Escuela Técnica Superior de Ingeniería Agronómica y de Montes

Departamento de Agronomía

Respuesta de *Aphis gossypii* Glover (Homoptera: Aphididae) y sus enemigos naturales entomófagos a la colonización de plantas de melón por hongos entomopatógenos endófitos

Tesis presentada por Dña. Natalia González Mas para optar por el grado de Doctora por la Universidad de Córdoba

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Córdoba, junio de 2019

TITULO: *RESPUESTA DE APHIS GOSSYPII GLOVER(HOMOPTERA:APHIDIDAE) Y SUS ENEMIGOS NATURALES ENTOMÓFAGOS A LA COLONIZACIÓN DE PLANTAS DE MELÓN POR HONGOS ENTOMOPATÓGENOS ENDÓFITOS*

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Response of *Aphis gossypii* Glover (Homoptera: Aphididae) and its entomophagous natural enemies to the colonization of melon plants by endophytic entomopathogenic fungi

Thesis presented by Mrs. Natalia González Mas to qualify for the degree of Doctor by the University of Cordoba

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University of Cordoba
Cordoba, June 2019

TÍTULO DE LA TESIS: Respuesta de *Aphis gossypii* Glover (Homoptera: Aphididae) y sus enemigos naturales entomófagos a la colonización de plantas de melón por hongos entomopatógenos endófitos.

DOCTORANDO: NATALIA GONZÁLEZ MAS

INFORME RAZONADO DEL/ LOS DIRECTOR/ES DE LA TESIS

El Prof. D. Enrique Quesada Moraga, Doctor Ingeniero Agrónomo, Profesor Catedrático de Producción Vegetal de la Universidad de Córdoba, en el Departamento de Agronomía de la E.T.S.I.A.M., y Responsable del Grupo PAIDI AGR 163 "Entomología Agrícola".

INFORMA: que el trabajo titulado **"Respuesta de *Aphis gossypii* Glover (Homoptera: Aphididae) y sus enemigos naturales entomófagos a la colonización de plantas de melón por hongos entomopatógenos endófitos"** realizado bajo mi dirección por **Dña. Natalia González Mas**, lo considero ya finalizado y que se han completado con éxito todos los objetivos planteados en dicho trabajo de investigación.

Que dicha Tesis Doctoral se va a presentar como compendio de publicaciones, las cuales se indican a continuación:

González-Mas N, Sánchez-Ortiz A, Valverde-Garcia P, Quesada-Moraga E (2019) Effects of endophytic entomopathogenic ascomycetes on *Aphis gossypii* Glover Life-History Traits and interaction with melon plants. Insects. https://doi.org/10.3390/insects10060165. Factor de Impacto: 1.848, posición Q1 (21/96) en "Entomology".

González-Mas N, Quesada-Moraga E, Plaza M, Fereres A, Moreno A (2019) Changes in feeding behaviour are not related to the reduction in the transmission rate of plant viruses by *Aphis gossypii* (Homoptera: Aphididae) to melon plants colonized by *B. bassiana* (Ascomycota: Hypocreales). Biological Control 130, 95-103, https://doi.org/10.1016/j.biocontrol.2018.11.001. Factor de Impacto: 2.112, posición Q1 (13/96) en "Entomology".

Gonzalez-Mas N, Cuenca-Medina M, Gutierrez-Sanchez F, Quesada-Moraga E (2019) Bottom-up effects of endophytic *B. bassiana* on multitrophic interactions between the cotton aphid, *Aphis gossypii*, and its natural enemies in melon. Journal of Pest Science. https://doi.org/10.1007/s10340-019-01098-5. Factor de Impacto: 4.402, posición Q1 (2/96) en "Entomology".

Por todo ello, puede ser presentado para su exposición y defensa como Tesis Doctoral en el Departamento de Agronomía de la Universidad de Córdoba.

Fdo.: Dr. D. Enrique Quesada Moraga

Córdoba, junio de 2019

MENCIÓN DE DOCTORADO INTERNACIONAL

La presente Tesis Doctoral cumple los requisitos establecidos por la Universidad de Córdoba para la obtención del título de Doctor con Mención Internacional:

- Estancia internacional predoctoral de 3 meses (del 22 de mayo al 18 de agosto de 2017) en el grupo de investigación "Fundamental and applied research in chemical ecology" de la Universidad de Neuchâtel (Neuchâtel, Suiza), bajo la supervisión del Profesor Ted Turlings, director del Centro de Competencia de Ecología Química (Centre of Competence In Chemical Ecology) de la Universidad de Neuchâtel.

- La Tesis Doctoral cuenta con el informe previo de dos doctores externos con experiencia acreditada pertenecientes a alguna institución de educación superior o instituto de investigación diferente a España:

    - Dr. José A. Pereira, Investigador Sénior del Instituto Politécnico de Bragança, Centro de Investigação de Montanha (CIMO), Portugal.

    - Dr. Eustachio Tarasco, Dipartimento di Scienze del Suolo, della Pianta e degli Alimenti, Università degli Studi di Bari Aldo Moro, Italy.

- Como miembro del tribunal, un doctor perteneciente a alguna institución de educación superior o centro de investigación no español forma parte del tribunal evaluador de la tesis: Dra. Paula Baptista, Departamento Biologia e Biotecnologia, Instituto Politécnico de Bragança, Portugal.

- Parte de la Tesis Doctoral, de acuerdo a la normativa, se ha redactado y se presentará en dos idiomas, castellano e inglés.

La Doctoranda

Fdo.: Natalia González Mas

TESIS PRESENTADA POR COMPENDIO DE ARTÍCULOS

Esta Tesis cumple el requisito establecido por la Universidad de Córdoba para su presentación como compendio de artículos. Consta de 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 referencias en la última relación publicada por Journal Citation Reports (SCI):

González-Mas N, Sánchez-Ortiz A, Valverde-Garcia P, Quesada-Moraga E (2019) Effects of endophytic entomopathogenic ascomycetes on *Aphis gossypii* Glover Life-History Traits and interaction with melon plants. Insects. https://doi.org/10.3390/insects10060165. Factor de Impacto: 1.848, posición Q1 (21/96) en "Entomology".

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La Doctoranda

Fdo.: Natalia González Mas

Los trabajos incluidos en esta Tesis Doctoral han sido financiados por los Proyectos de Investigación AGR-P11-7681, de la Junta de Andalucía, y AGL 2016-80483-R, del Ministerio de Economía y Competitividad. La doctoranda ha contado con la ayuda de una beca FPU del Ministerio de Educación, Cultura y Deporte para la realización de la presente Tesis Doctoral con referencia: FPU13/06342.

Agradecimientos

En primer lugar, dar las gracias a mi director Enrique Quesada, por darme la oportunidad de formar parte de su grupo de investigación. Por haberme transmitido su entusiasmo por la investigación y por su confianza, apoyo y motivación desde mi llegada y a lo largo de todos estos años.

A todos mis compañeros de laboratorio:

Mis compañeros de despacho, María y Meelad que sobre todo vosotros habéis vivido conmigo mis momentos todos los altibajos, alegrías y decepciones, por estar ahí y animarme y ayudarme siempre. A Inma por su predisposición a ayudar siempre.

A mis compañeros y amigos Fernando y María, por vuestra ayuda y colaboración para llevar a cabo esta tesis y por sus ánimos, yo también he aprendido mucho de vosotros. Aunque ha habido momentos en los que os he querido matar ahora os quiero mucho ;)

A los recién llegados Toñi y Rafa, que llevan poco tiempo pero que han colaborado en hacer este final más llevadero.

A Sandra y María Victoria, por su ayuda y ánimos, sobre todo al principio. Cómo echo de menos cruzar la puerta y encontrarte Sandrita.

A todos los compañeros que habéis pasado por el laboratorio y por mi vida, y sin los que esta tesis no habría salido igual seguramente: Lola, Carmen, Álex, Gloria, Enrique, Adrián y Dani.

A Alberto Fereres y a Arancha Moreno, y a todos los compañeros del ICA-CSIC de Madrid, María, Elisa, Jaime, Michelle, Mauricelia, Pilar, Andrés, Casimiro y Laura, por todo lo aprendido allí y ayuda prestada durante mi estancia y a lo largo de estos años. Me hicisteis sentir doblemente en casa. ¡Muchísimas gracias!

Al profesor Ted Turlings, por darme la oportunidad de llevar a cabo una estancia en su laboratorio en Suiza, y por todo lo aprendido allí, todos mis compañeros y amigos que hice durante mi estancia en Suiza: Hao, Luca, Sonia, Charlyne, Diane, Carlos, Apostolos, Juan, Patrick, Pamela, Audrey, Carla y Alfonso. Por hacérmelo más fácil y todos los buenos ratos pasados. A Pili, sin ti si que esta estancia no hubiera sido igual. Por hacerme sentir cerca de casa y esas confesiones en el lago con los cisnes de testigo. Doy gracias por haberte conocido amiga y ojalá coincidamos otra vez.

A Araceli Sánchez, por su paciencia y buen humor trabajando siempre. Por todo lo que he aprendido durante mis visitas al IFAPA de Jaén.

A Pablo Valverde, muchísimas gracias por tu ayuda y ánimos. No sé que haríamos sin ti.

Y, ya por último, a mi familia, mis padres, mi abuela y mi hermana, a los que siento que les he quitado tiempo de estar juntos con esto de haberme asentado en el sur, pero que sin vuestra ayuda y apoyo incondicional esto no habría sido posible. ¡Muchísimas gracias!

Resumen:

La evolución de la agricultura hacia un modelo más sostenible requiere reemplazar progresivamente los insecticidas químicos de síntesis por otras alternativas de control de plagas de insectos más respetuosas con el medioambiente y el ser humano, para garantizar la seguridad e inocuidad alimentarias. El control microbiano de plagas es una de estas alternativas, y dentro de los microorganismos entomopatógenos, los hongos entomopatógenos (HE), por su presencia natural y modo de acción por contacto, presentan un gran potencial para el control de varios grupos de insectos de gran relevancia agrícola, como los fitomizos, y muy en particular los pulgones. Durante el siglo XXI, se han puesto de manifiesto distintas asociaciones de los HE con las plantas con nuevas funciones ecológicas, en la rizosfera, en el filoplano, y como endófitos, que han ampliado las posibilidades de su empleo para el control de plagas, y de forma más amplia, en producción vegetal. Sin embargo, permanecen desconocidos los posibles efectos directos e indirectos de la colonización endofítica de las plantas por los HE no sólo sobre el insecto hospedante, sino sobre las señales químicas producidas por las plantas, y, por tanto, sobre las relaciones insecto fitófago-planta o incluso en las de los insectos fitófagos con sus enemigos naturales

En la presente tesis doctoral se ha estudiado el efecto de la colonización endofítica de plantas de melón por cepas de *Beauveria bassiana* (Balsamo) Vuil. y *Metarhizium brunneum* Petch, tanto sobre la biología del "pulgón del algodón" *Aphis gossypii* Glover, (Homoptera: Aphididae), importante plaga polífaga de cultivos hortícolas, como sobre su comportamiento alimenticio y capacidad de transmitir virus persistentes y no persistentes, y además, el impacto de esta colonización sobre las señales químicas producidas por la planta y las relaciones del pulgón con sus enemigos naturales entomófagos, para su posible empleo en estrategias de control integrado del áfido.

El capítulo II pone de manifiesto que la cepa EABb 01/33-Su de *B. bassiana* reduce significativamente la población de *A. gossypii* tanto cuando los especímenes se alimentan a expensas de hojas pulverizadas y colonizadas, como cuando lo hacen a expensas de hojas colonizadas distantes de las pulverizadas, mientras que las cepas EABb 04/01-Tip de *B. bassiana* y EAMa 01/58-Su de *M. brunneum* solo redujeron la población en la primera circunstancia (aunque a un nivel de significación del 10% también en la

segunda). La eficacia como endófita de la cepa EABb 01/33-Su para manifestar su máxima virulencia en tejidos distantes a los tratados en plantas de melón ha hecho que fuera seleccionada para ser utilizada a lo largo de los experimentos llevados a cabo en los capítulos III y IV. En este capítulo además, se destaca una falta de efecto de la colonización endófitica de plantas de melón con las tres cepas seleccionadas sobre fecundidad total *per capita* de los pulgones 5 días después de la exposición, aunque se detecta una modificación en el comportamiento reproductivo de los pulgones que se alimentan a expensas de plantas colonizadas, que, con respecto a los especímenes sanos, aceleran su actividad reproductiva para alcanzar con anterioridad su máximo potencial biótico. En este capítulo, se describen en primicia diferencias cualitativas y cuantitativas en el perfil de compuestos volátiles liberados por las plantas colonizadas endofíticamente e infestadas posteriormente por *A. gossypii* en comparación con los compuestos que emiten las plantas testigo. Sin embargo, la colonización endofítica de plantas por AMEs no influye en su elección por parte de los pulgones.

En el capítulo III se ha estudiado la influencia de la colonización endofítica sobre el comportamiento alimenticio de los pulgones y a la transmisión de virus persistentes y no persistentes. Mediante el empleo de la técnica de gráficos de penetración eléctrica (EPGs), aplicada por primera vez a pulgones que se alimentan a expensas de plantas colonizadas con hongos entomopatógenos, ha sido posible detectar la actividad del estilete del insecto y relacionarla con los procesos de transmisión de virus en plantas de melón colonizadas con la cepa EABb 01/33-Su de *B. bassiana*. Los resultados revelan que dicha colonización no afecta al comportamiento alimenticio de *A. gossypii* asociado a la inoculación del Cucumovirus no persistente, *Cucumber mosaic virus* (CMV), y al Polerovirus persistente *Cucurbit aphid-borne yellows virus*, pero modifica algunas relacionadas con el proceso de adquisición de virus no persistentes, posiblemente asociadas con alteraciones en el comportamiento alimenticio del pulgón debidas a un mesófilo con estructuras fúngicas o los metabolitos secretados por ellas o incluso por la activación de las defensas de la planta. Además, los ensayos de transmisión llevados a cabo revelan que la incidencia de los dos virus estudiados se ve significativamente reducida cuando las plantas se encuentran colonizadas endofíticamente con la cepa EABb 01/33-Su de *B. bassiana* antes de producirse su infestación con pulgones virulíferos, lo que

posiblemente se debe a la inducción de resistencia sistémica consecuencia de la colonización de la planta por el hongo.

Finalmente, en el capítulo IV se determina el efecto de la colonización endofítica de los AMEs sobre las relaciones tritróficas de los pulgones, no solo con las plantas colonizadas, sino con los enemigos naturales entomófagos de *A. gossypii*, el depredador generalista *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) y el parasitoide de pulgones *Aphidius colemani* (Dalman) (Hymenoptera: Braconidae), cuando estos depredan o parasitan a pulgones que se alimentan a expensas de ellas. La cantidad de hembras ápteras de *A. gossypii* consumidas por *C. carnea*, así como el tiempo de consumo, no se vieron afectadas significativamente tras su exposición directa a una suspensión de conidios de la cepa EABb 01/33-Su, en comparación con lo observado con los pulgones testigo. Sin embargo, las crisopas no consumieron por completo los pulgones con indicios de infección fúngica, como posible mecanismo de seguridad por parte del neuróptero. Además, se ha demostrado que no existe un efecto sobre la eficacia depredadora de *C. carnea* cuando se alimenta de pulgones que se habían alimentado previamente de plantas de melón colonizadas endofíticamente con *B. bassiana*, aunque se detecta una tendencia a una reducción en el consumo de presas y un incremento en el tiempo de consumo en comparación con el control. Sin embargo, los resultados obtenidos en los ensayos de elección muestran una preferencia significativa de las crisopas por los pulgones de *A. gossypii* que se alimentan de plantas de melón colonizadas con la cepa EABb 01/33-Su, con respecto a las plantas testigo, lo que podría estar relacionado con los resultados del capitulo II, ya que algunos de los compuestos detectados en las plantas que se encontraban colonizadas endofíticamente podrían afectar al comportamiento de los insectos actuando como atrayentes.

