lo que permitiría obtener mejores resultados en la implementación de estrategias de GIP (Akutse et al., 2014; Jaber y Araj, 2018; Gonzalez-Mas et al., 2019a).

I.4. Los ascomicetos mitospóricos entomopatógenos en el control microbiano de la "rosquilla negra" *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae)

I.4.1. *Spodoptera littoralis*: distribución, morfología, biología y daños

Spodoptera littoralis (Boisduval) (Lepidoptera: Noctuidae) es un insecto fitófago de gran importancia. El nombre común más empleado para referirse a esta especie es el de "cotton leafworm" (Lanzoni et al., 2012), siendo igualmente aceptados "Egyptian cotton worm", "African cotton leafworm", "Mediterranean climbing cutworm", "Mediterranean brocade moth", "tobacco caterpillar", "tomato caterpillar", "ver du coton" y "noctuelle méditerranéenne" (CABI, 2020; EPPO, 2020). Como puede apreciarse, la mayoría de ellos hacen mención bien a su distribución geográfica, bien a su incidencia sobre algún cultivo de interés. En cambio, en castellano es denominada simplemente "rosquilla negra", debido a que la larva "*es negruzca y se arrolla en espiral*" (Dominguez, 2004). Este apelativo fue acuñado por agricultores en Almería durante las primeras observaciones de plagas de este insecto en la península ibérica (El Saadi, 1980).

El citado lepidóptero se encuentra ampliamente distribuido a lo largo de la cuenca mediterránea, por algunos países de Europa (principalmente en el norte, sur y territorio insular), la totalidad del continente africano y algunas zonas de Asia menor (EPPO, 2020) (Figura I.4).

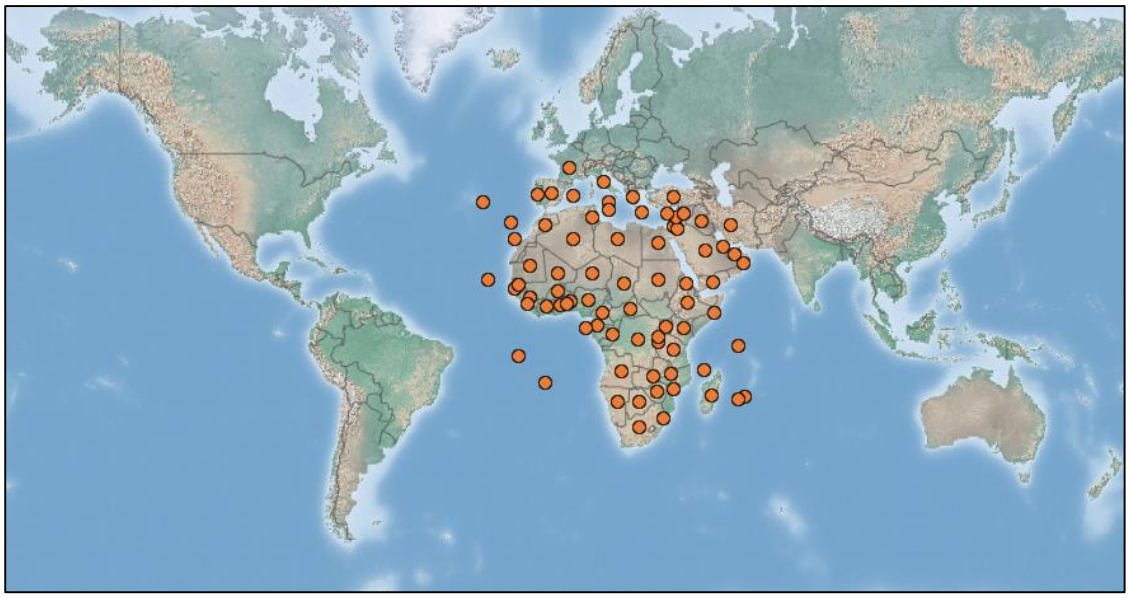

Figura I.4. Distribución mundial de *Spodoptera littoralis*. Fuente: EPPO, 2020.

Los huevos tienen forma esférica y algo aplanada, con un diámetro de 0.6 mm (Figura I.5A); durante la oviposición son colocados en plastones alargados de 1-1.5 cm, donde son dispuestos en filas más o menos regulares y formando de una a tres capas, hasta llegar a un total de 200 a 500 huevos por plastón, ovipositando cada hembra cerca de un millar de huevos durante su vida. Los plastones son recubiertos por escamas del abdomen de la hembra adulta, que esta desprende tras la puesta, quedando de esta manera protegidos. El color de los huevos es, por lo general, amarillo blanquecino, aunque cambia a negro justo antes de la eclosión (Pinhey, 1975; Hosny et al., 1986; Dominguez, 2004). Las larvas son polipoides y eruciformes, contando con seis estadios larvarios y creciendo hasta alcanzar los 35-45 mm de longitud (Figura I.5B). Carecen de pilosidad, tienen una forma cilíndrica, se estrechan hacia la parte posterior y varían de tonalidad a lo largo de su desarrollo (gris negruzco, verde oscuro, marrón rojizo, amarillo blanquecino), si bien su color más característico es el negro. Presentan bandas longitudinales de tonalidades oscuras y claras a ambos lados del cuerpo; lado dorsal con dos manchas semilunares oscuras lateralmente en cada segmento, excepto el protórax; manchas en los segmentos abdominales primero y octavo más grandes que en los otros, interrumpiendo las líneas laterales en el primer segmento (Pinhey, 1975; Dominguez, 2004; Alfaro, 2005). Cuando están recién formadas, las pupas son de color verde con un tono rubescente en el abdomen, aunque después de unas horas se tornan marrones con un matiz rojo oscuro, casi ocráceo (Figura I.5C). La forma general de la crisálida es cilíndrica, con dimensiones de 14-20 × 5 mm, y se estrecha hacia los segmentos posteriores del abdomen. El último segmento termina en dos fuertes ganchos rectos (El Saadi, 1980). El adulto es una polilla con cuerpo parduzco virando a grisáceo, de 15 a 20 mm de longitud (Figura I.5D), y con una envergadura alar de 30-38 mm. Las alas anteriores son de color gris a marrón rojizo, con líneas más pálidas a lo largo de las venas (en los machos, aparecen áreas azuladas en la base y punta del ala) y con un patrón que se asemeja al número cuatro; el ocelo, marcado por dos o tres franjas oblicuas blanquecinas. Las alas posteriores son de color blanco grisáceo, iridiscentes,

con márgenes grises y generalmente carecen de venas más oscuras. El cuerpo es muy peludo, presentando dos mechones centrales y otros dos laterales, más pequeños, en el tórax (Dominguez, 2004; CABI, 2020).

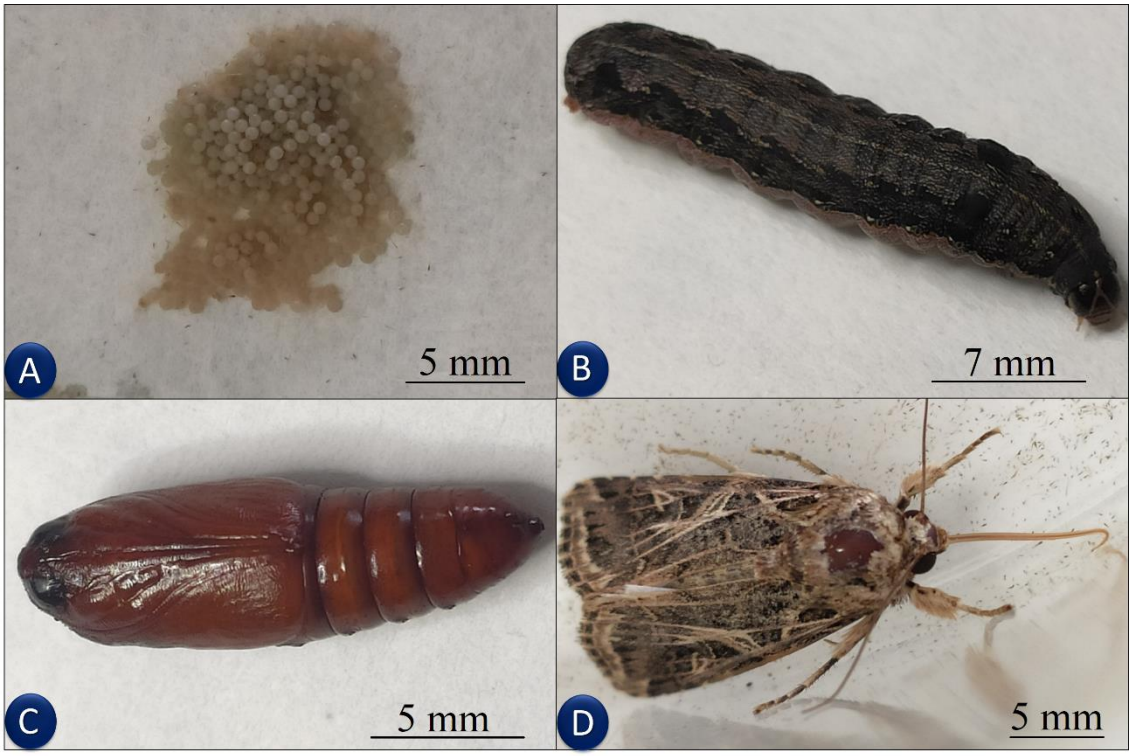

Figura I.5. Estados de desarrollo de *Spodoptera littoralis.* A. Huevo. B. Larva. C. Pupa. D. Imago. Fuente: propia.

Tras la oviposición, que se realiza de manera preferente en el envés de la hoja y en cultivos recién regados, tiene lugar la maduración de los huevos y posterior eclosión, la cual se produce al cabo de 3-5 días, aunque puede dilatarse más en el tiempo si la temperatura es baja (Pinhey, 1975; Hosny et al., 1986; Dominguez, 2004). Las larvas huyen del exceso de luminosidad, procurando refugiarse en sitios frescos (como restos vegetales u hojarasca) si las temperaturas son altas, y permanecen allí largos ratos enrolladas; por el contrario, cuando cae la noche o la temperatura desciende, se encaraman a la planta y dan rienda suelta a su voracidad. En ocasiones, adquieren un marcado gregarismo durante su alimentación. A lo largo de unos veinte días, alcanzan su sexto y último estadio larvario, momento en que ingieren la máxima cantidad de tejido vegetal y pueden atacar a los frutos. Para pupar, tienen la costumbre de enterrarse en el suelo o sustrato unos centímetros, construyendo con gránulos de tierra una celda suboval y convirtiéndose en crisálida en unas horas. En este estado permanece unos quince días, tras los cuales emerge el imago, que procede a la cópula con prontitud, realizando la puesta a su término y reiniciando, de esta manera, el ciclo (Dominguez, 2004). Habitualmente cuenta con tres generaciones anuales, aunque algunos autores han

observado un voltinismo mucho más acelerado en condiciones favorables para su desarrollo (Avidov y Harpaz, 1969; Dominguez, 2004).

Los daños económicos que esta especie origina en los cultivos son únicamente de naturaleza directa, y la inevitable consecuencia de la alimentación de sus larvas: estas obtienen su sustento de tejido foliar preferentemente, respetando la epidermis superior de la hoja al inicio de su desarrollo (Dominguez, 2004) y horadando profundas oquedades en el margen foliar cuando se encuentran en estadios larvarios avanzados (Russell et al., 1993). Se estima que cada larva puede consumir 5.3 g de tejido foliar (equivalente a una superficie de 264 cm^2) a lo largo de toda su vida, de los cuales no menos del 50% serán ingeridos durante su sexto y último estadio larvario (Russell et al., 1993). Aunque los principales ataques se efectúan en el filoplano, ello no es óbice para que las orugas puedan alimentarse de otros órganos de la planta, como la flor o el fruto (Russell et al., 1993; Dominguez, 2004), lo que deprecia en gran medida producciones hortícolas de gran valor, como el tomate o el pimiento (Avidov y Harpaz, 1969; Dominguez, 2004).

Sus hábitos polífagos y destacada voracidad convierten a este noctuido en una amenaza reseñable para numerosos cultivos de gran importancia económica, tales como el algodón, el tomate, el pimiento, la lechuga, el melón, la patata, la berenjena, la alcachofa, la fresa, el espárrago, la espinaca, el tabaco y diversos cultivos herbáceos y ornamentales, pudiendo constituir severas plagas tanto en el campo como en sistemas de cultivo bajo abrigo (Sannino, 2003; Alfaro, 2005; Lanzoni et al., 2012; EPPO, 2020). Se conocen cerca de 90 posibles hospedantes para este fitófago (Salama et al., 1970), si bien no existen suficientes informes sobre el daño que causa en cada uno.

Diferentes autores han estimado pérdidas de rendimiento variables, existiendo metodologías para mesurar las mismas (Hosny et al., 1986), si bien el perjuicio económico depende en gran medida de la estación en que se produzca el ataque de *S. littoralis*, así como del cultivo y su sensibilidad a la defoliación (Russell et al., 1993). En especies vegetales cuyo rendimiento apenas se ve afectado por esta, como es el caso del algodonero, el menoscabo en la cosecha puede ser insignificante aun si el 20% de la filosfera ha sido devorada por este insecto (Hosny et al., 1986; Russell et al., 1993). En hortícolas, sin embargo, es capaz de ocasionar verdaderas devastaciones, especialmente por los severos ataques al fruto (Avidov y Harpaz, 1969). Por otra parte, esta especie puede tener hasta siete u ocho generaciones anuales, dependiendo de las condiciones (Avidov y Harpaz, 1969), lo que aumenta la gravedad de las infestaciones y dificulta el control y la toma de decisiones. En España, las observaciones iniciales de *S. littoralis* se remontan a antes de la guerra civil, aunque se limitaban a ejemplares aislados, no teniéndose constancia de daños económicos; sin embargo, en 1937 aparecen por vez primera auténticas plagas de esta especie en Almería, acentuadas por la ausencia de medidas de control. Desde allí se extendería por las otras provincias de Andalucía, así como por el Levante y Tarragona, suponiendo una amenaza para numerosos cultivos (Dominguez, 2004). Actualmente se encuentra en otras provincias como Cáceres y Cuenca, así como en el territorio insular (Alfaro, 2005).

I.4.2. Control de *Spodoptera littoralis*

El manejo de esta especie, análogamente a otros lepidópteros fitófagos, se basa en las medidas de control tradicionales, si bien el grado de implantación de cada una de ellas frente a la rosquilla negra ha sido desigual:

-Métodos legales: la European and Mediterranean Plant Protection Organization lista a este insecto entre sus organismos de cuarentena A2 (EPPO, 2019) y ha incidido en aspectos como su identificación (EPPO, 2015) o la prevención de su dispersión (EPPO, 1990). Actualmente, la normativa europea le concede una importancia muy limitada por su generalizada presencia en territorio comunitario, de modo que los esfuerzos en materia de sanidad vegetal se centran en otras especies del mismo género, como *Spodoptera frugiperda* (Smith) y *S. litura* (Fabricius) (Lepidoptera: Noctuidae).

-Métodos agronómicos y de manejo: una forma de combatir al noctuido en algunas regiones donde causa graves infestaciones ha sido el empleo de sistemas de trampeo, que pueden ser efectivos frente a la rosquilla negra y, en ocasiones, sustituir a los tratamientos con insecticidas (El Saadi, 1980). De este modo, se han desarrollado técnicas basadas en capturas masivas (Teich et al., 1985; Salem y Salama, 1985), confusión sexual (Kehat et al., 1983; Dunkelblum et al., 1987) o tratamientos-cebo (De Souza et al., 1992). Otro método de manejo citado por diversos autores es la retirada manual de insectos o huevos de las plantas infestadas (Hosny et al., 1986; Russell et al., 1993; Dominguez, 2004). Aunque pueden reducir significativamente la presencia de *S. littoralis* en el campo, su rentabilidad es puesta a menudo en tela de juicio (Hosny et al., 1986; Russell et al., 1993).

-Resistencia genética: diferentes autores han descubierto fuentes de resistencia frente al azote del insecto (Hagenbucher et al., 2013). La investigación en este campo se ha centrado en gran medida en el cultivo del algodón (Hagenbucher et al., 2013), aunque no faltan las publicaciones que abordan otras especies vegetales (Berlinger et al., 1997). Ciertos trabajos incidían en la relación de la morfología foliar y la alimentación de la rosquilla negra, que evita engullir hoja provista de una excesiva pilosidad o un elevado número de tricomas (Kamel, 1965), mientras que otros han evaluado el potencial de algunos compuestos naturales —especialmente terpenoides— que interfieren en el desarrollo del noctuido (Hagenbucher et al., 2013). Uno de los más estudiados, el gosipol, inhibe la actividad de la proteasa y la amilasa en esta especie más de un 70% (Meisner et al., 1977). Esta sustancia actúa como repelente, llevando a las larvas a evitar las hojas ricas en ella y al adulto a reducir su oviposición (Anderson et al., 2011; Hagenbucher et al., 2013).

-Control químico: la aplicación de tratamientos con insecticidas químicos ha sido el método de control más utilizado frente a la rosquilla negra (Ghribi et al., 2012); sin embargo, la inducción en este insecto de resistencia a diversas materias activas de uso frecuente supone una importante limitación para ello y amenaza su continuidad: después de décadas de empleo continuo e ininterrumpido de una plétora de insecticidas químicos en Egipto, este lepidóptero ha desarrollado con prontitud resistencia a la totalidad de ingredientes activos, perdiendo los tratamientos su eficacia al cabo de dos o tres años de aplicación aun al mezclar insecticidas de diferentes familias (El-Sebae, 1977; Metcalf, 1984). Los organofosforados (Issa et al., 1984a), piretroides (Issa et al.,

1984b) y reguladores del crecimiento (Mosallanejad y Smagghe, 2009) son solo algunos de los grupos de insecticidas entre los que se han detectado poblaciones de *S. littoralis* resistentes.

-Control biológico: *S. littoralis* cuenta con un total de 135 enemigos naturales descritos, de los cuales 73 (54.1%) son parásitos, 32 (23.7%) son depredadores y los 30 restantes (22.2%) son microorganismos entomopatógenos o con actividad antagonista frente al insecto (CABI, 2020). La mayor parte de la investigación concerniente a los enemigos naturales de este fitófago se ha llevado a cabo en España (CABI, 2020).

Como se refirió anteriormente, las variedades vegetales de las plantas cultivadas que producen y acumulan altas concentraciones de compuestos químicos, como los terpenoides, pueden tener efectos sobre la alimentación o el desarrollo de *S. littoralis*, suscitando interés el empleo de líneas resistentes (Hagenbucher et al., 2013). Como consecuencia lógica, se ha evaluado el efecto de estas mismas sustancias, así como otras de similar naturaleza, aplicadas al fitófago mediante tratamiento directo (pulverización, inmersión, por vía tópica o de cualquier otra forma); de este modo, ciertos monoterpenos, fenilpropenos y sesquiterpenos podrían ser alternativas muy prometedoras para el desarrollo de bioinsecticidas (Abdelgaleil et al., 2020), así como determinados aceites esenciales y otros compuestos de origen natural (Ammar et al., 2020; Pavela et al., 2020).

Por otro lado, numerosos son los entomófagos que se han barajado como candidatos para el manejo de este insecto, desde himenópteros parasitoides como *Meteorus pulchricornis* (Wesmael) y *Homolobus truncatoides* van Achterberg (Hymenoptera: Braconidae) (Caballero et al., 1990) hasta depredadores pertenecientes a órdenes diversos, como el de los coleópteros [destacando *Coccinella undecimpunctata* L. (Coleoptera: Coccinellidae), *Paederus fuscipes* Curtis (Coleoptera: Staphylinidae), *Calosoma chlorostictum* Dejean (Coleoptera: Carabidae)], hemípteros [*Orius albidipennis* Reuter (Hemiptera: Anthocoridae), *Creontiades pallidus* (Rambur) (Hemiptera: Miridae)], dermápteros [*Labidura riparia* (Pallas) (Dermaptera: Labiduridae)] o himenópteros [*Polistes gallicus* (L.) (Hymenoptera: Vespidae)] (Hamed y Hassanein, 1984; Delvare y Rasplus, 1994; CABI, 2020), y, fuera de la clase de los insectos, incluso arácnidos (Perez-Guerrero et al., 2013).

Entre los enemigos naturales de la rosquilla negra merece la pena mencionar a *Hyposoter didymator* (Thunberg) (Hymenoptera: Ichneumonidae), avispa solitaria y endoparasitoide koinobionte que ha sido señalado por diversos autores como un firme candidato para el control biológico de *S. littoralis* (Hatem et al., 2016). El himenóptero se encuentra ampliamente distribuido por algunos países europeos como es el caso de España, donde se ha catalogado como especie autóctona (Cabello, 1989; Caballero et al., 1990). Este insecto parasita de forma natural a las larvas de diferentes familias del orden de los lepidópteros, evidenciando preferencia para ello, por lo general, por estadios larvarios tempranos (Hatem et al., 2016). La mayoría de sus hospedantes conocidos pertenecen a la familia de los noctuidos (16 especies), aunque hay constancia asimismo de su parasitación natural a una especie de piérido, una de ninfálido y una de lasiocámpido (Bahena, 1997); entre ellas figuran géneros de gran relevancia agrícola por su fitofagia como *Spodoptera* y *Helicoverpa* (Bahena, 1997; Bahena et al., 1998;

Hatem et al., 2016). En el campo, este parasitoide ejerce un nivel de control natural de diferentes insectos fitófagos muy variable que, en ocasiones, puede alcanzar el 100% (Cabello, 1989), de lo que se deduce su eficacia y el consecuente interés de su empleo como agente de control biológico.

Cuando la hembra adulta de *H. didymator* localiza un candidato oportuno al que hacer hospedante de su progenie, esto es, una larva de un lepidóptero de la especie y el estadio de desarrollo precisos, punciona el cuerpo de aquel con su oviscapto, prefiriendo la región dorso-lateral para tal fin, y oviposita normalmente un único huevo, que permanece flotando en el hemocele (Bahena et al., 1999). Simultáneamente, inyecta toxinas secretadas en una glándula, un virus de la familia Polydnaviridae y otras sustancias que deprimen el sistema inmunológico del insecto hospedante, reduciendo así las posibilidades de que acontezca una respuesta inmune que impida la supervivencia de su prole (Volkoff et al., 1995; Bahena et al., 1999). Al cabo de uno o dos días ocurre la eclosión, emergiendo del huevo una larva de *H. didymator*, que cuenta con tres estadios larvarios. Comienza esta a absorber la hemolinfa de su hospedante a través de su apertura oral, desarrollando mandíbulas con las que masticar los órganos y tejidos a su alrededor en su segundo estadio (Bahena et al., 1999). Cuando ha devorado por completo a su anfitrión, se abre camino al exterior, deshaciéndose con presteza del tegumento de aquel y comenzando a tejer un capullo, el cual concluye uno o dos días después (Schneider et al., 2004). Al término de la metamorfosis, se habrá convertido en un adulto que procederá, tan pronto halle un imago del sexo opuesto, a la cópula (Figura I.6). La duración del ciclo vital del parasitoide depende del insecto hospedante a expensas del cual se haya alimentado, si bien el tiempo de desarrollo larvario ronda los diez días, y el de pupado, los siete (Hatem et al., 2016).

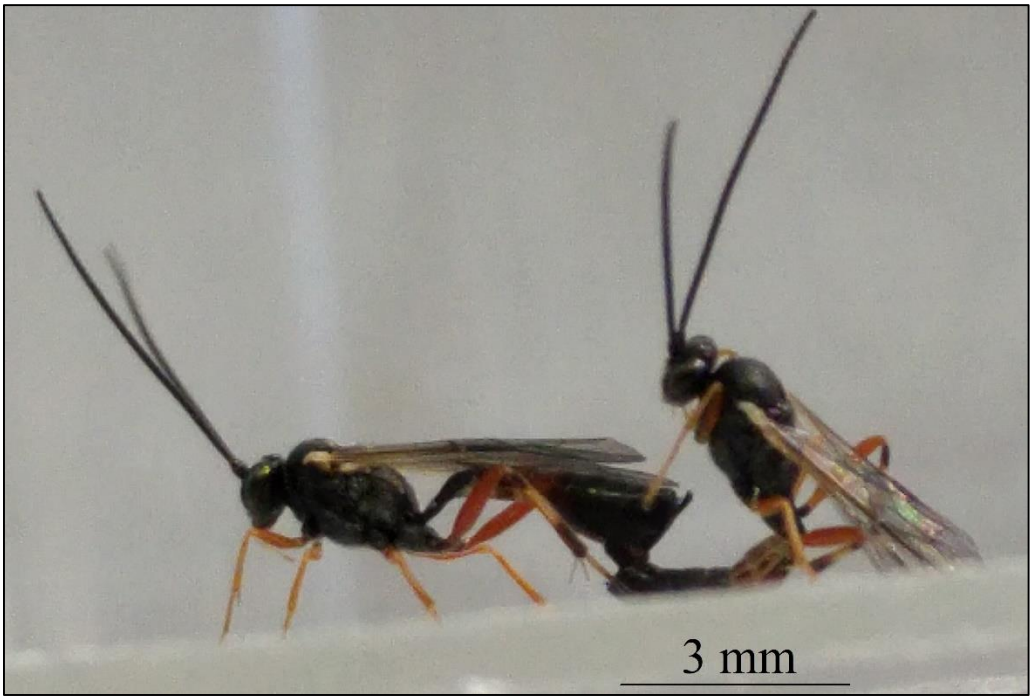

Figura I.6. Fotografía de dos adultos del parasitoide *Hyposoter didymator* durante su cópula. Fuente: propia.

Pese a los altos ratios de parasitismo de este icneumónido en diferentes especies de lepidópteros (Bahena, 1997; Bahena et al., 1998; Hatem et al., 2016), también suele ocurrir un grado variable de mortalidad natural entre las larvas del hospedante, elevado en algunas especies y bajo o nulo en otras (Bahena et al., 1998); unido a ello, varios autores han observado la ocurrencia de parasitismo incompleto, esto es, muerte prematura de larvas que han sido parasitadas sin que el desarrollo larval de *H. didymator* pueda completarse (Bahena et al., 1998; Hatem et al., 2016). Este fenómeno parece relacionado con cierto porcentaje de encapsulación y, de manera poco frecuente, superparasitismo (Bahena et al., 1998).

No son los artrópodos entomófagos los únicos enemigos naturales de la rosquilla negra que han impelido el interés de los científicos: la bacteria *B. thuringiensis*, utilizada durante años para el control biológico de *S. littoralis* en campo mediante pulverización directa y por medio de cultivos transgénicos basados en la proteína Cry, es uno de los agentes de biocontrol estudiados con más profusión (Sanchis et al., 1989; Tabashnik et al., 2003; Ben Farhat-Touzri et al., 2013). La respuesta de la rosquilla negra a sus toxinas depende en buena parte de la cepa bacteriana (Boukedi et al., 2018), evidenciando el fitófago una baja susceptibilidad a aislados de gran recorrido comercial (Moore y Navon, 1973; Sneh et al., 1981; Moussa et al., 2020); ello ha llevado a algunos autores a examinar otras aplicaciones del bacilo para aumentar su eficacia (Boukedi et al., 2018). También ha tenido un destacable recorrido la investigación del empleo de virus entomopatógenos frente a la rosquilla negra (Santiago-Alvarez y Vargas-Osuna, 1988; Vargas-Osuna y Santiago-Alvarez, 1988; Hatem et al., 2011; Fard et al., 2020), mientras que otros autores han indagado acerca de la efectividad de nematodos entomopatógenos para controlar al noctuido (Adel et al., 2012; Shaik et al., 2020).

Finalmente, está creciendo el interés por estudiar la aplicación simultánea de más de un agente de biocontrol, o bien de agentes de biocontrol e insecticidas químicos, como posibles opciones para el manejo sostenible del fitófago, existiendo en ocasiones sinergia o aditividad en las combinaciones o, por el contrario, antagonismo en los tratamientos (Sneh et al., 2009; Dader et al., 2020; Fard et al., 2020; Shaik et al., 2020).

I.4.2.1. Control de *Spodoptera littoralis* mediante ascomicetos mitospóricos entomopatógenos

En los últimos tiempos está aumentando el interés por el empleo de los HE como alternativa para la lucha contra *S. littoralis*. Son numerosos los estudios en los que se han ensayado diferentes metodologías para el control del fitófago mediante AME, las más habituales siendo la inoculación mediante inmersión o inundación, la aplicación tópica, la pulverización o la ingestión de alimento que ha sido tratado con el hongo previamente, en la mayoría de trabajos dirigiendo los tratamientos a la larva (Amer et al., 2008; El-Garhy, 2013; Resquin-Romero et al., 2016a; Resquin-Romero et al. 2016b; Rios-Moreno et al., 2018; El Husseini, 2019) y, ocasionalmente, a otros estados de desarrollo como la pupa, ya sea directamente sobre los individuos (Ahmed y El-Katatny, 2007) o en tratamientos de suelo (Garrido-Jurado et al., 2020), donde las larvas de *S. littoralis* se entierran para pupar (Dominguez, 2004). La inundación del sustrato

con suspensiones de AME provoca una elevada mortalidad en prepupas y pupas del lepidóptero, así como distintos efectos subletales en las crisálidas y los adultos de la misma generación, destacando la deformación de los individuos en el primer caso y una menor fertilidad en el segundo (Garrido-Jurado et al., 2020). Las técnicas enumeradas producen ratios de mortalidad variables en *S. littoralis* dependiendo del procedimiento experimental y las cepas fúngicas, pudiendo alcanzar el 100% en situaciones favorables (El Husseini, 2019) o procurar una mayor mortalidad mediante combinaciones de aislados (Resquin-Romero et al., 2016b).

Últimamente se están ampliando horizontes sobre la investigación concerniente al control de los AME sobre la rosquilla negra, como la aplicación de diversos hongos endófitos frente a esta especie, como *Sarocladium strictum* (W. Gams) Summerb. (Hypocreales: *Incertae sedis*) (El-Sayed et al., 2020), o de especies de AME tan recurrentes en la literatura científica como puedan ser *M. brunneum* o *B. bassiana* (Resquin-Romero et al., 2016a). **Así, la inoculación de la planta con AME conllevaría su ulterior colonización endofítica, localizándose el AME en el interior de los tejidos vegetales, principalmente en el espacio intercelular (Garrido-Jurado et al., 2017); la alimentación de las larvas de *S. littoralis* con tejido foliar colonizado por el hongo acarrearía la muerte del noctuido por una combinación de factores no del todo conocidos tales como la producción de metabolitos insecticidas o la inducción de la respuesta defensiva de la planta (Resquin-Romero et al., 2016a).**

I.4.2.1.1. Efectos tróficos involucrados en el control de *Spodoptera littoralis* con ascomicetos mitospóricos entomopatógenos endófitos y sus enemigos naturales entomófagos

Algunos autores han explorado cómo la aplicación de tratamientos con microorganismos entomopatógenos hacia *S. littoralis* puede afectar a los enemigos naturales que son expuestos a las larvas tratadas, pero los trabajos son muy escasos y a veces se orientan a organismos entomopatógenos distintos de los hongos, como los nematodos (Atwa et al. 2013) o las bacterias (Sneh et al., 2009). En lo que a AME concierne, se ha observado que, al ofrecer larvas de *S. littoralis* tratadas con las cepas de *M. brunneum* EAMa 01/58-Su y BIPESCO5 al depredador *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae), no se vio afectada su esperanza de vida, aunque las crisopas evidenciaron una preferencia significativa hacia las presas no tratadas, viendo reducido su peso al alimentarse exclusivamente con larvas que habían recibido hongo (Rios-Moreno et al., 2018). Sin embargo, en este trabajo no se detectaron metabolitos insecticidas en los cuerpos de los enemigos naturales, por lo que, presuntamente, las implicaciones tróficas no van más allá de un desarrollo subóptimo, siendo esta metodología calificada por los autores como “de bajo riesgo” para el depredador. Por otro lado, al infectar larvas de *S. littoralis* previamente parasitadas por el endoparasitoide solitario *Microplitis rufiventris* Kok. (Hymenoptera: Braconidae) con el AME *B. bassiana* se observó que, si bien el desarrollo de la larva del himenóptero en tales condiciones fue posible, tuvieron lugar ciertos efectos derivados del tratamiento fúngico en el tiempo de desarrollo de los estados preimaginales del bracónido y el peso de las pupas de este, aumentando el valor promedio de ambas variables (El-Maghraby et al., 1988).

Por tanto, los AME y el parasitoide *H. didymator* se presentan como dos de las principales alternativas para el control biológico de *S. littoralis*, por inundación o a través de la vía endofítica los primeros, importante enemigo natural que debe ser conservado e incluso utilizado por inundación en agricultura bajo abrigo el segundo. De esta forma, resulta crucial el estudio en un sistema tritrófico de la interacción entre el entomopatógeno, AME, y el entomófago, parasitoide, tanto cuando el primero es aplicado al fitófago por contacto, como cuando es ingerido por este a partir de tejidos colonizados endofíticamente (Figura I.7).

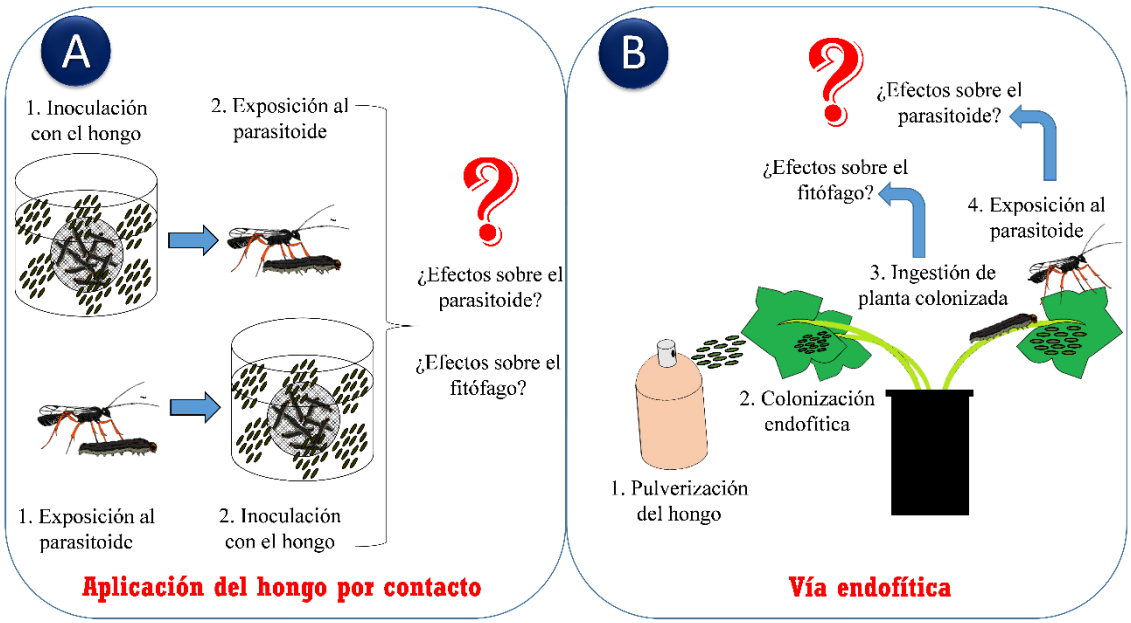

Figura I.7. Aplicación conjunta de ascomicetos mitospóricos entomopatógenos (AME) y el endoparasitoide *Hyposoter didymator* para el control de la rosquilla negra, *Spodoptera littoralis*, mediante dos estrategias diferentes. A. Aplicación del hongo por contacto, en dos escenarios: (1) inoculación con el AME de las larvas de *S. littoralis* y (2) subsecuente exposición al parasitoide *H. didymator*, o (1) exposición al parasitoide y (2) subsecuente inoculación con el AME. B. Aplicación por vía endofítica en un sistema tritrófico planta de melón-*S. littoralis*-*H. didymator*: (1) pulverización de la planta con el AME, (2) colonización endofítica de la planta por el AME, (3) ingestión de planta colonizada por larvas de *S. littoralis*, y (4) exposición de las larvas de *S. littoralis* al parasitoide *H. didymator*.

I.5. Enfermedades de las plantas

En el campo de la patología vegetal se emplea el término “enfermedad” para referirse a los procesos fisiológicos ocasionados en las plantas por agentes bióticos de escala microbiana y naturaleza patogénica, en contraposición a aquellas alteraciones causadas por factores abióticos, que reciben el nombre de fisiopatías —sirvan de ejemplo los desequilibrios nutricionales o la incidencia de episodios meteorológicos desfavorables para la fenología del cultivo (Ainsworth, 1981)—; no obstante lo anterior, no faltan las aproximaciones más clásicas que pueden incurrir en clasificaciones más amplias, considerando asimismo a las fisiopatías como enfermedades o a determinados

organismos no microbianos (por ejemplo, las plantas holoparásitas) como patógenos (Dominguez, 2004; Agrios, 2005; Katan, 2017).

Atendiendo al criterio primero, las plantas "enfermas" (es decir, aquellas que sufren la infección de un organismo fitoparásito al que son susceptibles) crecen, se desarrollan y producen en grado inferior a las sanas, exhiben síntomas de naturaleza diversa e, incluso, pueden llegar a morir (Agrios, 2005). En consecuencia, los organismos fitopatógenos ocasionan unas pérdidas globales de la producción potencial que rondan el 13-16% (Oerke, 2006; Pimentel, 2007), resultando común que ocurran pérdidas mayores e incluso que la cosecha sea testimonial (Gulya et al., 2019).

A nivel legislativo, en el control químico de enfermedades se aplica el marco regulatorio de la UE, sumamente restrictivo en el empleo de fitosanitarios como se mencionó previamente, lo que pone de manifiesto la necesidad de desarrollar nuevos métodos de control no químicos para poder alcanzar un control sostenible desde los puntos de vista económico y ambiental.

I.5.1. Efecto de la asociación de los ascomicetos mitospóricos entomopatógenos con las plantas sobre el control de enfermedades

La literatura científica es muy prolija en lo que al control biológico de organismos fitopatógenos se refiere, habiéndose evaluado el potencial de un sinnúmero de agentes de naturaleza y modos de acción diversos (Kohl et al., 2019); era mera cuestión de tiempo, pues, que los HE —y entre ellos los AME por su representatividad— suscitasen el interés de los investigadores. Los primeros estudios que abordan esta temática datan de finales del siglo XX, siendo *B. bassiana* el AME más frecuentemente utilizado (Ownley et al., 2004). De este modo, resulta conocido el antagonismo de este entomopatógeno frente a hongos y oomicetos fitopatógenos como *Gaeumannomyces tritici* (J. Walker) Hern. Restr. and Crous (Renwick et al., 1991), *Fusarium oxysporum* Schlecht. (Flori y Roberti, 1993; Shternshis et al., 2014), *Armillaria mellea* (Vahl) P.Kumm., *Rosellinia necatrix* Prill., *Botrytis cinerea* Pers. (Bark et al., 1996), *Pythium ultimum* Trow, *P. debaryanum* Hesse, *Parastagonospora nodorum* (Berk.) Quaedvlieg, Verkley and Crous (Vesely y Koubova, 1994), *Rhizoctonia solani* Kuhn (Lee et al., 1999; Ownley et al., 2000; Shternshis et al., 2014), *Alternaria porri* (Ellis) Cif. (Gothandapani et al., 2015) o *Plasmopara viticola* (Berk. and M.A. Curtis) Berl. and de Toni (Jaber, 2015; Rondot y Reineke, 2019), entre otros.

Como se deduce del párrafo anterior, los estudios donde se ha evaluado el potencial de los AME contra hongos y oomicetos son, ciertamente, numerosos. Existe, no obstante, una reducida cantidad de artículos en los que los organismos fitopatógenos que se pretende controlar pertenecen a otros Reinos, como el de las bacterias, caso de *Xanthomonas axonopodis* pv. *malvacearum* (Smith) (Griffin et al., 2006; Ownley et al., 2008), o de los virus, como el virus del mosaico amarillo del calabacín (Zucchini yellow mosaic virus, ZYMV) (Jaber y Salem, 2014), el virus del mosaico del pepino (Cucumber mosaic virus, CMV) o el virus del amarilleo de las cucurbitáceas transmitido por pulgones (Cucurbit aphid-borne yellows virus, CABYV) (Gonzalez-Mas et al., 2019b).

No es *B. bassiana* el único AME valorado como candidato a agente de biocontrol contra organismos fitopatógenos: en la literatura pueden hallarse múltiples referencias al potencial de otros AME, tales son los casos del entomopatógeno *Beauveria brongniartii* (Sacc.) frente a los fitopatógenos *P. ultimum*, *P. debaryanum* y *P. nodorum* (Vesely y Koubova, 1994); de *M. brunneum*, *M. flavoviride* Gams and Rozsypal y *M. robertsii* Bisch., Rehner and Humber frente a *Fusarium culmorum* (Smith) Sacc. (Keyser et al., 2016); de *M. brunneum*, *B. pseudobassiana* (Bals.) Vuill. y *B. varroae* Rehner and Humber frente a *Phytophthora megasperma* Drechs., *P. inundata* Brasier, Sanch. Hern. and Kirk y *Verticillium dahliae* Kleb. (Lozano-Tovar et al., 2013; 2017) y *Akanthomyces lecanii* (Zimm.) Spatafora, Kepler and B. Shrestha y *M. anisopliae* frente a *A. porri* (Gothandapani et al., 2015).

La protección que aportan los AME frente a los organismos fitopatógenos puede deberse a mecanismos o modos de acción tan diversos como la secreción fúngica de compuestos con actividad antibiótica o biocida (Sasan y Bidochka, 2012; Lozano-Tovar et al., 2013; Quesada-Moraga et al., 2014a; Jaber y Ownley, 2018; Vega, 2018), la inducción de resistencia sistémica o la activación de defensas en la planta (Bultman y Murphy, 2000; Jaber, 2015; Shrivastava et al., 2015; Rondot y Reineke, 2019; Quesada-Moraga, 2020), la promoción del crecimiento vegetal (Sasan y Bidochka, 2012; 2013) o diferentes manifestaciones de antagonismo que incluyen el micoparasitismo y la competencia por nutrientes y espacio (Ownley et al., 2010; Lozano-Tovar et al., 2013; Jaber y Ownley, 2018; Quesada-Moraga, 2020).

Los primeros trabajos publicados en este campo incluían únicamente experimentos de laboratorio a escala *in vitro* muy alejados de las condiciones de campo (Ownley et al., 2004), empleando metodologías muy comunes para el enfrentamiento de ambos organismos, entomopatógeno y fitopatógeno, en medio de cultivo, como el cultivo dual (Lozano-Tovar et al., 2013), o bien para la evaluación del efecto de extractos del entomopatógeno sobre el fitopatógeno (Ownley et al., 2004). Así, los estudios hacían énfasis en cómo el antagonismo de los AME limitaba el crecimiento y el desarrollo de los fitopatógenos en medio de cultivo, provocando la lisis celular de aquellos o afectando a parámetros como la germinación de conidios, el crecimiento de micelio o la esporulación (Renwick et al., 1991; Vesely y Koubova, 1994; Bark et al., 1996; Reisenzein y Tiefenbrunner, 1997). No obstante lo anterior, estudios de esta clase siguen siendo habituales (Lozano-Tovar et al., 2013).

Con el paso de los años, han ido apareciendo cada vez más experimentos *in planta*, lo que no es óbice para que en ellos se incorpore, al menos, algún ensayo *in vitro* de manera facultativa (Griffin et al., 2006; Jaber y Salem, 2014; Jaber, 2015; Lozano-Tovar et al., 2017; Rondot y Reineke, 2019).

Pese al destacable potencial de los AME para la lucha contra los microorganismos fitopatógenos, puesto de manifiesto por la comunidad científica a lo largo de las tres últimas décadas (Ownley et al., 2004), no es menos cierto que continúa resultando necesario un avance cualitativo para desarrollar soluciones tecnológicas viables que permitan abordar tanto el control a medio plazo de enfermedades en condiciones de campo como, en un futuro, el control simultáneo de insectos fitófagos y organismos fitopatógenos (Jaber y Ownley, 2018).

I.5.2. Efecto de los ascomicetos mitospóricos entomopatógenos en el control integrado de patógenos de suelo

Se conoce como "patógenos de suelo" o "patógenos telúricos" al heterogéneo conjunto compuesto por aquellos organismos fitopatógenos (hongos, oomicetos, nematodos, virus y plantas parásitas, fundamentalmente) cuyo ciclo depende, al menos parcialmente, del medio edáfico (Katan, 2017). La principal característica definitoria de los mismos es su supervivencia durante periodos de tiempo prolongados en este, que actúa como su reservorio; en él permanecerán activos y predispuestos para la infección de la planta cuando las condiciones resulten adecuadas para ello (Katan, 2017). Sin embargo, algunas de las especies de este grupo pueden disponer de vías alternativas para la patogénesis, como la dispersión aérea, habitual en el género *Plasmopara* (Agrios, 2005). Para garantizar su supervivencia a largo plazo en el suelo, cuentan con estructuras especializadas que les permiten sobrevivir durante años en este en caso de no encontrar un hospedante, como oosporas en el caso de oomicetos, microesclerocios en *Verticillium* spp. o semillas en *Orobanche* spp. (Pegg y Brady, 2002; Agrios, 2005; Katan, 2017).

La lucha contra estos patógenos se ha visto tradicionalmente condicionada por su localización, así como por su dispersión en el suelo (Katan et al., 2012; Katan, 2017). Ello dificulta la aplicación de los tratamientos químicos o biológicos, lo que determina que el manejo de la mayoría de estos patógenos se lleve a cabo mediante métodos orientados a la propia planta en vez de al patógeno, como la resistencia genética (Katan et al., 2012) o los tratamientos de semilla (Molinero-Ruiz et al., 2003b).

Los estudios relativos al control de patógenos de suelo mediante AME se han abordado, tradicionalmente, desde múltiples vertientes:

-*In vitro*: en primer lugar, hay trabajos donde se ha evaluado el efecto de los AME sobre los fitopatógenos, ya sea de manera directa (competencia entre ambos organismos vivos en medio de cultivo) o indirecta (efectos de extractos crudos o purificados de AME sobre los fitopatógenos). Describen metodologías recurrentes de enfrentamiento AME-fitopatógeno como el cultivo dual o la incorporación de extractos crudos o purificados del AME en el medio de crecimiento del fitopatógeno. Los principales mecanismos subyacentes de reducción del crecimiento, desarrollo y reproducción de los fitopatógenos, así como referencias de interés, ya se describieron en el epígrafe correspondiente, por lo que no se incidirá más a este respecto. Estos estudios pueden considerarse el primer paso para el control de las enfermedades de las plantas mediante el empleo de los AME.

-Tratamientos de semilla o plántula: una de las principales estrategias de aplicación de los AME es la del tratamiento de semillas o plántulas desinfestadas en el momento de la siembra o de manera previa a ella. Estas metodologías han sido reproducidas con éxito por diversos autores, alcanzando niveles adecuados de protección frente a un extenso abanico de hongos, oomicetos y bacterias (Ownley et al., 2004; Griffin et al., 2006; Ownley et al., 2008). Se piensa que uno de los principales factores que contribuyen a la respuesta favorable de la planta a la enfermedad es la promoción del crecimiento vegetal proporcionada por los AME, así como la activación de respuestas defensivas (Sasan y Bidochka, 2012; 2013; Jaber y Ownley, 2018).

-Tratamientos de suelo: otros estudios, *in planta*, han abordado el control de las enfermedades mediante tratamientos de suelo, dirigiéndolos de este modo al lugar de origen de la infección. Como ejemplos destacados, Lozano-Tovar et al. (2017) diseñaron una metodología de aplicación al suelo de extractos purificados de *B. bassiana* y *M. brunneum* para proteger a plantas de olivo de *V. dahliae*. Los tratamientos con los extractos redujeron la severidad de los síntomas de manera significativa, evidenciando un marcado antagonismo y reduciendo la germinación de microesclerocios y la formación de hifas y microesclerocios. De forma similar, la aplicación del AME *M. robertsii* en el sustrato donde se cultivaron plantas de judía inoculadas con el hongo de suelo *Neocosmospora phaseoli* (Burkh.) L. Lombard and Crous (Sasan y Bidochka, 2013) ocasionó una menor expresión de síntomas en las plantas tratadas. La mayoría de trabajos publicados coinciden en señalar como mecanismos principales de reducción de la enfermedad en estas circunstancias a la competencia directa o antagonismo en el suelo o la rizosfera, o bien al efecto antibiótico de los extractos (Sasan y Bidochka, 2013; Lozano-Tovar et al., 2017), así como a las propiedades beneficiosas de los AME en el crecimiento de la planta, como un mayor vigor o un desarrollo radicular más pronunciado (Sasan y Bidochka, 2012; 2013). Las metodologías descritas no solo revisten un pronunciado interés desde el punto de vista teórico, sino que podrían convertirse en una opción de peso para el manejo de patógenos como *V. dahliae* de olivo (Lozano-Tovar et al., 2013; 2017).

-Tratamientos aéreos: por último, en estudios recientes se ha evaluado la pulverización aérea de la filosfera con suspensiones de conidios del AME como una vía alternativa de aplicación del agente de biocontrol (Jaber, 2015; Rondot y Reineke, 2019). El fundamento de esta praxis es la colonización endofítica de la planta por el entomopatógeno subsecuente a la pulverización foliar (Landa et al., 2013; Garrido-Jurado et al., 2017) y la protección que a la planta confiere el AME (Jaber y Ownley, 2018). Diversos autores han empleado esta técnica y constatado la colonización de la parte aérea por el AME *B. bassiana*, consiguiendo disminuir de manera significativa la expresión de síntomas de mildiu causado por el oomiceto *P. viticola* (Jaber, 2015; Rondot y Reineke, 2019) y las virosis asociadas a ZYMV (Jaber y Salem, 2014), CMV y CABYV (Gonzalez-Mas et al., 2019b). Esta metodología alberga un gran potencial, pues permitiría eludir una de las principales dificultades en el manejo de las enfermedades de suelo, que es precisamente el dónde enfocar los tratamientos (Katan et al., 2012; Katan, 2017).

I.6. El girasol y sus principales patógenos

El girasol, *Helianthus annuus* L., es una planta herbácea anual de la familia de las compuestas originaria de América del Norte. Esta especie fue domesticada por los pueblos nativos en torno al 3000 a.C., adquiriendo un papel fundamental en la dieta de aquellos (Fernandez-Martinez et al., 2010; Velasco y Fernandez-Martinez, 2015). Al igual que otros cultivos de gran relevancia, esta asterácea fue introducida en el Viejo Mundo a principios del siglo XVI de mano de exploradores españoles (Velasco y Fernandez-Martinez, 2015). Aunque su empleo originario en Europa se limitaba al aprovechamiento ornamental de la inflorescencia para fines recreativos y de la semilla

para su consumo, la necesidad de disponer de aceites vegetales para alimentación en el este de Europa fue el motivo de que, a partir del siglo XIX se estableciera en Rusia un importante programa de mejora genética de girasol. Este programa, liderado por el científico V. Pustovoit, tenía como principal objetivo incrementar el contenido de aceite en la semilla. A lo largo del siglo XX el cultivo de girasol oleaginoso se extendió desde Rusia hacia el resto de Europa (Seiler y Gulya, 2016).

El girasol tiene una importancia indiscutible en Europa desde el punto de vista económico y es clave en la seguridad alimentaria global en cuanto a grasas vegetales. En 2018 se cultivó en el mundo una superficie de 26.7×10^6 ha de girasol, que produjeron 52 Mt de semilla para extracción de aceite (FAO, 2020). Casi tres cuartas partes de la producción mundial de girasol se concentran en el sur y este de Europa, particularmente en Ucrania (14.2 Mt) y la Federación Rusa (12.8 Mt), El resto de la producción de girasol se localiza en Argentina y China. Los países líderes, Ucrania, la Federación Rusa y Argentina; producen, respectivamente, el 25, 24, y 7% del total de semilla. El segundo grupo de productores está integrado por China, Rumania, Bulgaria, Francia, Turquía, Hungría y España, que producen el 27% también del total de semilla de girasol (FAO, 2020). En nuestro país, el girasol es el cultivo oleaginoso más importante y extendido después del olivo (MAPA, 2019). La superficie de girasol sembrada en España ha experimentado una moderada regresión en los últimos años, con un valor de 691276 ha en 2018, de las cuales el 11% se encuentran en regadío; por comunidades autónomas, destacan Castilla y León, Andalucía y Castilla-La Mancha, que acumulan en total el 94% de la superficie nacional dedicada a este cultivo. La producción en 2018 alcanzó un valor de 950346 t (MAPA, 2019).

El girasol cultivado atraviesa dos etapas fenológicas distintas: la vegetativa y la reproductiva. Dentro de la vegetativa se definen diferentes subgrupos, a saber: VE (desde el inicio del desarrollo hasta la emisión del primer par de hojas mientras estas no superen los 4 cm de longitud) y V1, V2… VN, para designar a la planta con un número N de hojas verdaderas con una extensión de al menos 4 cm. Cuando se aprecia la emisión del botón floral inmaduro tiene comienzo la etapa reproductiva, dividida en las fases R1-R9 hasta su senescencia, cuando las brácteas que conforman el capítulo han adquirido un tono marrón (Schneiter y Miller, 1981) (Figura I.8). El ciclo concluye con el llenado del grano, siendo el fruto un aquenio ovalado en cuyo interior se halla el grano. Cada fase afecta de diferente manera al rendimiento final del cultivo, resultando la germinación y nascencia y el momento de llenado de grano de vital importancia (Alberio et al., 2015). El aceite se extrae del grano de la semilla; su contenido depende de la variedad, del manejo del cultivo y de las condiciones edafoclimáticas, entre otros factores (Mantese et al., 2006).

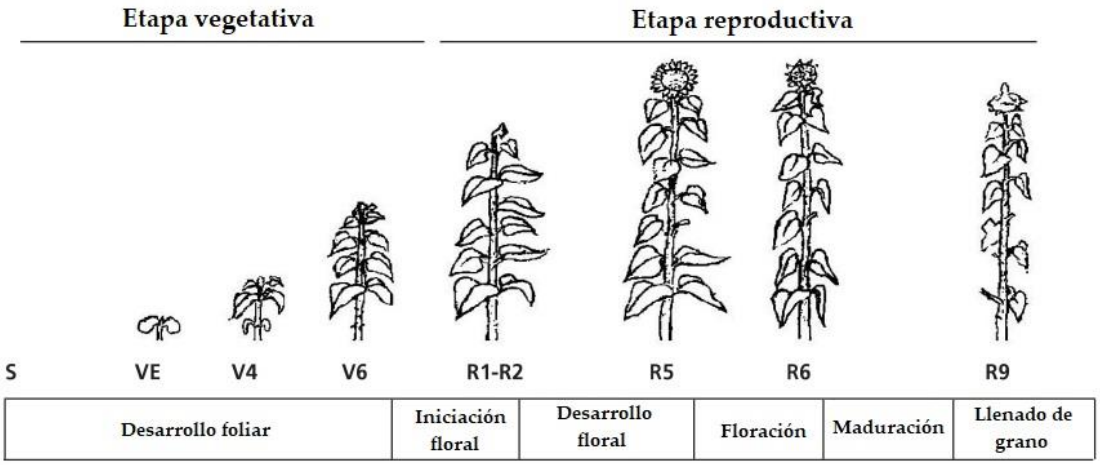

Figura I.8. Representación de la fenología del cultivo del girasol. S. Siembra. VE. Primera fase vegetativa. V4, V6. Fases vegetativas subsecuentes, denominadas conforme a su número de hojas verdaderas. R1-R9. Fases reproductivas, desde la iniciación floral hasta el llenado de grano. Fuente: Adaptado de Alberio et al., 2015.

El girasol es considerado un cultivo muy sostenible por el bajo nivel de insumos que requiere, incluyendo un aporte de nitrógeno reducido (Agreste, 2014), y por su buena adaptación al secano de climas continentales (Debaeke et al., 2017a). Asimismo, las emisiones de gases de efecto invernadero que origina este cultivo son escasas, reduciendo de este modo su huella de carbono, y las variedades cultivadas en todo el mundo son de origen no transgénico (Debaeke et al., 2017a). Sin embargo, el girasol no está exento de la influencia de nuevas condiciones de cultivo ante el cambio climático y de limitaciones bióticas a su producción, entre otros (Debaeke et al., 2017b).

Dentro de los limitantes de naturaleza biótica tienen particular relevancia las enfermedades, que hace 40 años se estimaron como causantes de la pérdida del 12% de la producción potencial (Zimmer y Hoes, 1978). Se ha descrito un elevado número de patógenos de este cultivo, aunque no todos ellos tienen relevancia en términos económicos (Gulya et al., 1997).

I.6.1. *Verticillium dahliae* Kleb.

El ascomiceto *Verticillium dahliae*, agente causal de la marchitez por Verticillium o verticilosis del girasol, es un patógeno vascular que afecta a un vasto rango de plantas cultivadas (MAPAMA, 2016): se conocen más de 200 posibles hospedantes para este hongo, algunos de los cuales poseen una destacable importancia económica, como el tomate, el tabaco, la patata, la lechuga, el algodonero, el olivo, la berenjena, la alcachofa, la coliflor y el girasol (Pegg y Brady, 2002). Este patógeno supone habitualmente una limitación para la producción agrícola allá donde se encuentre, pues afecta gravemente al rendimiento de los cultivos y puede permanecer en el suelo en forma de microesclerocios (estructuras de resistencia) durante más de 10

años (Pegg y Brady, 2002; MAPAMA, 2016). Su importancia es mayor, además, en climas templados (Gulya et al., 1997).

En el caso del girasol, este patógeno se encuentra distribuido por Estados Unidos, Argentina, Europa (Pegg y Brady, 2002) y algunas regiones de Canadá (Putt, 1958; Fick y Zimmer, 1974), considerándose uno de los patógenos de mayor importancia en este cultivo en la mayoría de países (Gulya et al., 1997; Pegg y Brady, 2002; Martin-Sanz et al., 2018b). En la última década se ha observado una mayor incidencia de esta enfermedad en varios países del sur de Europa, entre ellos España y Francia (Garcia-Ruiz et al., 2014; Debaeke et al., 2017a; Martin-Sanz et al., 2018b), lo que pone de manifiesto su creciente importancia.

El suelo, donde permanece entre campañas de cultivo en forma de microesclerocios, es el principal reservorio de este ascomiceto (Gulya et al. 1997). Los microesclerocios germinan al ser estimulados por los exudados de las raíces y producen hifas infectivas (Fitzell et al., 1980). Tras la penetración de las hifas infectivas en la raíz de la planta el hongo avanza a través de sus tejidos hasta alcanzar los vasos del xilema, donde continúan formándose hifas y conidias fúngicas que permiten la dispersión interna y ascendente de *V. dahliae* en el girasol. Finalmente el patógeno se distribuye por toda la planta, pudiendo aislarse de raíces, tallo, hojas, peciolo y capítulo (Sackston et al., 1957; Pegg y Brady, 2002). Los primeros síntomas se hacen visibles en las hojas basales, produciéndose al principio la aparición de pequeñas manchas y deviniendo, al tiempo, en una marcada clorosis de un intenso tono amarillento que se extiende desde el margen de la hoja hacia su interior siguiendo un patrón internervial, ocupando sectores de tamaño y conformación irregular y extendiéndose hacia las hojas superiores (Gulya et al., 1997). Por último, la clorosis torna en lesiones necróticas de tejido muerto, también marginales, que recubren progresivamente superficies foliares mayores, quedando habitualmente rodeadas de un halo plesionecrótico (Figura I.9). En el interior de los haces vasculares aparecen asimismo lesiones de una coloración parda o marrón oscura que, en cortes transversales del tallo, pueden apreciarse en forma de anillos. Su color puede adquirir tonalidades grisáceas en fases avanzadas de la enfermedad, consecuencia de la producción de microesclerocios, de apariencia negruzca y consistencia pulverulenta (Gulya et al., 1997). Por último, el capítulo de las plantas se reduce en tamaño (entre un 16 y un 42% de su diámetro). También se reduce el tamaño y peso de las semillas (Hoes, 1972), que pueden contener al hongo en su interior y dar lugar, de este modo, a la transmisión vertical de la enfermedad (MAPAMA, 2016). Por eso *V. dahliae* es también un patógeno de semilla. Las plantas infectadas pueden morir prematuramente en caso de afección severa o expresar un grado variable de severidad de síntomas, opción más común, reduciéndose su rendimiento (MAPAMA, 2016).

Figura I.9. Síntomas causados por *Verticillium dahliae* en girasol. Fuente: Martin-Sanz et al., 2017.

Las pérdidas de cosecha que ocasiona *V. dahliae* están sujetas a una gran variabilidad y dependen en buena medida del propio cultivo (variedad de girasol), la densidad de inóculo, las prácticas agronómicas (especialmente el riego, cultivos susceptibles previos o la presencia de hospedantes alternativos) y las condiciones edafoclimáticas (Gulya et al., 1997; Pegg y Brady, 2002; Klosterman et al., 2009; MAPAMA, 2016). En girasol, la disminución del rendimiento puede oscilar entre el 10% y el 100% (Hoes y Putt, 1962). En España, la gravedad de esta enfermedad suele ser menor que la de otras como el mildiu, mientras que *V. dahliae* es un importante limitante de la producción de semilla de girasol en Francia (Garcia-Carneros et al., 2015).

La resistencia genética es la medida de control más efectiva para la verticilosis del girasol (Garcia-Carneros et al., 2015; MAPAMA, 2016), ya que, hasta el momento, no se han descrito medios químicos ni biológicos efectivos en este cultivo. Los medios físicos, agronómicos y de manejo que se conocen se basan en prácticas como la racionalización del abonado, la eliminación de la flora adventicia, la solarización, la desinfestación de las herramientas y maquinaria, etc., las cuales no resultan, por sí mismas, suficientemente practicables, rentables o eficaces (Gulya et al., 1997; MAPAMA, 2016; Debaeke et al., 2017a). Cabe destacar que la rotación de cultivos no es eficaz para el control de la verticilosis del girasol por la prolongada persistencia de los microesclerocios en el suelo (Gulya et al., 1997).

La primera vez que se observó resistencia al patógeno en este cultivo fue en Manitoba en el año 1957 (Putt, 1958); esta era de carácter cualitativo y estaba conferida por el gen V_1 (Putt, 1964). Desde entonces, se han desarrollado diversos programas de mejora que han permitido la obtención de cultivares resistentes a la verticilosis (Putt, 1964; Fick y Zimmer, 1974), así como de nuevas fuentes de resistencia, incluyendo resistencia cuantitativa (Gulya et al., 1997; Galella et al., 2012; Garcia-Carneros et al., 2015). Por otro lado, sucesivos estudios han tenido como objetivo la caracterización patogénica, molecular y/o genética del patógeno (Galella et al., 2012; Garcia-Carneros et al., 2015; Martin-Sanz et al., 2018b), permitiendo determinar las poblaciones que superan (o no) los distintos genes de resistencia. Trabajos llevados a cabo por nuestro grupo de investigación han descrito dos grupos de poblaciones de *V. dahliae* de girasol: uno, más heterogéneo, incluye poblaciones del este de Europa, mientras que el segundo es más homogéneo y en él se agrupan aislados del oeste europeo y de Argentina (Martin-Sanz et al., 2018b). Actualmente en Europa existen tres razas de *V. dahliae* de girasol: V1 (controlada por el gen de resistencia V_1), V2-EE (este de Europa) y V2-WE (oeste de Europa) (Martin-Sanz et al., 2018b).

Pese a la indudable utilidad de la resistencia genética para el control de la verticilosis del girasol, la aparición de razas de *V. dahliae* capaces de superar las fuentes de resistencia conocidas en Estados Unidos, Argentina y España amenaza el control eficaz de esta enfermedad y apunta al interés de buscar otras herramientas de control que puedan complementar a la resistencia genética (Galella et al., 2004; Gulya, 2007; Garcia-Ruiz et al., 2014).

I.6.2. *Plasmopara halstedii* Farl. Berl. and de Toni

El oomiceto *Plasmopara halstedii* es el agente casual del mildiu del girasol, considerada la enfermedad más importante de este cultivo (Gulya et al. 1997). Este biotrofo obligado originario de América del Norte tiene una distribución cosmopolita, hallándose presente en los cinco continentes y encontrándose en prácticamente cualquier lugar donde se cultive el girasol (Zimmer y Hoes, 1978; Gulya et al., 1997), aunque ocasiona mayores pérdidas en las latitudes templadas (Gulya et al., 1997). *Plasmopara halstedii* es un patógeno de suelo, donde puede sobrevivir durante varios años en ausencia de girasol gracias a estructuras de resistencia denominadas oosporas (MAPAMA, 2016).

Este patógeno ocasiona dos tipos de infección en el campo. Las infecciones primarias tienen lugar cuando las zoosporas del patógeno emergen a partir de oosporas presentes en el suelo y alcanzan el sistema radical de las jóvenes plántulas, o bien mediante transmisión vertical a partir de semilla procedente de planta infectada; es entonces cuando se produce mortalidad de plántulas en pre- o en post-emergencia o, en su defecto, un severo retraso en el crecimiento (Molinero-Ruiz y Melero-Vara, 2010). Las plantas infectadas manifiestan una clorosis muy pronunciada en forma de mosaico, restringida al área internervial en las hojas de edad avanzada pero cubriendo las hojas jóvenes por completo (Molinero-Ruiz, 2019). En condiciones de humedad relativa alta y temperaturas suaves, el parásito desarrolla un crecimiento algodonoso en el envés de las hojas infectadas que corresponde al desarrollo de hifas y esporangióforos portadores de

zoosporangios; la esporulación coincide con la estructura del mosaico clorótico presente en el haz de la hoja (Molinero-Ruiz y Melero-Vara, 2010; Gulya et al., 2019). Las infecciones secundarias o aéreas, de carácter localizado, son causadas por zoosporas transportadas por el aire desde plantas infectadas (Gulya et al., 2019), o bien por esporangios que germinan al depositarse en hojas sanas (Molinero-Ruiz y Melero-Vara, 2010). En estos casos, resulta frecuente el desarrollo de síntomas de carácter leve: manchas cloróticas de un tono verdoso y una estructura poligonal, asociadas a la nervadura de la planta (Molinero-Ruiz y Melero-Vara, 2010). Las pérdidas de cosecha son ocasionadas principalmente por las infecciones primarias, teniendo las secundarias muy poca importancia desde el punto de vista económico (Gascuel et al., 2015; Gulya et al, 2019). Aparte de los síntomas anteriormente descritos, *P. halstedii* causa enanismo o achaparramiento de las plantas debido al acortamiento de los entrenudos (las plantas afectadas crecen entre 10 y 50 cm menos que las sanas), deformación ocasional de la planta y sus órganos, menor tamaño del capítulo (que, además, puede quedar en posición horizontal, con el disco floral hacia arriba), infertilidad parcial o total de las semillas, lesiones necróticas en hoja y tallo, agallas en la raíz (las cuales disminuyen la tolerancia de la planta a la sequía) y manchas foliares (Molinero-Ruiz y Melero-Vara, 2010; Gascuel et al., 2015; MAPAMA, 2016) (Figura I.10). A pesar de lo anterior, si las condiciones resultan más favorables para la planta que para el patógeno, la infección puede desarrollarse con sintomatología leve, o incluso de manera asintomática (Molinero-Ruiz y Melero-Vara, 2010).

Figura I.10. Síntomas y signos de *Plasmopara halstedii* en girasol. A. Lesiones foliares de origen clorótico. B. Enanismo y capítulos horizontales. C. Esporulación en cotiledones y hojas verdaderas. Fuente: MAPAMA. 2016 (A,B), propia (C).

La reducción global de rendimiento debida a esta enfermedad se sitúa en torno al 3.5%, aunque son frecuentes las pérdidas del 100% de la cosecha si las condiciones son muy favorables para el desarrollo de *P. halstedii* o se producen infecciones muy localizadas (Gascuel et al., 2015; Gulya et al., 2019); por el contrario, si la infección es leve o muy temprana, la muerte de plantas puede verse compensada por un mayor desarrollo de las demás y, de este modo, no se produce una disminución apreciable en el rendimiento (Gulya et al., 1997; Molinero-Ruiz y Melero-Vara, 2010). Los principales factores que condicionan el efecto de este patógeno sobre el rendimiento del cultivo son la cantidad de inóculo, el porcentaje y la distribución de plantas infectadas en el campo,

la edad de las plantas, los órganos afectados y las condiciones meteorológicas y edafológicas (Gulya et al., 1997; Molinero-Ruiz y Melero-Vara, 2010). Por otro lado, la disminución de rendimiento se debe, principalmente, a la muerte de plantas, el menor tamaño de los capítulos, el menor peso de las semillas y el menor contenido en aceite de estas (Gulya et al., 1997).

Este patógeno se caracteriza por tener una importante diversidad racial. Al comienzo de la década de los 70, es decir, poco tiempo después del inicio del uso generalizado de este cultivo para la obtención de aceite, se conocían únicamente dos razas fisiológicas de *P. halstedii*: la raza 1 o Europea y la raza 2, también conocida como Norteamericana o Red River (Gulya et al., 1997). Por aquel entonces, se controlaban de manera satisfactoria mediante el empleo de dos genes de resistencia: el Pl_1, que aportaba resistencia contra la raza 1, y el Pl_2, que la proporcionaba frente a ambas (Molinero-Ruiz y Melero-Vara, 2010). Aunque en principio se supuso que cada una de estas razas se encontraba restringida al área geográfica en el que eran conocidas, esa misma década se identificaron en Italia, Rumanía y Rusia poblaciones del patógeno con virulencia similar a la de la raza Norteamericana (Molinero-Ruiz y Melero-Vara, 2010). En la década de los 80 se puso de manifiesto que el perfil racial del patógeno era más complejo de lo que se había supuesto pues, en base a las fuentes de resistencia superadas, se descubrieron nuevas razas. En pocos años había nueve razas descritas (Gulya et al., 1997; Molinero-Ruiz y Melero-Vara, 2010). Conforme avanzaba el conocimiento, se evidenció la necesidad de contar con métodos más precisos de identificación racial de *P. halstedii*, desarrollándose a finales de los 90 el método de nomenclatura que sigue empleándose actualmente. Consiste en el uso de nueve líneas diferenciales de girasol, cada una incorporando distintas fuentes de resistencia a *P. halstedii* (con la excepción de la línea HA-304, susceptible a todas las razas), agrupadas en tres bloques o tripletes en un orden preestablecido. Tras la inoculación de los nueve diferenciales con un determinado aislado de *P. hasltedii*, se anota la respuesta susceptible o resistente de cada uno, trasladando los resultados a una fórmula de tres dígitos, cuyo resultado constituirá el nombre de la raza en cuestión (Gulya et al., 1998).

En la actualidad, se han descrito 42 razas diferentes de *P. halstedii* en todo el mundo (Viranyi et al., 2015; Sedlarova et al., 2016; Trojanova et al., 2018); este espectro racial está en continua evolución en las zonas productoras de girasol (Viranyi et al., 2015). En el caso de España, sucesivos estudios han aportado información de la distribución racial del patógeno desde los años 90 hasta 2006 (Molinero-Ruiz et al., 2002a; 2008); actualmente hay evidencia de la presencia de nuevas razas en nuestro país, entre ellas algunas altamente virulentas (Garcia-Carneros y Molinero-Ruiz, 2017; Molinero-Ruiz, 2019).

El método más eficaz de manejo de la enfermedad es el uso de genes de resistencia. La identificación de genes de resistencia frente a *P. halstedii* suele evolucionar de forma paralela, aunque no simultánea, al espectro de razas. Hasta 2008 se habían identificado 12 genes de resistencia a mildiu (Molinero-Ruiz et al., 2002b; 2003a; Vear et al., 2008). Desde entonces, se han descrito otros 10 genes: Pl_{14} (Bachlava et al., 2011) y Pl_{15} (Bertero de Romano et al., 2010) en Argentina, Pl_{13} (Mulpuri et al., 2009) y Pl_{16} (Liu et al., 2012) en USA, Pl_{17}, Pl_{19} y Pl_{21} de *H. annuus* silvestre (Vincourt et al., 2012; Qi et al., 2015; Zhang et al., 2017), Pl_{18} y Pl_{20} de *H.*

argophyllus Torr. and A.Gray (Qi et al., 2016; Ma et al., 2017) y Pl_{22}, probablemente de *H. tuberosus* L. (Badouin et al., 2017). Estos nuevos genes son efectivos a muchas de las nuevas razas descritas recientemente, pero ninguno de ellos lo es frente a todas.

El segundo método para controlar el mildiu es el tratamiento preventivo de semilla de siembra con metalaxil-M. El metalaxil-M se ha desarrollado a partir del metalaxil, una fenilamida comercializada en los años 80 del siglo XX para el control de oomicetos (Cohen y Coffey, 1986). En 2003 el metalaxil fue sustituido por el metalaxil-M, de la misma familia química pero que, en su composición, solamente incluye el enantiómero bioactivo. Actualmente en España es obligatorio sembrar girasol cuya semilla haya sido tratada con metalaxil-M (MAPA, 2020) pero, al igual que en otros países europeos, se han descrito poblaciones de *P. halstedii* con resistencia a metalaxil (Molinero-Ruiz et al., 2003b), a metalaxil-M (Molinero-Ruiz et al., 2008) o a ambos simultáneamente (Molinero-Ruiz et al., 2008).

Por último, el manejo del mildiu del girasol también debe tener en cuenta las prácticas culturales, como corresponde en un contexto de gestión integrada de plagas (MAPAMA, 2016). Dentro de ellas, la decisión del adecuado momento de siembra es la que más puede influir para reducir la incidencia de mildiu (Molinero-Ruiz y Melero-Vara, 2010). Teniendo presente que las prácticas culturales, por sí solas, no suelen alcanzar un grado aceptable de control de la enfermedad (Molinero-Ruiz y Melero-Vara, 2010), que el control químico se ve amenazado por la detección de aislados de *P. halstedii* resistentes al metalaxil-M (Molinero-Ruiz et al., 2003b; 2008) y que la resistencia genética se halla limitada por el amplio y cambiante perfil racial del patógeno, manejar el mildiu de girasol en campo resulta difícil y requiere de la combinación inteligente del uso de las distintas herramientas disponibles.

I.6.3. *Cadophora helianthi* (L. Molinero-Ruiz, A. Martin-Sanz, C. Berlanas and D. Gramaje)

Un patógeno de girasol que está adquiriendo una especial relevancia en los últimos tiempos es el hongo *Cadophora helianthi*. El patógeno fue detectado por primera vez en plantaciones de girasol de Rusia y Ucrania en 2017, donde se observó una enfermedad en este cultivo con una sintomatología muy similar a la de *V. dahliae* y una pérdida casi total de la producción (Martin-Sanz et al., 2018a) (Figura I.11). En un principio, se identificó erróneamente como *Cadophora malorum* (Kidd and Beaumont) W. Gams (Martin-Sanz et al., 2018a), pero más tarde se descubrió que era una nueva especie con rasgos morfológicos y moleculares particulares (Crous et al., 2019).

Figura I.11. Síntomas causados por *Cadophora helianthi* en girasol. Fuente: Martin-Sanz et al., 2018a.

La investigación sobre este hongo de suelo reviste un interés especial, puesto que, al haberse descrito en 2018, no existe información sobre su etiología, distribución geográfica, importancia económica ni, por supuesto, medidas de control. Sin embargo, se ha constatado que se encuentra presente en los dos principales países productores de girasol, Rusia y Ucrania, que los síntomas que causa son fácilmente confundibles con los de la verticilosis y que puede afectar a prácticamente la totalidad de la cosecha si las condiciones durante la campaña del cultivo son propicias para el patógeno (Martin-Sanz et al., 2018a; Molinero-Ruiz, 2019).

I.7. Empleo de los ascomicetos mitospóricos entomopatógenos en el girasol

El desarrollo de nuevas estrategias para la lucha integrada en el cultivo del girasol se ha convertido en una necesidad imperiosa para garantizar su sostenibilidad a largo plazo, no solo debido a la dificultad para controlar algunos de sus patógenos actuales (como *V. dahliae, P. halstedii* o *C. helianthi*), sino también para hacer frente a otras amenazas asociadas al cambio climático, que disminuirá el rendimiento de este cultivo en Europa entre un 5 y un 30% en la próxima década (Debaeke et al., 2017b) y tendrá una influencia significativa en su sanidad, propiciando la aparición de nuevos patógenos y de cepas más agresivas, alterando la incidencia o la distribución de sus

enfermedades y modificando las interacciones patógeno-hospedante (Coakley et al., 1999; Molinero-Ruiz, 2019).

Los AME se hallan ampliamente distribuidos por agroecosistemas de diversa condición (Garrido-Jurado et al., 2015b; Quesada-Moraga, 2020), habiéndose descrito su presencia natural en plantaciones de girasol (Garrido-Jurado et al., 2015b). A pesar de ello, las referencias sobre su empleo en girasol son muy escasas.

El uso de los AME en girasol se ha limitado, predominantemente, al control biológico de plagas de insectos: de esta manera, ciertos AME como *M. brunneum*, *M. anisopliae* y *B. bassiana* pueden resultar candidatos muy prometedores frente a fitófagos que amenazan este cultivo como son los gusanos de alambre (*Agriotes* spp. Eschscholtz) (Coleoptera: Elateridae) (Ortiz-Bustos et al., 2016), el curculiónido *Tanymecus dilaticollis* Gyll. (Coleoptera: Curculionidae) (Takov et al., 2013) o el tortrícido *Cochylis hospes* Walsingham (Lepidoptera: Tortricidae) (Barker, 1999). También existe un trabajo en el que se evaluó la eficacia de una cepa comercial de *Isaria fumosorosea* Wize, sola y en aplicación conjunta con insecticidas químicos, para el control del minador *Liriomyza trifolii* (Burgess) (Diptera: Agromyzidae), sobre variedades ornamentales de gerberas y girasoles para producción de flor cortada en vivero, obteniéndose niveles de control variables y dependientes tanto del cultivo como de la combinación del agente de biocontrol con los diferentes insecticidas, no resultando, en ocasiones, recomendable esta práctica (Wekesa et al., 2011).

Otro efecto que los AME pueden tener sobre el girasol es la promoción de su crecimiento. Algunos autores han referido mejor asimilación de nutrientes, mayor desarrollo vegetativo y floración temprana en plantas de esta especie cultivadas en cámara de crecimiento tras la aplicación de cepas de *B. bassiana*, *M. brunneum* e *Isaria farinosa* (Holmsk.) (Sanchez-Rodriguez et al., 2016; Raya-Diaz et al., 2017a; 2017b).

I.8. Objetivos de la presente Tesis Doctoral

Los objetivos de la presente Tesis Doctoral son los que a continuación se enumeran:

1.- Evaluar la compatibilidad de diferentes estrategias de aplicación simultánea del parasitoide *H. didymator* y el ascomiceto mitospórico entomopatógeno *M. brunneum* para el control de la rosquilla negra, *S. littoralis*, en bioensayos *in vitro*, así como los efectos directos e indirectos de la aplicación del entomopatógeno sobre el parasitoide.

2.- Evaluar la compatibilidad de diferentes estrategias de aplicación simultánea del parasitoide *H. didymator* y el ascomiceto mitospórico entomopatógeno *M. brunneum* para el control de la rosquilla negra, *S. littoralis*, en bioensayos *in planta*, para explorar tanto la aplicación directa del hongo sobre el fitófago como la vía endofítica, con un estudio histopatológico sobre la naturaleza de las interacciones fitófago-hongo-parasitoide.

3.- Evaluar la capacidad antagonista *in vitro* de diferentes cepas de los ascomicetos mitospóricos entomopatógenos *B. bassiana* y *M. brunneum* frente a los patógenos de girasol *V. dahliae* y *C. helianthi*, así como su potencial para reducir síntomas de verticilosis del girasol en invernadero y su persistencia en el sustrato y en el interior de plantas tras ser aplicados mediante tratamientos de suelo.

4.- Evaluar el efecto de los ascomicetos mitospóricos entomopatógenos *B. bassiana* y *M. brunneum* sobre la severidad de síntomas del mildiu de girasol en cultivo axénico, así como en el crecimiento de girasol, y los patrones de colonización endófita en las mismas condiciones.

Los resultados relativos al primer objetivo se desarrollan a lo largo del capítulo II, que consiste en una versión adaptada del artículo "Compatibility between the endoparasitoid *Hyposoter didymator* and the entomopathogenic fungus *Metarhizium brunneum*: a laboratory simulation for the simultaneous use to control *Spodoptera littoralis*", publicado en el número 76 de la revista Pest Management Science entre las páginas 1060 y 1070, con doi: 10.1002/ps.5616. Esta revista es D1: 7/101 en "Entomology" con un factor de impacto de 3.750.

Los resultados concernientes al segundo objetivo se desarrollan a lo largo del capítulo III, que consiste en una versión adaptada del artículo "Entomopathogenic fungal endophyte-mediated tritrophic interactions between *Spodoptera littoralis* and its parasitoid *Hyposoter didymator*", actualmente publicado online en la revista Journal of Pest Science, con doi: 10.1007/s10340-020-01306-7. Esta revista es D1: 2/101 en "Entomology" con un factor de impacto de 4.578.

Los resultados correspondientes al tercer objetivo se desarrollan a lo largo del capítulo IV, que consiste en una versión adaptada del artículo "Evidence of soil-located competition as the cause of the reduction of sunflower verticillium wilt by entomopathogenic fungi", publicado en el número 69 de la revista Plant Pathology entre las páginas 1492 y 1503, con doi: 10.1111/ppa.13230. Esta revista es Q1: 21/91 en "Agronomy" con un factor de impacto de 2.169.

Los resultados relacionados con el cuarto objetivo se desarrollan a lo largo del capítulo V, que consiste en una versión adaptada del artículo "Updated characterization of races of *Plasmopara halstedii* and entomopathogenic fungi as endophytes of sunflower plants in axenic culture", aceptado con minor revision en la revista Agronomy. Esta revista es Q1: 18/91 en "Agronomy" con un factor de impacto de 2.603.

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CAPÍTULO II. COMPATIBILITY BETWEEN THE ENDOPARASITOID *Hyposoter didymator* AND THE ENTOMOPATHOGENIC FUNGUS *Metarhizium brunneum*: A LABORATORY SIMULATION FOR THE SIMULTANEOUS USE TO CONTROL *Spodoptera littoralis*

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Compatibility between the endoparasitoid *Hyposoter didymator* and the entomopathogenic fungus *Metarhizium brunneum*: a laboratory simulation for the simultaneous use to control *Spodoptera littoralis*

Pedro Miranda-Fuentes, Enrique Quesada-Moraga,* Hani K Aldebis and Meelad Yousef-Naef

Abstract

BACKGROUND: The cotton leafworm, *Spodoptera littoralis*, is one of the most destructive pests in the Mediterranean basin, being predominantly controlled using synthetic chemical pesticides. Strain EAMa 01/58-Su of the fungus *Metarhizium brunneum* and the parasitoid *Hyposoter didymator* are promising biological control agents for this pest. In this study, we assessed the compatibility between these two agents to control *S. littoralis* under joint attack scenarios.

RESULTS: Firstly, the direct and indirect effects of the fungus towards parasitoid adults were studied. The fungus significantly decreased life expectancy of the parasitoid (mortality = 62.5%; mean lethal concentration = 1.85×10^6 conidia ml^{-1}; average survival time = 92.2 h) when applied at high concentrations (10^8 conidia ml^{-1}), whereas it did not affect the reproductive potential of the parasitoid females during the 3 days after treatment. Secondly, the combinations between the two agents to control *S. littoralis* under different simultaneous use scenarios (inoculation of *S. littoralis* larvae with the fungus before being exposed to parasitoid females and *vice versa*) were investigated, with additive effect in all cases. A significant effect on fitness (preimaginal development time and reproductive potential) of the F1 parasitoid generation were detected. Moreover, parasitization significantly reduced the total hemocytes in *S. littoralis* hemolymph compared with the control, promoting fungal infection. Finally, parasitoids showed a significant preference for non-inoculated *S. littoralis* larvae.

CONCLUSIONS: We demonstrated compatibility (additive effect) between fungus and parasitoid under different joint attack scenarios to control *S. littoralis* in laboratory conditions. However, this will be supported by our ongoing greenhouse and field studies.

Keywords: integrated management; biological control; intra-host relationships; *Metarhizium brunneum*; parasitoids; cotton leafworm

1 INTRODUCTION

Phytophagous insects are a major constraint to crop production and often cause huge yield losses. One of the most destructive and ubiquitous insect pests in the Mediterranean basin is the cotton leafworm, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae). This species is widespread in many northern and southern European countries, particularly Spain, Greece and Italy, and has been detected in Africa and Asia Minor.[1,2] The polyphagous habit of *S. littoralis* makes it a noxious pest on numerous economically-important crops in both greenhouses and open fields: tomato, pepper, eggplant, lettuce, artichoke, strawberry, asparagus, spinach, ornamentals and herbs.[2,3] Chemical control has been the traditional control method for *S. littoralis*.[4] However, chemical control is not a sustainable approach for the future. The environmental impact of chemical compounds threatens food and water security[5,6] and their use has led to the development of resistance in *S. littoralis* to several active ingredients, mainly among orgaphosphorus,[7] IGRs[8] and pyrethroids.[9] Moreover, chemical insecticides may be harmful for the natural enemies of insect pests.[10] As a result, research has increasingly focused on non-chemical measures for control of *S. littoralis* with a particular emphasis on biological control.[11] Biological control agents

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II. Compatibility between the endoparasitoid *Hyposoter didymator* and the entomopathogenic fungus *Metarhizium brunneum*: A laboratory simulation for the simultaneous use to control *Spodoptera littoralis*

Pedro Miranda-Fuentes, Enrique Quesada-Moraga, Hani Kassim Aldebis and Meelad Yousef-Naef

II.0. Abstract

Background: The cotton leafworm, *Spodoptera littoralis*, is one of the most destructive pests in the Mediterranean basin, being predominantly controlled using synthetic chemical pesticides. Strain EAMa 01/58-Su of the fungus *Metarhizium brunneum* and the parasitoid *Hyposoter didymator* are promising biological control agents for this pest. In this study, we assessed the compatibility between these two agents to control *S. littoralis* under joint attack scenarios.

Results: Firstly, the direct and indirect effects of the fungus towards parasitoid adults were studied. The fungus significantly decreased life expectancy of the parasitoid (mortality=62.5%; mean lethal concentration=1.85×10^6 conidia ml^{-1}; average survival time=92.2h) when applied at high concentrations (10^8 conidia ml^{-1}), whereas it did not affect the reproductive potential of the parasitoid females during the three days after treatment. Secondly, the combinations between the two agents to control *S. littoralis* under different simultaneous use scenarios (inoculation of *S. littoralis* larvae with the fungus before being exposed to parasitoid females and *vice versa*) were investigated, with additive effect in all cases. A significant effect on fitness (preimaginal development time and reproductive potential) of the F1 parasitoid generation was detected. Moreover, parasitization significantly reduced the total haemocytes in *S. littoralis* haemolymph compared with the control, promoting fungal infection. Finally, parasitoids showed a significant preference for non-inoculated *S. littoralis* larvae.

Conclusions: We demonstrated compatibility (additive effect) between fungus and parasitoid under different joint attack scenarios to control *S. littoralis* in laboratory conditions. However, this will be supported by our ongoing greenhouse and field studies.