En cuanto al efecto sobre parasitoides, no se han observado diferencias significativas en el número de momias de *A. colemani* formadas, porcentaje de emergencia y ratio sexual de los adultos emergidos cuando las hembras ápteras de su insecto hospedante *A. gossypii* se encontraban alimentándose en hojas de melón colonizadas endofíticamente con la cepa EABb 01/33-Su en comparación con el testigo, lo que finalmente refuerza el empleo de hongos entomopatógenos en combinación con otros enemigos naturales como depredadores y parasitoides en programas de control integrado de pulgones.

Esta tesis sienta las bases del empleo de hongos entomopatógenos endófitos para el control de pulgones, no solo por el efecto directo de los propágulos fúngicos sobre los áfidos, sino también por los efectos directos e indirectos sobre los mismos que acarrea la colonización endofítica de las plantas por las cepas fúngicas. Además, la tesis ilustra las ventajas de la estrategia, no solo por extender su eficacia a la capacidad de los pulgones para transmitir virus, sino por su compatibilidad en control integrado con entomófagos, parasitoides y depredadores.

Palabras clave: hongos entomopatógenos, *Beauveria bassiana*, *Metarhizium brunneum*, colonización endofítica, efectos subletales, Gráficos de Penetración Eléctrica, comportamiento alimenticio, *Cucumber mosaic virus*, *Cucurbit aphid-borne yellows virus*, *Chrysoperla carnea, Aphidius colemani,* depredador, parasitoide, interacciones multitróficas, control biológico, Control Integrado

Abstract:

Agricultural sustainability requires progressively replacing synthetic chemical insecticides by more environmentally friendly pest control measures in order to guarantee food safety and food security. Microbial control is one of these alternatives, and among entomopathogenic microorganisms, entomopathogenic fungi (EF), due to their natural presence and mode of action by contact, show high potential for the control of several groups of insects of great agricultural relevance, such as sap-sucking insects, especially aphids. During the 21st century, different associations of the EF with the plants in the rhizosphere, in the phylloplane, and as endophytes, have been discovered, new ecological roles that have expanded the possibilities of their use for pest management, and more broadly, in crop production. However, research on the direct and indirect effects of the endophytic colonization of the crop by EF is needed, with emphasis in the effect of such colonization on the plant semiochemical composition and tritrophic relationships between the insect pest and the crop and between the insect pest and its natural enemies.

In this thesis, the effect of the endophytic colonization of melon plants by two strains of *Beauveria bassiana* (Balsamo) Vuil and one of *Metarhizium brunneum* Petch, has been studied on both the fitness of the "cotton aphid" *Aphis gossypii* Glover, (Homoptera: Aphididae), a key pest of horticultural crops, as well as on its feeding behavior and ability to transmit persistent and non-persistent viruses. In addition, the present research focuses on the impact of this colonization on the chemical signals produced by the plant and on the relationships between the aphid and its natural entomophagous enemies for the development of IPM strategies. Hence, in chapter II, it is shown that *B. bassiana* EABb 01/33-Su strain significantly reduces the population of *A. gossypii* both when the specimens were fed on sprayed and endophytically colonized leaves (SCL), and when they fed only on non-sprayed colonized leaves (NSCL), distant from the sprayed ones. Meanwhile, strains EABb 04/01-Tip of *B. bassiana* and EAMa 01/58-Su of *M. brunneum* only reduced the aphid population when fed on SCL leaves, whereas feeding on NSCL also causes a significant reduction of the aphid population at a significance level of 0.1 (10%). Strain EABb 01/33-Su was shown to have the better endophytic behavior and therefore it was selected to be used throughout the experiments carried out in chapters III and IV. In Chapter II, it is also revealed a lack of effect of the endophytic colonization of melon plants with the three selected strains on total *per capita* fecundity of the aphids 5 days after the exposure, whereas aphids that feed on

colonized plants advanced their average onset of nymph laying. Finally, in chapter II, qualitative and quantitative differences in the blend of volatile compounds released by endophytically colonized plants and subsequently infested by *A. gossypii* in comparison with the compounds emitted by control plants have been detected. Noteworthy, endophytic colonization of melon plants by the selected fungal strains did not modify aphid choice.

In chapter III, the influence of melon endophytic colonization on the aphid feeding behavior and the transmission of persistent and non-persistent viruses has been studied. By using the electrical penetration graph (EPGs) technique, applied for the first time to aphids that feed on plants colonized with entomopathogenic fungi, it has been possible to detect the activity of the insect's stylet and relate it to the transmission processes of viruses in melon plants colonized with the *B. bassiana* strain EABb 01/33-Su. Melon endophytic colonization did not affect the feeding behavior of *A. gossypii* associated with the inoculation of the non-persistent Cucumovirus, *Cucumber mosaic virus* (CMV), and the persistent Polerovirus *Cucurbit aphid-borne yellows virus*, but modified the acquisition process of non-persistent viruses, which could be related to modifications of the feeding behavior of the aphid due to presence of fungal propagules or fungal metabolites in the mesophyll. In addition, the transmission bioassays carried out revealed that the incidence of the two target viruses was significantly reduced when the plants were endophytically colonized by the *B. bassiana* strain EABb 01/33-Su.

Finally, in chapter IV, the effect of melon endophytic colonization by EABb 01/33-Su strain on the tritrophic interaction of *A. gossypii* with the crop and with their natural enemies, the generalist predator *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) and the aphid parasitoid *Aphidius colemani* (Dalman) (Hymenoptera: Braconidae), was investigated. The number of apterous females of *A. gossypii* consumed by *C. carnea*, as well as the time of consumption, were not significantly affected by direct exposure to a conidial EABb 01/33-Su suspension as compared with the controls. Noteworthy, the lacewings did not completely consume the aphids with signs of fungal infection, as a possible safety mechanism developed by the predator. In addition, it has been shown the lack of a significant effect of endophytic colonization of the aphid hos plant, melon, on the predatory efficacy of *C. carnea*, whereas a trend towards a prey consumption decrease and consumption time increase was detected in comparison with the controls. Interestingly, the choice experiments revealed a significant preference of lacewings for aphids that fed on EABb 01/33-Su colonized

melon plants, which could be related to the overproduction of certain semiochemicals elicited by endophytic colonization with known predator attractant potential. In addition, no significant differences were observed in the number of mummies of *A. colemani* formed, percentage of emergence and sexual ratio of the emerged adults when the insect host *A. gossypii* fed on EABb 01/33-Su endophytically melon leaves, which strengthen the potential of the simultaneous use of EF and predators and parasitoids in *A. gossypii* IPM.

On the overall, the results of this thesis support the development of mycoinsecticides for aphid control due to the direct effect of fungal spray on the aphid population, but also due to direct and indirect effects related to the endophytic colonization of the plant by the fungal biocontrol agent, with emphasis in the reduction of the aphid virus-transmission potential. In addition, the results of the thesis highlight the compatibility of endophytic EF with parasitoids and predators.

Keywords: entomopathogenic fungi, *Beauveria bassiana*, *Metarhizium brunneum*, endophytic colonization, pre-mortality effects, Electrical Penetration Graph, probing behaviour, *Cucumber mosaic virus*, *Cucurbit aphid-borne yellows virus*, *Chrysoperla carnea, Aphidius colemani,* predator, parasitoid, multitrophic interactions, biological control, Integrated Pest Management

ÍNDICE

CAPÍTULO I. INTRODUCCIÓN

CAPÍTULO II. EFFECTS OF ENDOPHYTIC ENTOMOPATHOGENIC ASCOMYCETES ON THE LIFE-HISTORY TRAITS OF *APHIS GOSSYPII* GLOVER AND ITS INTERACTIONS WITH MELON PLANTS

Supplementary material

CAPÍTULO III. CHANGES IN FEEDING BEHAVIOUR ARE NOT RELATED TO THE REDUCTION IN THE TRANSMISSION RATE OF PLANT VIRUSES BY *APHIS GOSSYPII* (HOMOPTERA: APHIDIDAE) TO MELON PLANTS COLONIZED BY *BEAUVERIA BASSIANA* (ASCOMYCOTA: HYPOCREALES)

CAPÍTULO I. INTRODUCCIÓN

CAPÍTULO II. EFFECTS OF ENDOPHYTIC ENTOMOPATHOGENIC ASCOMYCETES ON THE LIFE-HISTORY TRAITS OF *APHIS GOSSYPII* GLOVER AND ITS INTERACTIONS WITH MELON PLANTS

CAPÍTULO I. INTRODUCCIÓN

1.- Introducción

En los agroecosistemas, que son mucho más simples que los ecosistemas naturales, existe una tendencia al monocultivo en amplias zonas con variedades vegetales en las que se pierde diversidad genética para mejorar caracteres de interés agrícola, con una menor diversidad de enemigos naturales y con una reducción del número de interacciones tróficas, que provoca una pérdida de resiliencia del ecosistema para responder a distintos estreses bióticos y abióticos (Gliessman 2000). En las circunstancias de escasez de relaciones tróficas de los agroecosistemas, su estudio es crucial para evitar que las intervenciones humanas puedan cambiar el signo de las interacciones que operan en favor de la sostenibilidad del proceso agrícola.

Casi toda la energía que consume la vida procede de la capacidad fotosintética de las plantas, que, como seres autótrofos, son la fuente de alimento de los animales herbívoros, entre los que se encuentran los insectos fitófagos, una amenaza para la agricultura. Las interacciones entre los insectos y las plantas son el resultado de un proceso de adaptación evolutiva, en el que los primeros deben enfrentarse a las defensas físicas y químicas, sustancias aleloquímicas, que les presentan las segundas. A su vez, en niveles tróficos superiores, las poblaciones de insectos fitófagos padecen el ataque de artrópodos entomófagos, depredadores y parasitoides, o microorganismos entomopatógenos. En estos sistemas tritróficos, las interacciones depredador-presa y parasitoide-hospedante pueden estar reguladas por sustancias volátiles producidas por las plantas e inducidas por la actividad de los propios insectos fitófagos (Dicke 2016).

La complejidad de estos sistemas tritróficos puede verse incrementada cuando intervienen los microbios, como ya se ha constatado

con determinados microorganismos no patogénicos del suelo, que pueden modular las interacciones entre los insectos y las plantas al desencadenar cambios bioquímicos en el metabolismo de estas (Badri et al. 2013).

Por ello, resulta cada vez más importante el estudio de las posibles modificaciones de las relaciones tritróficas asociadas al empleo de microorganismos, en especial, cuando estos son utilizados por inundación para el control de plagas, como ocurre con los entomopatógenos, y de forma urgente, si estos agentes de control de plagas llegan a establecer relaciones beneficiosas con las plantas, aspectos en los que se ha adentrado la presente tesis doctoral.

1.1. Control biológico e integrado de Plagas

Los principios de la Agricultura Sostenible, y de su herramienta, la Producción Agraria Integrada, impregnan las políticas agrarias en el mundo, con énfasis en el Control Integrado de Plagas, para reducir o eliminar el uso de los insecticidas convencionales y, por tanto, sus efectos negativos sobre el medio ambiente y la salud humana (Directiva 2009/128 / CE; Código EE.UU. § 136r-1; Reglamento (CE) 834/2007) (Quesada-Moraga et al. 2009).

El uso de insecticidas químicos de síntesis presenta numerosos inconvenientes tales como la aparición de resistencia a los mismos por parte de las poblaciones de insectos fitófagos, el resurgimiento de plagas, un efecto negativo en la fauna auxiliar, la presencia de residuos en los productos agrícolas, con los correspondientes plazos de seguridad y condiciones de reentrada a las explotaciones, así como aspectos legales, que, en su conjunto, incrementan los costes de tratamiento (Nauen y Elbert 2003; Whalon et al. 2016). El Control Integrado de Plagas persigue

mantener las poblaciones de insectos fitófagos por debajo del umbral de tolerancia mediante el empleo de las medidas que mejor se ajusten a los criterios ambientales, económicos y sociales de la actividad agrícola, para reducir al máximo la aplicación de insecticidas químicos de síntesis (Metcalf y Luckmann 1994; FAO 2018). El control biológico de plagas, que es una de las principales alternativas a los insecticidas químicos en control integrado de plagas, se basa en el uso de organismos entomófagos, vertebrados (pequeños mamíferos, aves) e invertebrados (depredadores y parasitoides), control macrobiano, así como de entomopatógenos, microorganismos causantes de enfermedad a los artrópodos (virus, bacterias, hongos, nematodos y protozoos), control microbiano, para mantener las poblaciones de insectos por debajo del umbral de tolerancia (Lacey y Shapiro-Ilan 2008; Kaya y Vega 2012; Abrol 2014; Lacey 2017).

1.2. Control microbiano de plagas

Existen varios grupos de microorganismos entomopatógenos, virus, bacterias, hongos, microsporidios, protozoos y nematodos. Dentro del grupo de los virus patógenos de insectos, destacan los baculovirus, los cuales solo infectan al filo Arthropoda, mayoritariamente de la clase Insecta, lo cual representa un alto grado de bioseguridad, tanto para los seres humanos como para la vida silvestre en general. Muestran además una elevada patogeneicidad y virulencia para gran número de especies de insectos que originan importantes plagas (Harrison y Hoover 2012). La familia Baculoviridae está compuesta por los géneros *Alphabaculovirus*, *Betabaculovirus*, *Gammavaculovirus* y *Deltabaculovirus* (Herniou et al. 2003, 2004; Jehle et al. 2006; Harrison y Hoover 2012). Los *Alphabaculovirus* y *Betabaculovirus* engloban respectivamente nucleopoliedrovirus y granulovirus que infectan especies del orden Lepidoptera. Por el contrario, los otros dos géneros, *Gammabaculovirus* y *Detabaculovirus* incluyen

nucleopoliedrovirus que infectan a especies de los órdenes Hymenoptera y Diptera respectivamente. El principal reservorio de los baculovirus es el suelo, y es necesario que sean transportados a la superficie foliar para ser ingeridos por insectos susceptibles, comenzando así el proceso infectivo, que a menudo provoca epizootias de gran impacto. Debido a su efectividad, han sido desarrollados comercialmente como insecticidas microbianos (Harrison y Hoover 2012).

Las bacterias entomopatógenas también ocupan un papel importante como agentes de control microbiano. La más conocida a nivel mundial es *Bacillus thuringiensis* Berliner(Bt), bacteria gram positiva que durante la esporulación produce un cuerpo parasporal o cristal de naturaleza proteínica, las proteínas Cry, que debe ser ingerido para causar la toxicidad; la composición peptídica y estructural de estas determinan su especificidad frente a los diferentes grupos de insectos (Jurat-Fuentes y Jackson 2012).

Los nematodos utilizados más asiduamente en el control biológico de plagas son especies de las familias Steinernematidae y Heterorhabditidae, las cuales se caracterizan por estar asociadas simbióticamente con enterobacterias; las especies de *Steinernema* sp. con *Xenorhabdus* sp., las de *Heterorhabditis* sp. con *Photorhabdus* sp. Su efectividad como agentes de control dependerá tanto del nematodo, de su capacidad para localizar, reconocer e invadir al hospedante, como de la virulencia de la bacteria en el hospedante (Griffin et al. 2005).

El responsable de todo es el tercer estadio juvenil, llamado juvenil infectivo, el único que se encuentra fuera del cuerpo del insecto, en el suelo; invade al hospedante, a través de las aberturas naturales, espiráculos, boca y ano, alcanza el hemocele, donde libera la bacteria que porta, esta se multiplica, proporciona las condiciones para que el nematodo culmine su desarrollo, con resultado de muerte del hospedante y liberación de nuevos infectivos (Lewis y Clarke 2012).