Keywords

Integrated management, biological control, intra-host relationships, *Metarhizium brunneum*, parasitoids, cotton leafworm

II.1. Introduction

Phytophagous insects are a major constraint to crop production and often cause huge yield losses. One of the most destructive and ubiquitous insect pests in the Mediterranean basin is the cotton leafworm, *Spodoptera littoralis* (Boisduval)

(Lepidoptera: Noctuidae). This species is widespread in many northern and southern European countries, particularly Spain, Greece and Italy, and has been detected in Africa and Asia Minor (Lanzoni et al., 2012; EPPO, 2019). The polyphagous habit of *S. littoralis* makes it a noxious pest on numerous economically-important crops in both greenhouses and open fields: tomato, pepper, eggplant, lettuce, artichoke, strawberry, asparagus, spinach, ornamentals and herbs (Sannino et al., 2003; Lanzoni et al., 2012). Chemical control has been the traditional control method for *S. littoralis* (Ghribi et al., 2012). However, chemical control is not a sustainable approach for the future. The environmental impact of chemical compounds threatens food and water security (Arias-Estevez et al., 2008; Adrees et al., 2015) and their use has led to the development of resistance in *S. littoralis* to several active ingredients, mainly among orgaphosphorus (Issa et al., 1984a), IGRs (Mosallanejad and Smagghe, 2009) and pyrethroids (Issa et al., 1984b). Moreover, chemical insecticides may be harmful for the natural enemies of insect pests (El-Wakeil et al., 2013). As a result, research has increasingly focussed on non-chemical measures for control of *S. littoralis* with a particular emphasis on biological control (Resquin-Romero et al., 2016). Biological control agents are important alternatives to chemical pesticides and one of the principal components of any Integrated Pest Management (IPM) programme (Menzler-Hokkanen, 2006). The European Directive on the sustainable use of pesticides (2009/128/EC) promotes the use of biological control as an environmentally friendly, sustainable and financially viable tool for pest control.

The endoparasitoid *Hyposoter didymator* (Thunberg) (Hymenoptera: Ichneumonidae) is also a promising biological control agent for consideration in any IPM programme for control of noctuid pest species including *S. littoralis* (Hatem et al., 2016). This solitary koinobiont wasp is indigenous in many European countries, including Spain, and it actively searches for and parasitizes larval stages of the genera *Spodoptera*, *Heliothis* and *Helicoverpa.* As an ichneumonid wasp, *H. didymator* has been described as a polydnavirus secretor, which is injected onto the host larva during oviposition (Volkoff et al., 1995). The polydnaviruses produced by hymenopteran parasitoids cause a suppression of the host immune response, affecting the presence of haemocytes in the host haemolymph (Volkoff et al., 2001). As defence units that modulate the cellular immune responses, haemocytes are a very important component of the insect immune system (Siddiqui and Al-Khalifa, 2014), and the affection of the immune system of the host may increase its susceptibility to other biocontrol agents (King and Bell, 1978). The well-known efficacy of *H. didymator* controlling several insect pests, including *S. littoralis*, have led different authors to emphasize on the interest of developing biocontrol strategies using this parasitoid (Hatem et al., 2016). Although several authors have reported difficulties rearing this parasitoid, and generally considered time-consuming and easily biassed towards males (Schneider and Viñuela, 2007), the recent develop of new rearing methods is leading to a more efficient production which could be used if a commercial *H. didymator* production is aimed (Hatem et al., 2016).

On the other hand, entomopathogenic fungi have great potential as biological control agents against many insect pests (Quesada-Moraga et al., 2014; Yousef et al., 2017). Their contact mode of action and ability to secrete insecticidal compounds put them at the vanguard of the global development of alternative control strategies

(Quesada-Moraga et al., 2014). Among them, it is worth mentioning the genus *Metarhizium*, which comprehends several species of a great efficacy as biocontrol agents, such as *Metarhizium anisopliae* (Metsch) (Hypocreales: Clavicipitaceae) and *Metarhizium brunneum* Petch (Hypocreales: Clavicipitaceae) (Brunner-Mendoza et al., 2019). As generalist entomopathogenic fungi, *Metarhizium* spp. have a broad host range, although their virulence, and thus, their efficacy as biocontrol agents depends largely on the strain more than the species (Goettel et al., 2005). Indeed, our previous studies have reported the efficacy of several isolates of entomopathogenic fungi for control of *S. littoralis*, both by direct inoculation of larvae with the fungus (Resquin-Romero et al., 2016) and by feeding larvae with leaves from endophytically-colonised plants (Sanchez-Rodriguez et al., 2018). The *M. brunneum* isolate EAMa 01/58-Su, in particular, has showed to be virulent against *S. littoralis* (Resquin-Romero et al., 2016) and other economically important insect pests (Yousef et al., 2017; 2018).

Combined use of multiple micro- and macro-biological control agents may enhance the effectiveness of any IPM programme. However, to ensure positive outcomes, it is important that the complex interactions between entomopathogens and arthropod natural enemies are fully understood before they are used together in IPM. Although hypocrealean fungi, including some *Metarhizium* species, have broad host ranges and may infect some non-target and/or beneficial insects, such as parasitoids, they are generally considered as organisms that have a low environmental risk (Van Lenteren et al., 2003). Indeed, many recent studies have demonstrated both the safety of these fungi to non-target insects and the potential for their combined use with arthropod natural enemies (Labbe et al., 2009; Rannback et al., 2015; Mohammed and Hatcher, 2017). However, interactions between natural enemies in a multitrophic context are complex and should be evaluated case by case if they are to be exploited effectively for pest control.

The objective of our study was to assess, at laboratory conditions, the compatibility of the entomopathogenic fungus *M. brunneum* and the parasitoid *H. didymator*, when used together to control *S. littoralis*. The main goal was to ascertain whether the joint use of both agents could help controlling the pest *S. littoralis* when the fungal infection occurred before or after parasitization. Furthermore, lethal and sublethal effects of the fungus on parasitoid adults and sublethal effects on F1 generation, the parasitoid capacity to discriminate between healthy and fungus-infected host larvae and effects of parasitism on host haemocyte counts were studied.

II.2. Materials and Methods

II.2.1. *Spodoptera littoralis* and *Hyposoter didymator* rearings

All insect cultures were maintained in a growth chamber at 26 ± 2 °C, 70 ± 5% RH and a photoperiod of 16:8 (L:D) h at the Department of Agricultural and Forestry Sciences of the University of Cordoba, Spain.

A stock colony of *S. littoralis* was established and reared using the method proposed by Poitout and Bues (1974) and modified by Santiago-Alvarez (1977). The

detailed rearing procedure is described in one of our own previous studies (Resquin-Romero et al., 2016).

The *H. didymator* colony was established in 2016 from pupae provided by Dr Anne-Nathalie Volkoff (University of Montpellier, France), and reared following the protocol described by Schneider and Viñuela (2007) with some modifications. Specifically, adult wasps (two males and one female) were placed in 12 × 5 cm methacrylate cages and provided with a solution of 10% honey. A circular hole (3 cm in diameter) covered with a net cloth allowed ventilation of the cage. For oviposition the parasitoids were routinely provided with third-instar (L3) larvae of *S. littoralis*; L3 is the best instar for obtaining high numbers of females and a low encapsulation rate (Schneider, 2002). Larvae of *S. littoralis* were introduced into the cages in groups of ten with small cubes of artificial diet (Santiago-Alvarez, 1977) and oviposition allowed to proceed for 24 h. After this time, the adult parasitoids were removed and the *S. littoralis* larvae transferred individually to cylindrical plastic boxes (4 cm diameter) and provided with the aforementioned diet *ad libitum* until their death due to parasitism and the subsequent emergence of L3 larvae of the parasitoid, which immediately spun cocoons and pupated. *Hyposoter didymator* pupae were incubated in the same conditions until adults emerged. Adults were fed with 10% honey. Emerging adult parasitoids were used in experiments or for rearing after they were sexed (female:male ratio = 1:2).

II.2.2. Inoculum preparation

The *M. brunneum* EAMa 01/58-Su strain was used in all bioassays; this strain was originally isolated from soil in which a wheat crop was growing at Hinojosa del Duque, Cordoba, Spain; it was deposited (accession number CECT 20764) in the Spanish collection of culture types (CECT) located at the University of Valencia, Spain. The fungus was sub-cultured from stored slant cultures onto malt agar in Petri dishes and grown for 12 days at 25 °C in darkness to provide inoculum for experiments. Conidia were scraped from the Petri plates into a sterile solution of 0.1% Tween 80, sonicated (Ultrasons HD 3000865; J.P. Selecta S.A.; Barcelona, Spain) for 5 min and then filtered through several layers of cheesecloth to remove any mycelia. The concentration of the conidial suspension was determined by counting using a haemocytometer (Malassez chamber; Blau Brand, Wertheim, Germany). The viability of the conidia was verified before the preparation of suspensions using germination tests in Sabouraud Dextrose Broth medium (BioCult B. Laboratories, Madrid, Spain). In all the experiments, germination rates were higher than 90%.

II.2.3. Direct (lethal) and indirect (pre-mortality) effects of *M. brunneum* EAMa 01/58-Su on adult *H. didymator*

To quantify lethal effects, a virulence bioassay of EAMa 01/58-Su was done against newly-emerged *H. didymator* adults. Four concentrations of conidia in suspension were prepared in a sterile solution of 0.1% Tween 80 (10^5, 10^6, 10^7 and 10^8 conidia ml^{-1}); the control was 0.1% Tween 80 without conidia. These concentrations were selected based on our previous studies (Resquin-Romero et al., 2016; Yousef et al., 2018). Newly-emerged adult parasitoids were cold-anesthetized and sprayed, in

replicate groups of ten, with conidial suspensions (or 0.1% Tween 80 [control]) in a Potter tower (Burkard Manufacturing Co. Ltd, Rickmansworth, United Kingdom), which deposited 1.54 ± 0.06 mg cm^{-2} at 0.7 bars of pressure. The quantity of conidial suspension used for each replicate was 1 ml and there were five replicates per treatment (n = 50 adult parasitoids per treatment in total). After treatment, replicate groups of parasitoids were placed in methacrylate cages (10 × 10 × 6 cm) with covers; each cage contained a circular hole (4 cm in diameter) covered with a net cloth for ventilation. They were all provided with a liquid diet daily consisting of 10% honey in water and incubated at 26 ± 2 °C, 50–60% RH in a photoperiod of 16:8 (L:D) h. Mortality was monitored daily for 5 days. Dead parasitoids were removed daily, processed as described by Quesada-Moraga et al. (2006), and inspected for fungal outgrowth as an indicator of fungal-induced mortality.

The mean lethal concentration (estimated concentration required to kill 50% of the test insects, LC_{50}) was estimated by Probit analysis (Finney, 1971), after assessing fit and overdispersion with other distributions such as logit, and not getting a better fit compared to Probit analysis. The values of average survival times (ASTs) were obtained by the Kaplan-Meier method and compared using the log-rank test calculated with SPSS 15.0 software for Windows (SPSS Inc., Chicago, IL).

To evaluate potential pre-mortality effects due to the fungus we compared reproductive potential of fungus-treated and untreated (control) female parasitoids in a second bioassay. Newly-emerged adult females were sprayed individually, as described above, with the two highest fungal concentrations among the assayed before, i.e. 10^7 and 10^8 conidia ml^{-1} (or 0.1% Tween 80 [control]). There were eight replicate parasitoids for each treatment and control, and they were incubated as described previously. One day after treatment, second-instar (L2) larvae (ten) of *S. littoralis* were offered to each female parasitoid in each cage for oviposition; this was repeated on the subsequent two days (30 larvae per female offered in total). The number of F1 generation pupal parasitoids that developed on *S. littoralis* larvae was used as an indication of reproductive potential of fungus-treated and untreated female parasitoids in the three days following inoculation.

The percentage of pupal parasitoids was subjected to ANOVA. Data analysis was done using Statistix® 10 (Analytical Software, Tallahassee, USA). Prior to analysis, data were checked for linear model assumptions: homogeneity of variances (Brown and Forsythe test), normality (Shapiro-Wilk test) and independence of residues (graphical test). In order to meet these assumptions, the variable parasitization, expressed as a percentage, was transformed using the arcsine transformation, $Y = arcsine\sqrt{\frac{Percentage}{100}}$. Means from different treatments were compared using a Tukey's test (α=0.05).

II.2.4. Compatibility of *M. brunneum* EAMa 01/58-Su and the endoparasitoid *H. didymator* for control of *S. littoralis*

Two bioassays were done to evaluate interactions between *M. brunneum* EAMa 01/58-Su and *H. didymator* when used together for control of *S. littoralis*.

II.2.4.1. Fungal infection of host larvae before parasitism

In the first bioassay, we evaluated the outcomes of dual infection/ parasitism when infection occurred before parasitism. Specifically, replicate groups of early L2 *S. littoralis* were inoculated by immersion for 60 seconds in a 10^8 conidia ml^{-1} fungal suspension (10 ml). Replicate groups of control larvae were immersed in the same volume of sterile 0.1% Tween 80. Twenty-four, 48 and 72 h after immersion individual treated and control *S. littoralis* larvae were offered to individual newly-emerged mated female parasitoids (females were kept with two males the same day they emerged from cocoon and were monitored for 24 h to ensure mating occurrence; only 48-h mated females were used in all the bioassays) and oviposition allowed to proceed for 24 h. A small cube of artificial diet was introduced into each oviposition cage. The assay included the following treatments: i) three treatments in which *S. littoralis* larvae were inoculated with the fungus and exposed to the parasitoid at different times (24, 48 and 72 h after inoculation); ii) three control treatments in which *S. littoralis* larvae were immersed in 0.1% Tween 80 and exposed to the parasitoid at 24, 48 and 72 h; iii) a control treatment in which *S. littoralis* larvae were inoculated with the fungus; iv) an absolute control treatment in which larvae were immersed in 0.1% Tween 80. There were ten replications of each treatment and control, each replication including a group of ten *S. littoralis* larvae that were inoculated and/or offered to a *H. didymator* female depending on the treatment as described before. After oviposition, the *S. littoralis* larvae were individually transferred to cylindrical plastic boxes (as described previously) and provided with artificial diet *ad libitum* until the emergence of *H. didymator* larvae and pupae. Fungus-induced mortality of *S. littoralis* larvae, the parasitoid reproductive potential and total mortality were all recorded. To determine whether mortality was due to the fungus, dead *S. littoralis* larvae were removed daily and were immediately surface-sterilised with 1% sodium hypochlorite followed by three rinses in sterile distilled water for 1 min each. They were then placed on sterile wet filter paper in sterile Petri plates, sealed with laboratory film, incubated at 25 °C and inspected for fungal outgrowth (Quesada-Moraga et al., 2006). The parasitoid reproductive potential, referred as *H. didymator* complete parasitism, was represented by the number of emerging parasitoid pupae (Hatem et al., 2016). Finally, total mortality was expressed as the sum of the two former variables (larvae with fungal outgrowth and larvae showing a complete parasitism) and the rest of *S. littoralis* larvae which died not evidencing fungal outgrowth nor complete parasitism, i.e. died by unknown reasons. The experiment was repeated twice with fresh fungal suspensions and a new parasitoid generation.

Total mortality (%), Parasitized larvae (%) and Larvae with fungal outgrowth (%) were analyzed using the linear mixed model: $Y = \mu + \text{treatment} + \text{experiment}$, where treatment was modeled as a fixed effect and experiment was modeled as a random effect. In order to improve the normality and homogeneity of variance of the datasets values were transformed using the arcsine transformation. The estimation method was Restricted Maximum Likelihood (REML) with Kenward-Roger's for degrees of freedom. Significance of the fixed effect (Treatment) was evaluated using the F-approximate test ($\alpha = 0.05$) and means from the different treatments were compared with Tukey's test ($\alpha = 0.05$) (Stroup, 2012). Data analyses were performed using JMP 14.2 (SAS Institute Inc., Cary, NC).

Furthermore, the reproductive potential of the F1 generation of parasitoids emerging from the 24 h treatment (fungus+parasitoid) *S. littoralis* larvae was also evaluated. For that, the newly-emerged adults (2 males and 1 female) were mated during 24 h and then offered ten L2 *S. littoralis* larvae as described previously. After 24 h, the parasitoids were removed and *S. littoralis* larvae were individualized and fed routinely. The number of F2 generation pupal parasitoids that developed on *S. littoralis* larvae was used as an indication of reproductive potential. There were six replications as only six females emerged in this treatment. As a control, the same procedure was done with those parasitoids from the 24 h parasitized control treatment (parasitoid alone).

Reproductive potential, expressed as % pupal parasitoids, was analyzed as expressed before: briefly, data were subjected to ANOVA, after using the arcsine transformation. Means from different treatments were compared using a Tukey's test (α=0.05).

II.2.4.2. Parasitism of host larvae before fungal infection

In the second bioassay, we evaluated the outcomes of dual infection/parasitism when parasitism occurred before infection. Specifically, individual early L2 *S. littoralis* larvae were offered to individual mated female parasitoids and oviposition allowed to proceed for 24 h, as described previously. The parasitoids were then removed and the parasitized *S. littoralis* larvae incubated in groups of ten with food. Twenty-four, 48, and 72 h after parasitization replicate groups of parasitized *S. littoralis* larvae were inoculated by immersion for 60 s a 10^8 conidia ml^{-1} fungal suspension (10 ml). Replicate groups of control larvae were immersed in a sterile solution of 0.1% Tween 80. All *S. littoralis* larvae were then incubated individually and routinely fed with artificial diet. Fungus-induced mortality of *S. littoralis* larvae, the parasitoid reproductive potential (represented by number of parasitoid pupae emerging) and total mortality were recorded as described previously. The assay included the following treatments: i) three treatments in which *S. littoralis* larvae were exposed to the parasitoid and inoculated with the fungus at different times (24, 48 and 72 h after parasitization); ii) three control treatments in which *S. littoralis* larvae were inoculated with the fungus at 24, 48 and 72 h; iii) a control treatment in which *S. littoralis* larvae were exposed to the parasitoid; iv) an absolute control treatment in which larvae were immersed in 0.1% Tween 80. There were five replications of each treatment and control, each replication including a group of ten *S. littoralis* larvae that were inoculated and/or offered to a *H. didymator* female depending on the treatment as described before.

Total mortality (%), Parasitized larvae (%) and Larvae with fungal outgrowth (%) were analyzed as the bioassay described before, but in this case the experiment was not replicated in time, therefore the model used was: $Y = \mu$ + treatment. Variables were also arcsine transformed. Means from the different treatments were compared with Tukey's test (α = 0.05).

Data from the dual infection/ parasitism bioassays (II.2.4.1 and II.2.4.2) were analyzed to determine whether there were synergistic, additive or antagonistic interactions between *M. brunneum* EAMa 01/58-Su and the parasitoid *H. didymator*. A χ^2 test was done as described by Hernandez et al. (2012). In this test, the expected

mortality due to the effect of both treatments (M_E) was calculated from the observed mortality with the formula used by Colby (1967): $M_E = M_P + M_F - (M_P \times M_F/100)$, where M_P and M_F represent, respectively, the mortality caused by the parasitoid and the fungus corrected according to Abbott (1925). The χ^2 was calculated using the formula $\chi^2 = (M_O - M_E)^2/M_E$, where M_O is the corrected observed mortality. The obtained values were compared with the χ^2 table values for 1 degree of freedom and $P > 0.05$. If the calculated values were lower than the values of the table, the interaction between treatments was considered additive; otherwise, the interaction could be synergistic or antagonistic depending on the relationship of M_O and M_P with M_E (Resquin-Romero et al., 2016).

II.2.5. Effects of the inoculation of *S. littoralis* on the preimaginal development time of the F1 generation of parasitoids

We assessed the development time of the preimaginal stages of *H. didymator* when the parasitoid developed at the expense of *S. littoralis* larvae inoculated with the EAMa 01/58-Su strain, in order to determine whether the presence of the fungus inside the host could affect the preimaginal development time of the F1 of the parasitoid. We reproduced the same methodology of the 24 h treatments (on the one hand, inoculation with the fungus and exposure to the parasitoid 24 h after inoculation; on the other hand, immersion in 0.1% Tween 80 and exposure to the parasitoid at 24 h as a control) described in II.2.4.1, including five replications instead of ten. Briefly, each replication of 10 larvae was inoculated with the fungus (treatment) or immersed in 0.1% Tween 80 (control) and, after 24 h, was offered to one *H. didymator* female in the same conditions than those described in II.2.4.1. After a 24 h offering time, the parasitoids were removed and *S. littoralis* larvae were individualized and routinely fed as described before. We selected three random parasitized *S. littoralis* larvae from both treatment (fungus and parasitoid) and control (Tween 80 and parasitoid) and monitored the larval and pupal development time. The larval development time was expressed as the time from the parasitization by the *H. didymator* female to the emergence of *H. didymator* L3 larvae from the host cadaver, whereas the pupal development time was the time from pupation (when the parasitoid larvae finished their spinning) to the emergence of *H. didymator* adults from the cocoons.

The larval development time and pupal development time were analyzed separately. Data were subjected to ANOVA. No transformations were needed to fulfill ANOVA's requirements. Means from different treatments were compared using a Tukey's test (α=0.05).

II.2.6. Impact of parasitization by *H. didymator* on the total haemocyte count in *S. littoralis* larvae

In this bioassay, which aim was to ascertain whether a depletion in *S. littoralis* could be caused by *H. didymator*, individual L3 *S. littoralis* larvae were offered to individual mated female parasitoids and oviposition allowed to proceed for 24 h. The parasitoids were then removed and the *S. littoralis* larvae fed with diet and incubated, as described previously. Control larvae were treated in the same way but were not

parasitized. There were three replicates, each of five larvae, for each treatment (parasitized or non-parasitized control) and each sampling day (from day one to five after parasitization). Haemolymph was only extracted from larvae in the treatment group that had actually been parasitized (three of the five larvae in each replication), which were first identified on the basis of their distinctive appearance (small size, reduced movement, paleness) and confirmed by dissection (presence of an egg or larva inside). For the control, three of the five larvae of each replication were selected randomly. Parasitized larvae did not die in the sampling period from days 1 to 5 after treatment.

Before collecting haemolymph, larvae were surface-sterilised with 70% ethanol followed by one rinse in sterile distilled water. Haemolymph was collected by laterally severing the anterior region of each larva with micro scissors and extracting the haemolymph from within using a micropipette. The haemolymph was mixed with an anticoagulant PBS buffer in a 2:1 ratio of PBS: haemolymph (v/v) to avoid haemocyte aggregation. A sample containing haemolymph (3 μl pooled from three *S. littoralis* larvae from each replication) was used to count haemocytes, and this procedure was repeated for each replication and day. Haemocyte counts were performed daily for 5 days after parasitization using a haemocytometer (Malassez chamber; Blau Brand, Wertheim, Germany).

The effect of treatment and time on the number of haemocytes were evaluated using a factorial linear model (ANOVA): $Y = \mu + \text{treatment} + \text{time} + \text{treatment} \times \text{time}$. Data was log transformed, $Y = \log_{10}(\text{nº haemocytes} + 1)$, to meet linear models assumptions.

II.2.7. Can *H. didymator* females distinguish between untreated and fungus-treated *S. littoralis* larvae?

A choice assay was done to evaluate whether female parasitoids showed a preference for fungus-treated or untreated *S. littoralis* larvae when offered both at the same time. Specifically, L2 *S. littoralis* larvae were inoculated (nine groups of five), by immersion (60 seconds) in a 10^8 conidia ml^{-1} suspension (10 ml) of the fungus. Control larvae (nine groups of five larvae) were immersed in a sterile solution of 0.1% Tween 80. The fungus-treated larvae were given a distinctive marking on the thorax using acrylic paint (Nail Polish Yesensy España S.L.; Madrid, Spain). After letting the paint dry, ten *S. littoralis* larvae (five inoculated + five non-inoculated) were offered to a mated female parasitoid for 5 hours (nine replicates in total). The parasitoids were removed and the larvae incubated individually and routinely fed with diet for 10 days. The parasitoid reproductive potential (represented by the number of emerged adults) was evaluated.

Choice test (oviposition preference for uninoculated vs. inoculated larvae of *S. littoralis*) was analyzed using a likelihood-ratio Chi-square test ($p \leq 0.05$) to determine whether the observed frequencies were significantly different to the expected ones under the hypothesis of no treatment effect (50% : 50%) (Zar, 2010). Data analysis was performed using JMP 14.2.

II.3. Results

II.3.1. Direct (lethal) and indirect (pre-mortality) effects of *M. brunneum* EAMa 01/58-Su on adult *H. didymator*

Mortality values of adult parasitoids ranged from 37.5% (10^5 conidia ml^{-1}) to 62.5% (10^8 conidia ml^{-1}). Furthermore, the following fungal outgrowth values were scored: 0% (10^5 conidia ml^{-1}); 6.6% (10^6 conidia ml^{-1}); 23.8% (10^7 conidia ml^{-1}); and 39.6% (10^8 conidia ml^{-1}). Mortality data were subjected to Probit regression analysis (slope = 2.9; $\chi^2 = 1.3$, with 3 df), which gave an LC_{50} value of 1.85×10^6 conidia ml^{-1}. The AST of adult parasitoids treated with the highest conidial concentration (10^8 conidia ml^{-1}), determined by Kaplan-Meier survival analysis, was 92.2 h, equivalent to a 20.7% reduction in AST compared with the control, which was 116.2 h. This difference was significant ($P < 0.05$) according to the log-rank test.

The reproductive potential of female parasitoids over three days was not significantly influenced by the fungal treatment based on the percentages of *S. littoralis* larvae parasitized between one and three days after fungal infection either the first day ($P = 0.6383$), the second ($P = 0.2446$) or the third ($P = 0.4593$) (Table II.1).

Table II.1. Percentage of *S. littoralis* larvae parasitized by *H. didymator* females treated with suspensions of the *M. brunneum* EAMa 01/58-Su conidia

Fungal concentration (conidia ml^{-1})	% Parasitized (mean ± SE)		
	24 h after treatment	48 h after treatment	72 h after treatment
0	55.00 ± 12.96a	65.00 ± 8.24a	75.00 ± 4.63a
10^7	41.25 ± 13.15a	41.25 ± 9.90a	68.75 ± 11.72a
10^8	57.50 ± 12.64a	61.25 ± 12.60a	58.75 ± 9.53a

Means within columns with the same letter are not significantly different from each other ($P < 0.05$) according to the Tukey's HSD test.

II.3.2. Compatibility of *M. brunneum* EAMa 01/58-Su and the endoparasitoid *H. didymator* for control of *S. littoralis*

II.3.2.1. Fungal infection of host larvae before parasitism

In the first bioassay, where *S. littoralis* larvae were inoculated with *M. brunneum* prior to being exposed to the parasitoid, treatments had a significant effect on the total mortality of *S. littoralis* larvae ($P < 0.001$), which ranged from 32.8% (when parasitism occurred 24 h after fungal inoculation) to 77.0% (when parasitism occurred 48 h after fungal inoculation) (Figure II.1). Parasitism of *S. littoralis* by *H. didymator* females was significantly affected by treatment ($P < 0.001$) with mean values of 21.4% (when parasitism occurred 24 h after fungal inoculation) to 64.9% (control: when parasitism occurred 48 h after experiment initiation). Fungal outgrowth from *S. littoralis* cadavers was not significantly affected by treatment ($P = 0.1695$), which ranged from 9.0% (when parasitism occurred 24 h after fungal inoculation) to 23.0% (control: fungal inoculation only). Mortality in the absolute negative control (no fungus + no parasitoid) was of 0% and was excluded from data analysis.

Finally, there was a significant effect of fungal treatment on the reproductive potential of the F1 generation of female parasitoids (P = 0.01); 48.8% of *S. littoralis* larvae were parasitized by F1 female parasitoids that originated from fungus-treated hosts compared with 65.9% by F1 female parasitoids originating from control hosts that had not been treated with fungus.

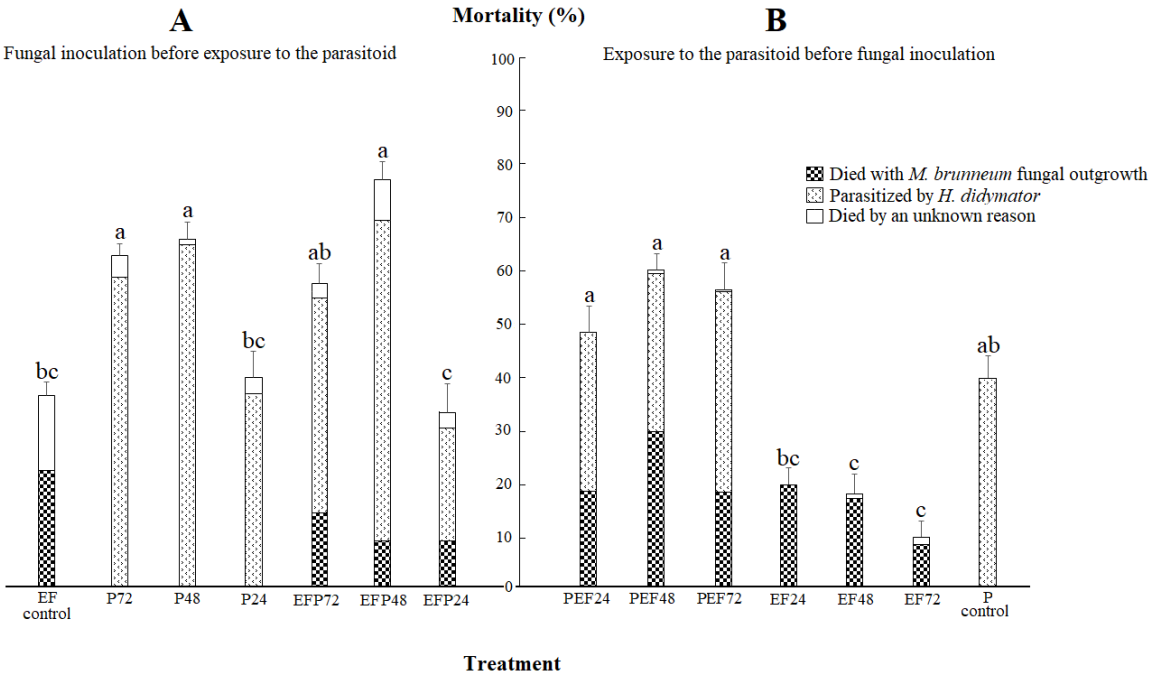

Figure II.1. Total percent mortality of *Spodoptera littoralis* larvae at 24- 48- and 72 h: number with *Metarhizium brunneum* fungal outgrowth + parasitized by *Hyposoter didymator* + died by an unknown reason. Letters show statistical comparisons between treatments within each assay (A or B) (Tukey's test; α=0.05). (A) Fungal inoculation before exposure to the parasitoid. EFP, *S. littoralis* larvae exposed to the parasitoid 24- 48- and 72 h after inoculation with the fungus. P, larvae only exposed to the parasitoid at 24- 48- and 72 h after starting the experiment. EF control, larvae only inoculated with the fungus. (B) Exposure to the parasitoid before fungal inoculation. PEF, *S. littoralis* larvae inoculated with the fungus 24- 48- and 72 h after exposure to the parasitoid. EF, larvae only inoculated with the fungus at 24- 48- and 72 h after starting the experiment. P control, larvae only exposed to the parasitoid. (A), (B) Both assays included an absolute negative control in which *S. littoralis* larvae were treated with aqueous 0.1% Tween 80 solution. The total mortality was of 0% and was not included in the analyses.

II.3.2.2. Parasitism of host larvae before fungal infection

In the second bioassay, where *S. littoralis* larvae were offered to the parasitoid prior to fungal inoculation, the total mortality of *S. littoralis* larvae was significantly influenced by treatment (P < 0.001) with values ranging from 10% (fungus inoculation alone at 72 h) to 60.3% (fungus inoculation 48 h after parasitism) (Figure II.1). Treatment also had a significant effect on the proportion of cadavers with fungal outgrowth (P = 0.0076), ranging from 8.0% (fungus inoculation alone at 72 h) to 30.0% (fungus inoculation 48 h after parasitism). However, there were no significant differences in reproductive potential (P = 0.3886), with parasitism values that ranged from 30.0% (fungus inoculation 24 h after parasitism) to 40.0% (control parasitized larvae). When averaging the results, larvae exposed to parasitoids had an average

mortality caused by the fungus of 22%, while for larvae not exposed to parasitoids, the value was 15.7%. Mortality in the absolute negative control (no fungus + no parasitoid) was of 0% and was excluded from data analysis.

We used a χ^2 test to assess whether there were synergistic, additive or antagonistic interactions between the fungus and the parasitoid. According to this test, the effect of their combined application on *S. littoralis* mortality was additive in both strategies (Table II.2).

Table II.2. Total percent mortality of *Spodoptera littoralis* larvae exposed to the parasitoid *Hyposoter didymator* and/or inoculated with the entomopathogenic fungus *Metarhizium brunneum* EAMa 01/58-Su within two different application strategies: (A) Fungal inoculation before exposure to the parasitoid; (B) Exposure to the parasitoid before fungal inoculation

Strategy	Treatment	Mortality mean (%) ± SE	Expected mortality	χ^2 calculated (gf=1)	χ^2 table value (P>0.05)	Effect on larvae
(A) Fungal inoculation before exposure to the parasitoid	EFP24	32.8 ± 5.5	29.2	0.4	3.8	Additive
	EFP48	77.0 ± 3.4	65.2	2.1	3.8	Additive
	EFP72	57.3 ± 3.7	48.2	1.7	3.8	Additive
	P24	39.5 ± 4.9	-	-	-	-
	P48	65.6 ± 3.2	-	-	-	-
	P72	62.5 ± 2.3	-	-	-	-
	EF control	36.0 ± 4.0	-	-	-	-
	Negative control	0.0 ± 0.0	-	-	-	-
(B) Exposure to the parasitoid before fungal inoculation	PEF24	48.0 ± 4.9	43.0	0.6	3.8	Additive
	PEF48	60.3 ± 3.2	51.2	1.6	3.8	Additive
	PEF72	56.0 ± 5.1	49.8	0.8	3.8	Additive
	EF24	20.0 ± 3.2	-	-	-	-
	EF48	18.0 ± 3.7	-	-	-	-
	EF72	10.0 ± 3.2	-	-	-	-
	P control	40.0 ± 4.5	-	-	-	-
	Negative control	0.0 ± 0.0	-	-	-	-

(A) EFP, *S. littoralis* larvae exposed to the parasitoid 24- 48- and 72 h after inoculation with the fungus. P, larvae only exposed to the parasitoid at 24- 48- 72 h after starting the experiment. EF control, larvae only inoculated with the fungus;
(B) PEF, *S. littoralis* larvae inoculated with the fungus 24- 48- and 72h after exposure to the parasitoid. EF, larvae only inoculated with the fungus at 24- 48- 72h after starting the experiment. P control, larvae only exposed to the parasitoid;
(A)(B) Negative control, larvae treated with aqueous 0.1% Tween 80 solution.

II.3.3. Effects of the inoculation of *S. littoralis* on the preimaginal development time of the F1 generation of parasitoids

The development time of the preimaginal stages of *H. didymator* individuals developed at the expense of both fungus-treated and non-treated *S. littoralis* larvae were scored. The fungal treatment applied to *S. littoralis* larvae had no significant effect on the development time of parasitoid larvae (P = 0.223), which was of 10.1 days (*H. didymator* larvae emerging from fungus-treated *S. littoralis* larvae) and 10.7 days (*H. didymator* larvae emerging from non-treated *S. littoralis* larvae). However, the fungal treatment had a significant effect on the pupal development time of the parasitoid,

causing a slight yet significant reduction (P = 0.01) in the pupal development time of *H. didymator* when *S. littoralis* larvae were inoculated with the fungus (6 day) versus the non-inoculated control (6.73 days).

II.3.4. Impact of parasitization by *H. didymator* on the total haemocyte count in *S. littoralis* larvae

The total haemocyte count (THC) varied significantly both with time after treatment (P = 0.0010) and by treatment (P < 0.0001) and there was a significant interaction between the two (time × treatment) (P = 0.0014). The haemolymph extracted from parasitized *S. littoralis* larvae had significantly fewer haemocytes than control larvae at 72 h (P < 0.0001), 96 (P = 0.0009) and 120 h (P < 0.0001) (Figure II.2).

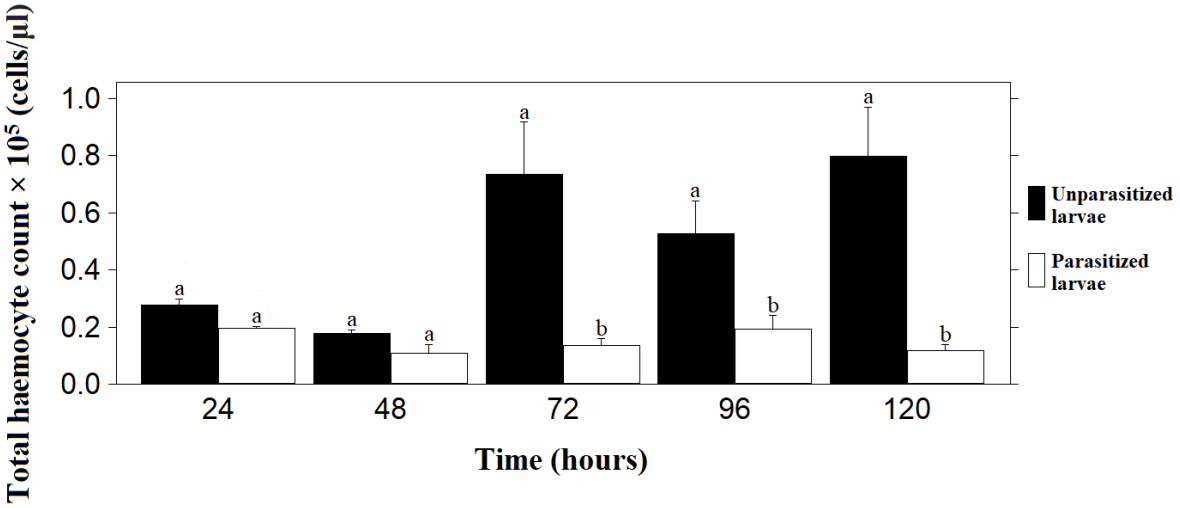

Figure II.2. Daily total haemocyte count in *Spodoptera littoralis* larvae parasitized by *Hyposoter didymator* over time. Letters show statistical comparisons between treatments within each evaluation time (α=0.05).

II.3.5. Can *H. didymator* females distinguish between untreated and fungus-treated *S. littoralis* larvae?

Parasitoids showed a significant preference for *S. littoralis* larvae that had not been inoculated with fungus (χ^2_{1df} = 3.98; P < 0.05). The percentage parasitism, determined from the number of F1 parasitoid pupae emerging was 33.3% from uninoculated *S. littoralis* larvae compared with 13.3% from larvae inoculated with the fungus.

II.4. Discussion

Interactions between entomopathogenic fungi and parasitoids have been reported in many papers with mixed results. Some indicate high compatibility between these two biological control agents with no negative effects of the fungus on the parasitoid

(Shrestha et al., 2018), or even describe the potential to use parasitoids as vectors of entomopathogenic fungi (Kryukov et al., 2018). However, other studies indicated antagonistic interactions between the two agents (Oreste et al., 2015). Despite this, the majority of investigations have shown that combined use of entomopathogenic fungi and parasitoids within IPM programmes is always effective with a suitable adaptation of release times, i.e. which agent is applied first, and the correct timing of applications (Shrestha et al., 2017).

In the present study, we measured direct lethal effects and pre-mortality effects of *M. brunneum* EAMa 01/58-Su on adult *H. didymator*. We also evaluated compatibility between these two agents when used together but released at different times and in different orders. The results showed that the parasitoid was susceptible to infection following direct contact with relatively high concentrations of conidia, with a 20.7% reduction in AST compared with the control. Few studies have addressed both direct lethal effects and pre-mortality effects of entomopathogenic fungi on parasitoids. However, our results confirm those obtained by Castillo et al. (2009) who found that direct application of the fungus *Beauveria bassiana* Bals. (Vuill) (Hypocreales: Clavicipitaceae) (10^8 conidia ml^{-1}) caused a 22% reduction in adult longevity of the eulophid endoparasitoid *Phymastichus coffea* LaSalle (Hymenoptera: Eulophidae). Furthermore, Matias da Silva et al. (2016) showed that adult stages of the braconid endoparasitoid, *Cotesia flavipes* Cam. (Hymenoptera: Braconidae), were susceptible to *B. bassiana* and *M. anisopliae*. Similarly, our previous work with the same strain (EAMa 01/58-Su) and the same concentration (10^8 conidia ml^{-1}) that we used in the present article, showed that the fungus caused a mortality of 21% on adults of the cosmopolitan parasitoid *Psyttalia concolor* Szepliget (Hymenoptera: Braconidae) (Yousef et al., 2018).

On the other hand, we calculated the LC_{50} of *H. didymator* inoculated with the EAMa 01/58-Su strain, which was of 1.85×10^6 conidia ml^{-1}. Although our results are consistent with those obtained for this strain and other parasitoids (Yousef et al., 2018), it is shown that the susceptibility of *H. didymator* to EAMa 01/58-Su is higher than those evidenced by different insect pests (Resquin-Romero et al., 2016; Yousef et al., 2017). This result is of a great importance to develop a suitable strategy for biological control as it allows to compare the susceptibility of the parasitoid with other insects. Moreover, the reproductive potential of *H. didymator* females, over three days, was not affected by direct applications of the fungus on parasitoid adults, even at high conidial concentrations (10^7 and 10^8 conidia ml^{-1}), as no significant differences in *S. littoralis* parasitization were scored during this time for any treatment (Table II.1). As the most productive copulation/egg-laying period for *H. didymator* females and males is 36 h after emergence (Hatem et al., 2016), our results show that fungus-treated *H. didymator* females would have plenty of time to parasitize *S. littoralis* larvae before being killed by the fungus (≈ 4 days after treatment), even if they were inoculated as soon as they emerged. It is worth stressing that direct contact between the fungus and the parasitoid represents the worst-case scenario under field conditions and could be prevented, or at least reduced, if fungus is applied after parasitization. These results are similar to those obtained by other authors, who found that the entomopathogenic fungus *B. bassiana* did not affect the reproductive potential of the parasitoid *Tamarixia triozae* (Burks) (Hymenoptera: Eulophidae) despite reducing their life expectancy (Tamayo-Mejia et al.,

2015). Furthermore, Labbe et al. (2009) reported that the use of commercial isolates of entomopathogenic fungi had no effect on survival rates of the parasitoid, *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae), and even increased parasitism rates. Other authors have reported that prior inoculation with entomopathogenic fungi could affect fitness of the parasitoid wasp *Trybliographa rapae* Westwood (Hymenoptera: Figitidae), reducing its life expectancy but increasing its oviposition rates as an adaptation in response to the presence of the fungus (Rannback et al., 2015).

Our compatibility bioassays, in which we assayed two different strategies (inoculation before and after parasitization) to control *S. littoralis*, demonstrated high compatibility between *M. brunneum* EAMa 01/58-Su and *H. didymator* since the effect of combined use of fungus and parasitoid on total mortality of *S. littoralis* larvae was additive in both scenarios. In the first scenario (fungal inoculation before exposure to the parasitoid), the time that parasitoids were released following fungal inoculation did influence the total mortality of *S. littoralis* larvae; the lowest mortality was obtained when parasitism occurred 24 h after fungal inoculation and highest mortality was obtained when parasitism occurred 48 h after fungal inoculation. Nonetheless, the mortality caused by both the parasitoid (=parasitization) and the fungus (=fungal outgrowth) was slightly higher when applied alone than when combined with the each other, yet the total mortality was higher when applied together. Of interest, the combined use of the two agents has an additive effect in all combinations with parasitoid time releasing-dependent mortality. Many studies have indicated that the time between fungal inoculation and subsequent parasitism is an important factor affecting the likelihood of both agents successfully completing their development within the same host (Furlong and Pell, 2005). Emami et al. (2013) showed that, increasing the release interval for the parasitoid *Aphidius colemani* Viereck (Hymenoptera: Braconidae) after *B. bassiana* application for control of green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) reduced the number of parasitoid pupae developing and the percent emerging as adults. The same result was reported by Mohammed and Hatcher (2017), who found that when *M. persicae* treated with the fungus *Lecanicillium muscarium* Zare & Gams (Hypocreales: Cordycipitaceae) were offered to the parasitoid *A. colemani* they were less likely to be parasitized if they were offered to the parasitoid 3-4 days after fungal infection than if they were offered 1-2 days after fungal infection. In our study, we think that the time between fungal infection and subsequent parasitism was not of great importance since the only day that parasitism was significantly lower (24 h), it was both in the combined treatment including fungus + parasitoid (EFP24) and in the treatment including parasitoid alone (P24). That led us to think that the most crucial factor for the parasitoid, and the reason why a lower parasitization was recorded the first day both in the EFP24 and P24 treatments, was the larval instar of the host. It has been shown that *H. didymator* only parasitizes second (L2) or third (L3) instar *S. littoralis* larvae (Schneider, 2002; Hatem et al., 2016). Earlier and later larval stages are considered as low-quality hosts because they provide few nutrients and have strong immune responses that prevent parasitoid development (King, 2002). In our study, we used early L2 *S. littoralis* larvae, which are suboptimal for *H. didymator* (King, 2002), which may explain why parasitization was higher in all treatments from the second day (48 h) onwards (72 h). On the other hand, the lack of significant differences on the larval death with fungal outgrowth in any treatment (including or not exposure to

parasitoid) suggests that parasitism does not interfere with the fungus. It is worth stressing that there was a certain percentage of mortality due to unknown reasons (neither complete parasitism nor larval mortality with fungal outgrowth) in most treatments (Figure II.1). However, its relative value was very low compared with total mortality except in the treatment including inoculation alone (EF control). As stated before, mortality due to unknown reasons was not scored in the absolute negative control (no fungus + no parasitoid) in any assay or repetition, what indicates that this mortality was caused by the biocontrol agents. In fact, there are two reasons to justify that mortality: in the treatments including only parasitoid, it is likely caused by incomplete parasitism, i.e. parasitization of *S. littoralis* without a complete develop of the parasitoid larva, causing the host premature death; incomplete parasitism causing *S. littoralis* larval death after exposure to *H. didymator* females has been described and may reach high values depending on the rearing method, larval age or instar and other factors (Hatem et al., 2016). On the other hand, we think that the high mortality without fungal outgrowth in the treatment including only inoculation with the fungus is likely due to the production of entomotoxic substances by the fungus. Resquin-Romero et al. (2016) inoculated *S. littoralis* larvae by an immersion in conidial suspensions (10^8 conidia ml^{-1}) of different strains of *M. brunneum* and *B. bassiana*, using the same methodology we presented here. The authors reported high rates of larval death without fungal outgrowth after inoculation with some of the strains, especially with the EAMa 01/58-Su strain, which was due to the toxins produced by the fungus (Resquin-Romero et al., 2016). Similar results were obtained by Yousef et al. (2018) when using the EAMa 01/58-Su strain against *P. concolor*, with fungal outgrowth values depending on the experimental methods.

In the second scenario (exposure to the parasitoid before fungal inoculation), the time between parasitism and subsequent fungal inoculation had no effect on the total overall mortality since no significant differences were scored any day in the same treatments (Figure II.1); nonetheless total mortality in those treatments including parasitoid was significantly higher than that scored in the treatments lacking them (EF24, EF48 and EF72). No significant differences in parasitism were scored for any day or treatment, what shows that further inoculation does not interfere with the development of *H. didymator*. However, significant differences in larvae with fungal outgrowth were scored, with its maximum value when parasitism occurred 48 hours before fungal application (PEF48). Interestingly, *S. littoralis* larval mortality due to the fungus was higher in this combined treatment than when the fungus was applied first (all treatment including fungus and parasitoid in the first scenario: EFP24, EFP48 and EFP72) or alone (EF control in the first scenario and EF24, EF48 and EF72 in the second scenario). Most studies on tritrophic interactions amongst parasitoids, their hosts, and entomopathogenic fungi, have focused on the negative effects of the fungus on parasitoid development within the same host; few studies have considered changes in the host susceptibility to the fungus after parasitoid oviposition (Furlong and Pell, 2005). Labbe et al. (2009) found that, in whiteflies, application of *B. bassiana* after parasitism by *E. formosa* had no effect on either the abundance of the parasitoid or parasitism rates. Furthermore, Mohammed and Hatcher (2017) showed that, in *M. persicae*, application of the fungus, *L. muscarium*, 3 - 7 days after parasitism by *A. colemani* had no effect on the proportion of aphids that were parasitized. It is possible

that the fungus may outcompete immature parasitoids within the host, but there are no reports of the fungus invading parasitoid tissues when they are both attacking the same host (Powell et al., 1986; Furlong and Pell, 2005); however, neither of these studies considered the influence of the parasitoid on host susceptibility to the fungus. King and Bell (1978) have shown that the noctuid moth *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) was more susceptible to the hypocrealean fungus, *Nomuraea rileyi* (Farl.) Kepler, Rehner & Humber (Hypocreales: Clavicipitaceae), if it was already parasitized by the braconid *Microplitis croceipes* (Cresson) (Hymenoptera: Braconidae). Furthermore, Powell et al. (1986) reported that *Metopolophium dirhodum* (Walker) (Hemiptera: Aphididae) aphids that had been parasitized for 2 days were more susceptible to infection by the entomophthoralean fungus *Pandora neoaphidis* Humber (Entomophthorales: Entomophthoraceae) than unparasitized ones; this is similar to our results for inoculation 48h after parasitism (PEF48). We hypothesise that this may occur because parasitism reduces immunity to subsequent infection; we showed that parasitized *S. littoralis* larvae had significantly fewer haemocytes than unparasitized larvae. Since haemocytes are a very important component of the insect immune system, a lower number of haemocytes may lead to a lower immune response (Siddiqui and Al-Khalifa, 2014). Furthermore, THC in control larvae increased over the five-day observation period, particularly between days 3 and 5; this may be because larvae moult from L2 to L3 between days 3 and 5. In general, THC increases with the larval age reaching a maximum in pre-pupae (Stoepler et al., 2013) although THC also tends to increase prior to each moult, decrease at moulting, and then increase again (Siddiqui and Al-Khalifa, 2014). Other studies have reported the same effect of parasitism on THC (Bauer et al., 1998). When comparing both scenarios, we observed that parasitism was the factor which has most contributed to *S. littoralis* total mortality. Since all *S. littoralis* larvae were offered to the parasitoid in early L2 instar in the second scenario, parasitization, and thus total mortality, was lower than in the treatments of the first scenario where larvae were offered from the second day (EFP48, EFP72, P48, P72). This finding is interesting and reinforces our idea that larval age is a crucial factor for achieving a sustainable *S. littoralis* control in the assayed conditions.

Our choice test experiment demonstrated a clear oviposition preference for un-inoculated *S. littoralis* larvae in *H. didymator* females, which is interesting as indicates that *H. didymator* is able to detect the presence of entomopathogenic fungi and tends to avoid them if possible, which had not been described before. However, in our no-choice scenarios (fungal inoculation before exposure to the parasitoid and *vice versa*) we showed that the presence of the fungus EAMa 01/58-Su does not seem to interfere with the further development of *H. didymator*. Furthermore, under a no-choice situation (i.e. presence of only inoculated or uninoculated larvae and a 24-h exposure time) the parasitization may be high regardless of the presence or absence of fungus (Figure II.1), so this natural avoidance of the fungus under a choice scenario (presence of both inoculated and uninoculated larvae and a reduced exposure time) may not be important for *S. littoralis* control if the conditions are appropriate.

Finally, we showed that the long-term consequences of the presence of fungus on the parasitoid were not very serious, resulting in only a slight reduction in parasitoid pupal development time and parasitization capacity of F1 *H. didymator* females. Potrich et al. (2017) also described a reduction in the egg-to-adult period of the parasitoid

Trichogramma pretiosum Riley (Hymenoptera: Trichogrammatidae) when used together with *M. anisopliae* against the Mediterranean flour moth, *Anagasta kuehniella* (Zeller) (Lepidoptera: Pyralidae).

This work contributes to a better understanding of intra-host interactions in *S. littoralis* and may be a first step for defining sustainable IPM strategies for this insect based on the joint use of *H. didymator* and *M. brunneum*. However, more research is necessary in order to assess the efficacy and compatibility of both biocontrol agents; thus, experiments evaluating the performance of both fungus and parasitoid controlling *S. littoralis* in real conditions (i.e. infesting a crop established in a greenhouse or directly in the field releasing both biocontrol agents) would complete the results presented so far.

II.5. Conclusions

The direct contact (worst case scenario) between the fungus and parasitoid adults could be dangerous for the parasitoid at relatively high concentrations of conidia. However, parasitoid reproductive potential was not affected during the the pre-mortality period (three days).

High compatibility between the two biocontrol agents has been demonstrated under different release scenarios; an additive effect was observed in all combinations. The time between fungal inoculation and subsequent parasitism and *vice versa* was not an important factor affecting the total mortality of *S. littoralis* larvae.

When applied together, fungal treatments did not affect the development time of parasitoid larvae. However, fungal treatment did significantly reduce the reproductive potential of the F1 parasitoid generation.

Parasitism reduces immunity of the cotton leafworm larvae to subsequent infection by the fungus when the fungus was applied 48 h after parasitoids release, resulting in improvement of fungal performance.

This work is the first step for better understanding the intra-host interactions between *H. didymator* and *M. brunneum* in *S. littoralis* when jointly used. The results can help improving the IPM strategies on force against this pest, but future studies must be performed to assess the efficacy and compatibility of both biocontrol agents under more realistic conditions before reaching a final conclusion.

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CAPÍTULO III. ENTOMOPATHOGENIC FUNGAL ENDOPHYTE-MEDIATED TRITROPHIC INTERACTIONS BETWEEN *Spodoptera littoralis* AND ITS PARASITOID *Hyposoter didymator*

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ORIGINAL PAPER

Entomopathogenic fungal endophyte-mediated tritrophic interactions between *Spodoptera littoralis* and its parasitoid *Hyposoter didymator*

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Abstract
The use of entomopathogenic fungi for pest control is gaining increasing attention. These fungi act as contact biological insecticides but also via endophytic colonization of targeted crops. In addition, the joint use of entomopathogenic fungi and natural enemies hold potential in many pest control programs. Here, we evaluated in vitro and *in planta* multitrophic interactions among the endophytic fungus *Metarhizium brunneum* colonizing melon (*Cucumis melo*) plant, the parasitoid *Hyposoter didymator* and the pest *Spodoptera littoralis*. In all experiments, total mortality of *S. littoralis* larvae was significantly affected by the presence of the parasitoid; the treatments including the parasitoid achieved the highest mortality rates both in vitro and *in planta*. Simultaneous exposure to the fungus and the parasitoid did not significantly increase the total mortality of *S. littoralis* larvae than the parasitoid alone. The time between exposure to fungus and parasitoid attack did not affect *S. littoralis* mortality, nor parasitism (parasitoid) or infection rates (fungus). However, the parasitoid showed a significant preference for larvae fed on control plants (24.4% parasitism) compared with larvae fed on fungus-colonized plants (4.4%). A histological study of *S. littoralis* larvae simultaneously attacked by *H. didymator* and *M. brunneum* showed, for the first time, the coexistence of both agents within the same host; parasitoid larvae developed inside the host despite fungal colonization. This provides key information about intra-host interactions between two important biological control agents when used together for *S. littoralis* control.

Keywords Integrated pest management · Biological control · Intra-host relationships · *Metarhizium brunneum* · Entomopathogenic fungi · Compatibility

Key Message

- Simultaneous use of biological control agents may provide different efficacy outputs. Thus, it should be addressed in a case by case study basis.
- *Hyposoter didymator* and *Metarhizium brunneum* are promising biocontrol candidates for simultaneous use against *Spodoptera littoralis*.
- The parasitoid alone caused the same mortality of *S. littoralis* larvae that when combined with the fungus.
- Simultaneous invasion by fungus and parasitoid was observed in *S. littoralis* larvae.

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III. Entomopathogenic fungal endophyte-mediated tritrophic interactions between *Spodoptera littoralis* and its parasitoid *Hyposoter didymator*

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III.0. Abstract

The use of entomopathogenic fungi for pest control is gaining increasing attention. These fungi act as contact biological insecticides but also via endophytic colonization of targeted crops. In addition, the joint use of entomopathogenic fungi and natural enemies holds potential in many pest control programmes. Here, we evaluated *in vitro* and *in planta* multitrophic interactions amongst the endophytic fungus *Metarhizium brunneum* colonizing melon (*Cucumis melo*) plant, the parasitoid *Hyposoter didymator*, and the pest *Spodoptera littoralis*. In all experiments, total mortality of *S. littoralis* larvae was significantly affected by the presence of the parasitoid; the treatments including the parasitoid achieved the highest mortality rates both *in vitro* and *in planta*. Simultaneous exposure to the fungus and the parasitoid did not significantly increase the total mortality of *S. littoralis* larvae than the parasitoid alone. The time between exposure to fungus and parasitoid attack did not affect *S. littoralis* mortality, nor parasitism (parasitoid) or infection rates (fungus). However, the parasitoid showed a significant preference for larvae fed on control plants (24.4% parasitism) compared with larvae fed on fungus-colonized plants (4.4%). A histological study of *S. littoralis* larvae simultaneously attacked by *H. didymator* and *M. brunneum* showed, for the first time, the coexistence of both agents within the same host; parasitoid larvae developed inside the host despite fungal colonization. This provides key information about intra-host interactions between two important biological control agents when used together for *S. littoralis* control.

Keywords

Integrated pest management, biological control, intra-host relationships, *Metarhizium brunneum*, entomopathogenic fungi, compatibility

III.1. Introduction

The extensive use of chemical pesticides to control herbivorous pest insects has been a constant over the centuries (Skinner et al., 2014). Whereas the first recorded use of insecticides is about 4500 years ago by Sumerians who used sulphur compounds to control insects and mites, synthetic chemical products were often used from the Second World War onwards (Unsworth, 2010). Nowadays, most field crops are sprayed with insecticides 1 – 5 times during the growing season, whereas some are sprayed up to 20 times (Nansen and Ridsdill-Smith, 2013). Meanwhile, extensive regulation across

nations and continents encourages a reduction in the use of pesticides and promotes the use of environmentally-friendly methods for pest control. Low pesticide-input Integrated Pest Management (IPM) systems are being implemented in the European Union Member States (Directive 2009/128/EC) and the United States (U.S. Code §136r–1), and include biological control as a key tool (Eilenberg et al., 2001; Skinner et al., 2014).

The solitary endoparasitoid *Hyposoter didymator* (Thunberg) (Hymenoptera: Ichneumonidae) has proved to be a good candidate for control of several noctuid pests of great economic importance in horticultural production and pine nurseries (Cabello, 1989; Bahena et al., 1999). These include the cotton leafworm, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae), a polyphagous and widely distributed pest (Lanzoni et al., 2012; Hatem et al., 2016). Not only does *H. didymator* achieve parasitism rates of over 50% in *S. littoralis* populations under controlled conditions (Miranda-Fuentes et al., 2020), but it also interferes with the immune system, reducing the number of haemocytes and making *S. littoralis* larvae more vulnerable to other biological control agents (Miranda-Fuentes et al., 2020).

The use of endophytic entomopathogenic fungi (EF) as microbial biological control agents is also receiving increased interest (Vega, 2018). Whilst the main route of entry of EF is directly through the insect integument, they can also systemically or transiently colonize the plant. Even in the later situation, this can improve the outcome of foliar applications of endophytic EF against chewing and sucking insects feeding on endophytically-colonized plants (Resquin-Romero et al., 2016a; Garrido-Jurado et al., 2017). As a result, the efficacy of several EF in controlling *S. littoralis* has been evaluated when applied against insects either directly as sprays (Resquin-Romero et al., 2016b; Miranda-Fuentes et al., 2020) or as endophytes via inoculation of plant tissues (Resquin-Romero et al., 2016a); mortality depends both on the method of inoculation and the particular fungal strain (Resquin-Romero et al., 2016a; 2016b).

Combined use of EF and arthropod natural enemies may enhance the efficacy of IPM programmes (Miranda-Fuentes et al., 2020). However, interactions amongst biological control agents applied simultaneously are often very diverse, complex and difficult to assess. Most authors consider that treatment with EF is safe for arthropod natural enemies, but only if both the dose and the timing are appropriately controlled (Mesquita and Lacey, 2001; Roy et al., 2008; Aqueel and Leather, 2013; Portilla et al., 2017). Some studies have reported poor compatibility and this is generally due to direct detrimental effects of the fungus on the arthropod natural enemy (Oreste et al., 2015). Therefore, compatibility between biological control agents may be heavily dependent on experimental procedures and the organisms involved, amongst other factors. Despite this, most research on the non-target effects of fungal treatments on arthropod natural enemies, only considers direct application to the natural enemies (Shrestha et al., 2017), or indirect application via exposure of natural enemies to treated prey/hosts (Mesquita and Lacey, 2001). Thus, possible effects on arthropod natural enemies caused by host consumption of EF-colonized plant tissue have been scarcely explored (Vega, 2018). Our recent study showed high compatibility between *H. didymator* and the EF *Metarhizium brunneum* Petch (Hypocreales: Clavicipitaceae) strain EAMa 01/58-Su, to control *S. littoralis* under laboratory conditions in a direct interaction between them

without the host plant (Miranda-Fuentes et al., 2020). However, we only explored direct fungal applications toward parasitoid adults and/or to *S. littoralis* larvae before offering them to parasitoids; hence, the effects of endophytic EF on the parasitoid or the noctuid, as well as the performance of *H. didymator* to control *S. littoralis* under *in planta* conditions, remain unexplored. As a consequence, the relationship between the two biological control agents and *S. littoralis* should be studied in a multitrophic context with the presence of a host plant and considering the endophytic property of the fungal strain used.

Hence, our main goal was to assess compatibility between the parasitoid *H. didymator* and the EF *M. brunneum* EAMa 01/58-Su to control *S. littoralis* both *in vitro* and *in planta* using host plants and different methods of fungal acquisition by *S. littoralis* (consumption of fungal-colonized plant tissue, consumption + contact with external fungal structures on plant leaves and larval inoculation with the EF). A secondary goal was to determine whether the parasitoid would be able to distinguish between *S. littoralis* larvae fed on plants colonized by the EF and larvae fed on uncolonized control plants. Finally, a histological study was also made to better understand intra-host relationships between the fungus and the parasitoid in *S. littoralis* larvae.

III.2. Materials and Methods

III.2.1. Biological materials: Insect cultures, plants and fungal strain

Individuals of *S. littoralis* and *H. didymator* used in these experiments were maintained in a climate chamber under controlled conditions: 26 ± 2 °C, 70 ± 5% RH and a 16:8 h light:dark regime. *Spodoptera littoralis* colony was established according to the rearing method of Poitout and Bues (1974), as modified by Santiago-Alvarez (1977). The parasitoid was reared on third-instar larvae of *S. littoralis* according to the methods of Miranda-Fuentes et al. (2020). Only newly-emerged (48 h since they emerged from cocoons) and mated female parasitoids were used in the experiments. To ensure mating had occurred, newly-emerged females were held with two males for 24 h prior to use in experiments (Miranda-Fuentes et al., 2020).

Melon (*Cucumis melo* L. cv. Galia) plants were used as host plants for *S. littoralis*, as in our previous research (Resquin-Romero et al., 2016a; Garrido-Jurado et al., 2020). Seeds were surface sterilized according to the technique of Garrido-Jurado et al. (2017), and then sown in 0.7 l pots filled with a soil substrate (Floragard, Germany) that had been sterilized twice in an autoclave at 121 °C for 20 min with a 24 h interval between each sterilization (Gonzalez-Mas et al., 2019). Plants were maintained in a growth chamber under the following controlled conditions: 24 ± 2 °C, 70 ± 5% RH and a 16:8 h L:D photoperiod. Irrigation and nutrition were supplied three times per week with a nutrient complex of 20:20:20 (N:P:K) Nutrichem 60 fertilizer (Miller Chemical & Fertilizer Corp., Hanover, PA, USA) according to the manufacturer's instructions. For all experiments, melon seedlings were used when they reached the four-leaf stage.

Metarhizium brunneum strain EAMa 01/58-Su from the culture collection of the Agronomy Department, University of Cordoba (Spain) was used in all experiments.

This strain was originally isolated from the soil beneath a wheat crop at Hinojosa del Duque (Cordoba, Spain) (Yousef et al., 2017). The strain was deposited in the Spanish collection of culture types (CECT), University of Valencia (Spain), under accession number CECT 20764. To prepare inoculum for experiments, the fungus was sub-cultured from stored slant cultures onto malt agar (BioCult Laboratories, Madrid, Spain) in Petri dishes and grown for 12 days at 25 °C in darkness. Conidia were scraped from Petri plates into a sterile solution of 0.1% Tween 80, sonicated (Ultrasons HD 3000865, J.P. Selecta S.A., Barcelona, Spain) for 5 min and then filtered through several layers of sterile gauze. The concentration of the resulting conidial suspension was adjusted to 1×10^8 conidia ml^{-1} using a haemocytometer (Malassez chamber, Blau Brand, Wertheim, Germany). Before use in experiments, conidial germination rate was confirmed as higher than 90% in germination tests on Sabouraud Dextrose Broth medium (Scharlab, S. L., Spain). If germination did not exceed 90% the suspensions were not used in experiments.

III.2.2. Inoculation and endophytic colonization of melon plants with *M. brunneum* EAMa 01/58-Su

Once plants reached the four-leaf stage, the adaxial and abaxial leaf surfaces of the two basal leaves were sprayed with a conidial suspension of strain EAMa 01/58-Su (1×10^8 conidia ml^{-1}; 1 ml per leaf) using an aerograph 27085 (piston compressor of 23 l min^{-1}, 15-50 PSI and 0.3 mm nozzle diameter, Artesania Latina S.A., Madrid, Spain) (Resquin-Romero et al., 2016a; Garrido-Jurado et al., 2017; 2020; Gonzalez-Mas et al., 2019). Uninoculated parts of the plants and the soil were carefully covered with plastic shielding bags and aluminium foil respectively to prevent them from being contaminated by run-off. Control plants were sprayed similarly with a sterile solution of 0.1% Tween 80. After spraying, treated and control plants were covered with transparent plastic shielding bags and maintained in a growth chamber in the conditions described before for 48 h to promote fungal colonization (Garrido-Jurado et al., 2020). To confirm that sprayed plants' phyllosphere had become colonized endophytically by the fungus, we sampled and evaluated unsprayed leaves (Garrido-Jurado et al., 2020); these leaves were removed from each treatment and control replicate in each experiment (Gonzalez-Mas et al., 2019) and surface sterilised by washing for 5 min in 1% sodium hypochlorite followed by two rinses (also 5 min) in sterile deionized water. Surface-sterilised leaves were then air dried under sterile air flow and ten leaf fragments (2 cm^2) excised from each leaf using a sterile scalpel of each plant (in total, 30 fragments per treatment), and plated onto selective culture medium (Sabouraud Dextrose Agar supplemented with 0.5 g l^{-1} chloramphenicol [SDAC] [Scharlab, S. L., Spain]; Gonzalez-Mas et al., 2019). Plates were sealed and incubated in darkness at 25 °C for 7 days. Colonization was expressed as the percentage of fragments from each leaf presenting visible growth of *M. brunneum*, which was observed using a light microscope (Nikon, Tokyo, Japan) and identified based on its morphological features according to Seifert et al. (2011). To confirm that surface sterilisation was effective, 40-µl aliquots from the last rinse water were plated on SDAC and incubated under the same conditions.

III.2.3. Reproductive potential of *H. didymator* on *S. littoralis* larvae fed on melon leaf discs endophytically colonized by *M. brunneum* EAMa 01/58-Su under *in vitro* conditions

Melon leaf discs (8 mm diameter) were cut from: the unsprayed leaves of melon plants treated with the fungus (to ensure plants were endophytically colonized and did not receive external fungal propagules during spraying); or leaves from untreated plants (control), as described previously. *Spodoptera littoralis* L2 larvae were placed individually in methacrylate cylindrical cages 4-cm in diameter provided with covers containing a circular hole (2 cm in diameter) covered with net cloth for ventilation and each allowed to feed on leaf discs for 48 h (one disc per larva every day), according to treatment/control (Resquin-Romero et al., 2016a). Larvae that consumed both discs completely were then allocated randomly to groups of nine (larvae that did not consume both leaf discs were discarded); each group was considered as a replicate. The following treatments were included: i) larvae fed on discs from inoculated plants and exposed to parasitoids; ii) larvae fed on discs from control plants and exposed to parasitoids; iii) larvae fed on discs from inoculated plants, and not exposed to parasitoids; iv) larvae fed on discs from control plants, and not exposed to parasitoids. There were eight replicate groups of nine *S. littoralis* larvae for each treatment and control. In the treatments exposed to parasitoids (i, ii), each replicate group was offered to a newly emerged and mated *H. didymator* adult female in methacrylate cages (17×11×11 cm), each with a 4 cm diameter hole covered with net cloth for ventilation, and was allowed to oviposit for 24 h after which time the parasitoid was removed; each cage was provided with 10% honey for the parasitoids, and a small cube of artificial diet for *S. littoralis*. Groups in unexposed treatments (iii, iv) were maintained in the same way but without parasitoids. Larvae were then placed individually into the aforementioned 4-cm diameter cylindrical cages, fed on artificial diet and monitored for 20 days.

During this time, dead *S. littoralis* larvae were removed daily and inspected for fungal outgrowth as an indication of fungal-induced mortality (as described by Quesada-Moraga et al. [2006]). Specifically, cadavers were surface sterilised by immersion in a 1% solution of sodium hypochlorite for 5 min followed by rinsing twice for 1 min each in sterile deionized water. Surface sterilised larvae were placed on sterile wet filter paper in sterile Petri dishes, sealed with laboratory film, incubated at 25 °C in darkness and inspected daily for fungal outgrowth for 10 to 15 days. We recorded the number of dead larvae with fungal outgrowth, the number of parasitized *S. littoralis* larvae (expressed as the number of parasitoid pupae) and *S. littoralis* total mortality (expressed as the sum of the former variables plus any dead larvae for which death could not be associated with fungus or parasitism). The three variables, i.e. death with fungal outgrowth, parasitism, and total mortality, were expressed as percentages.

III.2.4. Reproductive potential of *H. didymator* on *S. littoralis* larvae fed on melon plants colonized by *M. brunneum* EAMa 01/58-Su under *in planta* conditions

To assess the effect of *M. brunneum* on *H. didymator* under *in planta* conditions, and when applied simultaneously against *S. littoralis*, two bioassays were conducted.

In the first bioassay, we compared parasitism by *H. didymator* on *S. littoralis* larvae fed on *M. brunneum*-colonized melon plants for different periods of time, with parasitism of *S. littoralis* larvae fed on control plants. Melon plants were grown and treated with the fungus as described previously. Control plants were treated in the same way but were not inoculated with the fungus. Eight L2 *S. littoralis* larvae were confined with one single leaf on each plant in *ad hoc* cages set up similar to those described by Gonzalez-Mas et al. (2019). Specifically, the cages (17×11×11 cm) were high-density polyethylene boxes with a ventilation hole sealed with fine-mesh netting and another hole through which the leaves were introduced. Only one leaf per plant was used and it remained attached to the mother plant. The area of cage in contact with the plant was lined with foam rubber to avoid leaf damage. Larvae fed *ad libitum* on the leaf for 24, 48 or 72 h, depending on the treatment/control. A further treatment was also included (positive control): *S. littoralis* L2 larvae were inoculated by immersion for 60 s in 10 ml of a 1×10^8 conidia ml^{-1} suspension of the EAMa 01/58-Su strain as described by Miranda-Fuentes et al. (2020). Once inoculated, these larvae were confined, as described previously, on leaves of unsprayed control plants (eight larvae per leaf) and fed *ad libitum* on the leaf for 24, 48 or 72 h depending on treatment.

The treatments included were: i) larvae fed on sprayed leaves from treated plants (endophytism + contact with external fungal propagules received during plant spraying); ii) larvae fed on unsprayed leaves from treated plants (endophytism only, as these leaves were carefully protected during plant spraying); iii) larvae inoculated by a 60-s immersion in a conidial suspension and fed on control plants (inoculation), and iv) larvae fed on leaves from control plants (control), followed by exposure to a female of *H. didymator* or without exposure to the parasitoid. Three replications of eight larvae each were included for each treatment and day (24, 48 and 72 h).

After feeding on leaves for 24, 48 or 72 h, larvae were removed from the plant, placed in 12×5×5 cm methacrylate cages and offered to a single *H. didymator* mated female and allowed for oviposition for 24 h. Parasitoids were then removed and *S. littoralis* larvae placed individually in the aforementioned 2-cm diameter cylindrical cages, fed on artificial diet and monitored for 20 days. Dead larvae were removed daily, processed as described by Quesada-Moraga et al. (2006) and inspected for fungal outgrowth. As in the previous experiment, we recorded the number of dead larvae with fungal outgrowth, the number of parasitized larvae and the total mortality, expressed as percentages.

The second bioassay simulated a saturation system, in which the *S. littoralis* larvae were confined throughout the experiment, with both the parasitoid and the plant which had been treated with the fungus. Specifically, the same treatments were made (endophytism + contact, endophytism only, inoculation and control, all of them with and without exposure to the parasitoid) in an analogous way. However, the *S. littoralis* larvae remained in the boxes with the *H. didymator* adults and the plant leaves as their only food source until the experiment was evaluated on day 20. Adult parasitoids were routinely provided with 10% honey. Dead *S. littoralis* larvae were removed daily and inspected for fungal outgrowth as described previously. Each treatment and control had three replicates, each with eight *S. littoralis* larvae. We assessed the same three

variables as in the previous experiments (death with fungal outgrowth, parasitism, and total mortality, expressed as percentages).

III.2.5. Host preferences of *H. didymator* offered *S. littoralis* larvae fed on fungus-colonized or uncolonized leaves

A choice assay was done to determine whether female parasitoids could distinguish between *S. littoralis* larvae fed on colonized or control leaves. We used the methodology described by Miranda-Fuentes et al. (2020) with slight modification. Endophytically-colonized and control melon plants were prepared as described previously. Unsprayed leaves from treated plants (i.e. leaves that were endophytically colonized but were protected during spray, thus being free from external fungal propagules), and leaves from control plants were removed and offered to L2 *S. littoralis* larvae. Briefly, single leaves were placed in Petri dishes with ten L2 larvae and a small ball of cotton wool soaked with sterile deionized water to maintain humidity; each dish was considered as a replicate and there were nine replicates for both the treatment and the control. Larvae were allowed to feed *ad libitum* for 48 h during which time they were monitored. Then, five larvae were selected from each replicate of each treatment and control and were marked using acrylic paint (Yesensy España S.L., Madrid, Spain) to distinguish them. Both the treatment and the control were marked using the same paint, but with slightly different shaped marks. Previously, we had thoroughly checked that this methodology did not alter *H. didymator* preference nor parasitism rates in any way (Miranda-Fuentes et al., 2020). Once the marker had dried, groups of ten *S. littoralis* larvae (five from the treatment + five from control) were each offered to a mated female parasitoid (nine replicates in total). The larvae were exposed to *H. didymator* females for a reduced, 5-h, period instead of 24 h to ensure the parasitoids could choose their preferred hosts, as Miranda-Fuentes et al. (2020) showed that, if the exposure time was long enough, females tended to finally parasitize all the available larvae, including those treated with fungus that were avoided in choice conditions. After this the parasitoids were removed and the larvae incubated individually, fed on artificial diet and monitored for 20 days. Parasitoid reproductive potential (represented by the number of *H. didymator* pupae) was determined.

III.2.6. Histological examination

We made a histological study of *S. littoralis* larvae exposed to both parasitoid and EF in order to observe intra-host relationships and characterize host invasion by fungus and parasitoid. Amongst the fungal inoculation methods evaluated (endophytism, endophytism + contact and direct inoculation by immersion), we chose the last one as it enables the main route of entry way of EF, simulates a field splay application and caused the highest mortality with fungal outgrowth from the cadavers in previous experiments, whereas endophytism is mainly related to death by other causes, e.g. metabolite production (Garrido-Jurado et al., 2020). Moreover, this method had been assayed previously when evaluating compatibility between *H. didymator* and *M. brunneum* EAMa 01/58-Su in laboratory conditions (Miranda-Fuentes et al., 2020), and their results in terms of parasitization and mycosis had been thoroughly characterized.

Cohorts of eight *S. littoralis* larvae (L2) were exposed to parasitoid females for 24 h and subsequently inoculated with the fungus by immersion in a suspension of 1×10^8 conidia ml^{-1} *M. brunneum* EAMa 01/58-Su for 1 min as described in the previous section. *Spodoptera littoralis* larvae exposed to parasitoids but not inoculated were included, as well as larvae inoculated but not exposed to parasitoids. Finally, we included a negative control in which larvae were not inoculated nor parasitized. After exposure to parasitoids and/or inoculation, larvae were fed *ad libitum* on artificial diet for 24, 48 or 72 h. After each incubation period, *S. littoralis* larvae were collected and fixed by immersion in 10% neutral buffered formalin for 24 hours. Larvae were dehydrated, embedded in paraffin and 4 µm thick sections cut. Thereafter, samples were stained with both hematoxylin and eosin (HE) and Periodic acid-Schiff stain (PAS) and evaluated for the presence of internal *M. brunneum* conidia or hyphae and/or the parasitoid *H. didymator* under light microscopy.

III.2.7. Data analyses

In the *in vitro* experiment, total mortality, parasitism and death due to fungus (visible outgrowth) data, expressed as percentages, were analysed using a generalized linear mixed model with binomial distribution and logit link function. Significance of the treatment was analysed with F-test and Tukey's multiple comparisons ($\alpha < 0.05$) (SAS Proc GLIMMIX).

In the first *in planta* bioassay, the three aforementioned variables were analysed using a factorial generalized linear mixed model for repeated measures (binomial distribution and logit link function). Treatment with fungus, parasitoid, time, and their second and third level interactions were modelled as fixed effects. Correlation between repeated measures was modelled with the autoregressive covariance matrix. Significance of the fixed effects was analysed with F-test and Tukey's multiple comparisons ($\alpha < 0.05$) (SAS Proc GLIMMIX). In the saturated system, a similar factorial generalized linear model was used, with fungus, parasitoid and their interaction as fixed effects.

Lastly, in the preference assay, the parasitoids' preferences were analysed using a generalized linear model with binomial distribution and logit link function with F-test and Tukey's for multiple comparisons ($\alpha < 0.05$) (SAS Proc GLIMMIX).

III.3. Results

III.3.1. Reproductive potential of *H. didymator* on *S. littoralis* larvae fed on melon leaf discs endophytically colonized by *M. brunneum* EAMa 01/58-Su under *in vitro* conditions

Metarhizium brunneum EAMa 01/58-Su successfully colonized sprayed plants; the average percentage recovery of the fungus from plated leaf fragments was over 90% in all replicates.

There was a significant effect of treatment on total mortality ($P < 0.0001$), with values ranging from 17.4% (when larvae were fed on endophytically-colonized plants

[E]) to 86.2% (when larvae were fed on endophytically-colonized plants and exposed to parasitoids [E, P]). Total mortality in the two treatments that included parasitoids were not significantly different to each other (Figure III.1). Total mortality in the control (larvae fed on control plants) was 0% and excluded from the analysis. There was a significant effect of treatment on parasitism ($P < 0.0001$), with a rate of 32.6% in the treatment including parasitoid + colonized plant (E, P), versus 67.7% in the treatment including the parasitoid alone (P). Mortality as a result of the fungal infection (i.e. with visible outgrowth) had an average value of 10.8% in the treatment combining parasitoid and endophytically-colonized plant (E, P), whereas it was 0% in the treatment with only the endophytically-colonized plant (E) (Figure III.1).

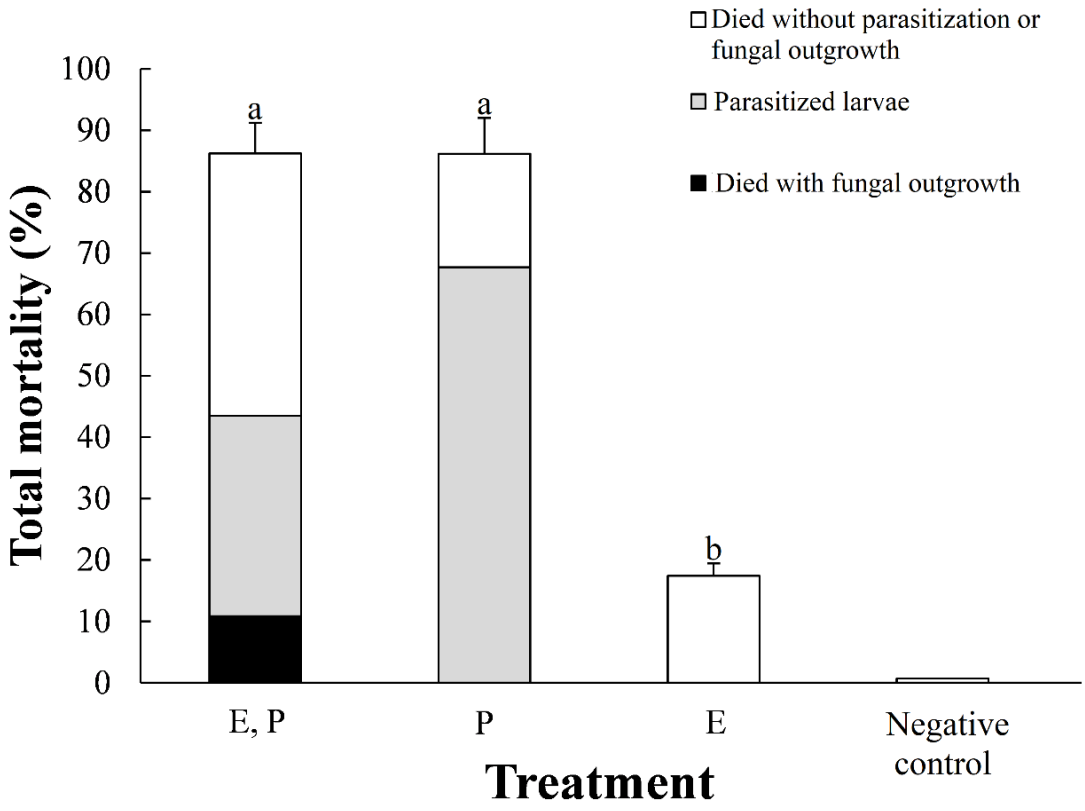

Figure III.1. Total percent mortality of *Spodoptera littoralis* larvae (mean ± standard error) exposed to the parasitoid *H. didymator* and fed on melon leaf discs colonized by *Metarhizium brunneum* EAMa 01/58-Su. E, P = parasitoid plus endophytically-colonized plant; P = parasitoid alone; E = endophytically-colonized plant alone; Negative control = no parasitoid or endophytic colonization. Percentage mortality in parasitized larvae represents complete parasitism by the solitary endoparasitoid *H. didymator* on *S. littoralis* larvae as measured by the number of parasitoid pupae. Letters show statistical comparisons between total mortality in the different treatments; columns with different letters are statistically significantly different from each other (Tukey's test; $\alpha = 0.05$). Total mortality in the control was 0% and not included in the analyses.

III.3.2. Reproductive potential of *H. didymator* on *S. littoralis* larvae fed on melon plants endophytically-colonized by *M. brunneum* EAMa 01/58-Su under *in planta* conditions

The fungus successfully colonized the leaves from inoculated plants; the average percent recovery of the fungus from plated leaf fragments was over 90% in all replicates.

In the first bioassay, in which *S. littoralis* larvae were fed on melon plants colonized by the fungus for different periods times and then offered to the parasitoid, total mortality was significantly affected by the presence of the parasitoid ($P < 0.0001$) (Figure III.2). However, total mortality was not affected by neither treatment with fungus nor time of exposure to fungus. The interactions fungus × time, fungus × parasitoid, parasitoid × time and fungus × parasitoid × time were not significant. Total mortality ranged from 5.6% (when larvae were fed on plants endophytically colonized by the fungus for 24 h [E 24]) to 100% (when larvae were immersed in a conidial suspension and offered to the parasitoid 72 h later [I, P 72]) (Figure III.2). The total mortality in the negative control (larvae fed on control plants) was 0% and was excluded from the analysis. The mean mortality in all treatments including parasitoids was of 73.4 ± 5.1%, in contrast with just 14.2 ± 3.4% in treatments that did not include the parasitoid.

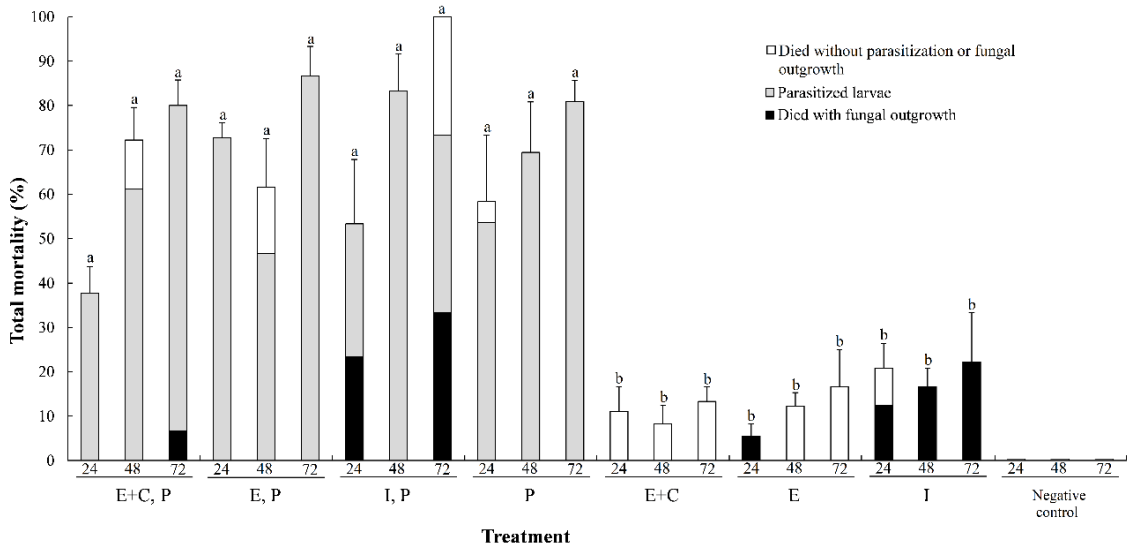

Figure III.2. Total percent mortality of *Spodoptera littoralis* larvae (mean ± standard error) exposed to *Metarhizium brunneum* EAMa 01/58-Su for 24, 48 or 72 h, and then offered to the parasitoid *Hyposoter didymator* in different treatments and combinations. E+C, P = endophytic fungus + conidial contact on the leaf, plus parasitoid; E, P = endophytic fungus plus parasitoid; I, P = immersion in a conidial suspension plus parasitoid; P = parasitoid only; E+C = endophytic fungus + conidial contact on the leaf; E = endophytic fungus only; I = immersion in a conidial suspension only. The numbers below the bars indicate the time of fungal exposure (hours) before larvae were offered to parasitoids. Letters show statistical comparisons between treatments; columns with different letters are significantly different to each other (Tukey's test; $\alpha = 0.05$). Percentage mortality in parasitized larvae represents complete parasitism by the solitary endoparasitoid *H. didymator* on *S. littoralis* larvae scored as the number of parasitoid pupae. An absolute negative control in which *S. littoralis* larvae were fed on control plant leaves was added; total mortality in this control was 0% and it was not included in the analyses.

Parasitism was not significantly affected by fungal treatment (P = 0.4982), nor was the time (P = 0.5079), or the interaction fungus × time significant (P = 0.6261). Parasitism values ranged from 30.0% (when larvae were immersed in a conidial suspension of the fungus and offered to the parasitoid 24 h later [I, P 24]) to 86.7% (when larvae were fed on endophytically-colonized plants for 72 h and then offered to the parasitoid [E, P 72]) (Figure III.2).

Mortality due to fungus (i.e. with visible outgrowth) was not significantly affected by the presence of the parasitoid (P = 0.9933) or the time (P = 0.6365). Fungal recovery values ranged from 5.6% (when larvae were fed on endophytically-colonized plants for 24 h [E 24]) to 33.3% (when larvae were immersed in a conidial suspension and then offered to the parasitoid 72 h later [I, P 72]). Larval death with visible fungal outgrowth did not occur in all treatments, including treatments with the fungus (Figure III.2).