Los microsporidios, protistas estrechamente relacionados con los hongos, son patógenos intracelulares obligados que infectan a los hospedantes por vía oral o transovarica. Aunque tienen impacto de manera natural sobre las poblaciones de insectos la aplicación práctica es muy limitada, su producción es difícil y costosa, por eso en la actualidad solo existe un producto registrado y comercializado para su uso como insecticida microbiano (Solter et al. 2012).

Los hongos entomopatógenos (HEs) destacan por su enorme potencial práctico, este amplio conjunto de especies muestra un modo de acción único, por contacto, además están presentes en casi todos los ecosistemas terrestres y hábitats donde regulan de forma natural las poblaciones de insectos (Lovett y St. Leger 2017).

2.-Los hongos entomopatógenos

2.1. Clasificación y diversidad

Se estima que existen alrededor de 700–750 especies del reino Mycota que actúan como patógenos de artrópodos, aunque este carácter adquiere frecuencia creciente en las divisiones Blastocladiomycota, Basidiomycota, Ascomycota, y Entomophthoromycota, división esta última que solo incluye HEs (Hibbett et al. 2007; Gryganskyi et al. 2012, 2013; Humber 2012; Wang et al. 2016) (Tabla 1).

En la división Entomophthoromycota se encuentran especies que originan importantes epizootias en las poblaciones naturales de artrópodos, en especial las familias Entomophthoraceae, Neozygitaceae y Ancylistaceae, pero al ser biotrofos obligados, resulta compleja su multiplicación en un medio artificial y, por tanto, su desarrollo comercial (Keller 2007; Pell et al. 2010; Boomsma et al. 2014).

Tabla 2. Clasificación de los hongos entomopatógenos. Modificado de Hibbet et al. (2007), Humber (2012), Gryganskyi et al. (2012; 2013), y la ARS collection of the entomopathogenic fungal cultures (http: //arsef.fpsnl.cornell.edu). En esta tabla no se indican los hongos entomopatógenos pertenecientes a las divisiones Blastocladiomycota y Basidiomycota.

División	**Clase**	**Orden**	**Familia**	**Género**
Entomophthoromycota	Basidiobolomycetes	Basidiobolales	Basidiobolaceae	*Basidiobolus*
	Neozygitomycetes	Neozygitales	Neozygitaceae	*Apterivorax, Neozygites, Thaxterosporium*
	Entomophthoromycetes	Entomophthorales	Ancylistaceae	*Ancylistes, Conidiobolus, Macrobiotophthora*
			Completoriaceae	Completoria
			Entomophthoraceae	SUBFAMILIA Entomophthoroideae: *Batkoa, Entomophaga, Entomophthora Eryniopsis, Massospora,* SUBFAMILIA Erynioideae: *Erynia, Eryniopsis, Furia, Orthomyces, Pandora, Strongwellsea, Zoophthora*
			Meristacraceae	*Meristacrum Tabanomyces*
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Paecilomyces*
	Sordariomycetes	Hypocreales	Clavicipitaceae	*Aschersonia, Hypocrella, Regiocrella, Metarhizium*
			Cordycipitaceae	*Cordyceps, Lecanicillium,Beauveria, Isaria*
			Ophicordycipitaceae	*Tolypocladium,Ophiocordyceps*

Por el contrario, los ascomicetos mitospóricos entomopatógenos (AMEs), con más de 600 especies que infectan insectos y ácaros, poseen ciclos de vida más complejos que incluyen etapas fuera del insecto hospedante, lo que les permite persistir durante prolongados periodos de tiempo en el suelo o asociados con las plantas a la espera de infectar al insecto (Quesada-Moraga y Santiago-Álvarez 2008; Chandler 2017). Estas especies fúngicas son de fácil manejo, lo que facilita su producción en masa, y su aplicación comercial como micoinsecticidas, en especial las familias Clavicipitaceae, Cordycipitaceae y Ophiocordycipitaceae, del orden Hypocreales, y Trichocomaceae, del orden Eurotiales (Lacey et al. 2015; Lacey 2017).

2.2. Patogénesis de los ascomicetos mitospóricos entomopatógenos

Los AMEs son los únicos agentes de control microbiano de plagas capaces de infectar al insecto hospedante por vía tegumentaria, al entrar en contacto con la cutícula del insecto los propágunos infectivos (Vega et al. 2012; Lacey et al. 2015). El proceso patogénico se desarrolla en tres fases principales: infección, crecimiento y reproducción (Quesada-Moraga y Santiago-Álvarez 2008; Vega et al. 2012).

En la **fase de infección**, se produce la adhesión de los conidios a la cutícula, propágulos infectivos, su germinación y la producción de una serie de enzimas hidrolíticas (proteasas, quitinasas y lipasas), que permiten al hongo atravesar el tegumento y acceder al hemocele en forma de hifas o cuerpos hifales (Quesada-Moraga y Santiago-Álvarez 2008; Vega et al. 2012). Durante la **fase de crecimiento**, las hifas o cuerpos hifales deben vencer la respuesta defensiva celular y humoral, y continuar su desarrollo en el hemocele donde invaden todos los tejidos del insecto hasta causarle la muerte. Esta puede ser el resultado de la combinación de distintas acciones del hongo como la utilización de los nutrientes, la invasión física de los

diferentes órganos del hospedante, y la producción de toxinas (Quesada-Moraga y Santiago-Álvarez 2008; Vega et al. 2012).

La fase de **reproducción** se inicia tras la muerte del insecto, durante la cual, si las condiciones microclimáticas son propicias, el hongo manifiesta crecimiento saprofítico (necrotrófico), lo que le permite utilizar el cadáver para volver a esporular en el exterior del mismo y liberar los nuevos propágulos infectivos (conidios), que se dispersan gracias a la acción del viento,la lluvia o los propios insectos y darán lugar a un nuevo ciclo de infección (Quesada-Moraga y Santiago-Álvarez 2008; Vega et al. 2012).

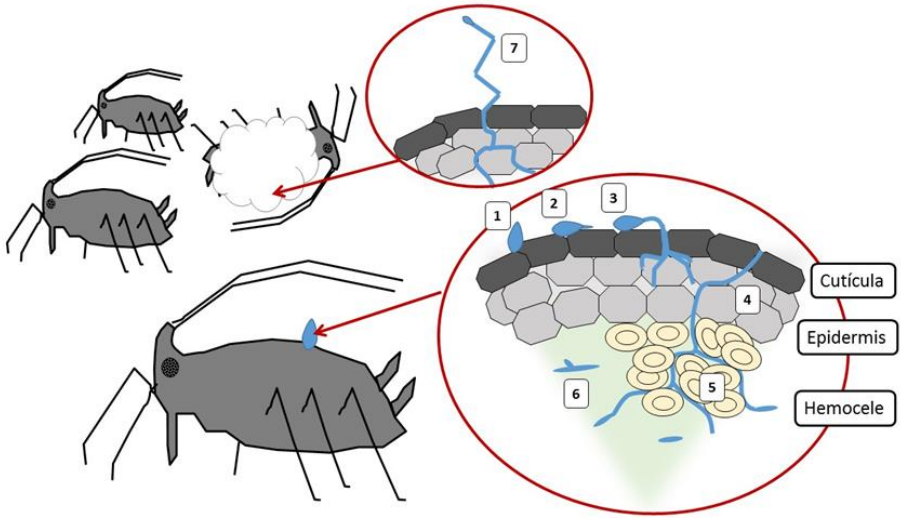

Figura 1. Modo de acción general de un ascomiceto mitospórico. (1) Adhesión del conidio a la epicutícula; (2) Germinación; (3) Penetración; (4) Hifas que atraviesan la cutícula y alcanzan el hemocele; (5) Reacción defensiva celular hemocitaria del insecto; (6) Cuerpos hifales en el hemocele.

3. Presencia natural de ascomicetos mitospóricos entomopatógenos

Durante el siglo XX se pensaba que los principales reservorios de AMEs eran el suelo y las poblaciones de artrópodos hospedantes a las que infectan (Inglis et al. 2001; Quesada-Moraga et al. 2007). Sin embargo, ahora se han descubierto sorprendentes asociaciones de los AMEs con las plantas, en el

filoplano, como **epífitos** (Meyling y Eilenberg 2006, 2007; Meyling et al. 2011; Schneider et al. 2012; Garrido-Jurado et al. 2015; Keyser et al. 2015; Howe et al. 2016), como **endófitos** en los tejidos vegetales (Quesada-Moraga et al. 2014; Vega 2018) e incluso como microorganismos competentes en la **rizosfera** (Fang y St. Leger 2010; Pava-Ripoll et al. 2011; Wyrebek et al. 2012; Barelli et al. 2018; McKinnon et al. 2018; Quesada-Moraga et al. 2019) (Figura2).

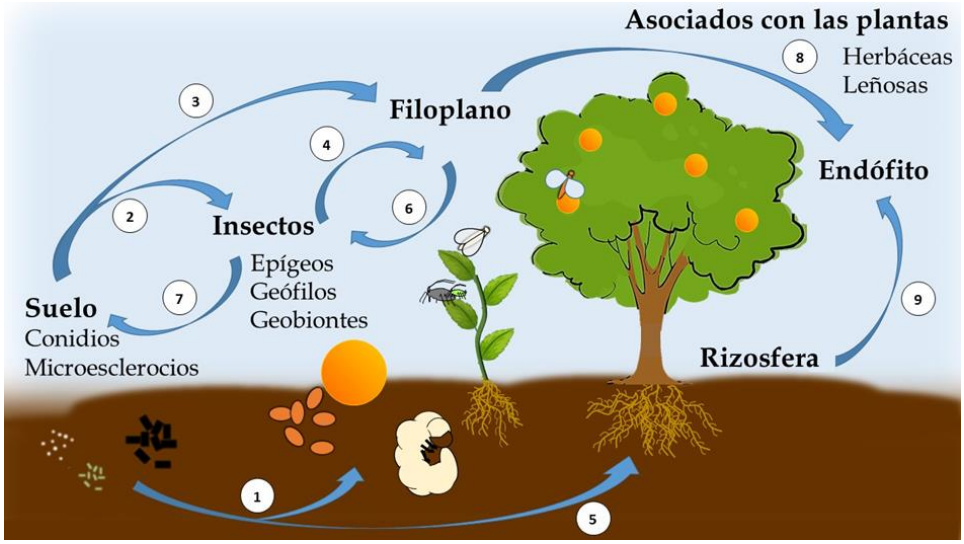

Figura 2. Presencia natural de los AMEs. El suelo supone el principal reservorio de los propágulos infectivos de los AMEs (conidios y microesclerocios). Desde este medio, los propágulos pueden alcanzar (1) insectos geófilos y geobiontes, (2) insectos epígeos, (3) el filoplano, tras la acción del viento (4) o debido la actividad de los mismos insectos, (5) o la rizosfera de las plantas. Además de en el suelo, los conidios pueden alcanzar a estos insectos en el filoplano (6). Una vez los insectos han sido infectados por un AME, se produciría su muerte y posterior esporulación en el exterior del cadáver, con lo que se liberarían nuevos propágulos infectivos, y gracias a la acción del viento, la lluvia o los propios insectos se dispersarían nuevamente (7) al suelo o (4) al filoplano. Estos hongos pueden formar diferentes tipos de asociaciones con las plantas. Pueden llegar a formar parte del microbiota del filoplano de diferentes tipos de vegetación (plantas herbáceas y leñosas, de hábitats naturales y transformados) o actuar como microorganismos competentes en la rizosfera. La presencia en el filoplano o en la rizosfera podría ser el preludio de una invasión endófita de los tejidos de la planta por estos hongos (8 y 9).

3.1. Presencia de AMEs en el suelo

Los AMEs son un componente importante de la microbiota del suelo (Meyling y Eilenberg 2007; Quesada-Moraga et al. 2007), tanto en suelos naturales como cultivados (Quesada-Moraga et al. 2007), donde los conidios están protegidos frente a condiciones extremas de temperatura, humedad y exposición a UV-B (Jaronski 2007). Además, el suelo es también el hábitat de insectos hospedantes potenciales, algunos de los cuales presentan altas densidades, lo que favorece el inicio del ciclo de patogénesis del hongo y su dispersión (Humber 2008).

La presencia, diversidad y dinámica poblacional en el suelo de los AMEs está determinada por factores como el grado de manejo de los ecosistemas, la existencia de microorganismos antagonistas en la microbiota del suelo, la riqueza y composición de la artropodofauna edáfica y su papel como fuente de hospedantes, además de factores abióticos como temperatura, humedad, radiación UV-B y condiciones edáficas (textura, pH, contenido de materia orgánica, capacidad de intercambio catiónico), sin olvidar la situación geográfica o el tipo de ecosistema (Meyling y Eilenberg 2007; Quesada-Moraga et al. 2007; Jaronski 2010; Garrido-Jurado et al. 2011; Lacey et al. 2015).

3.2. Presencia de AMEs en los artrópodos

Aunque algunas especies de AMEs como *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin pueden estar presentes en la epicutícula sin causar infección (Greif y Currah 2007), lo más frecuente es que se inicie el proceso patogénico, que permite a los AMEs obtener la energía fundamental para su nutrición a partir de los artrópodos, como microorganismos hemibiotróficos, primero biotrofos, durante las etapas de infección y crecimiento, luego necrotrofos, durante la etapa de reproducción. Cuando existe escasez de insectos hospedantes, o las condiciones ambientales no

son favorables, los AMEs producen esporas de reposo, se forman estructuras especiales como clamidosporas o microesclerocios, que les permiten permanecer en el suelo durante largos períodos de tiempo (Quesada–Moraga et al. 2007).

La condición de patógenos obligados resulta controvertida en los géneros como *Metarhizium*, *Beauveria*, *Lecanicillium* e *Isaria* (Luangsa-ard et al. 2005; Sung et al. 2007), pues, aunque *Beauveria* y *Metarhizium* actúan principalmente como patógenos de insectos, no se descarta la posibilidad de que se produzcan tipos de nutrición adicionales como sus asociaciones con las plantas (Vega 2012). Existe también cierta especialización, pues las especies de AMEs de la familia Ophiocordycipitaceae infectan larvas y pupas de insectos en el suelo, mientras que en la familia Clavicipitaceae son más frecuentes las asociaciones con las plantas o incluso la infección del orden Homoptera, donde las familias Coccidae y Aleyrodidae, cuyas formas inmaduras inmóviles, son las que han registrado una mayor diversidad de infecciones naturales por AMEs (Humber 2008).

3.3. Asociaciones de los AMEs con las plantas

A lo largo del siglo XXI se han descrito sorprendentes asociaciones de los AMEs con las plantas (Figura 3). Los propágulos de los AMEs pueden llegar a formar parte de la microbiota del filoplano de diferentes tipos de vegetación, por la acción del viento o a la actividad de los insectos, tanto en hábitats naturales como transformados (Meyling y Eilenberg 2006, 2007; Hesketh et al. 2010; Ormond et al. 2010; Meyling et al. 2011; Schneider et al. 2012; Garrido-Jurado et al. 2015, Howe et al. 2016; Quesada-Moraga et al. 2019).

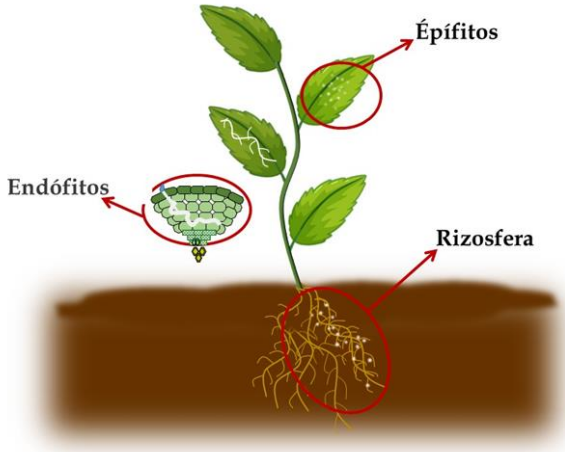

Figura 3. Asociaciones de los AMEs con las plantas. En el filoplano, como epífitos, en los espacios intercelulares, como endófitos, y como microorganismos competentes en la rizosfera.

La presencia en el filoplano podría ser el preludio del carácter endofítico de estos hongos, descrito por primera vez en maíz en 1991 (Bing y Lewis 1991), y durante el siglo XXI, en numerosas especies cultivadas y no cultivadas, tanto de forma natural o artificial (Wagner y Lewis 2000; Vega 2008; Vega et al. 2008, 2009; Quesada-Moraga et al. 2014; Vidal y Jaber 2015; Vega 2018; Quesada-Moraga et al. 2019) (Tabla 2).

Como endófitos, los AMEs colonizan de forma asintomática los tejidos de la planta (Saikkonen et al. 2006; Arnold y Lutzoni 2007), e incluso pueden promover su crecimiento, o protegerla frente a estreses de tipo biótico, plagas y enfermedades, o abiótico, déficit hídrico, nutricional etc. (Quesada-Moraga et al. 2019). El grado de colonización de los diferentes tejidos y órganos de la planta y su persistencia en el tiempo varía según el cultivo y la cepa de AME, pudiendo existir únicamente una colonización transitoria o llegar a transmitirse verticalmente y encontrarse en la semilla (Wagner y Lewis 2000; Landa et al. 2013; Quesada-Moraga et al. 2014; Garrido-Jurado et al. 2017).

Los recientes estudios sobre la adaptación y evolución de los AMEs hacia hábitos epífitos, endófitos o rizosféricos, sugieren que la dispersión de

la mayoría de los propágulos fúngicos tiene su origen en el suelo, su principal reservorio, y alcanza el filoplano de las plantas gracias a la acción del viento o la actividad de los insectos (Meyling y Eilenberg. 2006; Garrido-Jurado et al. 2015; Howe et al. 2016; Fernández-Bravo et al. 2017).

A este respecto, se ha puesto de manifiesto la presencia de los mismos grupos genéticos de AMEs en los tres hábitats muestreados, suelo, filoplano de planta leñosa y filoplano de planta herbácea bajo la copa de especies arbóreas, lo que indica el movimiento de estos desde el suelo a las fanerógamas adventicias y a los árboles (Garrido-Jurado et al. 2015). No obstante, también se ha detectado la presencia de algunos aislados en el filoplano con un mismo perfil genético y distante del resto, lo que pondría de manifiesto la existencia de genotipos de AMEs adaptados al filoplano, como verdaderos epífitos (Garrido-Jurado et al. 2015).

Otro tipo de asociación que pueden manifestar estos hongos con las plantas, en especial las especies pertenecientes al género *Metarhizium*, es como microorganismos competentes en la rizosfera (Hu y St. Leger 2002; Vega et al. 2009; Fang y St. Leger 2010; Pava-Ripoll et al. 2011; Wyrebek et al. 2012; Barelli et al. 2018; McKinnon et al. 2018). Esta capa de tierra que envuelve a las raíces parece promover la persistencia y actividad biológica de los AMEs, e incluso *Metarhizium anisopliae* (Metschnikoff) Sorokin (Ascomycota: Hypocreales) expresa genes diferentes cuando crece en los exudados de las raíces de plantas o cuando lo hace sobre la cutícula o hemolinfa de un insecto, lo que revela el desarrollo de un mecanismo de adaptación como organismos competentes de la rizosfera (Hu y St. Leger 2002; Wang et al. 2005; Pava-Ripoll et al. 2010). Esta evolución supondría una ventaja adaptativa para el género *Metarhizium* pues se ha comprobado que sus conidios persisten durante más tiempo en la rizosfera que en otras zonas del suelo, y pueden infectar a lo largo de más tiempo a las poblaciones de insectos hospedantes (Bruck 2005).

Tabla 2. Especies de plantas colonizadas de manera natural o artificial por hongos entomopatógenos endófitos. Modificada de Resquín–Romero (2016), Raya-Díaz (2017), Vega (2018) y actualizada. Continúa 1/2

Especie fúngica	Presencia	Planta hospedante	Referencias (*)
Beauveria bassiana	Natural	***Aesculus hippocastanum***, *Abies beshanzuensis*, *Ammophila arenaria*, *Carpinus carolinana*, *Coffea arabica*, *Dactylis glomerata*, *Datura stramonium*, *Elymus farctus*, *Espeletia* spp., *Eucalyptus globulus*, *Gossypium hirsutum*, Papaver somniferum, ***Phaseolus vulgaris***, *Pinus montícola*, *Pinus radiata*, *Pinus sylvestris*, *Quercus ilex*, *Schizachne purpurascens, Theobroma gileri*, *Zea mays*	Ramos et al. 2017; Barta 2018
	Artificial	***Aesculus hippocastanum,*** *Brassica oleracea, Brassica napus,* ***Calendula officcinalis****, Capsicum annuum, Coffea arabica, Corchorus capsularis, Corchorus olitorius, Cucumis melo, Cucurbita maxima, Cucurbita pepo, Cynara scolymus, Echinacea purpurea, Festuca arundinacea,Fragaria x ananassa,Glycine max, Gossypium hirsutum, Manihot esculenta, Medicago sativa,Musa sp., Nicotiana tabacum, Oryza sativa, Papaver somniferum, Phaseolus vulgaris, Phoenix dactylifera, Pinus radiata,* ***Saccharum officinarum****, Solanum lycopersicum, Sorghum bicolor, Theobroma cacao, Triticum aestivum, Triticum durum, Vitis vinifera, Vicia faba, Vigna unguiculata, Zea mays*	Barta 2018; Donga et al. 2018; Heinz et al. 2018
Beauveria pseudobassiana	Artificial	***Aesculus hippocastanum***	Barta 2018
Bionectria ochroleuca	Artificial	*Cynara scolymus*	
Clonostachys rosea	Natural	*Coffea arabica, Quercus myrsinifolia*	
	Artificial	*Quercus robur, Allium cepa*	
Cordyceps memorabilis	Natural	*Eucaliptus globulus*	
Cordyceps sinensis	Natural	*Holcus lanatus*, *Theobroma giler*	
Hirsutella aphidis	Natural	*Lolium perenne*	
Hypocrea lixii	Artificial	*Allium cepa*	
Isaria farinosa	Natural	*Pinus sylvestris*	
Isaria fumosorosea	Artificial	***Calendula officinalis****, Festuca arundinacea,* ***Phaseolus vulgaris****, Sorghum bicolor*	Dash et al. 2018; Heinz et al. 2018

Tabla 2. Especies de plantas colonizadas de manera natural o artificial por hongos entomopatógenos endófitos. Modificada de Resquín–Romero (2016), Raya-Díaz (2017), Vega (2018) y actualizada. Continúa 2/2

Especie fúngica	Presencia	Planta hospedante	Referencias (*)
Lecanicillium dimorphum, Lecanicillium cf. *Psalliotae*		*Phoenix dactylifera*	
Lecanicillium lecanii	Natural	*Ammophila arenaria*, *Carpinus caroliniana*, *Dactylis glomerata*, *Elymus farctus*, *Gossypium hirsutum*, ***Phaseolus vulgaris***	Dash et al. 2018
Metarhizium acridum	Artificial	*Cucumis sativus, Vigna unguiculata*	
Metarhizium anisopliae	Natural	*Cynodon dactylon*	
	Artificial	***Annona squamosa, Arachis hypogaea,*** *B. oleracea, B. napus, Camelia sinensis, Manihot esculenta, P. vulgaris, Quercus robur, Solanum lycopersicum, Sorghum bicolor, Vicia faba*	Hao et al. 2017; Pathan y Deshpande 2019
Metarhizium brogniartii	Artificial	*V. faba*	
Metarhizium brunneum	Natural	*Schizachne purpurascens*	
Metarhizium brunneum	Artificial	*B. oleracea, C. annuum, Cucumis melo,* ***Glycine max****, Helianthus annuus, Medicago sativa, S. lycopersicum, Solanum tuberosum, Sorghum bicolor,* ***Triticum aestivum****, Vicia faba, Zea mays*	Clifton et al 2018; Jaber 2018
Metarhizium guizhouense	Natural	*Solidago altissima*	
Metarhizium guizhouense	Artificial	*Zea mays*	
Metarhizium robertsii	Natural	*Aster vimineus, Hieracium pratense, Solidago altissima*	
Metarhizium robertsii	Artificial	*Panicum virgatum, Phaseolus vulgaris, Sorghum bicolor, Cucumis sativus, Vigna unguiculata*	
Paecilomyces formosus	Artificial	***Glycine max***	Bilal et al. 2018
Purpureocillium lilacinum	Artificial	*Gossypium hirsutum*	
Tolypocladium cylindrosporum	Natural	*Festuca rubra*, *Holcus lanatus*	

(*) Se indican solo referencias recientes, posteriores a Resquín–Romero 2016, Raya-Díaz 2017 y Vega 2018. Estas nuevas detecciones de endófitos en los diferentes cultivos se encuentran destacadas en negrita.

4. Aplicaciones en agricultura de las asociaciones con plantas de ascomicetos mitospóricos entomopatógenos

Las asociaciones con las plantas de los AMEs pueden dar lugar a distintas estrategias de protección de cultivos, pero también de producción vegetal, impensables hasta hace poco (Figura 4). Se abre un horizonte en el que la correcta valoración de un agente de control microbiano requiera información completa sobre las ventajas que proporciona a la planta, no solo en términos de su protección frente al ataque de plagas, sino también sobre la agronomía general de la misma.

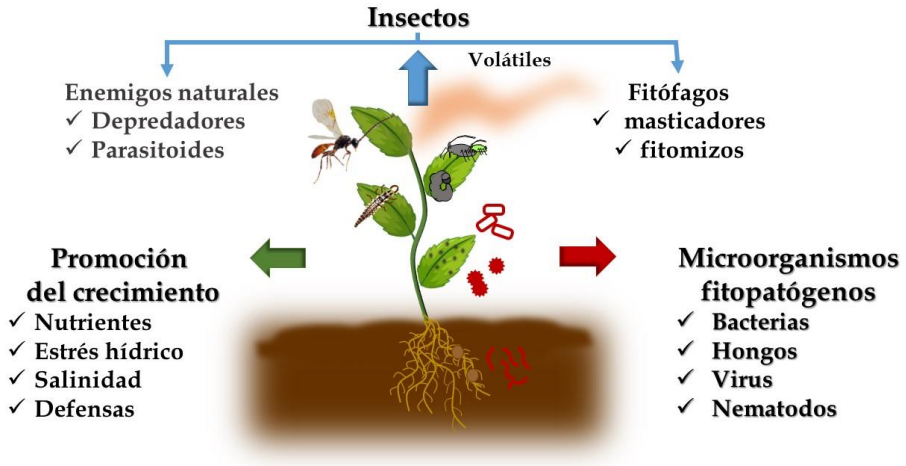

Figura 4. Aplicaciones de las asociaciones de los AMEs en agricultura. En su papel como endófitos, los AMEs pueden proteger a la planta de factores bióticos, como el ataque de insectos fitófagos masticadores y fitomizos, ya sea como un resultado directo de la colonización endofítica o como una defensa indirecta de la planta, al atraer enemigos naturales, sobre los que tiene escaso o nulo efecto. También frente a la invasión de microorganismos fitopatógenos como bacterias, hongos, virus o nematodos fitopatógenos (rojo). Además de proteger a la planta frente a los factores bióticos, se ha observado que algunas especies endófitas de AMEs actúan como promotores del crecimiento vegetal, ya que pueden aliviar el estrés nutricional, hídrico, la salinidad o activar las defensas de la planta (verde). Los volátiles que emiten las plantas, mediante los cuales se comunican fitófagos y enemigos naturales, juegan un papel fundamental en su protección.

4.1. Protección de la planta frente a factores bióticos

4.1.1. Plagas de insectos

Los AMEs tienen un gran potencial como como agentes de control microbiano de plagas, pues se ha demostrado su actividad sobre todos los órdenes de insectos fitófagos, por inoculación o inundación, aplicación a la planta o al suelo, e incluso por medio de dispositivos de atracción e infección.

Además, se ha demostrado que la aplicación de micoinsecticidas por pulverización a la planta, al suelo, o por tratamiento de semilla puede dar origen a una colonización endofítica transitoria o sistémica de la planta que provoca una mortalidad adicional a la inicialmente esperada por su aplicación directa sobre el insecto (Resquín-Romero et al. 2016; Garrido-Jurado et al. 2017).

Así, la acción combinada de la pulverización al cultivo y la colonización endofítica de la planta puede mejorar la eficacia del tratamiento con micoinsecticidas, además de minimizar los inconvenientes asociados a la aplicación convencional de los AMEs, en especial, su inactivación por los factores climáticos o su posible efecto sobre organismos no diana (Lacey et al. 2015).

La capacidad endófita natural de los AMEs ha permitido llevar a cabo con éxito de forma artificial la protección sistémica de cultivos frente al ataque de insectos con gran diversidad de formas de vida y hábitos alimenticios.

El empleo endofítico de *B. bassiana* ha permitido la protección sistémica de importantes cultivos frente a sus plagas clave, como los

barrenadores del maíz *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) y *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae) en plantas de maíz (Bing y Lewis 1991; Cherry et al. 2004), el taladro del tomate *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) en plantas de tomate (Powell et al. 2007), el picudo de la platanera *Cosmopolites sordidus* (Germar) (Coleoptera: Curculionidae) en plataneras (Akello et al. 2008a, b), la larva barrenadora y formadora de agallas del himenóptero cinípido *Iraella luteipes* (Thompson) (Hymenoptera: Cynipidae) en plantas de adormidera (Quesada-Moraga et al. 2009), o el gorgojo *Apion corchori* Marshall (Coleoptera: Curculionidae) en plantas de yute (Biswas et al. 2013).

Los barrenadores *H. zea* y *S. nonagrioides* también han podido ser controlados por los hongos *Purpureocillium lilacinum* (Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson (Ascomycota: Hypocreales) en plantas de algodón y por *Metarhizium robertsii* JF Bischoff, Rehner & Humber (Ascomycota: Hypocreales) e *Isaria fumosorosea* Wize (Ascomycota: Hypocreales) en sorgo respectivamente (Castillo-Lopez y Sword 2015; Mantzoukas et al. 2015). Otros insectos masticadores como el gusano blanco, *Anomala cincta* Say (Coleoptera: Melolonthidae) y el minador de la hoja *Ophiomyia phaseoli* (Tryon) (Diptera: Agromyzidae) también son susceptibles a plantas de maíz colonizadas por *Metarhizium pingshaense* Q.T. Chen & H.L. Guo (Ascomycota: Hypocreales) y de judía con *M. anisopliae* (Peña-Peña et al. 2015; Mutune et al. 2016).

Esta **protección sistémica** es aplicable de igual modo contra insectos fitomizos, en los que además de evaluar la mortalidad, pueden existir efectos subletales, como ocurre con el pulgón del algodón, *Aphis gossypii* Glover (Homoptera: Aphididae) sobre plantas de algodón colonizadas por los hongos *B. bassiana*, *Lecanicillium lecanii* (Zimm.) Zare & W. Gams (Ascomycota: Hypocreales) o *P. lilacinum* (Gurulingappa et al. 2010;

Castillo-Lopez et al. 2014), el pulgón verde de las leguminosas, *Acyrthosiphon pisum* (Harris) (Homoptera:Aphididae) y el pulgón negro de las habas, *Aphis fabae* Scopoli (Homoptera:Aphididae) sobre plantas de haba colonizadas por cepas de *M. anisopliae* y *B. bassiana*(Akello y Sikora 2012), o el pulgón verde del melocotonero *Myzus persicae* (Sulzer) (Homoptera: Aphididae) con plantas de pimiento colonizadas por el hongo *Metarhizium brunneum* Petch (Ascomycota: Hypocreales) (Jaber y Araj 2018), e incluso con la mosca blanca de la batata *Bemisia tabaci* (Gennadius) en plantas de melón colonizadas por *B. bassiana* (Garrido-Jurado et al. 2017).