In the second bioassay, a saturated model was simulated and *S. littoralis* larvae were confined with both the parasitoid and the fungus-colonized plant throughout the experiment. As in the first bioassay, total mortality was significantly affected by the presence of the parasitoid (P < 0.0001). However, fungus treatment did not significantly affect total mortality (P = 0.5472) nor was the interaction fungus × parasitoid significant (P = 0.2939). Total mortality ranged from 9.5% (when larvae were fed on endophytically-colonized plants [E]) to 96.3% (when larvae were fed on endophytically-colonized plants and then offered to the parasitoid [E, P]) (Figure III.3). Total mortality in the negative control (larvae fed on control plants) was 0% and excluded from the analysis. The mean mortality in all treatments that included a parasitoid was of 89.2 ± 7.1% compared with just 14.3 ± 3.3% in treatments without the parasitoid.

Parasitism was not significantly affected by treatment (P = 0.2393), with values ranging from 16.2% (when larvae were immersed in a conidial suspension of the fungus and then offered to parasitoid (I, P)] to 59.2% [when larvae were fed on control plants and then offered to the parasitoid [P]) (Figure III.3). The parasitism value in the treatment in which *S. littoralis* larvae were immersed in a conidial suspension of the fungus and then offered to parasitoid (I, P) was lower than the other three values (Figure III.3), although the differences were not statistically significant.

The fungus could only be recovered from those *S. littoralis* larvae that had been immersed in a conidial suspension and fed on control plants, irrespective of whether they were subsequently offered to the parasitoid (Figure III.3). Dead larvae with visible fungal outgrowth were higher when *S. littoralis* larvae were offered to the parasitoid after fungal treatment (I, P, 18.1%) than when they were treated with the fungus alone (I, 8.5%) (Figure III.3). However, the presence of the parasitoid did not significantly affect the proportion of dead larvae with fungal outgrowth (P = 0.4287) despite the values obtained.

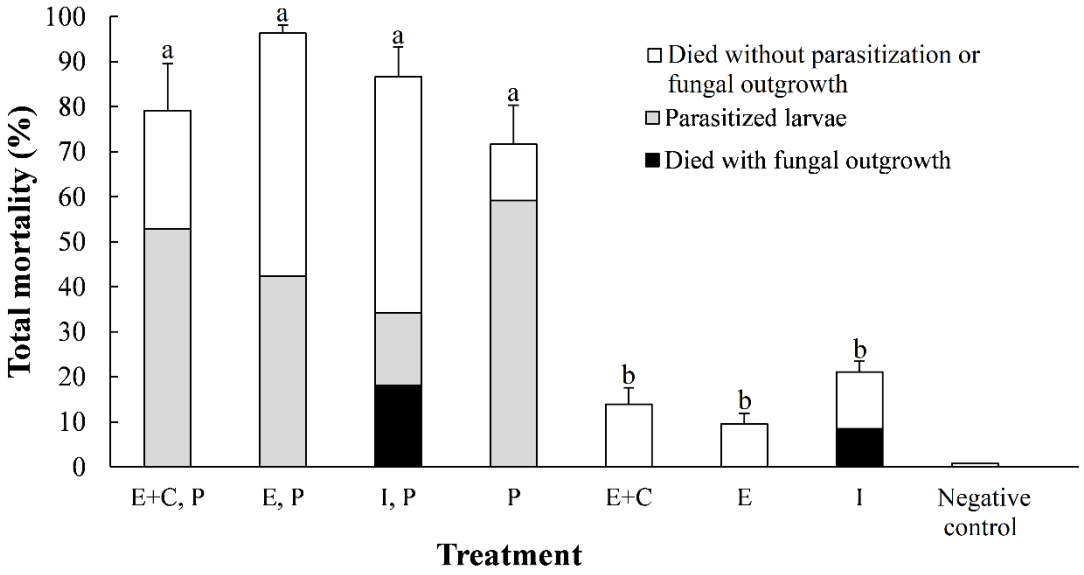

Figure III.3. Total percent mortality of *Spodoptera littoralis* larvae (mean ± standard error) exposed to *Metarhizium brunneum* EAMa 01/58-Su strain and then offered to the parasitoid *Hyposoter didymator* for 20 days in different treatments and combinations. E+C, P = endophytic fungus + conidial contact on the leaf plus parasitoid; E, P = endophytic fungus plus parasitoid; I, P = immersion in a conidial suspension plus parasitoid; P = parasitoid only; E+C = endophytic fungus + conidial contact on the leaf; E = endophytic fungus only; I = immersion in a conidial suspension. Letters show statistical comparisons of total mortality between treatments (Tukey's test; $\alpha = 0.05$). Percentage mortality in parasitized larvae represents complete parasitism by the solitary endoparasitoid *H. didymator* on *S. littoralis* larvae scored as the number of parasitoid pupae. Total mortality was 0% in the negative control (*S. littoralis* larvae were fed on control plant treated with aqueous 0.1% Tween 80 solution only) and was not included in the analyses.

III.3.3. Host preferences of *H. didymator* offered *S. littoralis* larvae fed on fungus-colonized or uncolonized leaves

Adult *H. didymator* females showed a significant preference for *S. littoralis* larvae fed on leaves from control plants compared with larvae fed on endophytically-colonized leaves ($P = 0.0279$). The percentage parasitism (expressed as the number of *H. didymator* pupae) was 4.4 ± 3.1% in larvae fed on endophytically-colonized leaves compared with 24.4 ± 6.4% in larvae fed on control leaves.

III.3.4. Histological examination

Histological examination of *S. littoralis* larvae exposed separately to the parasitoid *H. didymator* or the EF *M. brunneum* is shown in Figure III.4, whereas the joint exposure to both agents is shown in Figure III.5. Finally, the control treatment in which *S. littoralis* was not exposed to any of the agents is shown in Figure III.4A.

Spodoptera littoralis larvae exposed to the parasitoid became parasitized, as indicated by the presence of *H. didymator* eggs with visible embryos (Figure III.4B) and fully developed *H. didymator* larvae (Figure III.4C), depending on the time after parasitism. When inoculated with EF, conidia attached to the cuticle of *S. littoralis* were observed within 24 h of inoculation (Figure III.4D), and fungal structures were present

inside *S. littoralis* within 72 h (Figure III.4E); in both cases they were stained positively by PAS (Figure III.4D, Figure III.4E).

The simultaneous presence of both EF and parasitoids was observed when the two control agents were applied together. Figure III.5A shows a general view of a *S. littoralis* larva that has been actively invaded by both agents; the regions where the presence of each agent are apparent can be seen at higher magnification in Figure III.5B and Figure III.5C. Hyphal bodies of the fungus are present throughout the *S. littoralis* thorax (Figure III.5B), whereas the *H. didymator* larva is present in the *S. littoralis* abdomen (Figure III.5C).

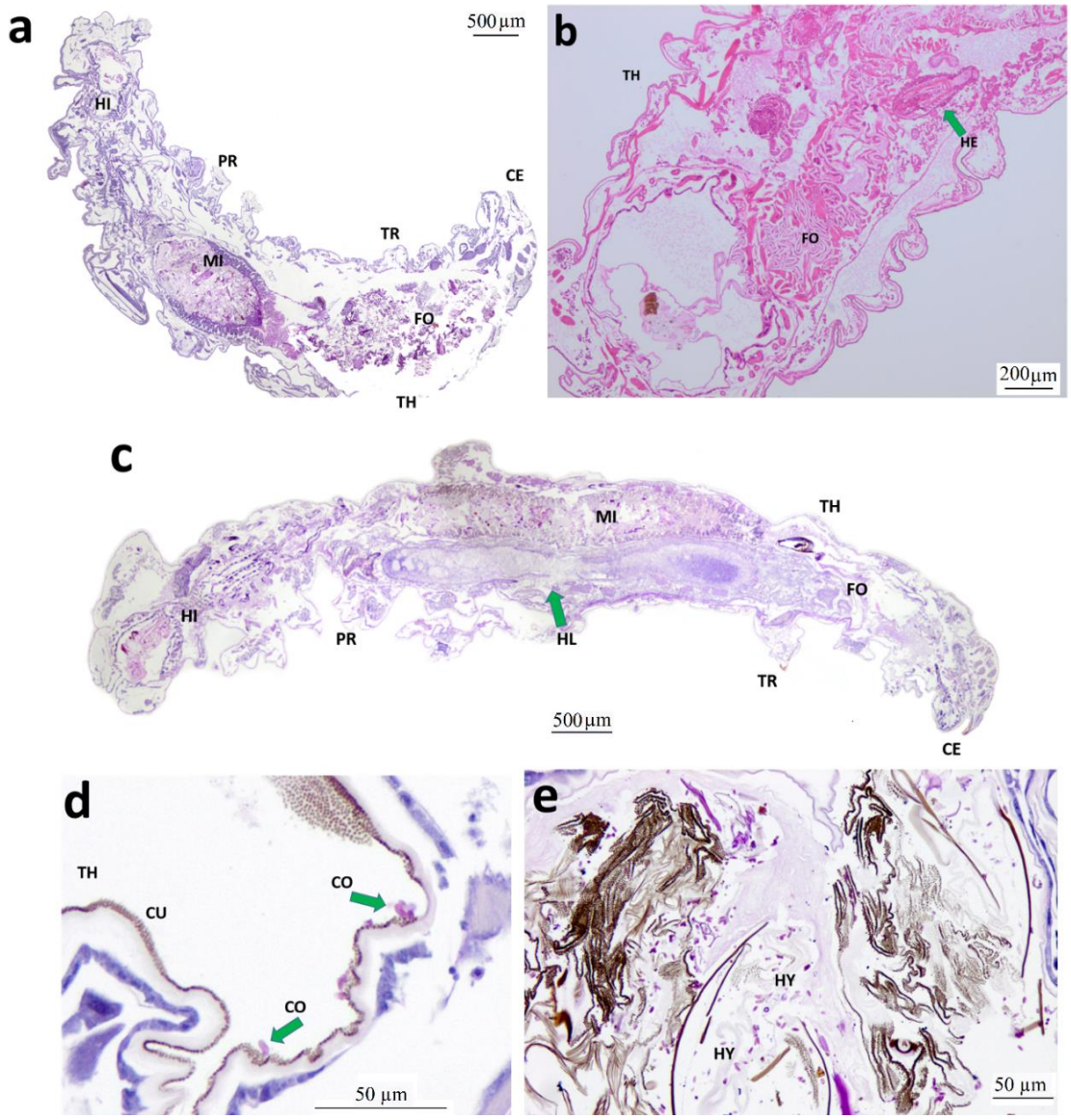

Figure III.4. Longitudinal sections of *Spodoptera littoralis* second instar larvae that have been exposed to either the parasitoid *Hyposoter didymator* (B, C) or treated with the entomopathogenic fungus *Metarhizium brunneum* EAMa 01/58-Su (D, E). A = *S. littoralis* larva from the control treatment. B = *S. littoralis* larva in the early stages of parasitism by *H. didymator*. C = *S. littoralis* larva parasitized by *H. didymator*. D = *S. littoralis* larva treated with the entomopathogenic fungus *M. brunneum* showing conidia attached to the cuticle. E = growth of *M. brunneum* inside the larva. CE = cephalic capsule, TH = thorax, TR = true legs, PR = prolegs, FO = foregut, MI = midgut, HI = hindgut, HE = *H. didymator* egg, HL = *H. didymator* larva, CU = cuticle, CO = conidia, HY = hyphal bodies.

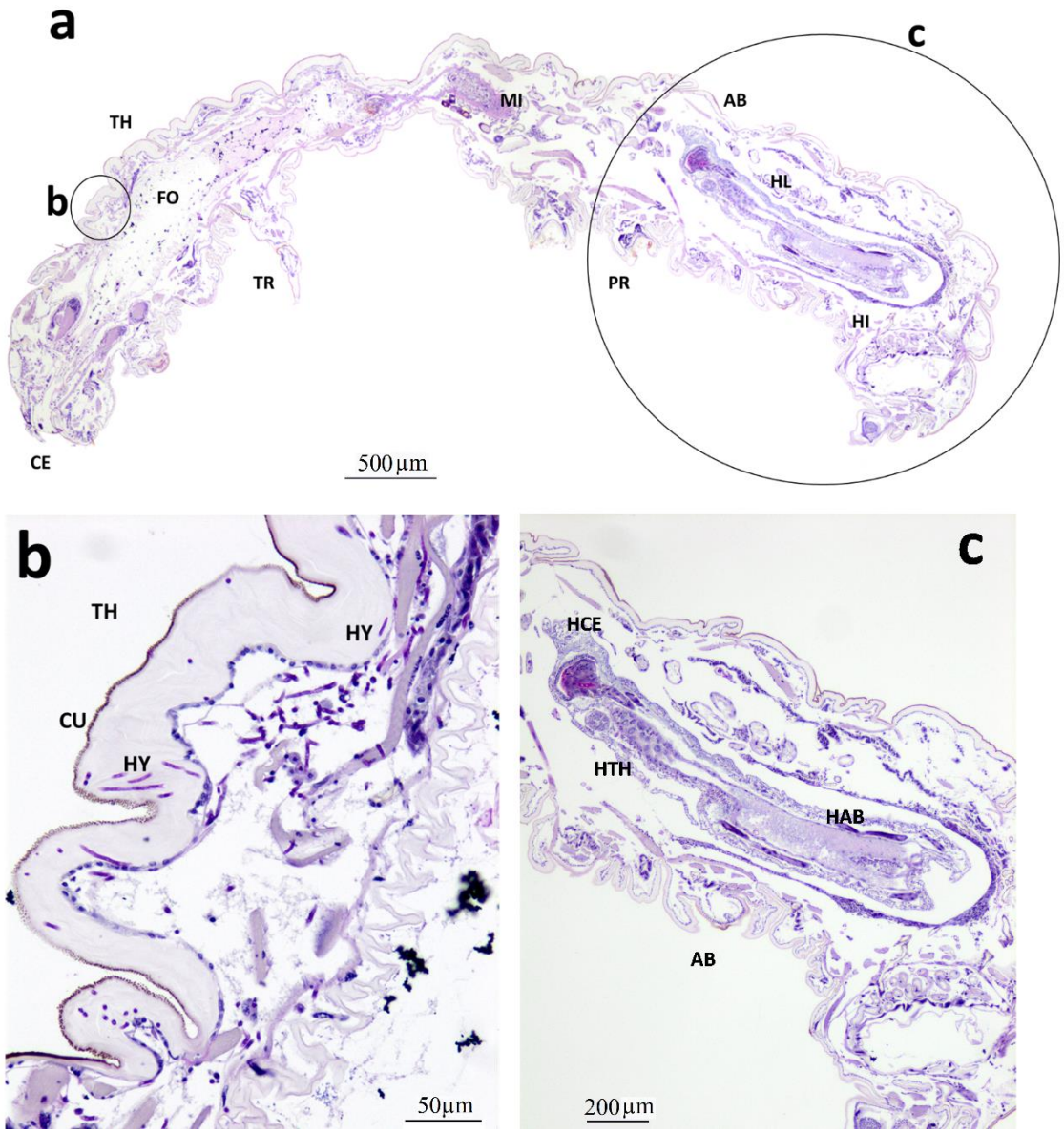

Figure III.5. *Spodoptera littoralis* second instar larva that has been invaded simultaneously by the entomopathogenic fungus *Metarhizium brunneum* and the endoparasitoid *Hyposoter didymator*. A = general view. B = detail of *M. brunneum* hyphal bodies growing inside a *S. littoralis* larva. C = detail of an *H. didymator* larva within a *S. littoralis* larva. CE = cephalic capsule, TH = thorax, AB = abdomen, TR = true legs, PR = prolegs, FO = foregut, MI = midgut, HI = hindgut, HL= *H. didymator* larva, CU = cuticle, HY = hyphal bodies, HCE = *H. didymator* cephalic capsule, HTH = *H. didymator* thorax, HAB = *H. didymator* abdomen.

III.4. Discussion

We explored the potential of *M. brunneum* EAMa 01/58-Su, applied by direct contact and/or as an endophyte, to control *S. littoralis* larvae alone or in combination with the solitary endoparasitoid *H. didymator*. This work, which is a follow-on from Miranda-Fuentes et al. (2020), is the first study involving a multitrophic system with an endophytic EF, *H. didymator* and the cotton leafworm.

Firstly, the fungus showed remarkable endophytic property in melon plant, with colonization of inoculated plants exceeding 90% in all experiments. This is consistent with our previous results, in which we obtained even higher colonization values in other strains (Garrido-Jurado et al., 2017; 2020). Since fungal re-isolation from plant tissues is a standardized and accepted method to assess endophytic property of endophytic EF (Vega, 2018), this confirms the potential of the EAMa 01/58-Su strain to be used as a temporal or transient endophyte since it remains in the leaf for at least 96 h, as was already determined in one of our previous studies (Garrido-Jurado et al., 2017).

In our first experiment (*in vitro*), total mortality of *S. littoralis* larvae in the combined treatment, with both fungus and parasitoid, was not affected by the presence of the fungus (i.e. not different to when the parasitoid was used alone). Similar results were seen by Martinez-Barrera et al. (2020) investigating different strategies for the combined use of the EF *Beauveria bassiana* (Balsam.) Vuill (Hypocreales: Clavicipitaceae) and the parasitoid *Coptera haywardi* (Hymenoptera: Diapriidae) for control of *Anastrepha obliqua* (Macquart) (Diptera: Tephritidae). These authors reported that the total mortality of *A. obliqua* was 88% when exposed to both control agents, which was not significantly different to the mortality achieved in treatments that included the parasitoid alone. In our study, consumption of colonized leaf discs significantly increased the percent mortality without parasitism or fungal outgrowth, whereas it reduced the *H. didymator* parasitism rate. Our previous studies showed similar effects of the fungus on parasitism rates of *S. littoralis* when applied by direct contact (Miranda-Fuentes et al., 2020). Furthermore, while Baverstock et al. (2005) reported enhanced aphid control when the EF *Pandora neoaphidis* (Remaudiere and Hennebert) Humber (Zygomycetes: Entomophthorales) and the parasitoid *Aphidius ervi* (Haliday) (Hymenoptera: Aphidiinae) were combined, the fungus reduced performance of the parasitoid overall. In some treatments, there were remarkable percentages of larval death not caused by complete parasitism nor fungal outgrowth. The possible underlying reasons for those deaths were incomplete parasitism (parasitoid stinging without oviposition or without successful develop of parasitoid larva), which was reported before in low proportions for *H. didymator* (Bahena et al., 1998; Hatem et al., 2016), and fungal endophytic activity, as reported by Garrido-Jurado et al. (2020).

In the first *in planta* experiment, total overall mortality of *S. littoralis* larvae was higher in all the treatments that included parasitoids compared with the treatments with fungus alone. Of interest, neither fungal exposure time nor application method had a significant effect on mortality. Our recent published work demonstrated a similar effect of fungal exposure time on total mortality of *S. littoralis* larvae when simultaneously applied with *H. didymator* (Miranda-Fuentes et al., 2020). In contrast, several authors reported that fungal exposure time was a significant factor affecting performance of the parasitoid *Aphidius colemani* Viereck (Hymenoptera: Braconidae) against *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) when applied together with EF (Emami et al., 2013; Mohammed and Hatcher, 2017).

In this study, fungal pathogenesis evidenced by visible fungal outgrowth occurred in most treatments where the fungus was applied directly by immersion, whereas it was not frequent in treatments where fungus was present as an endophyte. In fact, previous studies showed that insects may be killed by endophytic EF with two

possible outputs of fungal outgrowth: in the first scenario, the insects may die without any fungal outgrowth, especially in chewing insects like is *S. littoralis* (Resquin-Romero et al., 2016a; Garrido-Jurado et al., 2020). The second scenario is the one of piercing-sucking insects in which fungal outgrowth was reported from the cadavers of *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) after being fed with endophytically-colonized plants (Garrido-Jurado et al., 2017). Moreover, Resquin-Romero et al. (2016a) reported that direct inoculation with *M. brunneum* EAMa 01/58-Su was more effective for *S. littoralis* control than consumption of fungus-colonized plant material; the absence of fungal outgrowth in the latter scenario was likely to be as a result of the production of insecticidal substances by the fungus. Furthermore, the most common causes that many authors have pointed as the main causes of larval death without fungal outgrowth are enzymatic activity (e.g. caspases), fungal or plant metabolite production (e.g. destruxins, phenols and saponins), thus causing cell death or apoptosis in the insects fed on colonized plants (Adel et al., 2000; Butt et al., 2013; Resquin-Romero et al., 2016a; Garrido-Jurado et al., 2017; 2020; Zhu et al., 2018; Russo et al., 2019), yet we have to elucidate the mechanisms underlying in our experiments. However, endophytic activity remains a complementary tool that may enhance the efficacy of the EF, which main route of entry is through the integument (Resquin-Romero et al., 2016a; Garrido-Jurado et al., 2017), because of the additional mortality achieved after insects feed on endophytically-colonized plant. It is important to stress that, whereas fungal outgrowth values were greater in all treatments that included the parasitoid compared with the fungus alone, the differences were not significant. Although, fungal outgrowth only occurred in the treatment including parasitoid in our *in vitro* experiment, so we cannot discard any kind of inner interaction between the two biological control agents. This is in agreement with our previous results, in which the parasitoid enhanced fungal performance by depleting insect defences, significantly reducing the number of haemocytes (Miranda-Fuentes et al., 2020).

In our second *in planta* experiment, L2 *S. littoralis* larvae were kept with the parasitoids and fungus-colonized plants as their only food source for 20 days. Nonetheless, higher *S. littoralis* total mortality than in the other experiments was not observed despite longer exposure to both EF and the parasitoid. This is in agreement with our previous *in planta* experiment, in which we observed that time did not significantly affect any of the measured variables. As *H. didymator* only parasitizes early larval instars (L2 to L3) of *S. littoralis* and is unable to parasitize older instars (Hatem et al., 2016), *S. littoralis* larvae were exposed to *H. didymator* females during the whole time lapse in which they were susceptible hosts for this parasitoid. These results confirm that *H. didymator* maximum performance is achieved the first days after parasitoid releasing, in agreement with previous research (Hatem et al., 2016; Miranda-Fuentes et al., 2020). On the other hand, *M. brunneum* EAMa 01/58-Su only presents transient endophytic colonization and is only present endophytically for ≈96 h after treatment, as reported by Garrido-Jurado et al. (2017). Thus, it is very likely that our fungal strain, used as an endophyte and not directly applied on *S. littoralis* larvae, only controls the cotton leafworm during the first days after it is sprayed on plants.

In our choice test, the parasitoid *H. didymator* showed a significant oviposition preference for *S. littoralis* larvae that had not fed on plant tissue colonized by EF.

Whereas parasitization percentages were lower than in previous experiments, it was due to the reduced 5-h exposure time to parasitoids. Moreover, although we cannot discard that the fungus affected the parasitoid performance in parasitized larvae previously fed on colonized leaves, in most of our experiments, fungal treatments did not significantly affect parasitization, and the differences were quantitatively lower, in contrast with the 550% we obtained in our choice experiment (4.4 versus 24.4%). Similarly, Miranda-Fuentes et al. (2020) treated *S. littoralis* larvae by immersion in conidial suspensions of *M. brunneum* EAMa 01/58-Su and also reported a significant preference for untreated larvae. The authors suggested that this preference for untreated hosts was as a result of the parasitoid's ability to detect and avoid the fungus. Similarly, Mesquita and Lacey (2001) reported shorter ovipositor probing of infected aphid hosts by the parasitoid *Aphelinus asychis* Walker (Hymenoptera: Aphelinidae) followed by rejection and absence of oviposition; this was due to strong internal cues. In contrast, Gonzalez-Mas et al. (2019) found that the oviposition preference of the parasitoid *A. colemani* was not affected when offered aphids fed on EF-colonized plants. In the case of combined use of EF and *H. didymator* to control the cotton leafworm in the field, it is not known how this preference outcomes would be like: in a similar scenario, Mesquita and Lacey (2001) stated that parasitoids would avoid potential hosts that were exposed to fungus and search the ones who avoided fungal treatment, what would be positive for long-term parasitoid survival. However, this must be thoroughly studied in the field.

Finally, the histological study allowed a better understanding of the intra-host interactions inside *S. littoralis*, as well as characterize this species invasion by this parasitoid and EF. This is the first observation of *S. littoralis* simultaneously exposed to a parasitoid and an EF, as well as the first histological examination of *H. didymator*. Yu et al. (2008) performed a similar examination of *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) larvae parasitized by the parasitoid *Cotesia vestalis* (Haliday) (Hymenoptera: Braconidae). The *H. didymator* egg we observed was a mature egg with a distinguishable cephalic capsule; its morphology was identical to that of the *C. vestalis* embryo presented by the aforementioned authors. Bahena et al. (1999) also made a microscopical examination of *H. didymator* larvae parasitizing *Mythimna umbrigera* (Saalmuller) (Lepidoptera: Noctuidae); they observed the same ellipsoidal shape and morphology for the *H. didymator* egg. However, their microscopy technique only allowed an external view of the chorion compared with our section in which the embryo within was also clearly visible.

When *S. littoralis* larvae were immersed in conidial suspensions, the conidia attached to *S. littoralis* cuticle immediately after inoculation, and had invaded and spread inside the insect's body within 72 h. Results are similar to those of Garrido-Jurado et al. (2017) for *B. tabaci* nymphs exposed to an endophytic strain of the EF *B. bassiana*, which also showed fungal colonization of the insect despite the fungus being applied as an endophyte and not by direct inoculation. Our results show that both agents can develop inside *S. littoralis* without mutual interference until advanced stages of development, as evidenced by the large size of the parasitoid larva and the spread of hyphal bodies. This is in agreement with Miranda-Fuentes et al. (2020), who reported compatibility between *H. didymator* and the EAMa 01/58-Su strain of *M. brunneum*, with additive effect on the noctuid control and even enhanced fungal performance due to parasitization, as *S. littoralis* larvae previously parasitized and then treated with

fungus showed higher rates of death with fungal outgrowth compared with fungus-treated, unparasitized larvae. According to the results obtained in our other experiments, most *S. littoralis* larvae simultaneously exposed to EF and *H. didymator* were finally killed by the parasitoid larva, which completed its life cycle without further difficulties. This is supported by the high rates of complete parasitism observed in all experiments regardless of whether the fungus treatment was via endophytism or direct contact. Despite this, long-term survival of *H. didymator* populations could be jeopardized by continuous presence of the fungus because *H. didymator* adults emerging from fungal-infected *S. littoralis* larvae are significantly less fertile, as was reported by Miranda-Fuentes et al. (2020) in the same conditions that our histological study was performed. Furthermore, in some combined treatments, parasitism was less (though not significantly so) than when parasitoids were offered control larvae, as showed in our *in planta* experiments. Jaber and Araj (2018) also found that endophytic colonization of plants by EF did not affect parasitism rates of *Aphidius colemani* Viereck (Hymenoptera: Braconidae). Furthermore, Akutse et al. (2014) found that feeding *Liriomyza huidobrensis* (Blanchard) (Diptera: Agromyzidae) larvae on EF-colonized plants did not affect the performance of the parasitoids *Phaedrotoma scabriventris* (Hymenoptera: Braconidae) and *Diglyphus isaea* (Hymenoptera: Eulophidae). As we proved that simultaneous development of fungus and parasitoid inside *S. littoralis* larvae is possible, inner interactions between the two agents may occur, yet future research will elucidate intra-host relationships and their possible outcomes in field applications.

In conclusion, this work is a first step towards understanding fungal-mediated intra-host relationships between *H. didymator* and *S. littoralis*. Importantly, the results from the three different experiments presented here are consistent with each other, and with previous research (Resquin-Romero et al., 2016a; Martinez-Barrera et al., 2020; Miranda-Fuentes et al., 2020). This may be an indicator that similar results would be obtained in other systems. Use of *M. brunneum* EAMa 01/58-Su as an endophyte may have great potential in IPM programmes, as bottom-up effects elicited by endophytic EF are a low risk for natural enemies (Gonzalez-Mas et al., 2019). However, our ongoing research at greenhouse and field level will help to better understand these multitrophic relashionships.

III.5. Acknowledgments

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CAPÍTULO IV. EVIDENCE OF SOIL-LOCATED COMPETITION AS THE CAUSE OF THE REDUCTION OF SUNFLOWER VERTICILLIUM WILT BY ENTOMOPATHOGENIC FUNGI

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ORIGINAL ARTICLE

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Evidence of soil-located competition as the cause of the reduction of sunflower verticillium wilt by entomopathogenic fungi

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Abstract

The increasing limitation of agrochemicals for disease control is a major challenge for European agriculture and a spur to developing environmentally friendly approaches such as biological control. Entomopathogenic fungi, which have been used in the control of insect pests for a long time, also have other uses, such as being antagonists of fungi, including plant pathogens. We determined the in vitro effect of three strains of *Metarhizium brunneum* and two of *Beauveria bassiana* against *Verticillium dahliae* and *Cadophora helianthi*, causal agents of sunflower wilts. Both *M. brunneum* and *B. bassiana* were able to inhibit the mycelial growth of the sunflower pathogens and, according to the dual culture and microscopy results, two types of antagonism were observed as being dependent on the strain: competition and/or antibiosis. Greenhouse experiments showed that, after soil treatments with entomopathogens and plant inoculation by root immersion in conidial suspensions of *V. dahliae*, the entomopathogens were able to efficiently persist in the soil, and three of the four strains even significantly reduced the severity of symptoms in sunflowers. Interestingly, molecular analysis showed that all the strains were able to establish themselves as endophytes in sunflowers in the absence of *V. dahliae*. When the plants were inoculated with *V. dahliae*, we detected the pathogen, but not the entomopathogen, in the sunflowers by molecular methods. The results of this work suggest that the protection conferred by *M. brunneum* and *B. bassiana* against verticillium wilt might not be plant-located, but is probably the consequence of their competition with *V. dahliae* in the soil.

KEYWORDS

biological control, entomopathogens, integrated pest management, plant pathogens, soilborne pathogens, sunflower diseases

1 | Introduction

Sunflower (*Helianthus annuus*) is the fourth largest oilseed crop worldwide. It is grown across all five continents but is particularly relevant in Europe, where over 20% of the world's production is located (FAOSTAT, 2020). Sunflower production is constrained worldwide by diseases. Although yield decreases largely depend on the pathogen, losses of up to 100% are frequently recorded when infections are severe (Gulya *et al.*, 2019). Several sunflower pathogens have been described, but only about a dozen of them are important in economic terms (Gulya *et al.*, 1997).

Verticillium dahliae, the causal agent of verticillium wilt and leaf mottle, is a soilborne ascomycete and a well-known vascular pathogen of several crop species that has been described in

IV. Evidence of soil-located competition as the cause of the reduction of sunflower verticillium wilt by entomopathogenic fungi

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The increasing limitation of agrochemicals for disease control is a major challenge for European agriculture and a spur to developing environmentally friendly approaches such as biological control. Entomopathogenic fungi, which have been used in the control of insect pests for a long time, also have other uses, such as being antagonists of fungi, including plant pathogens. We determined the *in vitro* effect of three strains of *Metarhizium brunneum* and two of *Beauveria bassiana* against *Verticillium dahliae* and *Cadophora helianthi*, causal agents of sunflower wilts. Both *M. brunneum* and *B. bassiana* were able to inhibit the mycelial growth of the sunflower pathogens and, according to the dual culture and microscopy results, two types of antagonism were observed as being dependent on the strain: competition and/or antibiosis. Greenhouse experiments showed that, after soil treatments with entomopathogens and plant inoculation by root immersion in conidial suspensions of *V. dahliae*, the entomopathogens were able to efficiently persist in the soil, and two of the four strains even significantly reduced the severity of symptoms in sunflowers. Interestingly, molecular analysis showed that all the strains were able to establish themselves as endophytes in sunflowers in the absence of *V. dahliae*. When the plants were inoculated with *V. dahliae*, we detected the pathogen, but not the entomopathogen, in the sunflowers by molecular methods. The results of this work suggest that the protection conferred by *M. brunneum* and *B. bassiana* against verticillium wilt might not be plant-located, but is probably the consequence of their competition with *V. dahliae* in the soil.

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IV.1. Introduction

Sunflower (*Helianthus annuus*) is the fourth largest oilseed crop worldwide. It is grown across all five continents but is particularly relevant in Europe, where over 20% of the world's production is located (FAOSTAT, 2020). Sunflower production is constrained worldwide by diseases. Although yield decreases largely depend on the pathogen, losses of up to 100% are frequently recorded when infections are severe (Gulya et al., 2019). Several sunflower pathogens have been described, but only about a dozen of them are important in economic terms (Gulya et al., 1997).

Verticillium dahliae, the causal agent of verticillium wilt and leaf mottle, is a soilborne ascomycete and a well-known vascular pathogen of several crop species that has been described in more than 200 different hosts (Pegg and Brady, 2002). Some of the most important hosts in economic terms are tomato, tobacco, potato, lettuce, cotton, olive tree, eggplant, artichoke, cauliflower, and sunflower. This pathogen is able to persist in the soil via microsclerotia for up to 14 years, affecting the yield of subsequent crops (Pegg and Brady, 2002). Yield losses due to *V. dahliae* are variable and largely depend on the affected crop, so that losses may range from 10% to 100% (Gulya et al., 1997; Klosterman et al., 2009), which makes this fungus one of the most important pathogens in many of its hosts, including sunflower (Martin-Sanz et al., 2018b).

The management of verticillium wilt is highly dependent on the host plant, and none of the available alternatives provide complete control of the pathogen (Pegg and Brady, 2002). Chemical control of verticillium wilt is based on fumigation or soil amendments with chemical (e.g. propamocarb) or organic (proteins, volatile fatty acids) compounds, yet this is only useful in certain crops (e.g. strawberry or pepper) and is limited by legal, economic, and/or environmental constraints (Rekanovic et al., 2007; Klosterman et al., 2009). Biological control, carried out by using fungi (a wide range of genera including *Fusarium* spp., *Phoma* spp., and many others) or bacteria (mainly pseudomonads), has been addressed by many authors (Varo et al., 2016), although none of the proposed methods have so far proven to be effective for disease control in field applications (Klosterman et al., 2009). Genetic resistance to verticillium wilt has been described and is widely used in crops like alfalfa, cotton, potato, tomato, strawberry, sunflower, oilseed rape, and lettuce (Klosterman et al., 2009).

In the case of sunflower, *V. dahliae* is one of the most important pathogens and is widespread in the USA, Argentina, Europe (Pegg and Brady, 2002; Gulya et al., 2019; Molinero-Ruiz, 2019), and some regions of Canada (Erreguerena et al., 2019). The only control measure available in sunflower is genetic resistance (Pegg and Brady, 2002), which was first reported in this crop in Manitoba in 1957 (Putt, 1958) and was found to be race specific and based on single genes (Fick and Zimmer, 1974). Since then, and due to further breeding and genetics programmes, resistance to verticillium wilt has been widely explored in sunflower, and several cultivars with resistance to the main pathogenic races have been obtained or identified, including new resistance sources (Radi and Gulya, 2007). However, the appearance of new races of *V. dahliae* that overcome the known resistance sources in the USA, Argentina, and Spain greatly threatens this disease control method (Gulya, 2007; Garcia-Ruiz et al., 2014).

Despite the undeniable importance of *V. dahliae*, there are recent reports of emerging sunflower diseases that can threaten the crop in some cultivation areas (Martin-Sanz et al., 2018a). *Cadophora helianthi* is of particular importance in Ukraine and Russia, where 26% and 21%, respectively, of the world's sunflower oil is produced (FAOSTAT, 2020). Initially, *C. helianthi* was mistakenly identified as *C. malorum* (Martin-Sanz et al., 2018a), but later it was reported as a new species with special morphological and molecular traits (Crous et al., 2019). Because no reports on *C. helianthi* existed until 2018, no control measures are yet available. Moreover, its geographic distribution and economic importance remain unknown, although it has been

associated with increasing incidence of sunflower wilt in Russia (Martin-Sanz et al., 2018a).

Traditionally, chemical control of plant diseases has relied heavily on synthetic chemical compounds. However, these are not effective against certain diseases including verticillium wilt (Klosterman et al., 2009). Furthermore, chemical treatments can induce the resistance of the pathogen to active ingredients (Molinero-Ruiz et al., 2003; 2008). Biological control is a topical option for effective disease management and, within it, entomopathogenic fungi (EF) are a feasible alternative. The latter are pathogenic to insects, are naturally present in a wide number of environments, and are often associated with plants, via the rhizosphere or even as plant endophytes or epiphytes (Garrido-Jurado et al., 2015). Certain EF have proved to be useful tools for controlling many insect pests within integrated pest management (IPM) strategies (Quesada-Moraga et al., 2014). Thus, more than 170 fungal strains, most of which belong to *Beauveria bassiana* and *Metarhizium anisopliae*, are registered worldwide (de Faria and Wraight, 2007). Their efficacy lies both in the mortality caused by the mechanical damage produced during the propagation of fungal structures through the insect's organs and haemolymph, and in the secretion of insecticidal compounds (Quesada-Moraga et al., 2014).

However, EF can play other ecological roles, such as promoting plant growth (e.g. *Metarhizium brunneum* and *B. bassiana* promoting sorghum and sunflower growth; Raya-Diaz et al., 2017a; 2017b); inducing systemic resistance (e.g. *B. bassiana* eliciting defensive responses in tomato; Shrivastava et al., 2015); producing compounds used in the pharmaceutical industry (e.g. terpenoids, steroids, and phenols; Schulz et al., 2002); enhancing plant nutrition (e.g. *M. brunneum* and *B. bassiana* improving Fe nutrition in sorghum and sunflower; Raya-Diaz et al., 2017a; 2017b); and as antagonists of other fungi (Ownley et al., 2010; Keyser et al., 2016). Recent works have reported some EF as acting as antagonists of plant soilborne pathogens affecting, among others, olive and wheat, both *in vitro* (e.g. *M. brunneum* and *B. bassiana* inhibiting *Phytophthora inundata*; Lozano-Tovar et al., 2013) and *in vivo* (e.g. *Clonostachys rosea* and *Metarhizium* spp. reducing wheat infection by *Fusarium culmorum*; Keyser et al., 2016). The mechanisms underlying the antagonism between EF and other fungi are mainly associated with nutrient competition and antimicrobial metabolite production (Lozano-Tovar et al., 2013; 2017), although the nature of those metabolites has not yet been explored. In sunflower, better vegetative growth and nutrient absorption due to the inoculation with EF was reported (Raya-Diaz et al., 2017a), as well as a successful control of two sunflower insect pests: the maize leaf weevil *Tanymecus dilaticollis* (Coleoptera: Curculionidae; Takov et al., 2013), and the banded sunflower moth *Cochylis hospes* (Lepidoptera: Tortricidae; Barker, 1999). However, EF interactions with sunflower pathogens have not been studied so far.

The aim of this study was to determine whether five strains of two different EF species (three strains of *M. brunneum* and two of *B. bassiana*) could be suitable candidates as biological control agents against the sunflower pathogens *V. dahliae* and *C. helianthi*. Thus, we carried out both *in vitro* experiments, in which the EF were dually plated against both pathogens, and *in planta* ones, in which we monitored the effect of the EF on the severity of verticillium wilt symptoms in sunflowers. We also

assessed the time lapse of EF in the substrate and performed a molecular detection of the fungi in the plants.

IV.2. Materials and Methods

IV.2.1. Fungal isolates

All the isolates of the sunflower pathogens *V. dahliae* and *C. helianthi* and EF *M. brunneum* and *B. bassiana* included in this study are shown in Table IV.1. The five EF strains tested were selected from the culture collection in the Agricultural Entomology laboratory of the Department of Agronomy, University of Cordoba (Spain) and are deposited in the Spanish Collection of Culture Types at the University of Valencia (Spain) (Raya-Diaz et al., 2017a). These strains were selected on the basis of their efficacy against insect pests or plant pathogens (Lozano-Tovar et al., 2013) and their previous use in sunflower (Raya-Diaz et al., 2017a).

All *V. dahliae* isolates used were previously characterized in a multidisciplinary study (Martin-Sanz et al., 2018b) and were selected on the basis of their different geographical origin and pathogenic race (Garcia-Carneros et al., 2014; Martin-Sanz et al., 2018b). *Cadophora helianthi* isolates were selected from the plant pathogen culture collection in the Field Crop Disease Laboratory at the Institute for Sustainable Agriculture from CSIC (IAS-CSIC) in Cordoba, Spain (Molinero-Ruiz, 2019).

IV.2.2. Dual cultures

Dual cultures were carried out to challenge the sunflower pathogens *V. dahliae* and *C. helianthi* against five strains of the EF *B. bassiana* and *M. brunneum* (Table IV.1). Active cultures were obtained on fresh potato dextrose agar (PDA; BD) by streaking a loop followed by incubation at 25 °C in the dark. Pieces (2 mm^2) of actively growing seven-day-old colonies of either *V. dahliae* or *C. helianthi*, and EF, were dually plated on malt extract agar (MA; Scharlab S. L.) at a distance of 3 cm and incubated under the aforementioned conditions. The dual cultures were evaluated until the control treatment colonies reached the edge of the plate, 37 days after plating. The growth of every colony was recorded periodically by directly drawing its contour on the plate. After the last evaluation, photographs of every plate were taken. Pictures were analysed with the free software GIMP v. 2.8.22 (https://www.gimp.org/). The percentage of the mycelial growth inhibition (IMG) was calculated from the last measurement, corresponding to the definitive growth over the plate. The IMG was expressed as:

$$\text{IMG} = 100 \times \frac{\text{Colony diameter in the control} - \text{Colony diameter in the treatment}}{\text{Colony diameter in the control}}$$

Experiments were carried out, independently, for *V. dahliae* and *C. helianthi*, in completely randomized factorial designs with three replications (plates) for *V. dahliae* and four for *C. helianthi*, the EF strains and fungal isolates being the two factors.

Table IV.1. Fungal isolates used in this work, with a list of the experiments in which they were used

Fungal species	Isolate	Host of isolation	Origin	Experiments
Verticillium dahliae	VdS0113	Sunflower	Cadiz (Spain)	Greenhouse experiment
	VdS1014	Sunflower	Valu lui Traian (Romania)	Dual cultures
	VdS0216	Sunflower	Montech (France)	Dual cultures
	VdS0316	Sunflower	Macerata (Italy)	Dual cultures
	VdS0616	Sunflower	Slava Rusa (Romania)	Dual cultures
	VdS0916	Sunflower	Kastamonu, Ahmetbey (Turkey)	Dual cultures
	VdS1016	Sunflower	Manvelivka, Dnepropet (Ukraine)	Dual cultures
Cadophora helianthi	CadoSR02-16	Sunflower	Orenburg (Russia)	Dual cultures
	CadoSR03-16	Sunflower	Orenburg (Russia)	Dual cultures
	CadoSU01-17	Sunflower	Kiev (Ukraine)	Dual cultures
Metarhizium brunneum	EAMa 01/58-Su	Wheat	Cordoba (Spain)	Greenhouse experiment, dual cultures
	EAMb 01/158-Su	Olive	Seville (Spain)	Dual cultures
	EAMb 09/01-Su	Wheat	Seville (Spain)	Greenhouse experiment, dual cultures
Beauveria bassiana	EABb 01/33-Su	Olive	Cadiz (Spain)	Greenhouse experiment, dual cultures
	EABb 04/01-Tip	*Iraella luteipes*	Seville (Spain)	Greenhouse experiment, dual cultures

IV.2.3. Hyphal interaction

Interactions between hyphae of both the sunflower pathogens and the EF were observed under the microscope. For this purpose, a sterile microscope slide was covered with a thin layer of MA and isolates VdS0113 of *V. dahliae* or CadoSU01-17 of *C. helianthi* (Table IV.1), and the EF strains were placed dually at a distance of 3 cm perpendicular to the slide. The slides were incubated at 25 °C in the dark and monitored until the growth front of both fungi was perceived. They were then observed under a microscope.