Es de destacar que en pocas ocasiones se ha conseguido observar un crecimiento fúngico o micosis en los cadáveres de insectos masticadores alimentados con plantas colonizadas con AMEs (Powell et al. 2007; Akello et al. 2008b; Vidal y Jaber 2015; Klieber y Reineke 2016; Garrido-Jurado et al. 2017; Vega 2018). Sin embargo, existen varios mecanismos independientes a la infección típica mediante los cuales los AMEs endófitos pueden causar un efecto letal sobre los insectos fitófagos (Vidal y Jaber 2015; Vega 2018). Este efecto podría ser debido a los metabolitos producidos por parte del AME endófito en la planta, o bien a las defensas de esta inducidas por el AME (Vega 2018).

La colonización endofítica por AMEs se relaciona con la inducción de resistencia sistémica y el correspondiente efecto sobre insectos fitófagos, aunque sin evidencias científicas moleculares sobre el mismo (Akello y Sikora 2012; Martinuz et al. 2012; Castillo-Lopez et al. 2014; Castillo-Lopez y Sword 2015; Jaber y Araj 2018; Vega 2018). Por otro lado, los metabolitos fúngicos hallados en la planta se han relacionado con el fenómeno de la antibiosis (Lewis y Cossentine 1986; Bing y Lewis 1991; Cherry et al. 2004; Akello et al. 2008a; Quesada-Moraga et al. 2009; Gurulingappa et al. 2010; Akutse et al. 2013; Castillo-Lopez et al. 2014; Golo et al. 2014; Muvea et al.

2014; Mantzoukas et al. 2015; Lefort et al. 2016; Mutune et al. 2016; Ríos-Moreno et al. 2016; Garrido-Jurado et al. 2017); en consecuencia, las destruxinas detectadas en plantas de distintas especies, colonizadas por *M. robertsii* y *M. brunneum*, explicarían la ausencia de crecimiento fúngico en cadáveres de insectos masticadores alimentados con ellas (Golo et al. 2014; Ríos-Moreno et al. 2016; Garrido-Jurado et al. 2017).

4.1.2. Enfermedades de las plantas

La colonización endofítica de distintos cultivos por cepas de *B. bassiana* (Flori y Roberti 1993; Ownley et al. 2004; Rubini et al. 2005; Griffin et al. 2006; Kim et al. 2007; Ownley et al. 2008a,b; Vega et al. 2009; Kim et al. 2010; Jaber y Salem 2014; Jaber 2015; Gan et al. 2017; Jaber y Alananbeh 2018) o por algunas pertenecientes a especies del género *Lecanicillium* (Askary et al. 1998; Benhamou y Brodeur 2000, 2001; Kim et al. 2007, 2008; Goettel et al. 2008), puede proteger a la planta frente a microorganismos fitopatógenos.

Así, la colonización endofítica por la aplicación de conidios de *B. bassiana* sobre semillas permite controlar con éxito el *damping-off,* o caída de plántulas, causado por los hongos fitopatógenos de suelo, *Rhizoctonia solani* Kuhn (Basidiomycota: Cantharellales) y *Pythium myriotylum* Drechsler (Oomycota: Pythiales), tanto en tomate (Ownley et al. 2004; Clark et al. 2006) como en algodón (Griffin 2007; Ownley et al. 2008).

La asociación endófita de *B. bassiana* se ha mostrado eficaz para el control de enfermedades causadas por especies de *Fusarium* spp.; *Fusarium oxysporum* f. sp. cepae (Hanzawa) W. C. Snyder & H. N. Hansen (Ascomycota: Hypocreales) responsable de la podredumbre basal de la cebolla (Flori y Roberti, 1993), *Fusarium oxysporum* f. sp. *lycopersici* (Saccardo) W.C. Snyder & H.N. Hansen (Ascomycota: Hypocreales) causante de la marchitez vascular del tomate (Prabhukarthikeya et al 2014; Culebro-Ricaldi et al.

2017), *Fusarium culmorum* (W.G. Smith) Saccardo (Ascomycota: Hypocreales) en plantas de trigo (Jaber 2018), y *F. oxysporum*, *F. culmorum* y *Fusarium moniliforme* J. Sheldon (Ascomycota: Hypocreales) en plantas de tomate (Jaber y Alananbeh 2018). Asimismo, se ha observado que al aplicar los conidios de *B. bassiana* por vía foliar sobre plantas de vid, se reduce la incidencia del mildium *Plasmopara viticola* (Berkeley & M.A. Curtis) Berlese & De Toni (Oomycota: Peronosporales) (Jaber 2015).

El efecto protector de *B. bassiana* como endófito se extiende a las bacterias fitopatógenas, como *Xanthomonas axonopodis* pv. *malvacearum* (Xam) en plántulas de algodón (Griffin et al. 2006; Ownley et al. 2008), e incluso a los virus fitopatógenos, con una reducción significativa de la incidencia y la severidad del virus no persistente "Zucchini yellow mosaic virus" (ZYMV; Potyvirus, Potyviridae) en plantas de calabacín (Jaber y Salem 2014).

Varias especies del género *Lecanicillium* también han demostrado tener actividad *in vitro* contra numerosos hongos fitopatógenos, como oidio (Verhaar et al. 1997, 1998; Askary et al. 1998; Dik et al. 1998; Miller et al. 2004), roya (Spencer y Atkey 1981; Leinhos y Buchenauer 1992), y mohos verdes (Benhamou y Brodeur 2000) o *Pythium* (Benhamou y Brodeur 2001), aunque pocos estudios han constatado esta actividad *in vivo*. Así, la resistencia inducida contra *Pythium ultimum* Trow (Oomycota: Pythiales) (Benhamou y Brodeur 2001) y el oidio *Sphaerotheca fuliginea* (Schlechtendal) Pollacci (Leotiomycetes: Erysiphales) (Hirano et al. 2008) ha sido relacionada con la colonización endofítica de las raíces de plantas de pepino por *Lecanicillium* sp. y *Lecanicillium muscarium* (Petch) Zare & W. Gams (Ascomycota: Hypocreales), respectivamente. Además, la supresión del oidio *S. fuliginea* por el hongo endófito *Lecanicillium longisporum* (Petch) Zare & W. Gams (Ascomycota: Hypocreales)ha sido también demostrada en plantas de pepino (Kim et al. 2010).

Otros AMEs han revelado su eficacia como antagonistas de agentes causantes de enfermedades. El AME endófito *M. robertsii* ha demostrado reducir la incidencia de la podredumbre de raíz causada por *Fusarium solani* f. sp. phaseoli (Burkholder) W.C. Snyder & N.H. Hans (Ascomycota: Hypocreales) en plantas de judía (Sasan y Bidochka 2013) o *M. brunneum* reducir la severidad de la enfermedad causada por *F. culmorum* en plantas de trigo (Jaber 2018), y *F. oxysporum*, *F. culmorum* y *F. moniliforme* en plantas de tomate (Jaber y Alananbeh 2018). En ensayos llevados a cabo *in vitro*, varias cepas de *M. brunneum* y *B. bassiana* y sus extractos han revelado actividad contra fitopatógenos como *Verticillium dahliae* Klebahn (Ascomycota: Glomerellales) *Phytophthora megasperma* Drechsler (Oomycota: Peronosporales), *Phytophthora inundata* Brasier, Sánchez Hernández & S.A. Kirk(Oomycota: Peronosporales) y *Cadophora malorum* (Kidd & Beaumont) W. Gams (Ascomycota: Helotiales) (Vega et al. 2009; Lozano-Tovar et al. 2013, 2017; Miranda-Fuentes et al. 2018). Otras especies, como *Clonostachys rosea* (Link) Schroers, Samuels, Seifert & W. Gams (Ascomycota: Hypocreales) o *Byssochlamys spectabilis* (Udagawa & Shoji Suzuki) Houbraken & Samson (Ascomycota: Eurotiales), forma sexual de *Paecilomyces variotii* Bainier (Ascomycota: Eurotiales), han demostrado reducir la incidencia de *F. culmorum* en plantas de trigo y *F. moniliforme* en plantas de *Lolium rigidum* (Gaudin) Weiss ex Nyman (Poaceae) respectivamente (Keyser et al. 2016; Rodrigo et al. 2017).

Asimismo, una cepa endófita de la especie *Phialemonium inflatum* (Burnside) Dania García, Perdomo, Gené, Cano & Guarro (Ascomycota: Sordariales) ha demostrado suprimir la penetración, formación de nódulos y reproducción del nematodo *Meloidogyne incognita* (Kofoid & White) Chitwood (Tylenchida: Meloidogynidae) en plantas de algodón (Zhou et al. 2016).

El efecto antagonista causado por los AMEs puede deberse a diferentes estrategias llevadas a cabo por el hongo. En el caso de *B. bassiana* se ha descrito la antibiosis (Renwick et al. 1991; Vesely y Koubova 1994; Bark et al. 1996; Reisenzein y Tiefenbrunner 1997; Lee et al. 1999), la competencia (Ownley et al. 2004) y la resistencia sistémica inducida (Griffin et al. 2006; Ownley et al. 2008b). Por otro lado, se ha demostrado que las especies del género *Lecanicillium*, además de utilizar estos mecanismos, antibiosis y resistencia sistémica inducida (Benhamou y Brodeur 2000, 2001), pueden actuar como parásitas de hongos fitopatógenos (Askary et al. 1998). Sin embargo, son necesarios nuevos estudios en los que se investigue sobre los mecanismos específicos que expliquen el efecto antagonista de los AMEs sobre los patógenos de plantas *in vivo*.

Estas propiedades inherentes al empleo de AMEs como insecticidas hacen que aumente su valor como producto biológico, al poder contener las pérdidas del cultivo por plagas y enfermedades mediante la aplicación de un solo producto en una estrategia de IPM (Goettel et al. 2008; Vega et al. 2009; Ownley et al. 2010).

4.2. Protección de la planta frente a factores abióticos

Se ha observado que algunas especies endófitas de AMEs pueden mejorar la adaptabilidad ecológica de las plantas y su respuesta frente a estreses abióticos como sequía, salinidad, temperatura, radiación ultravioleta y estrés nutricional, además de proteger a la planta frente a plagas y enfermedades. De hecho, estos hongos pueden actuar como promotores del crecimiento vegetal (Kabaluk y Ericsson 2007; García et al. 2011; Dara 2013; Castillo-Lopez y Sword 2015; Sánchez–Rodríguez et al. 2015; Jaber y Enkerli 2016, 2017), desarrollo de las raíces (Wyrebek et al. 2011; Sasan y Bidochka 2012; Liao et al. 2014), influir en la nutrición de las plantas (Behie et al. 2012; Behie y Bidochka 2014), e incluso aliviar el estrés

hídrico (Dara 2016),la salinidad (Waller et al. 2005) o deficiencia de micronutrientes como el hierro (Fe) (Sánchez–Rodríguez et al. 2015, 2016).

Recientemente, se ha demostrado que la colonización endofítica de plantas de trigo blando y trigo duro por una cepa de *B. bassiana* tiene un efecto sobre el crecimiento, longitud de la raíz, rendimiento del cultivo, niveles de fitohormonas y absorción de nutrientes, además de causar la mortalidad de larvas de *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) que consumen el tejido foliar colonizado (Sánchez-Rodríguez et al. 2018).

El posible impacto de la colonización endofítica por AMEs o la presencia de estos en la rizosfera sobre la respuesta de la planta a estrés nutricional se ha estudiado *in vitro*. Los resultados señalan que varias cepas de *B. bassiana, M. brunneum* e *I. farinosa* son capaces de aumentar la disponibilidad de Fe de forma diferente en función del tamaño de partícula, así como de la cristalinidad de las fuentes de Fe. Las cepas de *B. bassiana* e *I. farinosa* aumentaron el pH del medio de cultivo, mientras que la de *M. brunneum* no produjo tal efecto (Raya-Díaz et al. 2017a). Además, las tres cepas aumentaron la disponibilidad de Fe, aunque la cepa más eficiente fue la de *M. brunneum,* que disminuyó el pH del medio calcáreo, lo que sugiere que utilizó una estrategia diferente (probablemente, mayor producción de ácidos orgánicos), a la utilizada por los otros dos hongos, que elevaron el pH del medio no calcáreo (Raya-Díaz et al. 2017a).

También se ha comprobado que la aplicación de *M. brunneum* al suelo produce efectos sobre el crecimiento vegetal y la biodisponibilidad de Fe en plantas que crecen sobre suelos calcáreos y no calcáreos, con el objetivo de optimizar su utilización. El suelo calcáreo, debido a su baja disponibilidad de Fe, indujo clorosis en las plantas de sorgo, a diferencia del suelo no calcáreo utilizado como testigo. Las dosis fúngicas más altas (5×10^6 y 5×10^8 conidios ml^{-1}) aliviaron los síntomas de clorosis Fe de las plantas de sorgo

crecido en el suelo calcáreo, y aumentaron la altura de la planta y la producción de inflorescencias de girasol cultivado en ambos suelos (Raya-Díaz et al. 2017b). Aunque los tres métodos de inoculación utilizados, foliar, suelo y semilla, condujeron a diferencias en el aislamiento de los tejidos vegetales, incrementaron la altura, el contenido de clorofila foliar, el Fe de la biomasa aérea, la longitud total de la raíz y la densidad de raíces finas, el tratamiento de suelo fue el más eficaz, por su mayor efecto y persistencia sobre el contenido de clorofila foliar cuando los síntomas de clorosis férrica eran más evidentes en las plantas testigo, y tuvo un mayor efecto sobre la longitud total de la raíz y densidad de las raíces finas (Raya-Díaz et al. 2017b).

M. robertsii es capaz asimismo de mejorar el crecimiento de raíces y la absorción de nutrientes en pastizales y plantas de judía (Sasan y Bidochka 2012), mientras que diferentes cepas endófitas de *B. bassiana, I. fumosorosea* y *M. brunneum* pueden promover el crecimiento de la planta y mejorar la salud y vigor de plantas de col cuando éstas se encuentran sometidas a condiciones de estrés hídrico (Dara 2016), e incluso, una cepa endófita de *M. anisopliae* ha permitido reducir el estrés salino en soja (Khan et al. 2012).

5. Efecto de la asociación de los AMEs con las plantas sobre las cadenas tróficas

5.1. Efecto sobre la relación insecto fitófago-planta

La mayoría de las respuestas comportamentales en los insectos, como la búsqueda de alimento, el apareamiento, preferencia de un lugar de oviposición o la interacción con los enemigos naturales, se encuentran reguladas por señales químicas olfativas producidas tanto por ellos como por las plantas (Dicke y Grostal 2001; Bruce et al. 2005; Sigsgaard 2005; Xu et al. 2018). Además, el perfil de volátiles emitido por las plantas puede

verse alterado por la colonización de estas por microorganismos, lo que puede modificar las relaciones insecto-planta e insecto-enemigo natural (Yue et al. 2001; Hempel et al. 2009; D'Alessandro et al. 2014; Shikano et al. 2017; Contreras-Cornejo et al. 2018; Tasin et al. 2018). Se ha demostrado que el comportamiento de los insectos puede verse afectado tanto por la infección por AMEs, como por la presencia de sus propágulos en la superficie de la planta (Pell y Vandenberg 2002; Meyling y Pell 2006; Hussain et al. 2010; Lam et al. 2010; Yanagawa et al. 2011; Mburu et al. 2013; Rashki y Shirvani 2013; Davis et al. 2013). Así, en un experimento de elección se ha demostrado que *B. bassiana* tiene un efecto sobre la elección de planta hospedante por parte del pulgón del algodón *A. gossypii*, el cual rehúsa las plantas de pepino en las que se encuentra presente *B. bassiana* y prefiere las plantas libres del mismo (Rashki y Shirvani 2013). A este respecto, las evidencias científicas disponibles no son concluyentes, unas indican repulsión de insectos por parte de plantas tratadas con AMEs (Rondot y Reineke 2017; Sword et al. 2017), otras, atracción de estos (Kepler y Bruck 2006).

Sin embargo, hasta la fecha se desconoce si la colonización endofítica por AMEs puede provocar alteraciones en las señales químicas producidas por las plantas, y, por tanto, en las relaciones insecto fitófago-planta o incluso en las de los insectos fitófagos con sus enemigos naturales (Figura 5).

Las chinches *Lygus hesperus* Knight y *Nezara viridula* (Linnaeus) son capaces de detectar y a continuación evitar las flores y frutos desarrollados en plantas cuyos tejidos se encuentran colonizados endofíticamente por *B. bassiana*, y prefieren las plantas testigo en experimentos de elección (Sword et al. 2017). Además, plantas en las que *B. bassiana* se había establecido

como endófito, provocaron un efecto disuasorio en adultos del gorgojo de la vid *Otiorhynchus sulcatus* (Fabricius), que prefirieron las del testigo (Rondot y Reineke 2017). Por el contrario, las larvas de este gorgojo demostraron verse atraídas por macetas que contenían plantas con *M. anisopliae* (Kepler y Bruck 2006).

Estos trabajos revelan la capacidad de los insectos para detectar la colonización endofítica de los tejidos vegetales por parte de los AMEs, conducta que podría estar regulada por variaciones en el perfil de compuestos volátiles de la planta, aunque la ausencia de evidencias científicas puede conducir a la especulación.

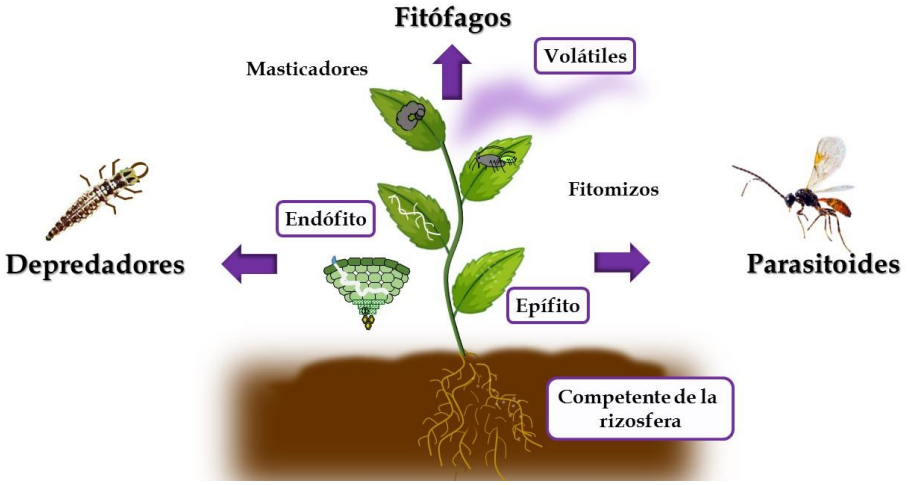

Figura 5. Efecto de la asociación de los AMEs con las plantas sobre las cadenas tróficas. La presencia de los AMEs en las plantas, ya sea como organismo competente de la rizosfera, epífito o endófito puede tener un efecto sobre insectos fitófagos (masticadores y fitomizos), así como sobre las poblaciones de enemigos naturales (depredadores y parasitoides). La producción de volátiles puede jugar un papel importante en estas interacciones.

5.2. Efecto sobre la relación insecto entomófago-insecto fitófago

La infección de insectos fitófagos por AMEs, iniciada bien por contacto, bien por vía endofítica, puede afectar a su comportamiento, y, por tanto, a sus relaciones intra e interespecíficas (Staples y Milner 2000; Roy et al. 2006; Meyling y Pell 2006). De hecho, se ha observado que varios insectos depredadores evitan entrar en contacto con AMEs, como *Anthocoris nemorum* (Linnaeus) (Meyling y Pell 2006) o *Coccinella septempunctata* Linnaeus (Ormond et al. 2011) que evitan insectos infectados por *B. bassiana*, aunque los mecanismos específicos que dan lugar a esta conducta se desconocen hasta el momento.

Aunque el empleo de agentes entomófagos y microorganismos entomopatógenos en el control biológico reduce los efectos sobre el medio ambiente y organismos no diana en comparación con la utilización de insecticidas convencionales, es necesario conocer la compatibilidad entre los mismos para el diseño de programas de control integrado de plagas (Roy et al. 2010).

De ahí, la existencia de numerosas investigaciones sobre la seguridad y la efectividad en el uso combinado de AMEs y otros agentes de control biológico (Roy y Pell 2000; Acevedo et al. 2007; Labbé et al. 2009; Ansari et al. 2010; Martins et al. 2014).

En general, se ha observado que la compatibilidad entre AMEs y entomófagos depende de la dosis fúngica y la secuencia de los tratamientos; dosis decrecientes y aplicación del entomófago antes que el AME reducen los efectos negativos sobre varios grupos de entomófagos como coccinélidos depredadores (James et al. 1995; Pingel y Lewis 1996; Todorova et al. 1996; Roy y Pell 2000; Smith y Krischik 2000; Pell y Vandenberg 2002; Roy et al. 2008), crisopas (Portilla et al. 2017), así como

sobre varias especies de parasitoides de pulgones (Brodeur y Rosenheim 2000; Mesquita y Lacey 2001; Kim et al. 2005; Aqueel y Leather 2013; Oreste et al. 2016; Shrestha et al. 2017).

Además, el grado de infección de la presa o el insecto hospedante y el intervalo de tiempo entre la aplicación fúngica y la liberación de los depredadores o parasitoides respectivamente, pueden afectar a su eficacia (Mesquita y Lacey 2001; Aqueel y Leather 2013; Ibarra-Cortés et al. 2018).

En general, se ha constatado que los tratamientos con HE pueden considerarse de bajo riesgo para depredadores y parasitoides, y por tanto, compatibles con los mismos. Los coccinélidos *C. septempunctata* y *Harmonia axyridis* (Pallas) consumen pulgones en un estado tardío de infección por *Pandora neoaphidis* Humber (Entomophthoromycota: Entomophthorales) y *Neozygites fresenii* (Nowakowski) Remaudière & S. Keller (Entomophthoromycota: Entomophthorales) (Pell et al. 1997; Roy et al. 1998, 2008; Simelane et al. 2008), el depredador *Chrysoperla carnea* (Stephens) se alimenta de larvas de *S. littoralis* previamente tratadas con *M. brunneum* (Rios-Moreno et al. 2018), y el parasitoide *Aphidius ervi* Haliday oviposita en pulgones infectados con *P. neoaphidis* (Baverstock et al. 2005).

En el caso de los depredadores, se ha detectado que, con cierta frecuencia, las crisopas no consumen por completo larvas de *S. littoralis* cuando éstas se encuentran infectadas por el hongo *M. brunneum*, bien para evitar zonas micosadas del cadáver, bien porque la infección fúngica puede reducir la calidad nutricional de la presa (Rios-Moreno et al. 2018). Otros trabajos también indican la capacidad de los depredadores para discriminar entre presas sanas e infectadas por AMEs (Pell y Vandenberg 2002; Meyling y Pell 2006; Rios-Moreno et al. 2018).

Sin embargo, existen pocos trabajos en los que se haya estudiado si la colonización endofítica de la planta por AMEs puede tener un efecto sobre integrantes del tercer nivel trófico, y los pocos trabajos que hay se han centrado en su efecto sobre los parasitoides.

En estos trabajos, no se ven afectados por la colonización endofítica por AMEs de la planta en la que se alimenta su insecto hospedante, ni en formación de momias, ni en emergencia, ni en ratio sexual (Akutse et al. 2014; Jaber y Araj 2018; Gathage et al. 2016). Además, solo se ha llevado a cabo un estudio sobre el uso combinado de AMEs endófitos y parasitoides para el control de pulgones (Jaber y Araj 2018).

6. Los ascomicetos mitospóricos entomopatógenos en el control del"pulgón del algodonero" *Aphis gossypii* (Homoptera: Aphididae)

El pulgón del algodonero *A. gossypii* ha sido utilizado como insecto modelo en los experimentos llevados a cabo en la presente tesis. En general, y centrando la descripción en la hembra virginópara áptera, esta tiene forma oval y un tamaño pequeño, aproximadamente 1-3 mm de longitud. El color de las formas adultas puede variar de amarillo pálido en colonias desarrolladas a altas temperaturas, hasta verdes o casi negros si se desarrollan con temperaturas más bajas, ya que se trata de una especie con un alto grado de polimorfismo. Incluso dentro de una misma colonia de pulgones procedentes de un mismo individuo se pueden observar variaciones considerables en color y tamaño (Rosenheim et al. 1995).

Es una especie cosmopolita, aunque se encuentra de manera particularmente abundante en los trópicos, y en casi toda su área de distribución es anholocíclica, aunque se ha observado que en ocasiones

puede comportarse como holocíclica y aparecer machos sexuados (Blackman y Eastop 1994; Barbagallo et al. 1998).

Se trata de una especie muy polífaga, ataca a más de 220 especies diferentes de plantas, cultivadas, ornamentales y adventicias, que pertenecen a más de 46 familias botánicas, aunque en las plantas cultivadas en las que suele ocasionar más daño son el algodón, las cucurbitáceas, los cítricos, las solanáceas y también el café y el cacao (Blackman y Eastop 2007). La especie muestra gran variabilidad en relación con la diversidad de plantas hospedantes y distribución geográfica (Blackman y Eastop 2007).

Este pulgón puede causar importantes pérdidas económicas en estos cultivos, como consecuencia del daño directo producido por su alimentación, lo que afecta a la producción, que se ve reducida, y a la calidad del fruto. Sin embargo, tienen más importancia los daños indirectos consecuencia de la transmisión de virus no persistentes y persistentes. La especie *A. gossypii* es capaz de transmitir más de 50 especies de virus, siendo los más comunes *Cucumber Mosaic Virus*CMV, *Cucurbit aphid-borne yellows virus*CABYV, el virus del mosaico amarillo del calabacín (*Zucchini yellow mosaic virus*, ZYMV) y el virus del mosaico 2 de la sandía (*Watermelon mosaic virus*-2, WMV) (Blackman y Eastop 1994).

Aunque existen numerosos métodos disponibles para el control de este áfido, el control biológico es la alternativa idónea para eliminar o reducir el empleo de insecticidas químicos de síntesis, a los que las poblaciones de pulgones desarrollan fácilmente resistencia.

Además, estos productos tienen un impacto negativo en el medio ambiente y la salud humana, por lo que las nuevas legislaciones promueven la reducción de su empleo e incluso prohibición, por lo que es imperante la aplicación de otras alternativas, entre las que destaca, sobre todo en cultivos protegidos, la utilización de agentes de control biológico macrobianos como himenópteros parasitoides o el díptero *Aphidoletes*

aphidimyza (Rondani), los cuales han conseguido controlar con bastante éxito las poblaciones de pulgones.

Entre los agentes de control microbiano para el control de pulgones destacan los AMEs, ya que infectan de forma natural a sus poblaciones y son los que poseen por ello mayor potencial para su control (Inglis et al. 2001; Pell et al. 2001; Evans 2003; Shah y Pell 2003; Nielsen y Hajek 2005; Völkl et al. 2007). La mayoría de las especies de HEs que se han descrito sobre pulgones pertenecen a las divisiones Ascomycota (orden Hypocreales, ej. *L. longisporum*, *B. bassiana*, *M. anisopliae*, y *Paecilomyces fumosoroseus*) y Entomophthoromycota (ej. *P. neoaphidis*, *Zoophthora radicans*, y *N. fresenii*). Existen numerosos estudios que demuestran que existen gran variedad de especies de AMEs que son capaces de controlar poblaciones del pulgón del algodón (*A. gossypii*) mediante su pulverización (Loureiro y Moino 2006; Vu et al. 2007; Kim y Roberts 2012; Jandricic et al. 2014). Además, su control se ha visto actualmente mejorado gracias al empleo de cepas de AMEs que muestran un comportamiento endofítico (Gurulingappa et al. 2010; Castillo-Lopez et al. 2014). Estos agentes de control biológico han demostrado reducir las poblaciones de pulgones debido a la mortalidad directa y efectos subletales reproductivos causados en las mismas (Kim 2007; Gurulingappa et al. 2010, 2011; Rashki y Shirvani 2013; Castillo-Lopez et al. 2014).

Figura 6. Colonia de pulgón del algodonero *Aphis gossypii* infectada por el AME *Beauveria bassiana*. Se indica mediante flechas rojas los especímenes infectados que muestran un crecimiento saprofítico externo.

7. Objetivos de la presente Tesis Doctoral

Los objetivos de la presente tesis doctoral son:

1.- Evaluar la capacidad endofítica de varias cepas de los HEs *B. bassiana* y *M. brunneum* en plantas de melón y determinar sus efectos sobre *A. gossypii*, tanto directos, mortalidad, como subletales reproductivos, así como la posible respuesta del pulgón frente a la planta colonizada de forma endofítica por las mismas.

2.- Evaluar el efecto de la colonización endofítica mediante *B. bassiana* sobre el comportamiento alimenticio de *A. gossypii* así como sobre la transmisión de virus en plantas de melón.

3.- Evaluar el efecto de la colonización endofítica de plantas de melón por cepas endófitas de AMEs sobre depredadores y parasitoides de *A. gossypii.*

Los resultados relativos al primer objetivo están expuestos en el capítulo II, que comprende el manuscrito González-Mas, N.; Sánchez-Ortiz, A.; Valverde-Garcia, P., Quesada-Moraga, E. 2019. Effects of endophytic entomopathogenic ascomycetes on *Aphis gossypii* Glover Life-History Traits and interaction with melon plants". Insects. https://doi.org/10.3390/insects10060165. Factor de Impacto: 1.848, posición Q1 (21/96) en "Entomology".

Los resultados del segundo objetivo se encuentran en el capítulo III, que corresponde con el manuscrito González-Mas, N., Quesada-Moraga, E., Plaza, M., Fereres, A., Moreno, A. 2019. Changes in feeding behaviour are not related to the reduction in the transmission rate of plant viruses by *Aphis gossypii* (Homoptera: Aphididae) to melon plants colonized by *Beauveria bassiana* (Ascomycota: Hypocreales)" Biological Control 130, 95-103,

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Los resultados del tercer objetivo se encuentran en el capítulo IV, que comprende el manuscrito Gonzalez‐Mas, N., Cuenca‐Medina, M., Gutierrez‐Sanchez, F., Quesada‐Moraga, E. 2019. Bottom-up effects of endophytic *Beauveria bassiana* on multitrophic interactions between the cotton aphid, *Aphis gossypii*, and its natural enemies in melon. Journal of Pest Science. https://doi.org/10.1007/s10340-019-01098-5. Factor de Impacto: 4.402, posición Q1 (2/96) en "Entomology".