IV.2.4. Greenhouse experiment

An experiment was conducted to assess the effect of EF on the development of verticillium wilt symptoms in sunflower under greenhouse conditions. According to the results obtained in the dual culture assays, all the EF isolates in Table IV.1, except EAMb 01/158-Su, were included in the experiment. The isolate VdS0113 of *V. dahliae*, whose pathogenicity against sunflower was tested in our previous works (Gonzalez-

Fernandez, 2015; Martin-Sanz et al., 2018b), and the sunflower breeding line RHA801, genetically susceptible to *V. dahliae* (Gonzalez-Fernandez, 2015), were used.

Sunflower seeds were surface-sterilized by a 10-min immersion in 10% sodium hypochlorite, rinsed twice with sterile deionized water and put into Petri dishes containing a thin layer of water-saturated perlite covered with sterile filter paper. Seeds were incubated in darkness at 25 °C for 48 h until radicles 2–5 mm long developed. Then, the seedlings were transplanted into high density polyethylene trays (19.5 × 26.5 × 6.5 cm) containing sterile vermiculite (one treatment per tray). Each tray was watered with 100 ml tap water and then maintained in a greenhouse at 24 °C and a 14 h photoperiod for 48 h until treatments with EF were carried out on plantlets at the VE development stage (Schneiter and Miller, 1981), four days after sowing (DAS).

The EF strains were applied similarly to the method described by Raya-Diaz et al. (2017b). They were plated in MA and incubated in the dark at 25 °C for 10–15 days. Fungal suspensions were prepared by scraping the mycelium with a sowing handle and suspending the fungus in 5 ml sterile deionized water. The suspensions were filtered through several layers of sterile cheesecloth to retain the mycelium, and homogenized by vortex. Concentrations were adjusted to 10^8 conidia ml^{-1} using a Neubauer chamber (Blau Brand). Each treatment (i.e. four plants; each plant a replication) was watered with 100 ml of the corresponding fungal suspension. Control plants were watered with 100 ml sterile deionized water. Plants were grown in the greenhouse under the aforementioned conditions for 1 month. During this time, they were given a nutrient solution (Hoagland and Arnon, 1950), 100 ml per plant, once a week and watered as needed.

Sunflowers were inoculated with *V. dahliae* 1 month after the treatment with the EF, 34 DAS, when plants were at V6 stage (Schneiter and Miller, 1981). Inoculation was carried out according to Martin-Sanz et al. (2018b) by manually uprooting the plants and immersing their root systems in 10^6 conidia ml^{-1} suspensions of *V. dahliae*, prepared as described above, for 30 min. Similarly, control plants were uprooted and immersed in sterile deionized water. After the inoculation, plants were individually transplanted into 0.7 l pots containing a mixture of sand:silt:peat (2:1:4) and incubated in the greenhouse under the aforementioned conditions for 1 month. The experiment was carried out in a completely randomized factorial design with four replications (plants), the two factors being *V. dahliae* (inoculated and noninoculated control) and EF (four strains and nontreated control). The severity of symptoms (Ss), expressed as the percentage of foliar tissue showing symptoms of wilting (Garcia-Ruiz et al., 2014), was assessed weekly for each plant until the end of the experiment, 4 weeks after inoculation with the pathogen and 64 DAS. By then, plants were at R5 (Schneiter and Miller, 1981). The sequential values were used to calculate the area under the disease progress curve (AUDPC) by the trapezoidal integration method (Campbell and Madden, 1990).

The population density of the EF isolates inside the pots was assessed weekly, from the day on which plants were inoculated with *V. dahliae* (34 DAS) until the end of the experiment (64 DAS) in accordance with the protocol described by Raya-Diaz et al. (2017a) with slight modifications: briefly, a sample of 3 g substrate was collected at a depth of 0–3 cm randomly from the four replications (plants) of each EF treatment

noninoculated with *V. dahliae* and suspended in 30 ml sterile deionized water and then shaken with an orbital shaker at 120 rpm for 90 min (Raya-Diaz et al., 2017a). The suspensions were diluted 10-fold in sterile deionized water and 100 µl aliquots were plated on Sabouraud dextrose agar, supplemented with 0.5 g l^{-1} chloramphenicol (SDAC; Scharlab, S. L.). Plates (four replicates for each treatment) were incubated at 25 °C for 5–7 days. Colonies identified as *M. brunneum* or *B. bassiana* were counted and cfu per g of substrate were calculated for each EF strain and replication.

IV.2.5. Microbiological and molecular detection of *V. dahliae* and entomopathogenic fungi in sunflower

The plant colonization of both *V. dahliae* and EF was assessed at the end of the greenhouse experiment, when the fourth and last evaluation of Ss was done, 64 DAS. For *V. dahliae*, we used the methodology described by Martin-Sanz et al. (2018b): briefly, 2-cm long fragments of the stem base of each plant were cut and surface-sterilized by a 10 min immersion in 10% sodium hypochlorite, then rinsed twice and dried under sterile air flow. The fragments were divided into 2–5 mm pieces, which were plated on PDA and incubated at 25 °C for 5–7 days. For the EF, we performed the isolation according to Gonzalez-Mas et al. (2019): leaves were removed, sterilized by a 2 min immersion in 1% sodium hypochlorite, rinsed twice and dried under sterile air flow. Then, fragments of 2 cm^2 were cut, plated on SDAC and incubated at 25 °C for 5–7 days.

For molecular diagnostic analyses, also at the end of the experiment 64 DAS, we surface-sterilized stem tissues as described above and lyophilized them. Total genomic DNA from lyophilized stem tissues of all the plants (noninoculated and nontreated controls, and plants only treated with the entomopathogen, only inoculated with *V. dahliae*, or both treated with the entomopathogen and inoculated with *V. dahliae*) was individually purified using the i-genomic Plant DNA Extraction NucleoSpin Plant II (Macherey-Nagel GmbH and Co. KG) according to the manufacturer's instructions. The quality and concentration of DNA samples were determined with a Qubit 3.0 fluorometer (Invitrogen). Finally, DNA samples were adjusted to a final concentration of 10 ng $µl^{-1}$ and stored at −20 °C until required. The presence of fungi as endophytes in sunflower tissues was confirmed by amplification of the region consisting of the 5.8S ribosomal DNA and internal transcribed spacers 1 and 2 using the primer set ITS5/ITS4 (White et al., 1990). Optimized PCR assays were carried out in a final volume of 25 µl containing 0.4 µM of each primer, 800 µM dNTPs, 2.5 µl 10 × PCR buffer (800 mM Tris-HCl, pH 8.3–8.4 at 25 °C, 0.2% Tween 20 w/v), 0.75 U Horse-Power Taq DNA polymerase (Canvax Biotech), 2.5 mM MgCl2, and 10 ng fungal DNA. The following profile was set for the amplifications: 3 min initial denaturation at 95 °C; 30 cycles of 30 s annealing at 56 °C, 2 min of extension at 72 °C and 30 s denaturation at 95 °C; and a final extension step of 10 min at 72 °C. Mycelial DNA of the fungi grown on PDA and sunflower DNA were used as positive controls, and water was used as a negative amplification control. All reactions were made in a T1 thermocycler (Whatman Biometra). Amplification products were separated by horizontal electrophoresis in 3.5% agarose gels containing 0.05 µl ml^{-1} GoodView nucleic acid stain (SBS Genetech Co.,

Ltd.) and visualized over a UV light source. A 100–2,000 bp BrightMAX DNA ladder (Canvax Biotech) was included in the electrophoresis.

IV.2.6. Data analysis

All the experiments in this work were repeated once and, after assessing the lack of any significant differences between the two replicates (McIntosh, 1983), data were pooled and analysed using Statistix 10 (Analytical Software).

Data expressed as percentages (IMG and Ss) were transformed using an arcsine transformation: $Y = arcsine\sqrt{\frac{Variable}{100}}$. Homoscedasticity (Brown and Forsythe test), normality (Shapiro–Wilk test) and randomization of residues (graphical test) were checked in order to perform an analysis of variance (ANOVA) of transformed IMG (dual culture experiments) and transformed Ss and AUDPC (greenhouse experiment). Dual culture experiments, as well as the greenhouse experiment, were statistically analysed according to completely randomized factorial designs. When significances were found for main factors and/or for their double interaction, means were compared using Fisher's least significant difference (LSD) test ($\alpha = 0.05$).

IV.3. Results

IV.3.1. Dual cultures

When cultured together with the sunflower pathogens, all the EF strains showed an antagonistic activity, as they significantly inhibited the mycelial growth of both *V. dahliae* ($P < 0.001$) and *C. helianthi* ($P < 0.0001$). Furthermore, in dual cultures involving *V. dahliae*, the mycelial growth of the pathogen was significantly dependent on the EF ($P < 0.0001$) and the pathogen isolate ($P < 0.001$), although the interaction EF × pathogen isolate was not significant ($P = 0.1761$). The percentage of IMG of *V. dahliae* ranged from 8.3% (*V. dahliae* VdS0216 co-cultured with EABb 04/01-Tip strain of *B. bassiana*) to 63.5% (*V. dahliae* VdS1016 co-cultured with EABb 01/33-Su strain of *B. bassiana*). Additionally, *M. brunneum* EAMb 01/158-Su presented the lowest IMG for four of the six *V. dahliae* isolates, whereas *B. bassiana* EABb 01/33-Su gave the highest inhibition against four of the six isolates (Table IV.2). Furthermore, EABb 01/33-Su strain caused an average IMG of 47.0% on the six *V. dahliae* isolates, EAMa 01/58-Su of 42.5%, EAMb 09/01-Su of 40.9%, EABb 04/01-Tip of 30.6%, and EAMb 01/158-Su of 24.3%. Lastly, the three *M. brunneum* strains caused an average IMG of 35.7% on the six *V. dahliae* isolates, whereas the two *B. bassiana* strains caused an average IMG of 38.6%.

In the case of *C. helianthi*, the EF significantly affected the pathogen's mycelial growth ($P < 0.0001$), which was not dependent on *C. helianthi* isolate ($P = 0.9808$); nor was the interaction between them significant ($P = 0.3386$). The IMG of *C. helianthi* ranged from 19.6% (EAMb 01/158-Su of *M. brunneum*) to 37.4% (EABb 01/33-Su of *B. bassiana*) (Table IV.3). The three *M. brunneum* strains caused an average IMG of

24.5% on the three *C. helianthi* isolates, whereas the two *B. bassiana* strains caused an average IMG of 34.8%.

Table IV.2. Effect of different strains of the entomopathogenic fungi (EF) *Metarhizium brunneum* and *Beauveria bassiana* on the vegetative growth of *Verticillium dahliae*, expressed as inhibition of the mycelial growth (IMG)

Isolate	EF	EF strain	IMG (%)[a]
VdS1014	*M. brunneum*	EAMa 01/58-Su	53.8 ± 7.0 a
		EAMb 01/158-Su	27.0 ± 9.2 b
		EAMb 09/01-Su	53.9 ± 6.7 a
	B. bassiana	EABb 01/33-Su	45.6 ± 6.8 ab
		EABb 04/01-Tip	41.8 ± 9.4 ab
VdS0216	*M. brunneum*	EAMa 01/58-Su	37.8 ± 10.1 a
		EAMb 01/158-Su	18.7 ± 5.9 ab
		EAMb 09/01-Su	23.9 ± 5.2 ab
	B. bassiana	EABb 01/33-Su	39.5 ± 10.2 a
		EABb 04/01-Tip	8.3 ± 4.3 b
VdS0316	*M. brunneum*	EAMa 01/58-Su	38.7 ± 1.9 ab
		EAMb 01/158-Su	23.4 ± 3.3 b
		EAMb 09/01-Su	38.7 ± 7.2 ab
	B. bassiana	EABb 01/33-Su	41.1 ± 5.7 a
		EABb 04/01-Tip	29.9 ± 5.9 ab
VdS0616	*M. brunneum*	EAMa 01/58-Su	44.0 ± 6.4 a
		EAMb 01/158-Su	28.8 ± 6.7 ab
		EAMb 09/01-Su	43.9 ± 6.1 a
	B. bassiana	EABb 01/33-Su	42.9 ± 11.4 ab
		EABb 04/01-Tip	22.0 ± 5.8 b
VdS0916	*M. brunneum*	EAMa 01/58-Su	33.8 ± 6.6 b
		EAMb 01/158-Su	24.3 ± 5.4 b
		EAMb 09/01-Su	46.1 ± 12.3 ab
	B. bassiana	EABb 01/33-Su	57.2 ± 6.5 a
		EABb 04/01-Tip	55.6 ± 3.5 a
VdS1016	*M. brunneum*	EAMa 01/58-Su	47.5 ± 5.9 ab
		EAMb 01/158-Su	23.4 ± 6.9 c
		EAMb 09/01-Su	34.8 ± 5.6 bc
	B. bassiana	EABb 01/33-Su	63.5 ± 8.2 a
		EABb 04/01-Tip	36.4 ± 9.7 bc

Note: Plates were incubated at 25 °C in the dark for 37 days, the time needed for the control of each *V. dahliae* isolate to reach the edge of the plate. For each *V. dahliae* isolate, means with a common letter are not significantly different according to Fisher's Least Significant Difference test. Analyses were performed on transformed data using an acrsine transformation.

[a] Mean ± SE of three replications of IMG expressed as percentages.

Finally, inhibition halos were observed when *M. brunneum* strain EAMb 01/158-Su was co-cultured with any of the pathogens. In addition, the EF strains EAMb 09/01-Su and EABb 01/33-Su were able to overgrow the mycelium of the pathogens. The EF strain EAMa 01/58-Su was able to facultatively cause inhibition halos and overgrow the pathogens. Types of antagonism are shown in Figure IV.1: inhibition of *V.*

dahliae and/or *C. helianthi* by *M. brunneum* (Figures IV.1A and IV.1C) and overgrowth of *B. bassiana* on *V. dahliae* and/or *C. helianthi* (Figures IV.1B and IV.1D).

Table IV.3. Effect of different strains of the entomopathogenic fungi (EF) *Metarhizium brunneum* and *Beauveria bassiana* on the vegetative growth of three isolates of *Cadophora helianthi*, expressed as inhibition of the mycelial growth (IMG)

EF	EF strain	IMG (%)[a]
M. brunneum	EAMa 01/58-Su	30.4 ± 3.2 ab
	EAMb 01/158-Su	19.6 ± 2.9 c
	EAMb 09/01-Su	23.5 ± 3.0 bc
B. bassiana	EABb 01/33-Su	37.4 ± 3.1 a
	EABb 04/01-Tip	32.2 ± 2.7 a

Note: Plates were incubated at 25 °C in the dark for 37 days, the time needed for the control of each *C. helianthi* isolate to reach the edge of the plate. Means with a common letter are not significantly different according to Fisher's Least Significant Difference test. Analyses were performed on transformed data using an acrsine transformation.

[a] Mean ± SE of four replications of IMG expressed as percentages.

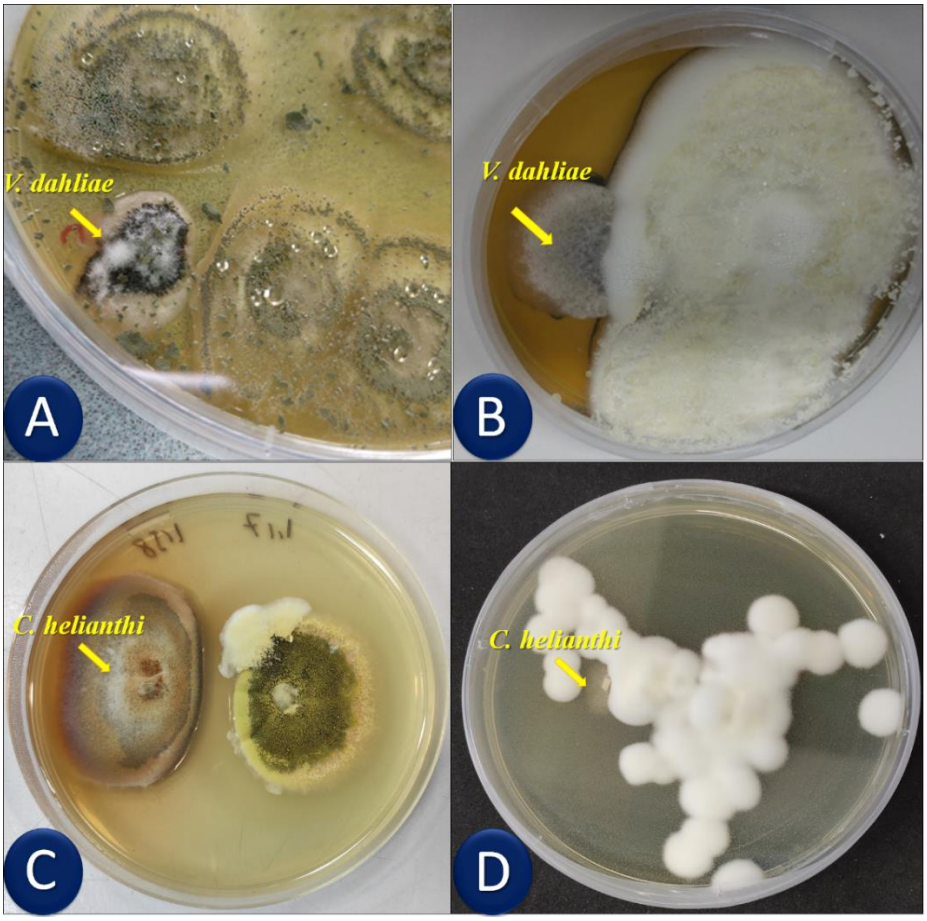

Figure IV.1. Antagonism exerted by entomopathogenic fungi *Metarhizium brunneum* and *Beauveria bassiana* on sunflower pathogens *Verticillium dahliae* and *Cadophora helianthi* in dual cultures. (A) Inhibition on *V. dahliae* VdS0216 by *M. brunneum* EAMa 01/58-Su; (B) overgrowth of *B. bassiana* EABb 01/33-Su on *V. dahliae* VdS0916; (C) inhibition on *C. helianthi* CadoSU01-17 by *M. brunneum* EAMb 01/158-Su; (D) overgrowth of *B. bassiana* EABb 01/33-Su on *C. helianthi* CadoSR02-16.

IV.3.2. Hyphal interaction

When slides of dual cultures were cut from the culture medium and observed under the microscope, with the exception of the lack of contact between EAMb 01/158-Su strain and either of the pathogens, contact between hyphae of *V. dahliae* or hyphae of *C. helianthi* and all the other EF strains was observed (Figure IV.S1). No antagonistic interactions were observed.

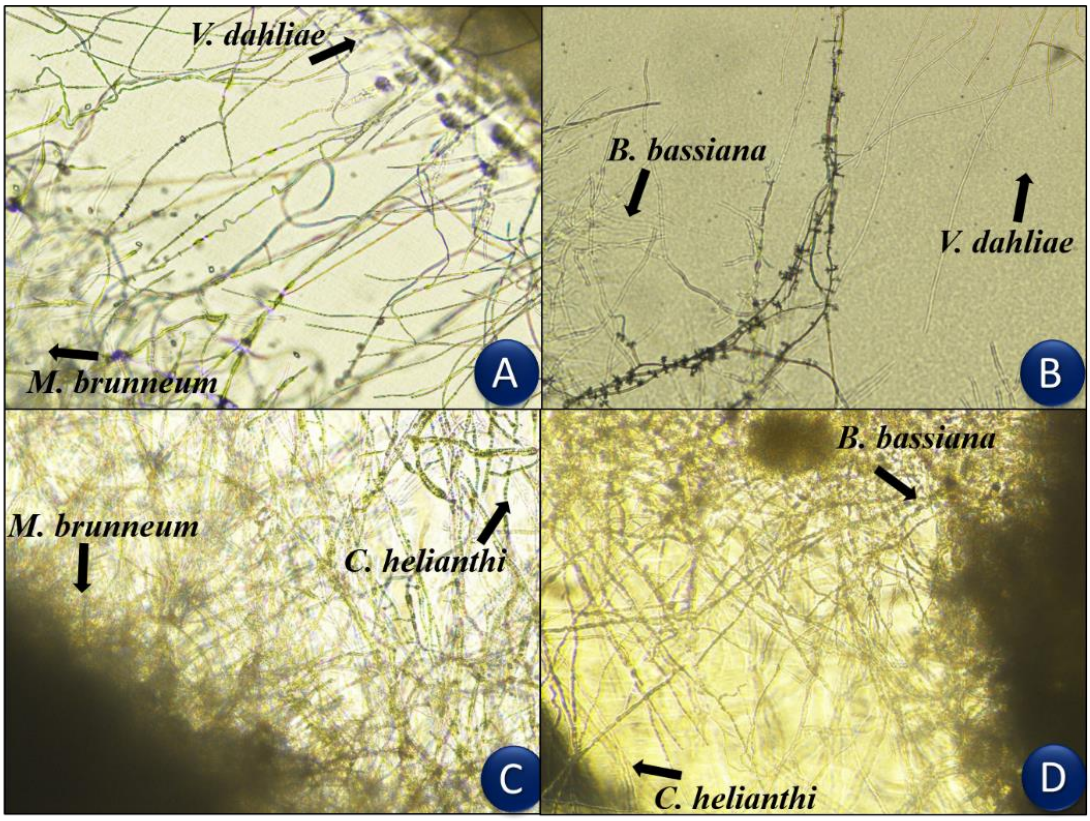

Figure IV.S1. Hyphal contact between an entomopathogenic fungus (*Metarhizium brunneum* or *Beauveria bassiana*) and *Verticillium dahliae* or *Cadophora helianthi* in dual cultures. (A) *M. brunneum* versus *V. dahliae*; (B) *B. bassiana* versus *V. dahliae*; (C) *M. brunneum* versus *C. helianthi*; (D) *B. bassiana* versus *C. helianthi*.

IV.3.3. Greenhouse experiment

Control plants not inoculated with *V. dahliae* did not show any symptoms of verticillium wilt and were excluded from data analysis. Initial symptoms of verticillium wilt were observed in the control plants inoculated with the pathogen 1 week after inoculation, and they reached 95% Ss at the end of the experiment (Figure IV.2A). Significant reductions in both Ss and AUDPC due to *V. dahliae* were associated with treatments with one strain of each EF species: *M. brunneum* EAMb 09/01-Su (53% and 1096, respectively) and *B. bassiana* EABb 01/33-Su (31% and 475, respectively) compared with the control plants (95% and 2226, respectively). The two remaining EF strains did not have a significant effect on verticillium wilt (either disease severity or AUDPC) (Figure IV.2A,B).

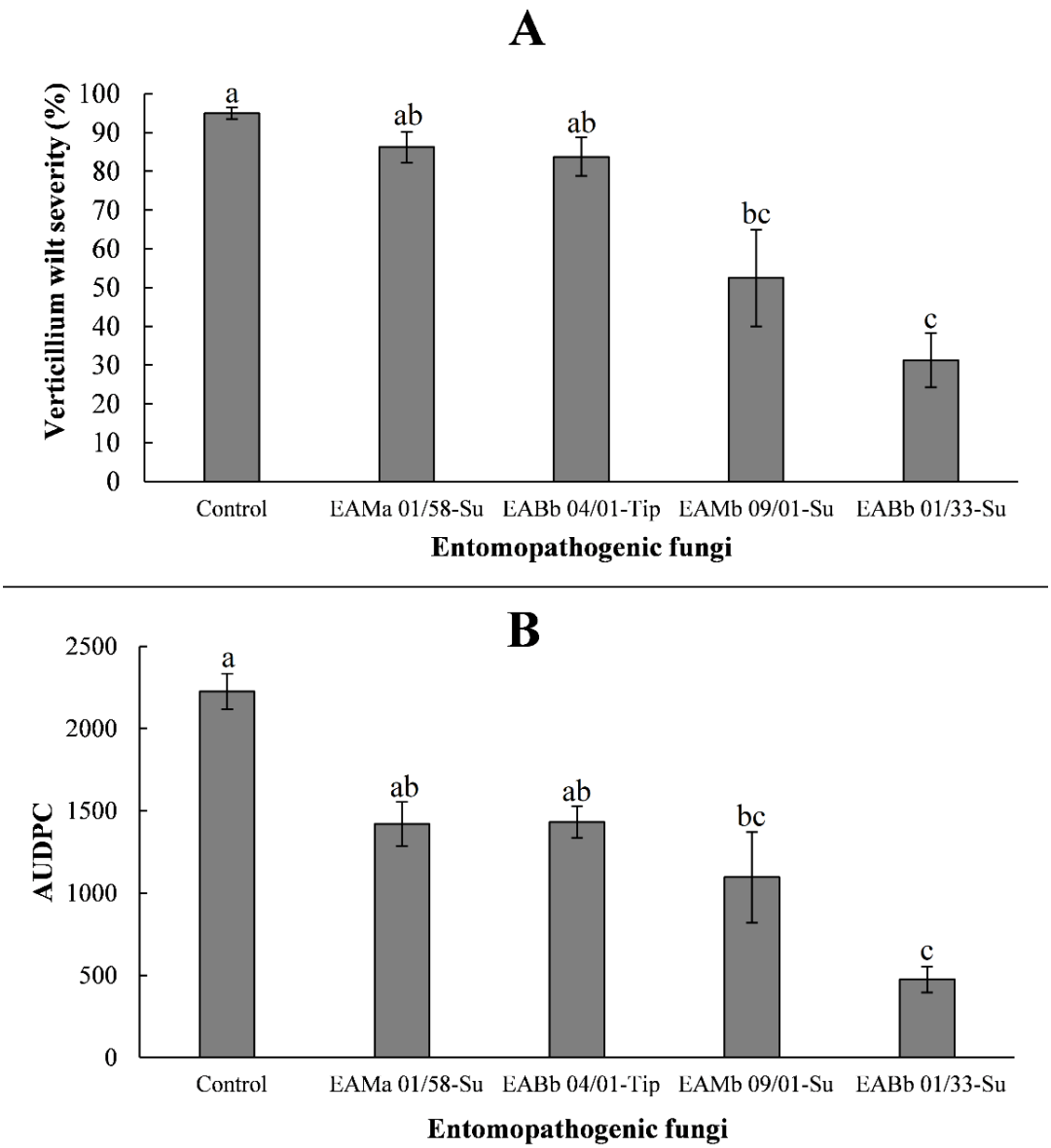

Figure IV.2. Verticillium wilt severity, expressed as (A) percentage of the foliar tissue affected (%) 4 weeks after pathogen inoculation (64 days after sowing); and (B) area under disease progress curve (AUDPC) at the same time, in sunflower plants inoculated with *Verticillium dahliae* and treated with different entomopathogenic fungi. Control plants were inoculated with *V. dahliae* alone. Letters on the bars indicate homogeneous groups according to LSD post hoc test ($P < 0.05$). Analyses of verticillium wilt severity (A) were performed on transformed data using an arcsine transformation.

In the first assessment of the population density of the EF isolates, the cfu g^{-1} substrate varied between 8.25×10^5 (EABb 01/33-Su) and 1.2×10^5 (EABb 04/01-Tip) at the moment of inoculation with *V. dahliae* (Figure IV.3A). Four weeks later, these populations decreased down to 3.75×10^4 (EABb 01/33-Su) and 0 (EABb 04/01-Tip). All the strains showed a marked decrease in populations 1 week after inoculation with *V. dahliae*, with the exception of EABb 04/01-Tip, which showed very low levels throughout the five sampling weeks, and decreased drastically during the last week (Figure IV.3A). In contrast, the severity of wilt symptoms in plants treated with EF and

later on inoculated with *V. dahliae* increased mainly between the first and second week, and varied little until the end of the experiment (Figure IV.3B).

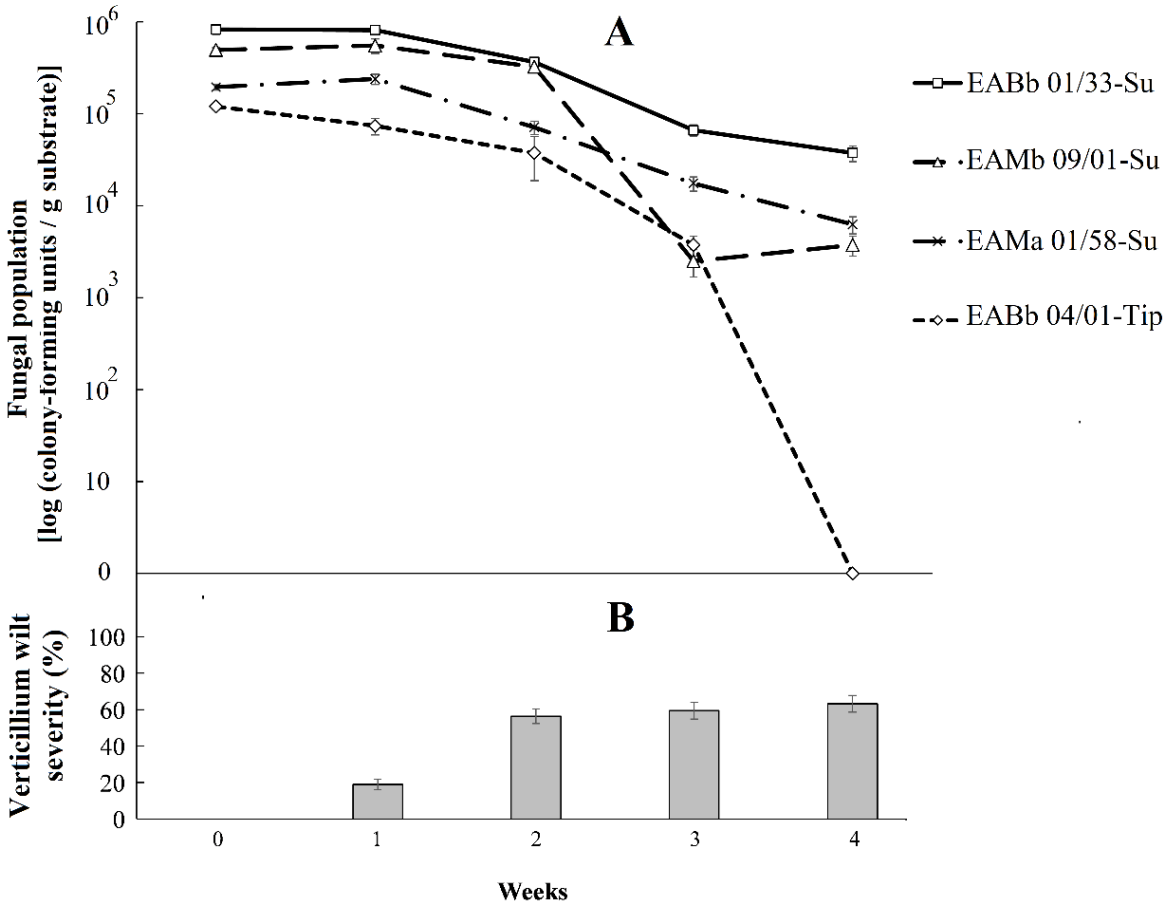

Figure IV.3. (A) Populations of four entomopathogenic fungi recovered from the substrate of pots in which sunflower plants were grown. Inoculation of sunflower plants with *Verticillium dahliae* was performed in week 0. Verticillium wilt symptoms were assessed between weeks 1 and 4. (B) Time course expression of verticillium wilt symptoms (severity of symptoms, %) in EF-treated plants from weeks 1 to 4 after inoculation with *V. dahliae*. Vertical bars represent the severity of symptoms in inoculated plants averaged across EF treatments.

IV.3.4. Microbiological and molecular detection of *V. dahliae* and entomopathogenic fungi in sunflower

At the end of the experiment, *V. dahliae* was successfully isolated from those plants that had been inoculated with the pathogen irrespective of the treatment with EF. The isolation percentages ranged from 32% to 64% in the treatments with EABb 01/33-Su and EAMb 09/01-Su, respectively (Table IV.4). Lastly, none of the EF was successfully isolated from sunflower leaves at the end of the experiment.

Table IV.4. Isolation of *Verticillium dahliae* (%) from sunflower plants inoculated with *V. dahliae* only (control) or with *V. dahliae* and one strain of entomopathogenic fungi, 4 weeks after the inoculation with the pathogen

Strain	Isolation of *V. dahliae* (%)
Control plants	60.7 ± 9.0
EABb 01/33-Su	32.1 ± 6.8
EABb 04/01-Tip	42.9 ± 14.3
EAMb 09/01-Su	64.3 ± 9.2
EAMa 01/58-Su	46.4 ± 14.7

Note: Data show mean ± SE.

With regard to the molecular analyses, amplifications of samples from plants inoculated with *V. dahliae* yielded a 550 bp fragment, which is diagnostic of the pathogen (Figure IV.4A). However, we were unable to confirm the presence of any of the EF strains in sunflowers inoculated with *V. dahliae*. The most interesting finding was that, in the absence of *V. dahliae*, bands of similar sizes to those of *B. bassiana* and *M. brunneum* were amplified, showing that the four EF strains succeed in endophytically colonizing the plants (Figure IV.4B). As expected, all the samples yielded the 750 bp band diagnostic of sunflower (Figure IV.4A,B).

IV.4. Discussion

In this work, we assessed the performance of five strains of EF as biological control agents against the sunflower pathogens *V. dahliae* and *C. helianthi*. Our results show that species of *Metarhizium* and *Beauveria* can play an active role as antagonists of those pathogens. Both the EF strain and the *V. dahliae* isolate had a significant effect on the mycelial growth of the pathogen when co-cultured with the EF, whereas under the same experimental conditions, the growth of *C. helianthi* was only dependent on the EF strain. This is not surprising, as *V. dahliae* affecting sunflowers in Europe exhibits a wide diversity that is highly dependent on its geographical origin (Martin-Sanz et al., 2018b). As for *C. helianthi*, it was recently reported as being a pathogen of sunflower (Crous et al., 2019). Although species diversity information is still needed, heterogeneity was not expected within just the three isolates included in our experiments. The most revealing finding was that, in spite of EF inhibiting the growth of both pathogens, no trend in their effect was observed as being associated with either genus (*B. bassiana* or *M. brunneum*), and one *B. bassiana* strain was the most effective one (EABb 01/33-Su) against *C. helianthi* and most of the *V. dahliae* isolates. Similarly, the antagonism of EF against the olive pathogens *V. dahliae*, *Phytophthora megasperma* and *P. inundata* was largely dependent on the particular strain more than on any other factor (Lozano-Tovar et al., 2013).

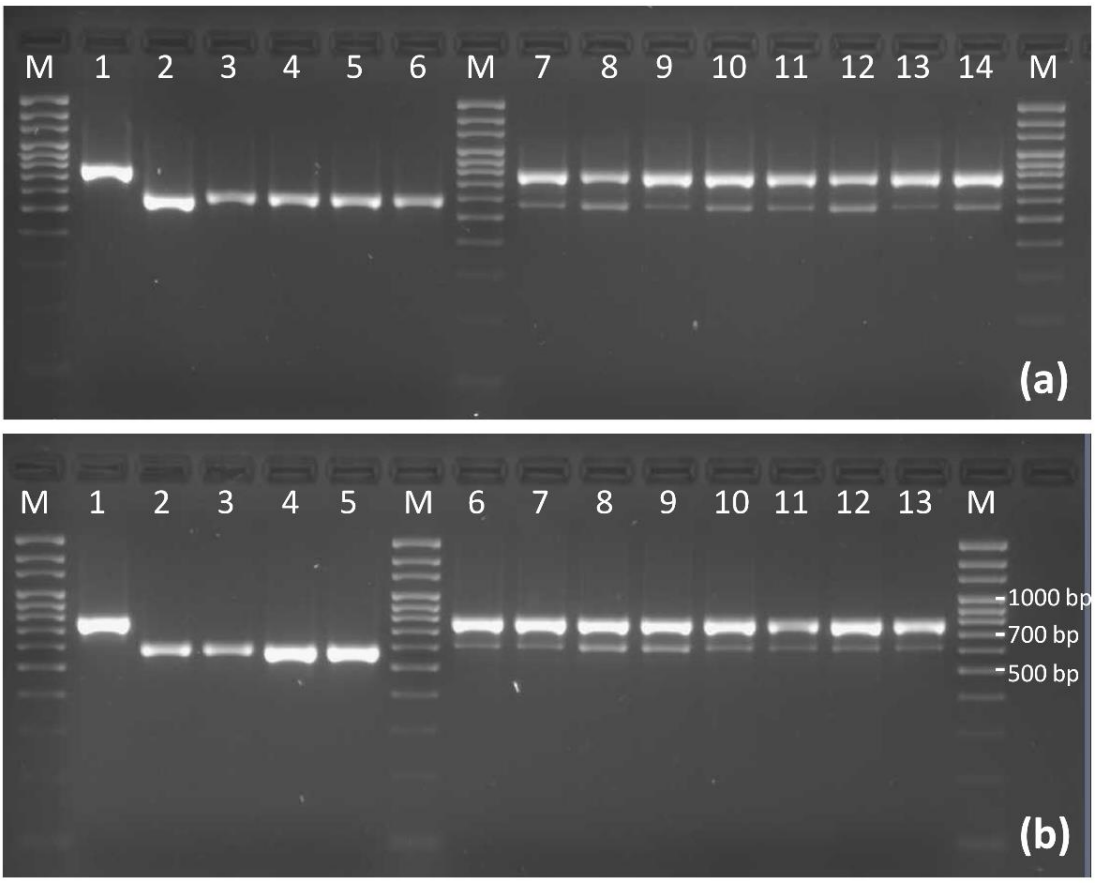

Figure IV.4. Band patterns obtained after PCR amplification of the ribosomal DNA region (5.8S rDNA and internal transcribed spacers 1 and 2) with the universal primer set ITS5 and ITS4 from individual sunflower plants. DNA samples were obtained from lyophilized stem tissue of sunflower plants (breeding line RHA801, R5 stage, 64 days after sowing) treated with: (A) four entomopathogenic fungi (EF) strains: *Beauveria bassiana* EABb 01/33-Su and EABb 04/01-Tip, and *Metarhizium brunneum* EAMa 01/58-Su and EAMb 09/01-Su (always in the same order). Lanes 1–6, DNA from sunflower breeding line RHA801, *V. dahliae* isolate VdS0113, and the four EF strains; lanes 7–14, DNA from two sunflowers only treated with the four EF strains. (B) EF followed by inoculation with *Verticillium dahliae* isolate VdS0113. Lanes 1–5, DNA from sunflower, and the four EF strains; lanes 6–13, DNA from two sunflowers treated with the four EF strains and inoculated with *V. dahliae* 1 month later. M, 100–2,000 bp BrightMAX DNA ladder (Canvax Biotech).

From the results of the dual culture experiments and the microscope observations, we identified two types of antagonism exerted by EF against plant pathogenic fungi. First, mycelial growth inhibition of the pathogens by two strains of *B. bassiana* (EAMb 09/01-Su and EABb 01/33-Su) and one of *M. brunneum* (EAMa 01/58-Su) was associated with overgrowth of the EF on both *V. dahliae* and *C. helianthi*. Not only do our EF strains have notoriously high growth rates (Quesada-Moraga et al., 2014; Raya-Diaz et al., 2017a; 2017b), but the ability of *M. brunneum* strain EAMa 01/58-Su to grow over the mycelia of olive root rot pathogens has already been reported (Lozano-Tovar et al., 2013). Similarly, Varo et al. (2016) carried out dual cultures to test the efficacy of several biological control agents against an isolate of *V. dahliae* that was pathogenic to olive. They identified two modes of antagonism: clear inhibition zones without *V. dahliae* mycelium, and the growth of the biocontrol agents over the pathogen. In our microscopy work, we observed contact and even intertwining

of the hyphae of the confronted fungi in the absence of any mechanical alteration or degradation, which is in agreement with competition as being the mode of action of *M. brunneum* and *B. bassiana*. Other authors have also proposed competition as at least one of the modes of action operating in disease suppression by EF (Ownley et al., 2010; Lozano-Tovar et al., 2013). Secondly, the presence of highly marked inhibition zones when EAMb 01/158-Su strain of *M. brunneum* was dually plated with the pathogens, and hyphae that were clearly distant from those of either *V. dahliae* or *C. helianthi* under the microscope, suggest that the detrimental effect of the strain is associated with the release of diffusible inhibitory substances. An antibiotic effect of *M. brunneum* against olive pathogens has already been reported by Lozano-Tovar et al. (2017). The chemical identification of compounds produced by EAMb 01/158-Su strain displaying antibiosis in our experiments is the subject of further work.

Regarding the development of verticillium wilt in sunflowers treated with *B. bassiana* or *M. brunneum* (strains EABb 01/33-Su and EAMb 09/01-Su, respectively) and thereafter inoculated with the pathogen, both entomopathogens were associated with significant disease reductions, with *B. bassiana* (EABb 01/33-Su) having the most pronounced effect. Although none of the EF strains were detected inside the plants in the presence of *V. dahliae*, an outstanding finding was that, in the absence of the pathogen, the four EF strains succeeded in establishing themselves in sunflowers. Despite these EF strains being well known as transient colonizers of different hosts when applied by foliar spraying (Resquín-Romero et al., 2016; Garrido-Jurado et al., 2017), in our work, with soil drenching applications, they were not only able to survive and settle in the substrate, but also to penetrate the plants and establish themselves as endophytes for weeks. Even though the entomopathogens were unable to compete against *V. dahliae* inside sunflowers, their ability to colonize the plants is an essential feature that could be useful against sunflower pathogens other than *V. dahliae* or even as plant growth promoters (Ownley et al., 2010). Also, the protection conferred by entomopathogenic fungi against verticillium wilt might be due, at least for the most part, to the direct competition between *V. dahliae* and *M. brunneum* (EAMb 09/01-Su) or *B. bassiana* (EABb 01/33-Su) in the soil. Both of these strains are highly adapted to the soil environment (Garrido-Jurado et al., 2017), which seems to favour their competition with *V. dahliae*. Varo et al. (2016) tested a wide variety of microorganisms against verticillium wilt in olive, reporting that both the antagonism in the soil/plant and systemic mechanisms could contribute to a relief of symptoms, thus controlling the disease. In this respect, Raya-Diaz et al. (2017a) reported that EF could promote sunflower growth and inflorescence production under controlled conditions due to an enhancement in Fe bioavailability. Whether this could be related to some extent to the triggering of systemic resistance in sunflower, consequently having a role in disease control, should be explored in future research.

In accordance with Raya-Diaz et al. (2017b), who determined that soil treatment was the best option for the application of EF because it resulted in good population recovery levels, we found that the four EF strains dramatically decreased during the first 4 weeks in the soil (from treatment to inoculation) and, thereafter, during the following 4 weeks. One of the strains (EABb 04/01-Tip) was even unable to remain in the soil as a stable population. Moreover, and as mentioned above, the persistence of EABb 01/33-Su and EAMb 09/01-Su populations in the soil could be the consequence of their

adaptation to this environment and a first requirement for controlling verticillium wilt. Also, the transient colonization of melon plants by these same strains after foliar applications has been reported (Garrido-Jurado et al., 2017), but this does not seem to be the operating mechanism in our experiment in which treatments were conducted by soil drenching. Although present in the soil, none of the three strains was reisolated from plant samples, suggesting that the observed *in vivo* effect of EF against *V. dahliae* is not plant-located, but is most likely the consequence of the intense competition waged by *B. bassiana* (EABb 01/33-Su) and/or *M. brunneum* (EAMb 09/01-Su) against the pathogen in the soil, as already mentioned. Moreover, as a result of the profuse growth of these EFs in comparison to that of *V. dahliae*, pathogen soil populations might be decreased, and root penetration and the development of eventual symptoms delayed in time, as suggested by the positive identification of *V. dahliae* in plants treated with the EF. In addition to the soil-mediated interplay between EABb 01/33-Su or EAMb 09/01-Su and *V. dahliae* suggested by the results of this work, further research should explore whether these strains are able to penetrate sunflower roots and inhibit *V. dahliae* in the host.