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CAPÍTULO II. EFFECTS OF ENDOPHYTIC ENTOMOPATHOGENIC ASCOMYCETES ON THE LIFE-HISTORY TRAITS OF *APHIS GOSSYPII* GLOVER AND ITS INTERACTIONS WITH MELON PLANTS

Este capítulo es una versión adaptada del artículo: Aceptado el 5 de junio de 2019 en la revista *Insects*.

insects

MDPI

Article

Effects of Endophytic Entomopathogenic Ascomycetes on the Life-History Traits of *Aphis gossypii* Glover and Its Interactions with Melon Plants

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Received: 16 May 2019; Accepted: 5 June 2019; Published: 10 June 2019

Abstract: Entomopathogenic fungi are sprayed commercially for aphid control in greenhouses. Recently, their ability to grow endophytically within plants was discovered, offering the opportunity for systemic biological control. Endophytic colonization of host plants could also influence life-table parameters and behavior of herbivores. We investigated lethal and pre-mortality effects of *Beauveria bassiana* and *Metarhizium brunneum* on *Aphis gossypii*; aphids either received inoculum while feeding on recently sprayed leaves (surface inoculum and endophytically-colonized) or while feeding on unsprayed but endophytically-colonized leaves. We used choice assays to identify any preferences for endophytically-colonized or control plants. Volatile emissions from endophytically-colonized plants and control plants were also compared. Aphid mortality rates ranged between 48.2 and 56.9 % on sprayed leaves, and between 37.7 and 50.0 on endophytically-colonized leaves. There was a significant effect of endophytic colonization on the rate of nymph production, but this did not result in an overall increase in the aphid population. Endophytic colonization did not influence host-plant selection even though there were qualitative and quantitative differences in the blend of volatiles released by endophytically-colonized and control plants. Although endophytic colonization did not change herbivore behavior, plants still benefit via indirect defense, resistance to plant pathogens or abiotic stress tolerance.

Keywords: Biological control; *Beauveria bassiana*; *Metarhizium brunneum*; endophyte; pre-mortality effects; tritrophic interactions

1. Introduction

Controlling aphids is a challenge for growers because, not only do aphids cause damage to crops by direct feeding, but also by transmission of economically costly plant viruses [1]. Aphids also have extremely high fecundities and short developmental times, allowing populations to grow very rapidly [2]. For these reasons, control commonly relies on intensive use of systemic chemical insecticides which, coupled with their reproductive rate, has led to the emergence of widespread resistance to currently-available modes of action [3]. Entomopathogenic fungi are the most important microbial control agents naturally regulating aphid populations and, therefore, have great potential as biological control agents of these pests [4].

Effects of Endophytic Entomopathogenic Ascomycetes on the Life-History Traits of *Aphis gossypii* Glover and Its Interactions with Melon Plants

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Abstract

Entomopathogenic fungi are sprayed commercially for aphid control in greenhouses. Recently their ability to grow endophytically within plants was discovered, offering the opportunity for systemic biological control. Endophytic colonization of host plants could also influence life-table parameters and behavior of herbivores. We investigated lethal and pre-mortality effects of *Beauveria bassiana* and *Metarhizium brunneum* on *Aphis gossypii*; aphids either received inoculum while feeding on recently sprayed leaves (surface inoculum and endophytically-colonized) or while feeding on unsprayed but endophytically-colonized leaves. We used choice assays to identify any preferences for endophytically-colonized or control plants. Volatile emissions from endophytically-colonized plants and control plants were also compared. Aphid mortality rates ranged between 48.2 and 56.9 % on sprayed leaves, and between 37.7 and 50.0 on endophytically-colonized leaves. There was a significant effect of endophytic colonization on the rate of nymph production, but this did not result in an overall increase in the aphid population. Endophytic colonization did not influence host-plant selection even though there were qualitative and quantitative differences in the blend of volatiles released by endophytically-colonized and control plants. Although endophytic colonization did not change herbivore behavior, plants still

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1. Introduction

Controlling aphids is a challenge for growers because, not only do aphids cause damage to crops by direct feeding, but also by transmission of economically costly plant viruses (Van Emden and Harrington 2007).

Aphids also have extremely high fecundities and short developmental times, allowing populations to grow very rapidly (Dixon 1998). For these reasons, control commonly relies on intensive use of systemic chemical insecticides which, coupled with their reproductive rate, has led to the emergence of widespread resistance to currently-available modes of action (Whalon et al. 2016). Entomopathogenic fungi are the most important microbial control agents naturally regulating aphid populations and, therefore, have great potential as biological control agents of these pests (Brodeur et al. 2018).

Most fungal species naturally infecting aphids are from the Entomophthoromycota and Ascomycota (order Hypocreales). Species in the Ascomycota have been commercialised for aphid control because they can be readily mass-produced *in vitro* and easily formulated for spray application (Burges 1998). Indeed, several mycoinsecticides for aphid control are available on the market and sold as environmentally-safe products that fit perfectly into current legislation requirements (Lacey et al. 2015). They can be applied by spraying, drenching or seed dressing (Quesada-Moraga et al 2009; Sánchez-Rodríguez et al. 2018), or can be established as transient endophytes in targeted crops where they protect the plants from pests (Resquín-Romero et al. 2016; Garrido-Jurado et al. 2017; Vega 2018).

Whilst the efficacy of transient endophytic colonization has been proved against some sucking insects, such as whiteflies (Garrido-Jurado et al. 2017), it should be noted that the immature developmental

stages of these species are attached to the plant and do not move within and between plants. In contrast, all stages of aphids (except eggs) are mobile and probe the phloem to feed (Tjallingii 1988; Moreno et al. 2011). Hence, they could avoid contact with fungal propagules. For this reason, it is important to determine whether endophytic colonization of plants by entomopathogenic fungi can cause aphid mortality. It also remains unclear whether the conidia that remain coating the leaves and stems combined with endophytic colonization can affect aphid survival.

Furthermore, pre-mortality effects are known to occur following direct exposure to entomopathogenic fungi (Kim 2007; Gurulingappa et al. 2011; Akello and Sikora 2012; Castillo-Lopez et al. 2014, Clifton et al. 2018); possible pre-mortality effects on reproductive capacity (i.e. per capita aphid fecundity) following exposure to endophytic entomopathogenic fungi have yet to be investigated.

Many behavioral responses in insects (foraging, finding mates and oviposition sites, and avoiding natural enemies) are mediated by olfactory chemical cues emitted by both the plants and the insects (Xu and Turlings 2018). Plant volatile emissions can also change when the plant is colonized by microorganisms, which can influence the responses of herbivorous insects and their natural enemies to those plants (Yue et al. 2001; Hempel et al. 2009; D'Alessandro et al. 2014; Shikano et al. 2017; Contreras-Cornejo et al. 2018; Tasin et al. 2018); whether endophytic colonization induces analogous effects remains unknown, even though it has been reported that entomopathogenic fungi do emit volatiles and that microbial volatiles may modify insect interactions (Davis et al. 2013; Bojke et al. 2018). In this study, we determined the lethal and premortality effects of several isolates of entomopathogenic fungi against the cotton aphid *Aphis gossypii* Glover

when the aphids were either exposed to leaves that were both sprayed with the fungal suspension and endophytically colonized or only endophytically colonized by the fungi. Aphid preference for uncolonized or endophytically-colonized melon plants was evaluated, as well as the volatile emissions from endophytically-colonized and uncolonized plants.

2. Materials and methods

2.1. Biological material: aphid colonies, plants and fungal isolates

A virus-free laboratory population of *A. gossypii* was provided by the Institute of Agricultural Sciences (ICA) CSIC (Madrid, Spain) for use in the assays. The aphids were then maintained in rearing cages on melon plants (*Cucumis melo* L. cv. Siglo) for several generations in an environmental growth chamber under controlled conditions: 25 ± 2 ˚C, 16:8 h light:dark regime, and 70% RH. For each experiment, newly emerged apterous adult females (24–72 h after last moult) were collected from the rearing cages using a camel-hair brush and used immediately in experiments.

Certified endophyte-free melon seeds were surface sterilized in 2% NaOCl (Sigma-Aldrich, MO, USA) for 2 min, rinsed twice with sterile Mili-Q water and dried under a sterile airflow. The soil substrate in which they were to be grown was sterilized in an autoclave for 20 min at 121 °C; this was done twice with a 24-h interval between each sterilization. Seeds were germinated in 9x9 cm pots using a mixture of equal parts of vermiculite (No. 3, Asfaltex S.A., Barcelona, Spain) and sterilized soil substrate (Floragard, Germany) and maintained in an environmental chamber under controlled conditions: 25 ± 2 ˚C, 16:8 h light:dark regime. Plants were watered three times a week and a

nutrient complex of 20-20-20 (N:P:K) Nutrichem 60 fertilizer (Miller Chemical & Fertilizer Corp., PE, USA) was added to the irrigation water in a proportion of 1 gl^{-1}.

Two isolates of *Beauveria bassiana* and one isolate of *Metarhizium brunneum* were used in the assays; all isolates had verified endophytic activity when inoculated onto melon plants (Resquín-Romero et al. 2016, Garrido-Jurado et al 2017). These isolates are deposited at the University of Córdoba Entomopathogenic Fungi Collection, Córdoba, Spain and full details can be found in Table 1. All isolates were grown over cellophane film in Petri dishes of Potato Dextrose Agar (PDA) (Becton, Dickinson and Company, USA); the cellophane between the agar and the fungus was used to prevent nutrients entering the conidial suspensions at harvest. Cultures were incubated for 15 days at 25 ˚C in darkness and conidial suspensions were prepared by scraping the fungus from the cellophane into a sterile aqueous solution of 0.01% Tween 80. The resulting suspension was filtered through several layers of sterile cheesecloth to remove mycelia, and sonicated for 5 min to homogenize the inoculum. Concentration of viable conidia used for inoculation was determined using a haemocytometer and appropriate dilutions were made in 0.01% Tween 80 to obtain a conidial concentration of 10^8 conidia/ml. Prior to experimentation conidial viability was determined on liquid Czapek-Dox broth plus 1% (w/v) yeast extract medium and only suspensions with >97.0% germination after 24 hours were used.

Table 1. Origin of the fungal isolates used in the assays.

Isolate *	**Fungal Species**	**Origin**	**Agroecosystem**	**Habitat**	**GenBank Accession Number**	**Spanish Type Culture Collection (CECT) Accession Number**
EABb 04/01-Tip	*B. bassiana*	Ecija (Sevilla, Spain)	Opium poppy crop	Insect (*Iraella luteipes*)	FJ972963	20744
EABb 01/33-Su	*B. bassiana*	El Bosque (Cádiz, Spain)	Traditional olive orchard	Soil	FJ972969	-
EAMa 01/58–Su	*M. brunneum*	Hinojosa del Duque (Córdoba, Spain)	Wheat crop	Soil	JN900390	20764

2.2. Inoculation of melon plants with entomopathogenic fungi and verification of endophytic colonization

For each experiment and each fungal isolate replicate groups of four-leaf-stage melon plants were treated. Two leaves per plant were sprayed with 2 mL of the fungal suspension with an aerograph 27085 (piston compressor of 23 L/min, 15–50 PSI and a 0.3 mm nozzle diameter, China). One millilitre of 10^8-conidial suspension was sprayed over each leaf, resulting in a spray deposition at the level of the target surface of approximately 0.1 µL/mm^2. Using the CFU method, we estimated that the suspension produced a deposition of 10,000 viable conidia/mm^2.

During application the remaining two leaves per plant were protected from the spray with a transparent plastic sheet and remained uninoculated. After inoculation, all plants were covered by another plastic sheet to promote fungal growth for 24 h. Control plants were treated in the same way but only sprayed with sterile water with 0.01% Tween 80.

To confirm endophytic colonization, leaves were collected when each experiment had finished to avoid damaging the plant and triggering plant defences which may have confounded the results. Inoculated and uninoculated leaves were sampled from each replicate plant, surface-sterilized with 1% NaOCl for 2 min, rinsed twice in sterile distilled water, and dried on sterile filter paper. Sections of approximately 2 cm^2 were cut with a sterile scalpel from each leaf and plated out independently in Petri dishes containing selective culture medium to determine the percentage colonized endophytically; the medium contained: 20 g of Agar Sabouraud Glucose Chloramphenicol (Cultimed Panreac, Spain), 500 mg L^{-1} streptomycin sulfate (Sigma-Aldrich Chemie, China), 500 mg L^{-1} ampicillin (Intron biotechnology,

China) and 500 mg L^{-1} dodine 65 WP (Barcelona, Spain). We also plated out the final rinse water from each leaf separately to confirm the effectiveness of the surface-sterilization procedure. All plates were incubated at 25 ˚C in darkness until fungal growth was observed. Only data obtained from confirmed endophytically colonized leaves were used in the results.

2.3. Effect of endophytic plant colonization by entomopathogenic fungi on aphid mortality and fecundity

To assess mortality and pre-mortality effects of the fungal isolates on aphids five melon plants per isolate were sprayed as described above. Forty-eight hours after spraying, five apterous adult aphids (7 days old) were transferred, using a camel-hair brush, to sprayed and unsprayed leaves in treatment and control melon plants respectively. Each group of five aphids was confined in a clip cage and mortality and fecundity were recorded every two days for one week. On each occasion, newly deposited nymphs were removed from each clip cage once they had been counted. When dead adult aphids were observed they were removed from the clip cage and immediately surface sterilized in 1% NaOCl for 30 s, followed by two rinses with sterile water. Cadavers were placed on sterile wet filter paper in sterile Petri plates sealed with Parafilm® and incubated at 25 °C in darkness until fungal growth on the surface of the insect cuticle was observed. The entire experiment was repeated using new plants, aphid specimens and fungal inoculum.

2.4. Effect of entomopathogenic fungal plant colonization on aphid behavior

Treated and control plants were produced as described previously. Aphid behaviour was assessed in an assay where, for each fungal isolate, aphids were offered a choice between an endophytically-colonized leaf and an untreated control leaf; both leaves remained attached to the plant (Figure 1). One unsprayed leaf from the fungus-inoculated plant (endophytically-colonized) and one from the control plant were placed under each of two holes (diameter 25 mm each) made in the bottom of a Petri dish (150 mm × 15 mm) and covered by the lid. The area in contact with the plant was lined with eva rubber to avoid damaging the leaves. This created an arena where the leaves were positioned 7 cm apart from each other and aphids had access to 5 cm^2 from each plant. The positions of endophytically-colonized and untreated leaves in the Petri dish (left or right) were randomized to avoid any positional bias during observations. Twelve 24-hour starved aphids were introduced into the middle of the Petri dish helped by a paintbrush and incubated at 25 ± 2 ˚C, 16:8 h light:dark regime, and 70% RH. After 24 h their position (on the endophytically-colonized leaf, on the control leaf or on neither leaf) was recorded. There were three replicate arenas for each fungal isolate. The experiment was repeated using new plants, aphid specimens and fungal inoculum each time.

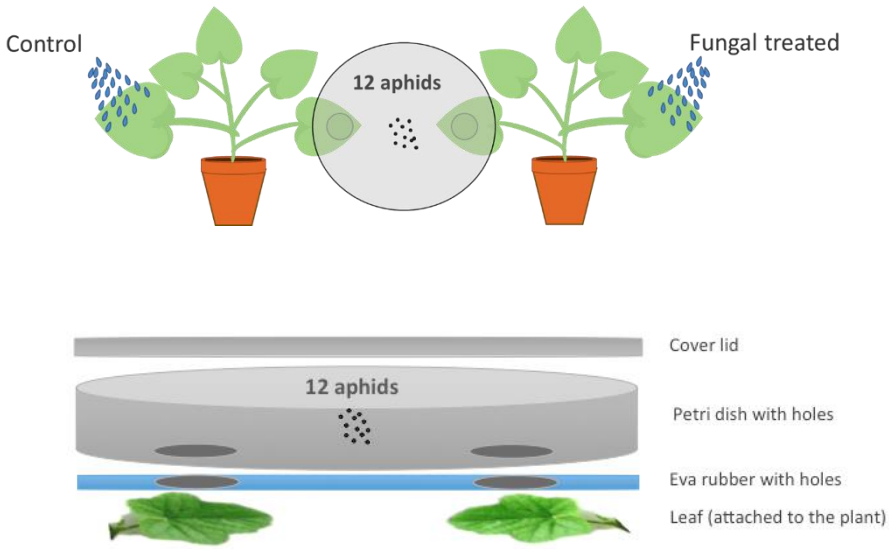

Figure 1. Design of an experimental arena for the choice bioassay. The set-up consists of one large Petri dish (150 mm x 15 mm) with two holes (diameter 25 mm each) in the bottom and covered by the lid. The area in contact with the plant was lined with eva rubber to avoid damaging the leaves. Aphids could choose between an unsprayed but endophytically-colonized leaf and a leaf from the control plant, or they could remain in the neutral area (no choice). The final position (endophytically-colonized leaf, control leaf, or neutral area) was recorded.