Few studies relating EF and insect pests of sunflower have been carried out so far. One by Takov et al. (2013) reported the presence of *B. bassiana* associated with a sunflower crop in Bulgaria. According to the authors, the indigenous EF caused high mortality rates in the sunflower pest *Tanymecus dilaticollis*. Similarly, Barker (1999) tested the efficacy of *Metarhizium anisopliae* (Metsch) and *B. bassiana* against the sunflower pest *Cochylis hospes* under laboratory conditions. In spite of the high efficacy in controlling sunflower insect pests reported in both works, the long-term survival of EF was also pointed out as being a priority for potential field applications.

In conclusion, both *M. brunneum* and *B. bassiana* are able to inhibit the mycelial growth of *V. dahliae* and *C. helianthi* and, according to the dual culture and microscopy results, two types of antagonism can operate: competition and/or antibiosis. In addition, although the entomopathogens were not found inside the plants in the presence of *V. dahliae*, they were when the pathogen was absent. In fact, they were able to establish themselves inside sunflowers, suggesting their potential utility in applications such as, for instance, those to control sunflower diseases other than verticillium wilt, or to promote sunflower growth. Finally, the ability of EF populations to persist in the substrate for several weeks, together with disease reduction observed in treatments with some strains, suggests that the protection they confer against verticillium wilt might not be plant-located, but is more likely to be the consequence of their competition with *V. dahliae* in the soil.

IV.5. Acknowledgments

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CAPÍTULO V. UPDATED CHARACTERIZATION OF RACES OF *Plasmopara halstedii* AND ENTOMOPATHOGENIC FUNGI AS ENDOPHYTES OF SUNFLOWER PLANTS IN AXENIC CULTURE

Este capítulo es una versión adaptada del artículo homónimo aceptado con minor revision en la revista Agronomy.

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MDPI

Article

Updated characterization of races of *Plasmopara halstedii* and entomopathogenic fungi as endophytes of sunflower plants in axenic culture

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Abstract: The management of downy mildew (*Plasmopara halstedii*) in sunflower, is heavily dependent on genetic resistance, whilst entomopathogenic fungi (EF) can reduce other sunflower diseases. In this work, we characterized *P. halstedii* from Spain and other countries collected in the past few years. Twenty-three races were identified (the most frequent in Spain being 310, 304, 705 and 715), with an increasing proportion of highly virulent races. Five isolates from countries other than Spain overcame the resistance in RHA-340. In addition, we assessed the efficacy of five EF against downy mildew and their effect on sunflower growth in axenic conditions. None of the entomopathogens reduced disease severity, nor did they have any effect on plantlet growth when applied together with *P. halstedii*. In contrast, three EF reduced some of the growth variables in the absence of the pathogen. Microbiological and molecular diagnostics suggest that the axenic system and the short experimental time used in this work do not favour the successful establishment of EF in the plantlets or their potential biocontrol effect. Our results show a shift in *P. halstedii* racial patterns and suggest that soil as a growth substrate and long infection times are needed for EF effectiveness against downy mildew.

Keywords: biological control; diseases of oilcrops; downy mildew; endophytic fungientomopathogens; genetic resistance; integrated pest management; pathogen races; soilborne pathogens

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1. Introduction

Downy mildew of sunflower (*Helianthus annuus* L.), caused by the obligate biotrophic oomycete *Plasmopara halstedii* Farl. Berl. & de Toni, is one of the most widespread diseases affecting this crop, and it is present in the five continents [1]. When *P. halstedii* zoospores emerge from dormant oospores present in the soil reaching seedling roots, primary, systemic infections of the host take place. Primary infections may cause damping-off or, if not, severe stunting. Infected plants manifest a pronounced chlorosis, which is restricted to the area bordering the main veins in the lowest leaves but may cover the entire foliar surface in young leaves [2]. When infected plants are exposed to high relative humidity and cool temperatures, the oomycete develop profuse cottony outgrowths from the underside of infected leaves, constituted by zoosporangia [3]. Sunflower downy mildew causes an average yield reduction of 3.5% worldwide, whereas crop losses of up to 100% are frequently scored when infections in the field are severe or highly localized [3,4].

The management of this pathogen in Europe is based on particular cultural practices, chemical pesticides (mainly, by seed treatments using the phenylamides metalaxyl, metalaxyl-M and mixtures of metalaxyl + mancozeb) [5,6], and the employment of genetically resistant sunflower hybrids [2]. Whereas cultural practices may not be effective

V. Updated characterization of races of *Plasmopara halstedii* and entomopathogenic fungi as endophytes of sunflower plants in axenic culture

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V.0. Abstract

The management of downy mildew (*Plasmopara halstedii*) in sunflower is heavily dependent on genetic resistance, whilst entomopathogenic fungi (EF) can reduce other sunflower diseases. In this work, we characterized *P. halstedii* from Spain and other countries collected in the past few years. Twenty-three races were identified (the most frequent in Spain being 310, 304, 705 and 715), with an increasing proportion of highly virulent races. Five isolates from countries other than Spain overcame the resistance into RHA-340. In addition, we assessed the efficacy of five EF against downy mildew and their effect on sunflower growth in axenic conditions. None of the entomopathogens reduced disease severity, nor did they have any effect on plant growth when applied together with *P. halstedii*. In contrast, three EF reduced some of the plant growth variables in the absence of the pathogen. Microbiological and molecular diagnostics suggest that the axenic system and the short experimental time used in this study did not favour the successful establishment of EF in the plants or their potential biocontrol effect. Our results show a shift in *P. halstedii* racial patterns and suggest that soil as a growth substrate and long infection times are needed for EF effectiveness against downy mildew.

Keywords

Biological control, diseases of oilcrops, downy mildew, endophytic fungi, entomopathogens, genetic resistance, integrated pest management, pathogen races, soilborne pathogens

V.1. Introduction

Downy mildew of sunflower (*Helianthus annuus* L.), caused by the obligate biotrophic oomycete *Plasmopara halstedii* Farl. Berl. & de Toni, is one of the most widespread diseases affecting this crop, and it is present in the five continents (EPPO, 2020). When *P. halstedii* zoospores emerge from dormant oospores present in the soil reaching seedling roots, primary, systemic infections of the host take place. Primary infections can cause damping-off or severe stunting. Infected plants manifest a pronounced chlorosis, which is restricted to areas bordering the main veins in the lower leaves, although they may cover the entire foliar surface in younger leaves (Molinero-Ruiz, 2019). When infected plants are exposed to high relative humidity and cool temperatures, the oomycete develops profuse cottony outgrowths from the underside of infected leaves, constituted by zoosporangia (Gulya et al., 2019). Sunflower downy mildew causes an average yield reduction of 3.5% worldwide. Crop losses of up to

100% are frequent when infections in the field are severe or highly localized (Gascuel et al., 2015; Gulya et al., 2019).

The management of this pathogen in Europe is based on particular cultural practices, chemical pesticides (mainly, by seed treatments using the phenylamides metalaxyl, metalaxyl-M and mixtures of metalaxyl + mancozeb) (Achbani et al., 1999; Molinero-Ruiz et al., 2008), and the employment of genetically resistant sunflower hybrids (Molinero-Ruiz, 2019). Whereas cultural practices alone may not be effective enough to control the disease, reports of *P. halstedii* isolates becoming resistant to metalaxyl and metalaxyl-M (Molinero-Ruiz et al., 2003; 2008) show that chemical control is not a sufficiently reliable tool against downy mildew in sunflower.

Genetic resistance is the most important control measure for this pathogen (Molinero-Ruiz et al., 2002; Molinero-Ruiz, 2019). Nonetheless, the use of resistant sunflower hybrids is threatened by the continuous identification of new *P. halstedii* populations of increased virulence that overcome the genes for resistance in the crop (Molinero-Ruiz et al., 2002; Garcia-Carneros and Molinero-Ruiz, 2017). Thus, information on the racial distribution of this pathogen is of crucial importance for its management by means of genetic resistance (Molinero-Ruiz, 2019). Although extensive research on the pathogenic characterization of *P. halstedii* was conducted in Spain between 1994 and 2006 (Molinero-Ruiz et al., 2002; 2008), the evolution of races of *P. halstedii* in recent years has been largely unknown in this country, with the exception of a few studies (Garcia-Carneros and Molinero-Ruiz, 2017).

On the other hand, biological control has triggered increasing interest in recent years, fostered by both researchers and agricultural policies worldwide, including the common agricultural policy in the European Union (Menzler-Hokkanen, 2006; Quesada-Moraga et al., 2020). Amongst biocontrol agents, entomopathogenic fungi (EF), which are fungal species that are pathogenic to insects, have achieved great relevance in economic terms, with a plethora of EF-derived biopesticides being registered (Quesada-Moraga et al., 2020). Several authors have reported the efficacy of EF as biocontrol agents against plant pathogens (Jaber, 2015; Keyser et al., 2016; Lozano-Tovar et al., 2017; Jaber and Ownley, 2018; Vega, 2018; Miranda-Fuentes et al., 2020a). In sunflower, EF can have three different effects, the control of insect pests being the most classical one among them. Some EF species like *Beauveria bassiana* Bals. (Vuill), *Metarhizium brunneum* Petch and *M. anisopliae* (Metsch) are good alternatives for controlling sunflower banded moth *Cochylis hospes* Walsingham (Barker et al., 1999), grey corn weevil *Tanymecus dilaticollis* Gyllenhal (Takov et al., 2013), and wireworms *Agriotes* spp. (Ortiz-Bustos et al., 2016). Also, the promotion of sunflower growth and nutrition by some strains of *B. bassiana*, *M. brunneum* and *Isaria farinosa* (Holmsk.) has been reported (Raya-Diaz et al., 2017a). Finally, we recently found that *B. bassiana* and *M. brunneum* not only inhibit the soilborne pathogenic fungi *Verticillium dahliae* Kleb. and *Cadophora helianthi* (L. Molinero-Ruiz, A. Martin-Sanz, C. Berlanas & D. Gramaje), but they also reduce verticillium wilt in sunflowers. Moreover, this reduction in symptoms seems to be the consequence of a soil-mediated competition between *V. dahliae* and the EF (Miranda-Fuentes et al., 2020a). Despite previous studies on the use of EF in sunflower, their efficacy against *P. halstedii* remains completely unexplored, and biological control of this pathogen has scarcely

been addressed (Nagaraju et al., 2012). However, several endophytic strains of *B. bassiana* were applied against *Plasmopara viticola* (Berk. & Curtis) Berl. & De Toni, thereafter colonizing treated plants and eventually reducing symptoms of grapevine downy mildew (Jaber, 2015; Rondot and Reineke, 2019).

Our study has focused on determining the effectiveness of both genetic resistance and biological control as sustainable methods for the management of sunflower downy mildew. Our objectives for this study were to: i) conduct a survey to update the racial characterization of isolates of *P. halstedii* present in Europe, with particular emphasis on those from Spain; ii) assess the efficacy of five strains of EF *M. brunneum* and *B. bassiana* against sunflower downy mildew; iii) assess the effect of EF on the growth of sunflower plants; iv) determine whether, or not, EF and *P. halstedii* simultaneously colonize inner tissues of sunflower.

V.2. Materials and Methods

V.2.1. Racial characterization of *Plasmopara halstedii*

A total of 58 fields, in which sunflowers showed downy mildew symptoms, were sampled between 2011 and 2020 in Spain (49), France (3), Italy (3), Portugal (2) and Romania (1) (Table V.1). Most of the samples were collected after unexpected disease outbreaks were observed. Sunflowers showing stunting and chlorosis in true leaves were collected from each field and taken to the laboratory. Samples consisting of tissue from more than one plant and collected in the same field location were treated as one. Following the methodology by our research group (ex. Molinero-Ruiz et al., 2002; 2008; Martin-Sanz et al., 2020), infected leaves were excised, placed in humid chambers and incubated in darkness at 15 °C for 24-48 h until profuse sporulation occurred on the leaves. Then, leaves showing sporulation were immersed in sterile deionized water and vigorously shaken for 5 min until a zoosporangial suspension was obtained. The suspension was filtered through two layers of sterile gauze and homogenized by vortex. The concentration of the suspension was adjusted to 4×10^4 zoospores ml^{-1} using a haemocytometer, specifically the Neubauer chamber (Blau Brand, Wertheim, Germany).

The racial characterization of the *P. halstedii* isolates was conducted by calculating the coded virulence formula (CVF) using the method described by Gulya et al. (1998). The CVF is a 3-digit number based on the resistant/susceptible reaction of three sets of three sunflower lines each (differentials) to the inoculation with *P. halstedii* zoospore suspensions. The sets are as follows: set 1 (HA-304; RHA-265; RHA-274), set 2 (PMI3; PM17; 803-1) and set 3 (HAR-4; QHP1; HA-335) (Gulya et al., 1998). Within each set, resistant reactions have a value of 0 and susceptible reactions have values of 1 (for differential 1), 2 (for differential 2) or 4 (for differential 3) (Gulya et al., 1998; Gilley et al., 2020). The addition of these values for each set results in the 3-digit CVF. In addition to the nine differentials, isolates of *P. halstedii* were also inoculated into the inbred line RHA 340, which carries a highly effective genetic resistance against *P. halstedii* (Miller and Gulya, 1988). Seeds of each of the sunflower genotypes were

germinated prior to inoculation with *P. halstedii* according to the methodology followed by our group (Miranda-Fuentes et al., 2020a).

Table V.1. List of sunflower samples collected in Europe, and included in this study, between 2011 and 2020

Sample number	Sample ID	N. of plants	Collection year	Sunflower Genotype	Geographic origin. Location (Province). Country [a]
1	DM01-11	2	2011	Transol/2-4	Alameda del Obispo (Cordoba). SP
2	DM02-11	1	2011	Transol/18-2	Alameda del Obispo (Cordoba). SP
3	DM03-11	2	2011	102/SIg	Marchena (Seville). SP
4	DM04-11	3	2011	Bosfora	Fuentes de Andalucía (Seville). SP
5	DM05-11	3	2011	Transol	Fuentes de Andalucía (Seville). SP
6	DM01-13	1	2013	C1/DBa	Las Cabezas de San Juan (Seville). SP
7	DM02-13	2	2013	B2/DBa	Las Cabezas de San Juan (Seville). SP
8	1978-R1	2	2014	- [b]	Tomejil (Seville). SP
9	DB0	-	2014	-	Las Cabezas de San Juan (Seville). SP
10	LGB	1	2014	LG560440	Bornos (Cadiz). SP
11	1SM	3	2014	Experimental line	Sao Matias (Beja). PO
12	2SEP	2	2014	Experimental line	Serpa (Beja), PO
13	DM01-15	2	2015	-	Cerro Perea (Seville). SP
14	DM02-15	1	2015	-	Fuentes de Andalucía (Seville). SP
15	DM03-15	1	2015	-	Fuentes de Andalucía (Seville). SP
16	DM04-15	4	2015	-	Jerez de la Frontera (Cadiz). SP
17	DM01-16	2	2016	-	Fuentes de Andalucía (Seville). SP
18	DM02-16	-	2016	-	Alcalá del Río (Seville). SP
19	DM01-17	2	2017	LG5485	Montellano (Seville). SP
20	B-B190418	4	2018	Experimental line	Utrera (Seville). SP
21	B-S040518-1	4	2018	Experimental line	Alcalá del Río (Seville). SP
22	B-S040518-2	4	2018	Experimental line	Viso del Alcor (Seville). SP
23	B-S100518	4	2018	Experimental line	El Arahal (Seville). SP
24	B-B180518	4	2018	Experimental line	Cañada Rosal (Seville). SP
25	B-B210518-1	-	2018	Solnet	Espera (Cadiz). SP
26	B-B210518-2	-	2018	Solnet	Alcalá Guadaira (Seville). SP
27	B-B210518-3	-	2018	Bonasol	Utrera (Seville). SP
28	B-B220518	1	2018	Solnet	Huévar (Seville). SP
29	B-S230518	2	2018	Syedison	Palma del Río (Cordoba). SP
30	B-B290518-1	1	2018	Solnet	Trigueros (Seville). SP
31	B-B290518-2	1	2018	Solnet	El Trobal (Seville). SP
32	B-B300518	-	2018	Solnet	Paradas (Seville). SP
33	B-LG310518-1	3	2018	Experimental line	Las Cabezas de San Juan (Seville). SP
34	B-LG310518-2	3	2018	Experimental line	Lebrija (Seville). SP
35	B-LG310518-3	2	2018	Experimental line	Maribáñez (Seville). SP
36	B-F050618	1	2018	-	Escacena del Campo (Huelva). SP
37	B-E050618-1	2	2018	Artic	Paterna del Campo (Huelva). SP
38	B-E050618-2	1	2018	Artic-T	Paterna del Campo (Huelva). SP
39	B-P080618	2	2018	-	Tulcea, RO
40	B-B120618	-	2018	Bonasol	La Campaña (Seville). SP
41	B-P130618-1	1	2018	64LE25	Cortona, IT
42	B-P130618-2	1	2018	64LE25-T	Cortona, IT
43	B-P130618-3	3	2018	XF16942	Cortona, IT
44	B-P130618-4	2	2018	P64LE99	Mauroux, FR
45	B-P130618-5	3	2018	P64LE25	Taybosc, FR
46	B-P130618-6	1	2018	P64LE25	Lectoure, FR
47	B-LG140618	2	2018	Experimental line	Écija (Seville). SP
48	B-S190618	2	2018	Experimental line	Olivares (Seville). SP
49	B-S280618	1	2018	-	Escacena del Campo (Huelva). SP
50	B-S230419 (SYT-1)	-	2019	M4	Villamanrique de la Condesa (Seville). SP
51	B-S080519-1 (2019HU01)	3	2019	-	Palma del Condado (Seville). SP
52	B-S080519-2 (2019-SE01)	3	2019	-	Gerena (Seville). SP
53	B-S230519-1 (GIB-1)	-	2019	M4	Villarrasa (Huelva). SP
54	B-S230519-2 (GIB-2)	-	2019	M4-T	Villarrasa (Huelva). SP
55	B-S190520-1	3	2020	M4	Fernán Núñez (Cordoba). SP
56	B-S190520-2	2	2020	M9	Pedro Abad (Cordoba). SP
57	B-S270520	1	2020	-	Andújar (Jaen). SP
58	Ph04-20	2	2020	M4	Calzada de Bureba (Burgos). SP

[a] SP = Spain, PO = Portugal, RO = Romania, IT = Italy, FR = France. [b] - = Unknown.

Briefly, a thin layer of perlite was put into Petri plates and covered with sterile filter paper previously moistened with sterile deionized water. Seeds were disinfested by a 5-min immersion in 10% sodium hypochlorite and three subsequent rinses in sterile deionized water and then they were air-dried in a sterile laminar flow cabinet for 5 min and placed into the plates. The seeds were incubated in darkness at 24 °C for 48 h until 2-8-mm long radicles emerged. Then, pericarps and seminal membranes were removed to prevent contamination.

For each differential and isolate, two replicates of 10 seedlings each were immersed in 20 ml of suspensions of 4×10^4 sporangia ml^{-1} for 4 h at 18 °C in darkness. Each replicate was transplanted in one 0.7-l pot filled with sterile perlite. Plants were incubated in a growth chamber at 20 °C and a 12-h photoperiod for 12 days, until expansion of the first pair of true leaves. Then, they were incubated for 48 h at 100% relative humidity under the same conditions of temperature and light to induce pathogen sporulation. The reaction of each differential line to each isolate of *P. halstedii* was assessed on the 14^{th} day after inoculation. It was recorded as being susceptible if profuse sporulation appeared on cotyledons and/or true leaves or resistant if no sporulation occurred (Molinero-Ruiz et al., 2008). The inoculation tests were performed twice, or even a third time if any of the reactions were not clear.

V.2.2. Effect of entomopathogenic fungi on sunflower downy mildew and on the growth of sunflower

The effect of five EF strains against *P. halstedii* was studied in two experiments that were conducted under controlled conditions. In the first one, the effect of entomopathogens on the development of symptoms of downy mildew in sunflowers was assessed. In the second experiment, we analysed whether EF have a particular effect on the development of sunflower plants in the absence of *P. halstedii*.

V.2.2.1. Biological materials: *Plasmopara halstedii* and entomopathogenic fungi

According to the results of the experiments described in V.2.1, the isolate 1SM of *P. halstedii* was used (Table V.1). This isolate was characterized as race 304 (Table V.2) and routinely maintained by subsequent inoculations of seedlings of the inbred line HA-304, which is susceptible to all races of the pathogen.

In addition, the five EF strains included in this study were EAMa 01/58-Su, EAMb 09/01-Su and EAMb 01/158-Su (*M. brunneum*), and EABb 01/33-Su and EABb 04/01-Tip (*B. bassiana*). They were selected based on their efficacy against other sunflower pathogens (Miranda-Fuentes et al., 2020a) and they are deposited in the Entomopathogenic Fungi Collection (CRAF) at the University of Cordoba (Spain) and the Spanish Collection of Culture Types (CECT) at the University of Valencia (Spain) (Raya-Diaz et al., 2017a). More information on their origin and other traits of interest is available in Miranda-Fuentes et al. (2020a).

V.2.2.2. Effect of entomopathogenic fungi on sunflower downy mildew

Seeds of HA-304 were germinated under controlled conditions as described in V.2.1 for 48 h until they had 2-8-mm long radicles, then their pericarps and seminal membranes were removed. The seedlings were sown in sterile glass tubes (one seedling per tube) each containing 40 ml of Hoagland and Knop's culture medium [components per liter of deionized water: agar (7.1 g), $Ca(NO_3)_2 \cdot 4H_2O$ (0.95 g), KNO_3 (0.61 g), $MgSO_4 \cdot 7H_2O$ (0.49 g), $NH_4H_2PO_4$ (0.12 g), $C_6H_5FeO_7$ (0.02 g), $MnSO_4 \cdot H_2O$ (2.273 mg), $ZnSO_4 \cdot 7H_2O$ (0.5 mg), $CuSO_4 \cdot 5H_2O$ (0.025 mg), $Na_2MoO_4 \cdot 2H_2O$ (0.025 mg), H_2SO_4 98% (0.5 µl); pH adjusted to 5.7 using NaOH].

Once the 48-h-old seedlings had been sown on the medium, they were treated with the EF and, thereafter, inoculated with *P. halstedii*. In the case of EF, conidial suspensions were prepared according to Miranda-Fuentes et al. (2020a): firstly, the five EF strains were plated by streaking a loop of conidial suspensions onto potato dextrose agar (PDA) (BD, New Jersey, USA) plates, and incubated at 24 °C in darkness for 7 days. Then, the mycelium of each strain was recovered using a sterile Digralsky spatula and suspended in 5 ml of sterile deionized water. The suspensions were filtered through sterile gauze to separate the mycelium and homogenized by vortex. The conidia concentration of the suspension was adjusted to 10^7 conidia ml^{-1}. The seedlings in the tubes were treated with 5 µl of EF conidial suspensions and inoculated with the same volume of zoosporangial suspension of *P. halstedii* 24 h later in sterile conditions. Zoosporangial suspensions were prepared as described in V.2.1, with the concentration adjusted to 4×10^4 zoospores ml^{-1}. Control plants were inoculated with *P. halstedii* as described 24 h after being treated with 5 µl of sterile deionized water instead of EF.

In order to allow both a higher humidity and gas exchange, the tubes were sealed with laboratory film into which an 8-mm hole was perforated. The experimental unit consisted of one seedling, and four replications were established for each treatment following a completely randomized design. Seedlings were incubated in a growth chamber at 24 °C with a photoperiod of 14 h of light for 14 days. Then, the severity of downy mildew in each plant was assessed by using a percentage scale from 0% (no symptoms) to 100% (cottony fungal growth completely covering the cotyledons and first true pair of leaves and also evident in the base of the stem). Additionally, we measured the height, length of root system and dry weight of both the shoot and root system of each plant at the end of the experiment. Dry weights were recorded once plants were dried at 60 °C for 96 h, right after the 14 days of incubation.

V.2.2.3. Effect of entomopathogenic fungi on sunflower growth

In order to assess the possible effects of treatments with EF on the sunflowers' growth in the absence of *P. halstedii*, an additional experiment was devised. Forty-eight-hour-old seedlings of the same inbred line used in the experiments described in V.2.2.2 (HA-304) were sown on Hoagland and Knop's culture medium as described above. Immediately afterwards, they were treated with the five EF strains. Specifically, plants were treated with 5 µl of conidial suspensions of each fungal strain, previously adjusted to 10^7 conidia ml^{-1}. Control plants were treated with 5 µl of sterile deionized water. The experimental design and the number of replications were similar to those

described in V.2.2.2. Plants were incubated for 14 days. At the end of the experiment, they were uprooted and the height, length of root system and dry weight of both the shoot and the root system of each plant was recorded. Although their height and root length were assessed immediately after the plants had been uprooted, dry weights were recorded once plants had been dried at 60 °C for 96 h, right after the end of the experiment.

V.2.2.4. Microbiological and molecular detection

The colonization of the plants by the five EF was assessed at the end of the experiments, i.e. 14 days after treatment with EF. The plants from each treatment were processed as follows: one 8-mm leaf disc was cut from the first pair of true leaves of each plant using a sterile puncher. One 1-cm fragment of roots and a similar fragment of the stem were also excised using a scalpel. Leaf discs and root and stem fragments were disinfested by a 5-min immersion in 1% NaClO and two rinses in sterile deionized water, then air-dried and plated onto Sabouraud Dextrose Agar + chloramphenicol (0.5 g l^{-1}) (Scharlab, S. L., Spain). The plates were sealed with laboratory film and incubated at 24 °C in darkness for 15 days and, during this time, the presence or absence of fungal growth of *M. brunneum* or *B. bassiana* in each fragment was assessed daily. Colonization was expressed as the percentage of plated samples showing EF growth.

Also, at the end of the experiments, molecular diagnostic analyses were conducted following the methodology of Miranda-Fuentes et al. (2020a). First, all the plants from both experiments were surface-sterilised and lyophilised. Then, total genomic DNA from each plant was extracted and purified using the i-genomic Plant DNA Extraction NucleoSpin® Plant II (Macherey-Nagel GmbH & Co. KG) kit following the manufacturer's instructions. Both the quality and concentration of the purified DNA were determined with a fluorometer (Qubit 3.0, Invitrogen) and the DNA samples were adjusted to a final concentration of 10 ng μl^{-1}. In the *P. halstedii*−EF experiment, the identity of *P. halstedii* was confirmed by amplification of the nuclear DNA coding for the large ribosomal unit (28S rDNA) using LR0R and LR6-O primers (Riethmüller et al., 2002). The presence of EF as endophytes in sunflower tissues was confirmed by amplification of the region consisting of the 5.8S ribosomal DNA and internal transcribed spacers 1 and 2 using the primer set ITS5/ITS4 (White et al., 1990). Optimized PCR assays were carried out in a final volume of 25 µl containing 0.4 µM of each primer, 800 µM dNTPs, 2.5 µl 10 × PCR buffer (800 mM Tris-HCl, pH 8.3–8.4 at 25 °C, 0.2% Tween 20 w/v), 0.75 U Horse-Power™ Taq DNA polymerase (Canvax Biotech), 2.5 mM $MgCl_2$ and 20 ng DNA. The following profile was used for the amplifications of the ITS region: 3 min initial denaturation at 95 °C; 30 cycles of 30 s denaturation at 56 °C, 2 min of annealing at 72 °C and 30 s extension at 95 °C; and a final extension step of 10 min at 72 °C. The profile for the amplification of the *P. halstedii* specific region was as follows: 3 min initial denaturation at 95 °C; 30 cycles of 1 min at 94 °C denaturation, 45 s at 50 °C annealing, extension for 1 min at 72 °C ; a final extension step of 7 min at 72 °C. As positive DNA controls, we used mycelial DNA samples of the five EF grown on PDA, of isolate 1SM of *P. halstedii*, and of sunflower. Sterile deionized water was used as a negative amplification control. Polymerase chain reactions were conducted using a T1 thermocycler (Whatman

Biometra). Lastly, amplification products were separated by horizontal electrophoresis in 3.5% agarose gels containing 0.05 µl ml^{-1} GoodView™ nucleic acid stain (SBS Genetech Co., Ltd.) and visualized over a UV light source. A 100–2000 bp BrightMAX™ DNA ladder (Canvax Biotech) was included in the electrophoresis.

V.2.2.5. Data analysis

All the experiments were conducted twice. In each of them, sunflower seed from different lots and different biological replicates of both the five EF strains and 1SM isolate (in the EF-*P. halstedii* experiment) were used. In each of the two replicate experiments, since the interaction between replicates when the experiments were combined was not significant (McIntosh, 1983), the data was pooled and analysed using Statistix® 10 (Analytical Software, Tallahassee, USA). Percentage severity of downy mildew was transformed using the arcsine transformation, $Y = arcsine\sqrt{\frac{severity}{100}}$ and subjected to Analysis of Variance (ANOVA). In the case of the length and the dry weight of the shoot and the root system, the data was not transformed. Prior to the analysis, the data were checked for ANOVA's requirements: homogeneity of variances (Brown and Forsythe test), normality (Shapiro-Wilk test) and independence of residues (graphical test). Means from different treatments were compared using Fisher's Least significant difference (LSD) test (α=0.05) when significant differences were obtained.

V.3. Results

V.3.1. Racial characterization of *Plasmopara halstedii*

The nine differential lines inoculated with all the isolates showed different patterns of resistant/susceptible reactions, thus allowing the racial characterization of all the *P. halstedii* isolates (Table V.2).

A total of 22 races were identified in Spain between 2011 and 2020. The most frequent races in the country were 310 (12.2% of the isolates), 304 (10.2%), 705 (8.2%), 715 (8.2%) and races 311, 315, 710 and 714 (6.1% each), whereas only one (2.0%) or two (4.1%) isolates of the rest of the races were found.

We identified highly virulent races of *P. halstedii* in Spain throughout the duration of the study since, as previously mentioned, the isolates mostly came from samples collected in fields where unexpected downy mildew outbreaks had occurred. Twenty-four of the isolates overcame the Pl_1 resistance gene in RHA-265, showing a 3 as the first CVF digit. Additionally, 20 isolates overcame both Pl_1 in RHA-265 and Pl_2 in RHA-274, and therefore showed a 7 as the first digit. Lastly, four of the isolates were able to only infect the susceptible line HA-304, thus having a 1 as first digit, and one isolate had a 5, since it was controlled by Pl_1 (RHA-265) but not by Pl_2 (RHA-274). Differentials PM17 and 803-1 were resistant to all the isolates and therefore their second digit was assigned a 0 (14 isolates, to which PMI3 was resistant) or a 1 (35 isolates, to which PMI3 was susceptible). The third digit of the CVF was the most diverse one. The three differentials of the third set were resistant to 12 isolates, which

gave a 0. Three isolates had a 3, due to the susceptibility of a HAR-4 and QHP1, and 14 isolates had a 4, since only HA-335 was susceptible in the third set. Finally, 13 of the isolates were assigned a 5, since they overcame the resistance of both HAR-4 and HA-335.

With regard to the eight isolates of *P. halstedii* obtained from countries other than Spain, almost all of them were of highly virulent races. The two isolates collected in Portugal were of races 304 and 700. The only isolate from Romania was of race 705. The three isolates from France belonged to race 715. Two of the isolates from Italy belonged to race 715, whereas the other one was of race 301. Furthermore, this race was not found in any of the isolates collected in Spain (Table V.2). In this group, we identified the highest virulence of *P. halstedii*. With the exception of 1SM and Ph22-18, all the remaining isolates were able to overcome Pl_1 (RHA-265), Pl_2 (RHA-274), Pl_{PMI13} (PMI3) and Pl_6 (HA-335), thus having a 7 and a 5 in the first and third digits, respectively. Importantly, the five isolates characterized as race 715 (two from Italy and three from France) were also able to infect differential RHA-340, previously considered resistant to all races. These results have been recently published (Martin-Sanz et al., 2020).

V.3.2. Effect of entomopathogenic fungi on sunflower downy mildew

The severity of downy mildew caused by isolate 1SM in the susceptible line HA-304 varied between 57% (strain EAMb 09/01-Su) and 97% (EAMa 01/58-Su), but no significant differences were observed when compared to the severity in the control plants inoculated only with *P. halstedii* (65%) (Figure V.1).

Additionally, the EF did not significantly influence sunflower plants in either shoot height, root length or dry weight of shoots and roots. Plant height and root length ranged from 7.3 (EABb 01/33-Su and EABb 04/01-Tip) and 6.9 cm (EAMb 01/158-Su), respectively, to 10.2 (EAMa 01/58-Su) and 9.3 cm (EAMb 09/01-Su). Dry weight of shoots varied between 0.55 g (EABb 01/33-Su) and 0.84 g (EAMb 09/01-Su). Dry weight of roots ranged from 0.13 g (EAMb 01/158-Su) to 0.21 g (EAMb 09/01-Su) (Table V.3).

The inoculation with *P. halstedii* seemed to interfere with plant colonization by EF, as none of the strains were consistently recovered from the stems, and particularly the leaves of the plants in the present of the oomycete. Similarly, the EF could not be diagnosed using PCR in any of the plants from the treatments with both EF and *P. halstedii*.

Table V.2. Racial characterization of isolates of *Plasmopara halstedii* collected in Europe from year 2011 to 2020

Sample ID	Sample number	Isolate reference number[1]	Race[2]	Reaction of RHA-340 line[3]	Sample ID	Sample number	Isolate reference number	Race	Reaction of RHA-340 line
DM01-11	1	Ph01-11	100	R	B-B290518-1	30	Ph11-18	110	R
DM02-11	2	Ph02-11	310	R	B-B290518-2	31	Ph12-18	310	R
DM03-11	3	Ph03-11	310	R	B-B300518	32	Ph13-18	114	R
DM04-11	4	Ph04-11	710	R	B-LG310518-1	33	Ph14-18	315	R
DM05-11	5	Ph05-11	710	R	B-LG310518-2	34	Ph15-18	311	R
DM01-13	6	Ph01-13	304	R	B-LG310518-3	35	Ph16-18	711	R
DM02-13	7	Ph02-13	304	R	B-F050618	36	Ph17-18	304	R
1978-R1	8	1978-R1	710	R	B-E050618-1	37	Ph18-18	312	R
DB0	9	DB0	700	R	B-E050618-2	38	Ph19-18	312	R
LGB	10	LGB	713	R	B-P080618	39	Ph20-18*	705	R
1SM	11	1SM*[4]	304	R	B-B120618	40	Ph21-18	711	R
2SEP	12	2SEP*	700	R	B-P130618-1	41	Ph22-18*	301	R
DM01-15	13	Ph01-15	310	R	B-P130618-2	42	Ph23-18*	715	S
DM02-15	14	Ph02-15	310	R	B-P130618-3	43	Ph24-18*	715	S
DM03-15	15	Ph03-15	310	R	B-P130618-4	44	Ph25-18*	715	S
DM04-15	16	Ph04-15	314	R	B-P130618-5	45	Ph26-18*	715	S
DM01-16	17	Ph01-16	705	R	B-P130618-6	46	Ph27-18*	715	S
DM02-16	18	Ph02-16	704	R	B-LG140618	47	Ph28-18	114	R
DM01-17	19	Ph01-17	317	R	B-S190618	48	Ph29-18	714	R
B-B190418	20	Ph01-18	305	R	B-S280618	49	Ph33-18	304	R
B-S040518-1	21	Ph02-18	715	R	B-S230419 (SYT-1)	50	Ph01-19	705	R
B-S040518-2	22	Ph03-18	714	R	B-S080519-1 (2019HU01)	51	Ph02-19	715	R
B-S100518	23	Ph04-18	315	R	B-S080519-2 (2019-SE01)	52	Ph03-19	715	R
B-B180518	24	Ph05-18	311	R	B-S230519-1 (GIB-1)	53	Ph06-19	505	R
B-B210518-1	25	Ph06-18	311	R	B-S230519-2 (GIB-2)	54	Ph07-19	705	R
B-B210518-2	26	Ph07-18	712	R	B-S190520-1	55	Ph01-20	304	R
B-B210518-3	27	Ph08-18	313	R	B-S190520-2	56	Ph02-20	714	R
B-B220518	28	Ph09-18	313	R	B-S270520	57	Ph03-20	715	R
B-S230518	29	Ph10-18	315	R	Ph04-20	58	Ph04-20	705	R

[1] An individual code was assigned to each isolate of *Plasmopara halstedii*, representing a population collected in a single sunflower field.

[2] Race was determined according to the coded virulence formula (CVF) described by Gulya et al. (1998), each of the three digits corresponding to the response of three sets of three sunflower differentials to inoculation with a *Plasmopara halstedii* isolate. The first differential within each set is coded with the number 1, the second differential with 2 and the third one with 4 if a susceptible reaction occurs, whereas resistant reactions are given a value of 0. The sum of all reactions in each set is obtained and its value assigned to each of the three digits of the formula.

[3] Reactions were quantified as resistant (R) or susceptible (S).

[4] Asterisks show isolates from countries other than Spain.

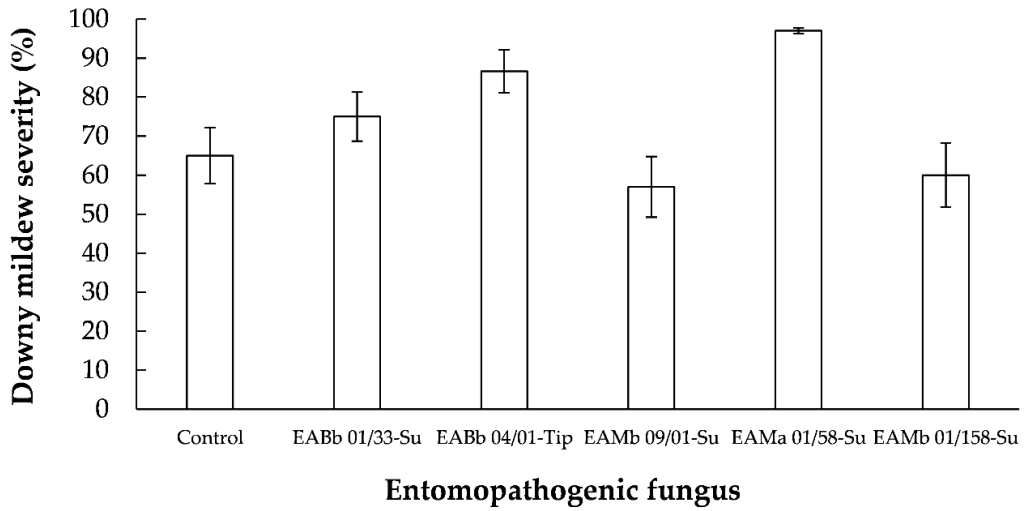

Figure V.1. Downy mildew severity (%) in sunflower plants inoculated with *Plasmopara halstedii* and treated with five entomopathogenic fungi. Control plants were inoculated with *P. halstedii* and treated with water. Vertical bars represent the standard error of the mean of eight replications. The severity of downy mildew in each plant was assessed by using a percentage scale from 0% (no symptoms) to 100% (cottony fungal growth completely covering the cotyledons and first pair of leaves, and also evident in the base of the stem).

Table V.3. Shoot height (cm), root length (cm) and shoot and root dry weight (g) of young sunflower plants treated with five entomopathogenic fungi and inoculated with isolate 1SM of *Plasmopara halstedii*. Seedlings were treated with 5 µl of conidial suspensions (10^7 conidia ml^{-1}) of each entomopathogenic fungus and, 24 h later, inoculated with the same volume of a zoospore suspension (4×10^4 zoospores ml^{-1}) of *P. halstedii*. The controls were treated with 5 µl of sterile deionized water instead of entomopathogenic suspensions, followed by inoculation with *P. halstedii* 24 hours later. Treatment and control plants were grown on Hoagland & Knop's culture medium in a growth chamber at 24 °C with a photoperiod of 14 h of light for 14 days

Entomopathogenic fungus (EF)	Shoot height [1] (cm)	Root length (cm)	Shoot dry weight [2] (g)	Root dry weight (g)
Control	9.06 ± 1.55 [3]	9.11 ± 0.86	0.62 ± 0.15	0.16 ± 0.05
EABb 01/33-Su	7.33 ± 1.26	8.78 ± 0.67	0.55 ± 0.12	0.15 ± 0.03
EABb 04/01-Tip	7.33 ± 1.44	9.20 ± 1.03	0.58 ± 0.16	0.18 ± 0.04
EABb 09/01-Su	9.83 ± 1.70	9.30 ± 0.67	0.84 ± 0.19	0.21 ± 0.04
EAMa 01/58-Su	10.23 ± 1.99	7.73 ± 0.86	0.58 ± 0.15	0.15 ± 0.03
EAMb 01/158-Su	8.69 ± 1.84	6.91 ± 0.86	0.58 ± 0.16	0.13 ± 0.04

[1] Lengths were assessed 14 days after treatment. [2] For dry weight evaluation, plants were dried at 60 °C for 96 h 14 days after treatment. [3] Mean value of eight replications ± standard error.

V.3.3. Effect of entomopathogenic fungi on sunflower growth

Plants were significantly affected by the treatments with EF in the four variables estimated: shoot height ($P = 0.0011$), root length ($P = 0.0424$), shoot dry weight ($P = 0.0007$) and root dry weight ($P = 0.0408$).

The strain EABb 01/33-Su caused the lowest values in the four variables of plant growth. Also, it was the only treatment significantly different from the others both in height and in dry weight of the shoot, whereas strains EAMb 09/01-Su and EABb 04/01-Tip were also significantly different from the other treatments in both length and weight of root system, respectively (Table V.4). The height of the plants and length of their roots ranged from 9.83 and 9.17 cm (EABb 01/33-Su), respectively, to 14.61 (averaged across all the treatments except EABb 01/33-Su) and 11.57 cm (averaged across the Control, EABb 04/01-Tip, EAMa 01/58-Su and EAMb 01/158-Su), respectively. Dry weight of shoots of the plants varied between 0.67 g (EABb 01/33-Su) and 1.11 g (average for all the remaining treatments). Dry weight of roots ranged from 0.24 g (EABb 01/33-Su) to 0.39 g (Control) (Table V.4).

The recovery percentages of the five EF when plants were only treated with the EF are shown in Table V.5. The five EF strains were isolated from the sunflower plants, although with percentages of isolation that varied between plant part and EF strain. Also, strains EABb 04/01-Tip and EAMb 01/158-Su were recovered from all parts of the plant (Table V.5). The molecular analyses showed that strains EABb 04/01-Tip and EAMa 01/58-Su successfully colonised the inside of the stems and were able to persist at least until the end of the experiment (14 days), since diagnostic bands of the EF were observed in samples of the four replications (individual plants). These EF-specific lower molecular weight bands were simultaneously visible with the 750 bp from the DNA of sunflower (Figure V.2). In the case of strains of *Metarhizium* EAMb 09/01-Su and EAMb 01/158-Su, the diagnostic band was only observed in one of the four replicates (Figure V.2). Finally, no endophytic ability was detected for the *B. bassiana* strain EABb 01/33-Su, since the band diagnosing this species was not observed in any of the plants (data not shown).

Table V.4. Height (cm), length of root system (cm) and dry weight (g) of shoot and root system of sunflower young plants treated with five strains of entomopathogenic fungi. Seedlings were treated with 5 µl of conidial suspensions [or 5 µl of sterile deionized water (Control)] and grown on Hoagland & Knop's culture medium in a growth chamber at 24 °C with a photoperiod of 14 h of light for 14 days

Entomopathogenic fungus (EF)	Shoot height [1] (cm)	Root length (cm)	Shoot dry weight [2] (g)	Root weight (g)
Control	14.72 ± 0.83 [3] a [4]	12.33 ± 0.93 a	1.15 ± 0.06 a	0.39 ± 0.03 a
EABb 01/33-Su	9.83 ± 1.54 b	9.17 ± 1.16 c	0.67 ± 0.09 b	0.24 ± 0.03 c
EABb 04/01-Tip	13.03 ± 0.94 a	11.10 ± 0.49 abc	0.98 ± 0.09 a	0.28 ± 0.02 bc
EABb 09/01-Su	15.44 ± 0.73 a	9.99 ± 0.62 bc	1.20 ± 0.13 a	0.32 ± 0.05 abc
EAMa 01/58-Su	15.07 ± 0.70 a	11.64 ± 0.29 ab	1.09 ± 0.07 a	0.32 ± 0.04 abc
EAMb 01/158-Su	14.80 ± 0.74 a	11.22 ± 0.49 abc	1.13 ± 0.05 a	0.33 ± 0.02 ab

[1] Lengths were assessed 14 days after treatment. [2] For dry weight evaluation, plants were dried at 60 °C for 96 h 14 days after treatment. [3] Mean value ± standard error. [4] Data were analysed separately for each of the four variables. In each column, means with a common letter are not significantly different according to Fisher's Least Significant Difference test.

V.4. Discussion

At least 42 races of *P. halstedii* have been identified around the world (Sedlarova et al., 2016). What is of interest in our study is that it shows that over 50% of those races, i.e. 22, have been found in Spain between 2011 and 2020, which constitutes an important increase in races compared to previous studies (Molinero-Ruiz et al., 2002; 2008), and reveals that the pathogen has evolved in terms of both diversity and pathogenicity. In fact, Viranyi et al. (2015) suggested that the increasing numbers of reports of new races indicated that *P. halstedii* shows evolution for virulence according to selection pressure from its host. The large number of races identified in Spain in this study demonstrates that racial profiles of downy mildew are shifting, as suggested by recent studies (Garcia-Carneros and Molinero-Ruiz, 2017; Molinero-Ruiz, 2019). Among all the isolates sampled in the country in this work, race 310, which was the most common one between 1994 and 2006 (Molinero-Ruiz et al., 2002; 2008), was frequently identified. Currently, race 310 is less frequent, since it was found only in 12.2% of the isolates sampled between 2011 and 2020. Moreover, this race was scarcely found from 2015 on, when several other and new races, e.g. 311 and 705, had a significant presence. In fact, in this study we describe for the first time the presence of 13 races of *P. halstedii* in Spain: 304, 305, 311, 313, 314, 315, 317, 704, 705, 712, 713, 714 and 715. Notwithstanding the limited number of sunflower isolates from countries other than Spain, we found that races 304 and 700 are present in Portugal, whereas race 705 is present in Romania, race 301 is present in Italy and race 715 is present in Italy and France. To the best of our knowledge, and although the four races were described in other European countries (Viranyi et al., 2015; Sedlarova et al., 2016) none of them had been reported in any of the four countries before (Viranyi et al., 2015; Spring, 2019).

Table V.5. Isolation of five endophytic entomopathogenic fungi (EF) on Sabouraud Dextrose Agar from roots, stem and leaves of sunflower young plants treated with the aforementioned EF or with sterile deionized water (Control). Seedlings were treated and grown on Hoagland & Knop's culture medium in a growth chamber at 24 °C with a photoperiod of 14 h of light for 14 days before tissue samples were plated for fungal isolation. From each plant, one sample each of roots, stem and leaves was plated

Entomopathogenic fungus	Roots	Stem	Leaves
Control	0.0 [1]	0.0	0.0
EABb 01/33-Su	87.5	0.0	0.0
EABb 04/01-Tip	30.0	20.0	10.0
EABb 09/01-Su	20.0	22.2	0.0
EAMa 01/58-Su	20.0	0.0	0.0
EAMb 01/158-Su	20.0	30.0	20.0

[1] Isolation was expressed as the percentage (%) of plated tissue samples that showed EF growth.

Particularly interesting is the identification of a number of races overcoming the Pl_6 resistance gene present in HA-335 and noted with a 4, 5, 6 or 7 as the last digit. According to our results for Spain, race 304 of *P. halstedii* existed in the country in as early as 2013. Similarly, the new race 705 had already been recovered in 2016 (Garcia-Carneros and Molinero-Ruiz, 2017). Overall, 55% of the isolates characterized in this

study (32 out of 58 isolates) were capable of infecting the resistant line HA-335. This was the case with several isolates from Spain and one (race 304) from Portugal, but also with all the isolates of Romania (race 705), Italy (race 715) and France (race 715). In addition, all those recovered in Spain in 2019 and 2020 were also pathogenic to HA-335 (races 304, 505, 705, 714 and 715). Of notice, sunflower RHA340 is resistant to isolates of race 715 from Spain whereas it is susceptible to isolates of race 715 from France and Italy. This shows that isolates having the same code (i.e. 715) can lead to different reactions in some genotypes that are widely used in sunflower breeding for resistance to downy mildew (i.e. RHA340) and suggests the need of updating the nine differentials that were internationally agreed upon more than 20 years ago to be utilized for race identification of *P. halstedii*. In any case, our results agree with those of other authors from the Czech Republic and Hungary (Sedlarova et al., 2016; Trojanova et al., 2018; Nisha et al., 2020) and confirm that the previously suggested widening of distribution of highly virulent *P. halstedii* races (Molinero-Ruiz, 2019) is a reality in Europe.

When we assessed the effect of five EF strains against downy mildew in sunflower in an axenic culture, none of them exerted a significant disease reduction effect. In a previous study in which we challenged *V. dahliae* and *C. helianthi* of sunflower with the same five EF and under *in vitro* conditions (plates with culture medium), we found that strains EABb 01/33-Su (*B. bassiana*) and EAMb 09/01-Tip (*M. brunneum*) were able to inhibit the mycelial growth of both sunflower pathogens (Miranda-Fuentes et al., 2020a). Furthermore, these strains significantly reduced verticillium wilt severity in sunflowers grown in the greenhouse for 9 weeks (Miranda-Fuentes et al., 2020a). Several studies proved that strains of the EF *B. bassiana* significantly reduced downy mildew incidence and severity in grapevine after leaf sprays (Jaber, 2015; Rondot and Reineke, 2019). Whereas none of the five EF strains had an effect on sunflower growth when combined with *P. halstedii*, some of them reduced some of the growth parameters when applied alone. Similarly, short-term lower vegetative growth was reported in sorghum (Raya-Diaz et al., 2017a) and wheat plants (Gonzalez-Guzman et al., 2020a; 2020b) 2 to 4 weeks after being treated with two of the strains we used in this study (*B. bassiana* EABb 04/01-Tip and *M. brunneum* EAMa 01/58-Su). Those authors suggested that detrimental effects of the EF on plants could be caused by direct competition between plant and EF for nutrients (Raya-Diaz et al., 2017a), as well as by fungal detraction of carbon from photosynthesis (Gonzalez-Guzman et al., 2020a). Furthermore, treatments with EF may cause stresses, thus jeopardizing the performance of crops (Partida-Martinez and Heil, 2011; Behie et al., 2017; Gonzalez-Guzman et al., 2020a; 2020b). However, most of the aforementioned stresses are produced in early stages of plant development and their effects disappear at the end of the plant cycle (Raya-Diaz et al., 2017a; 2017b; Gonzalez-Guzman et al., 2020a; 2020b), causing higher yields (Gonzalez-Guzman et al., 2020a; 2020b) or more pronounced vegetative growth (Raya-Diaz et al. 2017a; 2017b). Protection against plant pathogens can even be conferred in late stages of plant growth (Miranda-Fuentes et al., 2020a). Since our experiments were carried out in an *in vitro* axenic culture, within a short time-lapse (14 days) and with only 24 hours elapsing between the EF application and inoculation with *P. halstedii*, these conditions prevented the EF from fully expressing their potential. Our previous study reported the efficacy of EF against verticillium wilt following their application to the soil one month before inoculation

with *V. dahliae* (Miranda-Fuentes et al., 2020a). Also, these EF strains are highly adapted to the soil environment (Garrido-Jurado et al., 2017). The effect of the EF included in this study against sunflower downy mildew could probably be significant under experimental conditions that favour their survival and competence in the growth substrate, and these should be explored in future investigations.

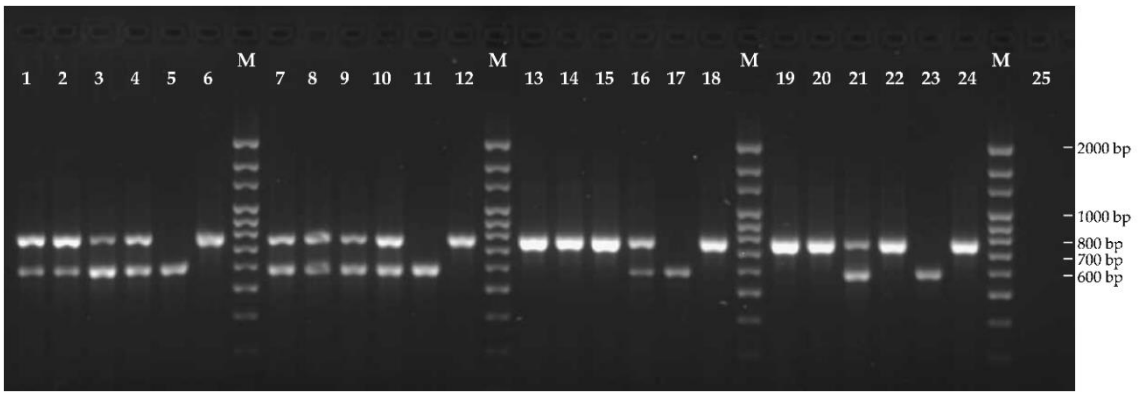

Figure V.2. Bands from PCR amplification of the ribosomal DNA region (5.8S rDNA and internal transcribed spacers 1 and 2) with the universal primer set ITS5 and ITS4 from individual sunflower plants only treated with entomopathogenic fungi (EF). The higher molecular weight band is sunflower-specific, and the lower molecular weight band is EF-specific. M, 100–2000 bp BrightMAX™ DNA ladder (Canvax Biotech); Lanes 1–4, DNA from four individual plants of sunflower breeding line HA-304 treated with *Beauveria bassiana* strain EABb 04/01-Tip; Lane 5, DNA of EABb 04/01-Tip; Lane 6, DNA of sunflower HA-304; Lanes 7–10, DNA from four individual plants of sunflower breeding line HA-304 treated with *Metarhizium brunneum* strain EAMa 01/58-Su; Lane 11, DNA of EAMa 01/58-Su; Lane 12, DNA of sunflower HA-304; Lanes 13–16, DNA from four individual plants of sunflower breeding line HA-304 treated with *M. brunneum* strain EAMb 09/01-Su; Lane 17, DNA of EAMb 09/01-Su; Lane 18, DNA of sunflower HA-304; Lanes 19–22, DNA from four individual plants of sunflower breeding line HA-304 treated with *M. brunneum* strain EAMb 01/158-Su; Lane 23, DNA of EAMb 01/158-Su; Lane 24, DNA of sunflower HA-304; Lane 25, negative control of PCR amplification.

Regarding the endophytic behaviour of the EF used in this study, *B. bassiana* EABb 04/01-Tip and *M. brunneum* EAMa 01/58-Su were able to consistently colonize the sunflower plants. It is worthwhile mentioning that we worked under axenic conditions and that the colonization of young plants by EF was assessed 14 days after treatment. The endophytic property of these EF strains was previously reported in other plant species and systems, such as sorghum (Raya-Diaz et al., 2017a; 2017b), melon (Miranda-Fuentes et al., 2020b) and wheat (Gonzalez-Guzman et al. 2020a; 2020b). In the case of sunflower, plant colonization by these strains has already been reported through both microbiological (Raya-Diaz et al., 2017a) and molecular (Miranda-Fuentes et al., 2020a) approaches. Most studies focus on transient colonization of plants by EF after foliar spraying (Resquin-Romero et al., 2016; Miranda-Fuentes et al., 2020b), but there is strong evidence of long-term endophytic colonization following soil treatment (Raya-Diaz et al., 2017a; Miranda-Fuentes et al., 2020a). However, colonization patterns tend to be erratic and inconsistent, especially when assessed through the re-isolation of the EF from plant tissue (Jaber, 2015; Rondot and Reineke, 2019). Both colonization and endophytic behaviour are largely dependent on the inoculation procedures, the host plant and the fungal species (Resquin-Romero et al., 2016; Raya-

Diaz et al., 2017a; 2017b; Rondot and Reineke, 2019; Gonzalez-Guzman et al., 2020a; 2020b).

This study provides information on two relevant topics, viz. the racial distribution of *P. halstedii* in Spain and other European countries in recent years and the role of EF as endophytic colonizers of sunflower and possible future biological control agents against downy mildew. Regarding the first topic, information on the races of *P. halstedii* is an important decision-making tool for farmers, who have the option of choosing the hybrids they grow based on their genetic resistance; and for sunflower breeders, who will find baseline information for their breeding programmes. With respect to the second topic, EF could have a great potential for the integrated management of sunflower diseases, as they have shown significant effects against *V. dahliae* and *C. helianthi* (Miranda-Fuentes et al., 2020a). This research on their efficacy against downy mildew is a first step in the biological control of the disease. Future research should elucidate whether some of the tested strains are effective against downy mildew under experimental conditions involving a soil system that favours their bioactivity. This issue appears to be a vital one due to the obligate nature of *P. halstedii* infections and to the strong dependence of *Metarhizium* and *Beauveria* species on soil ecology and characteristics.

V.5. Acknowledgments

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CAPÍTULO VI. DISCUSIÓN GENERAL

Los ascomicetos mitospóricos entomopatógenos (AME) han demostrado, en las últimas décadas, hallarse a la vanguardia entre los agentes de control biológico de plagas (Quesada-Moraga y Santiago-Alvarez, 2008; Quesada-Moraga, 2020). Como aspectos más destacables, pueden señalarse su gran virulencia frente a artrópodos fitófagos (Quesada-Moraga et al., 2014), su modo de acción por contacto pero también por la vía endofítica (Butt et al., 2013; Vega, 2018; Quesada-Moraga et al., 2020), la notable diversidad de cepas registradas para su empleo comercial (Li et al., 2010; Lacey et al., 2015; Quesada-Moraga et al., 2020), su rápida y creciente penetración en el mercado de los medios de defensa fitosanitaria (Lacey et al., 2015; Reportsmonitor.com, 2019), su compatibilidad con otros agentes de biocontrol (Gonzalez-Mas et al., 2019) y, por último, las aplicaciones derivadas de sus nuevas funciones ecológicas con impacto en el crecimiento vegetal y en la protección de la planta frente a otros estreses de tipo biótico, como enfermedades, o abiótico, como deficiencias nutricionales, hídricas o térmicas (Ownley et al., 2010; Jaber y Ownley, 2018; Quesada-Moraga, 2020).

La presente Tesis incide en el empleo combinado de los AME con otros agentes de biocontrol y en su antagonismo frente a microorganismos fitopatógenos y el efecto de control de las enfermedades que ocasionan. De este modo, mientras que el primer tema se desarrolla en los capítulos II y III, el segundo lo hace en los capítulos IV y V.

La compatibilidad entre agentes de biocontrol puede depender en gran medida no solo de los organismos implicados, sino también del sistema, de la estrategia de aplicación o, incluso, de los tiempos de liberación de cada agente (Baverstock et al., 2005; Emami et al., 2013; Mohammed y Hatcher, 2017; Shrestha et al., 2017; Gonzalez-Mas et al., 2019), de manera que algunos autores han puesto de manifiesto un mejor control de insectos fitófagos derivado de la aplicación conjunta (Kryukov et al., 2018), mientras que otros han referido lo contrario (Oreste et al., 2015). En la presente Tesis se ha empleado un sistema tritrófico integrado por un insecto fitófago de gran importancia agrícola (el noctuido polífago *Spodoptera littoralis*), una cepa de AME muy próxima a su desarrollo comercial (la cepa EAMa 01/58-Su de *Metarhizium brunneum*) y un himenóptero endoparasitoide candidato para el biocontrol del fitófago (*Hyposoter didymator*), con dos escenarios completamente diferentes: una simulación de laboratorio para la aplicación a diferentes tiempos del parasitoide y el hongo, este último aplicado por vía directa mediante inoculación del fitófago (capítulo II), y un modelo *in planta* con melón donde se han comparado dos vías de aplicación del AME, inoculación del fitófago y vía endofítica, y su incidencia sobre la mortalidad de *S. littoralis* y el potencial reproductivo del parasitoide (capítulo III).

En la primera aproximación a la aplicación simultánea, descrita en el capítulo II, se ha revelado que la cepa EAMa 01/58-Su reduce significativamente la esperanza de vida de los adultos de *H. didymator* cuando se inoculan mediante pulverización con suspensiones de conidios, correlacionándose la tasa de mortalidad y el aislamiento fúngico a partir de los cadáveres con la concentración de la suspensión empleada en el tratamiento. De forma similar, otros autores han señalado que la aplicación directa de suspensiones de hongos entomopatógenos sobre himenópteros parasitoides bracónidos y

eulófidos puede afectar a su longevidad en situaciones de "worst-case scenario" con aplicación por pulverización o inmersión en elevadas dosis de distintas cepas fúngicas en escenarios poco probables en situaciones reales (Castillo et al., 2009; Matias da Silva et al., 2016). Sin embargo, en el presente trabajo, el AME no afecta significativamente el potencial reproductivo de los parasitoides pulverizados con él durante los días previos a su muerte, tal como pusieron de manifiesto diversos autores en condiciones similares con otros himenópteros parasitoides eulófidos y afelínidos (Labbe et al., 2009; Tamayo-Mejia et al., 2015). Este resultado alberga gran importancia, puesto que el potencial reproductivo de *H. didymator* alcanza su máximo a las 36 h de la emergencia del adulto (Hatem et al., 2016), esto es, antes de que pueda morir debido al contacto con el entomopatógeno. No obstante lo anterior, ha de tenerse en cuenta que la aplicación directa del AME sobre el enemigo natural mediante pulverización es un caso extremo y poco probable en condiciones reales.

Cuando se aplicaron el hongo y el parasitoide de manera combinada en dos estrategias diferentes (inoculación de larvas de *S. littoralis* con el AME de manera previa a su exposición al parasitoide y vicecersa), se observó que la mortalidad de *S. littoralis* fue significativamente mayor en todos los tratamientos que incluían al parasitoide en comparación a aquellos que incluían únicamente el hongo, mientras que en los tratamientos combinados (hongo + parasitoide a diferentes tiempos) se obtuvieron los valores más altos de mortalidad del noctuido. Ni el orden ni el tiempo de aplicación de ambos agentes de biocontrol resultaron de gran importancia para el control del fitófago, no así la edad de las larvas de *S. littoralis*, que condicionó de manera crucial su susceptibilidad al entomopatógeno y, especialmente, al parasitoide, tal y como se ha descrito con anterioridad para el mismo sistema noctuido-parasitoide (Hatem et al., 2016). El efecto de la aplicación combinada de ambos agentes en el control de la rosquilla negra resultó aditivo en todos los escenarios de acuerdo al test chi cuadrado, sin importar el orden de aplicación ni el tiempo de diferencia entre la exposición a ambos. En contraste, otros autores han señalado que el tiempo de liberación de hongos entomopatógenos y parasitoides influye de manera significativa en el control del pulgón del melocotonero, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) (Emami et al., 2013; Mohammed y Hatcher, 2017).

Asimismo, se ha observado una reducción significativa en el número de hemocitos presentes en la hemolinfa de las larvas de *S. littoralis* parasitadas, lo que se relaciona con la mayor eficacia del entomopatógeno en aplicaciones posteriores a estas mismas larvas en comparación con su efecto sobre larvas sanas no expuestas al parasitoide. Este resultado, de gran aplicación práctica, fue descrito previamente en otros sistemas experimentales en los que diversos insectos fitófagos fueron parasitados por himenópteros y, posteriormente, expuestos a AME, teniendo la infección por los entomopatógenos un mayor efecto sobre los fitófagos que habían sido parasitados (King y Bell, 1978; Powell et al., 1986).

En el capítulo III se ha explorado la vía endofítica de la cepa EAMa 01/58-Su, con porcentajes de colonización superiores al 90% en las plantas de melón tratadas mediante pulverización foliar. Este resultado, similar a los descritos en el mismo grupo de investigación previamente (Resquin-Romero et al., 2016; Garrido-Jurado et al., 2017), pone de manifiesto la excelente aptitud del AME como endófito. Al igual que en

el capítulo II, en este capítulo se ha observado que el control de la rosquilla negra depende principalmente del parasitoide. A lo largo de tres experimentos diferentes, el primero *in vitro* con larvas de *S. littoralis* alimentadas a partir de discos foliares de planta colonizada por el AME y los otros dos con las larvas del lepidóptero confinadas junto al parasitoide en la propia planta, se han comparado tres vías de aplicación del hongo (endofítica más contacto con estructuras fúngicas, estrictamente endofítica e inoculación de las larvas al igual que en el capítulo II). Además, en el segundo experimento se han comparado distintos tiempos de liberación para ambos agentes, mientras que en el tercero se ha simulado un modelo saturado en el que las larvas han permanecido en contacto con ambos agentes hasta su muerte. No obstante, ni la vía de aplicación del hongo ni el tiempo han tenido influencia en el control del noctuido en ninguno de los tres experimentos de compatibilidad del capítulo III, dependiendo este fundamentalmente de *H. didymator*. Por el contrario, si analizamos las causas de la muerte de las larvas de rosquilla negra, sí que hay una clara influencia: la inoculación de las larvas con el AME ha sido el método que ha originado una mayor recuperación del hongo a partir de los cadáveres, mientras que el mayor parasitismo completo (es decir, con desarrollo posterior del parasitoide) se ha obtenido en los tratamientos en los que las larvas no se han visto expuestas al hongo, viéndose en ocasiones reducido en los tratamientos que incluían hongo.

En el capítulo III se presentan, asimismo, los resultados de un estudio histológico en el que se obtuvieron, a distintos tiempos, secciones longitudinales de larvas de *S. littoralis* parasitadas y tratadas con la cepa EAMa 01/58-Su. Aparte de poder seguir la evolución temporal de cada agente de biocontrol en el interior del noctuido, se ha comprobado la ocurrencia del desarrollo simultáneo de hongo y parasitoide, que en la mayoría de ocasiones termina con el segundo devorando al hospedante por completo, sin mayores impedimentos para el desarrollo de su ciclo. Sin embargo, en el capítulo II, en las mismas condiciones, se constató una disminución del potencial reproductivo en individuos de *H. didymator* desarrollados a expensas de larvas de rosquilla negra tratadas con el entomopatógeno, sin que en ningún modo el hongo impidiera al himenóptero alcanzar su estado imaginal.

Por último, en los capítulos II y III se ha observado una preferencia significativa de las hembras del parasitoide por aquellas larvas de *S. littoralis* que no han tenido contacto con la cepa EAMa 01/58-Su de *M. brunneum*, ya sea mediante inoculación de las larvas en una suspensión de conidios del AME (capítulo II) o por ingestión de hoja colonizada endofíticamente por el hongo (capítulo III). La razón más probable para justificar el rechazo del enemigo natural por las presas expuestas al entomopatógeno puede ser la capacidad del parasitoide para detectar y, en consecuencia, evitar al hongo, como sugirieron Mesquita y Lacey (2001): esta podría ser la explicación más obvia para lo observado en el capítulo II, en el que las larvas de *S. littoralis* fueron inoculadas con el AME y ofrecidas al parasitoide de manera casi inmediata. Tampoco puede descartarse que las hembras del himenóptero prefieran las larvas con un mejor estado de salud (Hatem et al., 2016), rechazando las que, al alimentarse de planta colonizada por el AME (capítulo III), han experimentado un desarrollo anómalo o subóptimo (Resquin-Romero et al., 2016). A pesar de ello, en ambos experimentos se dispuso un tiempo reducido (5 horas) para favorecer la elección del parasitoide, habiéndose constatado en los capítulos II y III que, si el tiempo de exposición a *H. didymator* es suficientemente

prolongado, el himenóptero terminará por parasitar a la mayor cantidad de larvas posible, al menos en los rangos estudiados (24 h y tandas de 10 larvas de *S. littoralis* por cada hembra de *H. didymator*). En consecuencia, la elección del parasitoide no debería suponer una limitación a su aplicación para el control de la rosquilla negra en condiciones reales, si bien debería estudiarse en profundidad.

En síntesis, los capítulos II y III de la presente Tesis constituyen un primer paso para abordar el control de *S. littoralis* mediante *H. didymator* y AME de manera conjunta. Aunque ya se había descrito el potencial de ambos agentes por separado para el control del noctuido (Hatem et al., 2016; Resquin-Romero et al., 2016), los trabajos presentados en los capítulos II y III son los primeros en los que se describe su empleo combinado. De igual manera, el capítulo II aporta la primera información sobre los efectos de un AME sobre *H. didymator*, mientras que el capítulo III presenta la primera observación histológica de este parasitoide. Los bioensayos del capítulo II presentan un escenario *in vitro* más teórico y los del capítulo III representan un escenario mucho más real *in planta*, aunque es necesario establecer experimentos en condiciones reales de invernadero que permitan discernir la eficacia de ambos agentes sobre el cultivo establecido, de cara a establecer estrategias factibles que puedan aplicarse en el campo, o bien en invernadero, dependiendo del sistema agrario que se contemple.

En los capítulos IV y V se ha explorado el potencial de los AME *B. bassiana* y *M. brunneum* como candidatos para el control biológico de enfermedades de girasol. En un cultivo extensivo como el girasol, y para luchar contra enfermedades causadas por patógenos de suelo, cualquier estrategia exitosa debe aplicarse en el momento de la siembra o poco después de esta. En el capítulo IV, las cinco cepas de los entomopatógenos inhibieron, en distinta medida, el crecimiento de los hongos patógenos de girasol *V. dahliae* y *C. helianthi* al enfrentarlos mediante cultivos duales. La respuesta de *V. dahliae* dependió del aislado del fitopatógeno utilizado, mientras que la ausencia de significación del factor aislado en la inhibición de *C. helianthi* se explicó por la escasa diversidad de los aislados del patógeno utilizados. En el caso de *V. dahliae*, los resultados obtenidos se hallan en consonancia con los presentados por otros autores en cuanto a rangos de inhibición, mecanismos de antagonismo y la respuesta del fitopatógeno a las mismas cepas de entomopatógenos usadas en el presente trabajo (Lozano-Tovar et al., 2013). En términos generales, la cepa EABb 01/33-Su (*B. bassiana*) fue la que inhibió en mayor medida a ambos fitopatógenos, mientras que la cepa EAMb 01/158-Su (*M. brunneum*) fue la de menor potencial antagonista. Cabe destacar que, de acuerdo a observaciones tanto visuales como al microscopio, el antagonismo de los cinco AME se asoció a dos mecanismos diferentes: antibiosis y competencia del AME sobre el fitopatógeno. Cuatro de las cepas de AME ejercieron solo uno de estos dos antagonismos, mientras que la cepa EAMa 01/58-Su causó una inhibición tanto por competencia como por antibiosis con el hongo fitopatógeno, tal como mencionaron Lozano-Tovar et al. (2013). De manera análoga, Varo et al. (2016) refirieron que un abanico muy amplio de agentes de biocontrol de naturaleza fúngica, incluyendo al género *Fusarium*, ejercieron estos dos modos de inhibición de aislados de *V. dahliae* patógenos de olivo.

Por otro lado, nuestros resultados relativos a la reducción significativa de síntomas de verticilosis en las plantas coinciden con las observaciones realizadas en

otros trabajos de control biológico de *V. dahliae* patógeno de olivo (Varo et al., 2016; Lozano-Tovar et al., 2017).

En contraste con el capítulo IV, ninguno de los cinco AME tuvo un efecto significativo sobre la severidad de síntomas del mildiu de girasol en el capítulo V. En este capítulo hubo diferencias metodológicas reseñables respecto al previo: el lapso de tiempo entre la aplicación de los entomopatógenos y la de *P. halstedii* fue muy reducido; los AME se aplicaron directamente a las plántulas y no al suelo; por último, se utilizaron distintos sistemas, macetas con sustrato versus cultivo axénico, por lo que las plantas no se incubaron hasta el final de su ciclo, cuando hubiera podido detectarse, en caso de existir, promoción de crecimiento asociada a la colonización endofítica por los AME (Sanchez-Rodriguez et al., 2016; 2018; Gonzalez-Guzman et al., 2020a; 2020b). Otros autores han demostrado que la pulverización foliar del AME *B. bassiana* es capaz de ocasionar un alivio de síntomas de mildiu en vid (Jaber, 2015; Rondot y Reineke, 2019). En el caso del mildiu de girasol, el único trabajo publicado en el ámbito del control biológico es el de Nagaraju et al. (2012), quienes proponían que los tratamientos de semilla con *Trichoderma harzianum* podían constituir una herramienta potencial para el control de la enfermedad. En vista de lo anterior, debe profundizarse en gran medida si se pretende que el manejo del mildiu de girasol mediante el uso de AME se convierta en una alternativa aplicable en el campo. En los experimentos plasmados en el capítulo V tampoco se ha observado que los AME beneficiaran a corto plazo el crecimiento vegetal del girasol cultivado *in vitro* ni que influyeran en su desarrollo cuando se aplicaron junto a *P. halstedii*. Por otro lado, en el corto periodo de tiempo durante el que se evaluó el desarrollo de las plantas, y en el caso de los tratamientos con la cepa EABb 01/33-Su, que es la cepa que ha demostrado mejor comportamiento endofítico en trabajos previos (Resquin-Romero et al., 2016; Garrido-Jurado et al., 2017), las plantas presentaron la pauta normal de retraso de crecimiento asociada a etapas iniciales de la colonización endofítica (Partida-Martinez y Heil, 2011; Sanchez-Rodriguez et al., 2016; 2018), mientras que en las cepas EABb 04/01-Tip y EAMb 09/01-Su dicha pauta solo tuvo lugar en el desarrollo del sistema radical. Análogamente, diferentes autores han informado del detrimento en el crecimiento de plantas de sorgo (Raya-Diaz et al., 2017a) y trigo (Sanchez-Rodriguez et al., 2018; Gonzalez-Guzman et al., 2020a; 2020b) los días siguientes a la aplicación de los entomopatógenos, fenómeno que se ha atribuido a la competencia por nutrientes entre planta y hongo, requeridos para el establecimiento del endófito (Raya-Diaz et al., 2017a), y a la sustracción por parte del AME de carbono procedente de la fotosíntesis (Gonzalez-Guzman et al., 2020a). No obstante, estos efectos revierten al final del ciclo de los cultivos, cuando, gracias al establecimiento del hongo endófito, se produce un aumento del crecimiento de las plantas tratadas (Raya-Diaz et al., 2017a; Sanchez-Rodriguez et al., 2018; Gonzalez-Guzman et al., 2020a; 2020b). En los experimentos del capítulo V no resultó posible mantener las plantas de girasol durante más tiempo para poder comprobar la posible promoción del crecimiento por parte de los AME a largo plazo y una vez superado el coste del endofitismo, ya que *P. halstedii* infecta a las plantas en fases muy tempranas impidiendo que estas finalicen el ciclo de cultivo. De hecho, transcurridas las dos semanas de los experimentos, las plantas control presentaban niveles de afección irreversibles y cercanos a la muerte. A la vista de nuestros resultados, la metodología empleada en este capítulo no proporciona a los

AME, adaptados al suelo (Garrido-Jurado et al., 2017), las mejores condiciones para su desarrollo y supervivencia.

En los capítulos III, IV y V se evaluó, mediante diferentes aproximaciones, la aptitud endofítica de los AME, así como su persistencia en el sustrato (capítulo IV). En el capítulo IV, tres de las cepas de AME pudieron aislarse a partir del sustrato dos meses después de aplicarlas a las plantas de girasol mediante tratamiento de suelo. No es la primera vez que se obtienen resultados similares con estas cepas: Raya-Diaz et al. (2017b) describieron que la presencia de las cepas EAMa 01/58-Su (*M. brunneum*) y EABb 04/01-Tip (*B. bassiana*) en el sustrato tras su aplicación podía durar meses, recomendando el tratamiento de suelo como método más eficaz para alargar su persistencia en el medio. Respecto al endofitismo, y aunque la presencia de estos mismos AME en el interior de los tejidos de la planta durante periodos de tiempo cortos ha sido demostrada tanto en los capítulos III (48 horas tras la aplicación) y V (15 días después) como en trabajos de otros autores (Resquin-Romero et al., 2016; Garrido-Jurado et al., 2017), en el capítulo IV se pone de manifiesto que la detección endofítica de los AME puede efectuarse al menos dos meses después del tratamiento de suelo con ellos, aunque únicamente mediante PCR. Particularmente interesante de este trabajo es el descubrimiento de que el endofitismo de los AME en planta de girasol únicamente ocurre cuando las mismas no han sido inoculadas con *V. dahliae*, lo que sugiere que ambos hongos, entomopatógeno y fitopatógeno, establecen una competencia en el suelo y apunta a este como el principal modo de acción de los AME frente a *V. dahliae* en girasol. Además, la inhibición de síntomas de verticilosis fue mayor en las plantas tratadas con aquellas cepas de AME que en los experimentos *in vitro* habían mostrado competencia con *V. dahliae* y/o *C. helianthi* en cuanto a nutrientes y espacio, pero no antibiosis. Esta revelación no resulta sorprendente, ya que los agentes de biocontrol capaces de crecer sobre los patógenos resultan antagonistas más eficaces que aquellos que presentan antibiosis (El-Katatny et al., 2011). De forma similar, la detección molecular de los AME en el capítulo V únicamente tuvo éxito en ausencia de *P. halstedii*, lo que posiblemente se deba a la competencia entre el oomiceto y los hongos entomopatógenos. Los resultados de detección molecular y aislamiento a partir de tejido vegetal no fueron congruentes entre sí, aunque ambos métodos permitieron identificar la colonización endofítica por parte de las cepas empleadas. Otros autores han obtenido resultados similares, advirtiendo sobre la brecha entre ambas metodologías, señalando la tendencia errátil de los aislamientos de tejido foliar (pues a menudo conllevan acusadas oscilaciones en el tiempo e incluso entre repeticiones de un mismo experimento) y recomendando las técnicas moleculares sobre las microbiológicas (Landa et al., 2013; Jaber, 2015; Rondot y Reineke, 2019). Al comparar los elevados y uniformes porcentajes de aislamiento de AME a partir de tejido foliar 48 horas después del tratamiento (capítulo III) con los resultados variables dos semanas después (capítulo V) y con la imposibilidad del aislamiento tras varios meses, pudiendo detectarse solo mediante PCR (capítulo IV), se pone de manifiesto ―pese a las diferentes metodologías― que la colonización endofítica por estos AME posee un carácter eminentemente transitorio, como habían apuntado Resquin-Romero et al. (2016) y Garrido-Jurado et al. (2017). Aun así, lo anterior no es óbice para que los AME puedan detectarse en las plantas tras periodos de tiempo prolongados a partir de tratamientos de suelo y semilla (Raya-Diaz et al., 2017a; 2017b; Gonzalez-Guzman et al., 2020a;

2020b), e incluso alcanzar la semilla de la nueva planta para transmitirse verticalmente a la siguiente generación (Quesada-Moraga et al., 2014).

En la caracterización racial de *P. halstedii* presentada en el capítulo V se han expuesto resultados de gran aplicación práctica: entre los años 2011 y 2020 se han encontrado un total de 23 razas diferentes en Europa, 22 de las cuales se hallan presentes en España. Este número, que supone más del 50% de razas de *P. halstedii* descritas en todo el mundo (Sedlarova et al., 2016), no solo pone de manifiesto la preocupante diversidad patogénica del oomiceto en nuestro país, con importantes implicaciones desde el punto de vista agrícola, sino que además revela la constante evolución racial del patógeno (Molinero-Ruiz et al., 2002; 2008). Como aspecto de interés, cabe destacar la reducción en la presencia de razas muy habituales en nuestro país hasta el año 2006, como la 310 (Molinero-Ruiz et al. 2002; 2008), y la alta frecuencia de otras con una mayor virulencia, incluyendo un total de 13 razas cuya presencia jamás se había descrito en España: 304, 305, 311, 313, 314, 315, 317, 704, 705, 712, 713, 714 y 715 (Molinero-Ruiz et al., 2002; 2008; Molinero-Ruiz, 2019). Del mismo modo, algunas de las razas de *P. halstedii* procedentes de Francia, Italia, Portugal y Rumanía no se habían descrito en ninguno de los referidos países con anterioridad (Viranyi et al., 2015; Sedlarova et al., 2016; Spring, 2019). No menos importante resulta el hecho de que más de la mitad de los aislados obtenidos en los muestreos son capaces de infectar a la línea de girasol resistente HA-335, muy utilizada en mejora de girasol para resistencia al mildiu. En definitiva, los perfiles raciales del patógeno en Europa (tendentes a una mayor diversidad y virulencia) muestran que, actualmente, el control del mildiu de girasol en el continente mediante resistencia genética requiere un conocimiento preciso de la composición y las características patogénicas de las poblaciones de *P. halstedii*.

En resumen, las investigaciones que se desarrollan a lo largo de esta tesis doctoral ponen de manifiesto la destacable versatilidad de los AME desde la óptica del control biológico, puesto que, por un lado, poseen una gran eficacia frente a insectos fitófagos y pueden ser combinados con otros agentes de biocontrol, como parasitoides (capítulos II y III), mientras que, por el otro, pueden defender a la planta en cierta medida frente al ataque de organismos fitopatógenos (capítulo IV), siendo aún necesario profundizar para determinar el efecto de su aplicación en este cultivo frente al mildiu, enfermedad en auge por la aparición de razas del patógeno *P. halstedii* cada vez más virulentas (capítulo V).

VI.1. Referencias

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CAPÍTULO VII. CONCLUSIONES

En el capítulo final de la presente Tesis se exponen las conclusiones generales derivadas de las investigaciones que se detallan en los capítulos II, III, IV y V. Asimismo, en cada una de ellas se indica el capítulo que la fundamenta.

1.- Los tratamientos directos con la cepa EAMa 01/58-Su de *Metarhizium brunneum* sobre el parasitoide *Hyposoter didymator* reducen su esperanza de vida con una relación directa dosis-mortalidad. Sin embargo, su potencial reproductivo no se ve afectado, con niveles de control de *Spodoptera littoralis* por los parasitoides tratados con el hongo entomopatógeno equivalentes a los obtenidos a partir de individuos no tratados. ⎪Capítulo II.

2.- Existen interacciones sinérgicas de pequeña entidad entre *H. didymator* y la cepa EAMa 01/58-Su, como una mayor eficacia del hongo en larvas de *S. littoralis* parasitadas debida a la depresión del sistema inmune que ocasiona el parasitoide, si bien a nivel estadístico todas las aplicaciones combinadas del entomopatógeno y el parasitoide han resultado ser aditivas en lo que al control (mortalidad) del noctuido se refiere, sin importar la estrategia de aplicación. ⎪Capítulo II.

3.- El empleo simultáneo de la cepa EAMa 01/58-Su y el parasitoide *H. didymator* para el manejo de *S. littoralis* es compatible en todos los sistemas y estrategias ensayados (tanto por aplicación directa del hongo como por la vía endofítica en plantas de melón colonizadas por el entomopatógeno), ya que el control (mortalidad) del noctuido que se alcanza es, al menos, igualmente efectivo que la aplicación del parasitoide solo, cuando no existe aditividad. Del mismo modo, ni el tiempo de liberación de cada agente ni su orden tienen impacto estadístico sobre la mortalidad del fitófago, con el parasitoide como factor más importante en el control de *S. littoralis*; aunque las hembras de *H. didymator* han evidenciado una tendencia inequívoca a evitar las larvas expuestas al hongo (ya sea por aplicación directa o por ingestión de tejido vegetal colonizado endofíticamente), con el suficiente tiempo de exposición acaban por parasitarlas igualmente. ⎪Capítulos II y III.

4.- En el interior de las larvas de *S. littoralis* expuestas a ambos agentes de biocontrol se produce el desarrollo simultáneo del entomopatógeno (en forma de cuerpos hifales y micelio) y la larva del parasitoide, observado en un estudio histológico, que debilita sobremanera al noctuido hasta su ineludible perecimiento, que en la mayoría de ocasiones acontece al ser devoradas por completo por la larva de *H. didymator* a pesar del hongo, pudiendo desarrollarse el himenóptero hasta su estadio imaginal pero sufriendo una disminución significativa de su potencial reproductivo. ⎪Capítulos II y III.

5.- Las cepas de *M. brunneum* y *Beauveria bassiana* presentan una destacada aptitud endofítica, tanto transitoria como a largo plazo, así como una prolongada capacidad de persistencia en el suelo (al menos dos meses desde el tratamiento con ellas); ello puede tener una gran utilidad para el control tanto de *S. littoralis* como de microorganismos fitopatógenos. ⎪Capítulos III, IV y V.

6.- Las cinco cepas de *M. brunneum* y *B. bassiana* utilizadas presentan un destacable antagonismo frente a los hongos patógenos de girasol *Verticillium dahliae* y *Cadophora helianthi* que, en el caso de *V. dahliae*, se traduce en valores significativos de inhibición del crecimiento micelial dependientes de la cepa de entomopatógeno y también del aislado del fitopatógeno. ⎪Capítulo IV.

7.- Las cepas de *M. brunneum* y *B. bassiana* utilizadas pueden ejercer dos tipos de antagonismo, observados tanto al microscopio como a simple vista: antibiosis y crecimiento sobre el fitopatógeno, dependiendo el mecanismo de la cepa del entomopatógeno, si bien una de ellas (EAMa 01/58-Su) es capaz de ocasionar ambos. ⎪Capítulo IV.

8.- Las cepas EABb 01/33-Su (*B. bassiana*) y EAMb 09/01-Su (*M. brunneum*), tras ser aplicadas al suelo, disminuyen significativamente la severidad de los síntomas de verticilosis en girasol posteriormente inoculado con *V. dahliae*. Dicha reducción de síntomas puede asociarse a una competencia entre la cepa del entomopatógeno y el aislado fitopatógeno que ocurre en el suelo y es previa a la penetración de este último en la planta. ⎪Capítulo IV.

9.- Ninguna de las cinco cepas de *M. brunneum* y *B. bassiana*, aplicadas en plántulas de girasol inoculadas con *P. halstedii* en cultivo axénico, reduce la severidad de mildiu ni afecta de forma significativa al crecimiento de las plantas. ⎪Capítulo V.

10.- La cepa EABb 01/33-Su, aplicada en ausencia de *P. halstedii*, origina un retraso inicial de los parámetros de desarrollo de la planta posiblemente asociado a su establecimiento endófito, aunque la transitoriedad de este fenómeno debería constatarse utilizando una metodología que permita mantener las plantas de girasol hasta el final de su ciclo. ⎪Capítulo V.

11.- Desde el año 2011 se ha identificado una llamativa evolución de la composición racial de *P. halstedii* tanto en España como en Europa, la cual se caracteriza por una gran diversidad de razas y un aumento de la virulencia de estas. ⎪Capítulo V.

Los resultados del capítulo II se han presentado en el artículo "Compatibility between the endoparasitoid *Hyposoter didymator* and the entomopathogenic fungus *Metarhizium brunneum*: a laboratory simulation for the simultaneous use to control *Spodoptera littoralis*", publicado en el número 76 de la revista Pest Management Science entre las páginas 1060 y 1070, con doi: 10.1002/ps.5616. Esta revista es D1: 7/101 en "Entomology" con un factor de impacto de 3.750.

Los resultados relativos al capítulo III se han presentado en el artículo "Entomopathogenic fungal endophyte-mediated tritrophic interactions between *Spodoptera littoralis* and its parasitoid *Hyposoter didymator*", actualmente publicado online en la revista Journal of Pest Science, con doi: 10.1007/s10340-020-01306-7. Esta revista es D1: 2/101 en "Entomology" con un factor de impacto de 4.578.

Los resultados concernientes al capítulo IV se han presentado en el artículo "Evidence of soil-located competition as the cause of the reduction of sunflower verticillium wilt by entomopathogenic fungi", publicado en el número 69 de la revista

Plant Pathology entre las páginas 1492 y 1503, con doi: 10.1111/ppa.13230. Esta revista es Q1: 21/91 en “Agronomy” con un factor de impacto de 2.169.

Los resultados concernientes al capítulo V se han presentado en el artículo “Updated characterization of races of *Plasmopara halstedii* and entomopathogenic fungi as endophytes of sunflower plants in axenic culture”, aceptado con minor revision en la revista Agronomy. Esta revista es Q1: 18/91 en “Agronomy” con un factor de impacto de 2.603.