2.5. Analysis of volatile compounds from aphid-infested, endophytically-colonized leaves

Treated and control plants were produced as described previously. Five adult aphids were retained in clip cages on one sprayed and one unsprayed leaf from each replicate treated and control plant and incubated for 6 days at 25 ± 2 ˚C, 16:8 h light:dark regime, and 70% RH There were 18 replicate treated plants per isolate and 18 control plants. After 6 days the leaves on which the aphids had been feeding were sampled and the volatiles evaluated. Homogenates of each leaf were prepared according to Sánchez-Ortiz et al. (2013). Briefly, 3 g

from each leaf was homogenized for 1 min at 19,500 rpm in 6 mL of distilled water using an A 11 IKA analysis mill (IKA, Werke GmbH & Co.KG, Staufen, Germany). The sample was allowed to equilibrate for 5 min at 25 °C; 1 mL aliquots of homogenate were placed in vials containing the same volume of a saturated $CaCl_2$ solution, sealed and stored at −20 °C prior to analysis.

Volatile compounds were extracted from the samples using solid-phase microextraction (SPME) and analyzed using a High-Resolution Gas Chromatograph with Triple Quadrupole systems Mass Spectrometry (HR-GC-TQ MS) (Bruker model Scion 456-GC-TQ MS system; Bruker, Massachusetts, USA). Method parameters were set based on previous work (Sánchez-Ortiz et al. 2013). Briefly, each sample was heated to 40 °C and allowed to equilibrate for 10 min; a preconditioned SPME fiber (50/30 μm DVB/CAR/PDMS Stableflex 23Ga, Autosampler, SUPELCO, Bellefonte, PA, USA) was then introduced into the vial and volatile compounds were adsorbed for 50 min. Desorption of volatile compounds was done directly into the GC injector at 250 °C for 5 min. A Supelcowax 10 capillary column (30 m × 0.25 mm, 0.25 μm, Sigma-Aldrich Co. LLC) was used with the following parameters: Helium as the carrier gas; injector and detector set at 250 °C; column held for 5 min at 40 °C and then the temperature programed to increase at a rate of 4 °C min^{-1} until it reached 200 °C.

Identification of the volatile compounds detected was done by comparing their retention times against available standards purchased from Sigma-Aldrich-Merck, matching them to the NIST Library, or by comparing their MS spectra with reference spectra from different libraries (resemblance percentage above 80%). The method used for the identification was as follows: EI mode (70 eV), ion source and transfer line temperatures were all fixed at 250 °C. Mass spectra were

obtained in full scan mode in the 300 to 400 mass-to-charge ratio range at a scanning speed of 7 scan/s. Chromatograms and spectra were recorded and processed using the Bruker MS Workstation version 8.2 (Bruker, MA, USA). Volatile compounds were organized according to their chemical structure with respect to aldehydes, alcohols, ketones, terpenoid and phenolic derivatives and they were semi-quantified using an external standard calibration for each chemical group using hexanal, 1-octen-3-ol, 6-methyl-5-hepten-2-one, farnesene and ethylbenzene as standards, respectively. Each compound was semi-quantified against the external standard calibration of the corresponding chemical group in mg/L of homogenate and analyzed three times in duplicate experiments.

2.6. Statistical Analysis

Aphid mortality (%) was analyzed using the generalized linear mixed model method, with logit link and binomial distribution: $\text{Mortality}_{ijk} = \mu + treatment_i + experiment_j$, where k indicates plant (replicate). Treatment was modelled as a fixed effect and experiment was modelled as a random effect. The estimation method was maximum likelihood with Laplace approximation. Significance of the fixed effect (Treatment) was evaluated using the F-approximate test ($\alpha = 0.05$) and means from the different treatments were compared to the control using the Dunnett's test ($\alpha = 0.05$). Mortality data obtained from these experiments were also subjected to Kaplan–Meier survival analysis (Kaplan and Meier 1958) to calculate Average Survival Time (AST) values in days and compared by the Log-rank test.

The number of nymphs produced each day per aphid was analyzed using a linear mixed model for repeated measures to evaluate main effects and interactions between treatment and day:

$$No.\,of\ nymphs\ per\ aphid_{ijk} = \mu + treatment_i + day_j + treatment \times day_{ij}$$

Correlation between repeated measures was modelled as an autoregressive covariance matrix. Each replicate (plant), denoted by *k*, was the experimental unit for repeated measures. Treatment and day were modelled as fixed effects. The factor experiment was initially included in the model as a random effect, but it was not significant and, therefore, was removed from the model and the two experiments were combined in the same analysis. The linear mixed model was estimated using the restricted maximum likelihood (REML) method, and means were compared using the Dunnett's test ($\alpha = 0.05$) (Stroup 2012).

Aphid choice (between endophytically-colonized and control plants) was analysed using a Pearson Chi-square test ($p \leq 0.05$) to determine whether the observed frequencies were significantly different to the expected ones (50% : 50%). Data on volatile compounds were analyzed using Shapiro–Wilk's and Levene's tests to evaluate linear model assumptions (normality and homogeneity of variance) and analysis of variance (ANOVA) applied at a significance level of 0.05. Mixed models were analyzed with proc GLIMMIX-SAS 9.3; Pearson Chi-square, Kaplan Meier and linear models were analyzed with SPSS 24.0.

3. Results

3.1. Endophytic colonization of melon plants

Microbiological techniques confirmed endophytic fungal colonization in 100% of the leaves that had received fungal sprays directly, but in only 20–40% of the leaf sections from unsprayed leaves from treated plants. Individual plants were considered as positive for endophytic colonization based on detection of fungus within any of the leaves sampled on that plant.

3.2. Effect of endophytic colonization on aphid mortality

The likelihood ratio tests to evaluate the effect of the experiment for both fungus sprayed leaves and unsprayed leaves were not significant ($\chi 2_{1df;}$ $p > 0.05$). For leaves sprayed with the fungal suspension (i.e. leaves with conidia on the surface and endophytically colonized), there was, overall, a significant effect of treatment on aphid mortality ($F_{3,28} = 5.63$; $p = 0.0038$; Table 2); there were significant differences between isolates and the control according to the Dunnett's test (EABb 01/33-Su: $t_{28df} = 3.31$; $p = 0.006$; EABb 04/01-Tip: $t_{28df} = 3.81$; $p = 0.0017$; EAMa 01/58-Su: $t_{28df} = 3.60$; $p = 0.0029$) (Table 2). For leaves that were only endophytically colonized (i.e. leaves not sprayed with the fungal suspension but on the same plant as fungus-sprayed leaves) the overall effect of treatment on mortality was also significant ($F_{3,28}=3.36$; $p = 0.0328$; Table 3). The mortality caused by isolate EABb 01/33-Su was significantly higher than the mortality in the control (Dunnett's test $t_{28df} = 3.01$, $p = 0.0135$; Table 3). There were no significant differences between the mortalities caused by isolates EABb 04/01-Tip and EAMa 01/58-Su and the mortality in the control at $\alpha=0.05$, although these differences were statistically significant at $\alpha = 0.1$ (Dunnett's test $t_{28df} = 2.22$, $p = 0.0796$ for strain EABb 04/01-Tip;

Dunnett's test $t_{28df} = 2.36$, $p = 0.0585$ for strain EAMa 01/58-Su; Table 3).

Table 2. Susceptibility of *A. gossypii* adults to entomopathogenic fungal isolates after exposure to leaves with conidia on their surfaces and which were also endophytically-colonized by those fungi.

Treatment	Mortality (%)	Kaplan-Meier Survival Analysis		
	Mean (± SE) [a]	AST [b] (± SE; days)	Confidence interval (95%)	
			Lower limit	Upper limit
Control	0.80 ± 0.06	6.56 ± 0.25 a	6.07	7.05
EABb 01/33-Su	48.15 ± 0.18 *	6.15 ± 0.28 b	5.60	6.71
EABb 04/01-Tip	56.92 ± 0.18 *	6.18 ± 0.25 b	5.70	6.66
EAMa 01/58-Su	53.68 ± 0.18 *	5.77 ± 0.28 b	5.22	6.33

[a] Means with * are significantly different from the control according to the Dunnett's test ($\alpha = 0.05$). [b] AST: Average survival time. Means within columns with the same lower-case letter are not significantly different to each other according to the log rank test ($p < 0.05$). Average survival time was limited at 7 days. Data are pooled from the two occasions on which the experiment was run.

3.3. Effect of endophytic colonization of plants on aphid fecundity

The likelihood ratio tests to evaluate the effect of the experiment for both fungus sprayed leaves and unsprayed leaves were not significant ($\chi 2_{1df;}\ p > 0.05$) and therefore the two experiments were combined. The analysis of the pooled fecundity data indicated that there was a significant effect of time on the number of nymphs deposited by aphids feeding both on fungus-sprayed leaves and unsprayed but endophytically-colonized leaves ($F_{2,69.8} = 52.01$; $p < 0.001$ and $F_{2,69.8} = 44.25$; $p < 0.001$ respectively). However, there was no significant effect of treatment ($F_{3,55.07} = 0.29$; $p = 0.84$) or a treatment*day interaction for the fungus-sprayed leaf ($F_{6,84.2} = 0.82$; $p = 0.56$) (Figure 2). Conversely, for the unsprayed but endophytically-colonized leaf, the effect of treatment and the treatment*day interaction

were significant, at α = 0.1 (treatment: $F_{3,56.6}$ = 2.35; p = 0.08; treatment*day interaction: $F_{6,81.7}$= 2.03; p = 0.07) (Figure 3). The maximum reproductive activity of aphids was attained earlier in aphids feeding on endophytically-colonized leaves than in the controls (Figure 3). In fact, significant differences were detected by day 3 of the assay, if the number of nymphs laid by aphids feeding on leaves endophytically-colonized by isolate EABb 01/33-Su is compared with the number laid by aphids feeding on control leaves ($t_{103.df}$ = 2.89; p = 0.01).

Nonetheless, although fecundity was accelerated, this did not result in an overall increase in the aphid population compared with the control (Figure 4). At the end of the assay (day 5), aphids feeding on endophytically-colonized leaves laid, in general, fewer nymphs than ones feeding on control plants. This was particularly apparent in aphids feeding on leaves endophytically-colonized by isolate EAMa 01/58-Su (t_{103df} = −2.49; p = 0.04).

Table 3. Susceptibility of *A. gossypii* adults to entomopathogenic fungal isolates after exposure to leaves that were endophytically-colonized by those fungi.

Treatment	**Mortality (%)**	**Kaplan-Meier Survival Analysis**		
	Mean (± SE) [a]	**AST** [b] **(± SE; days)**	**Confidence interval (95%)**	
			Lower limit	Upper limit
Control	13.74 ± 0.08	6.62 ± 0.21 a	6.22	7.03
EABb 01/33-Su	49.99 ± 14.09 *	5.90± 0.32 b	5.28	6.52
EABb 04/01-Tip	37.71 ± 13.61	6.41 ± 0.28 a	5.85	6.96
EAMa 01/58-Su	40.08 ± 13.56	6.11 ± 0.25 b	5.62	6.60

[a] Means with * are significantly different from the control according to the Dunnett's test (α = 0.05). [b] AST: Average survival time. Means within columns with the same lower-case letter are not significantly different to each other according to the log rank test (p < 0.05). Average survival time was limited at 7 days. Data were pooled from the two occasions on which the experiment was run.

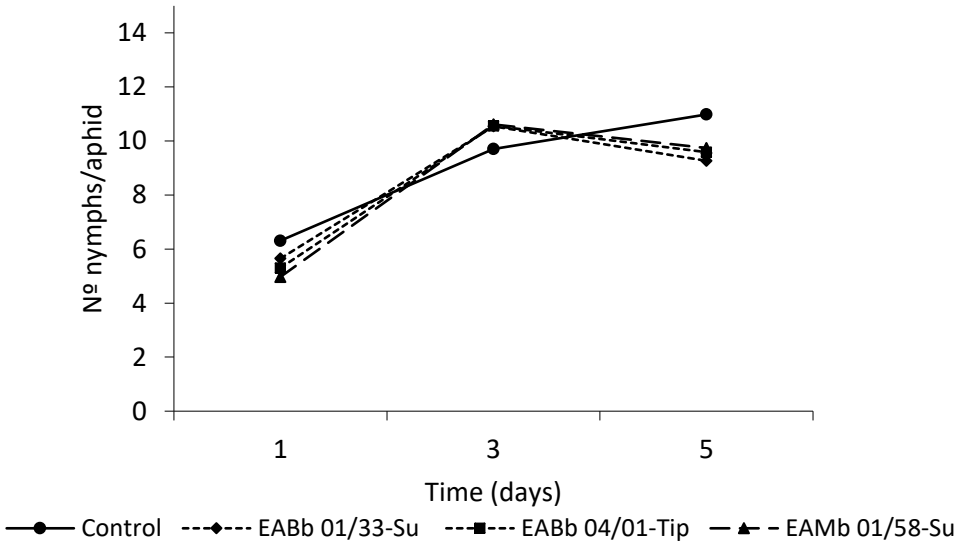

Figure 2. Per capita aphid fecundity over time (5 days) after aphid exposure to a sprayed leaf (with both surface conidia and endophytic colonization by one of three fungal isolates or the control). Evaluation began (day 1 on graph) 72 hours after leaves had been sprayed. Data represents the average of two trials.

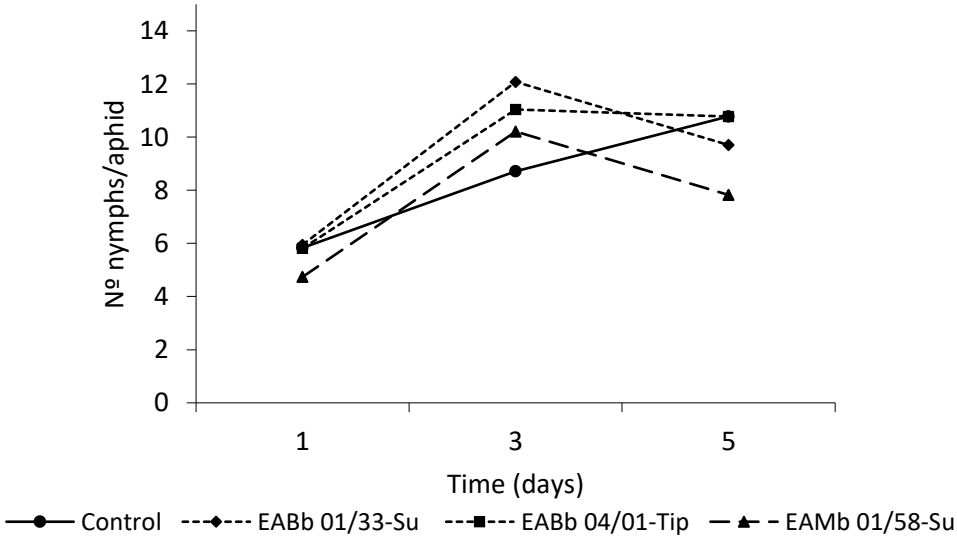

Figure 3. Per capita aphid fecundity over time (5 days) after exposure to an unsprayed leaf (endophytically-colonized by one of three fungal isolates or the control). Evaluation began (day 1 on graph) 72 hours after leaves had been sprayed. Data represents the average of two trials.

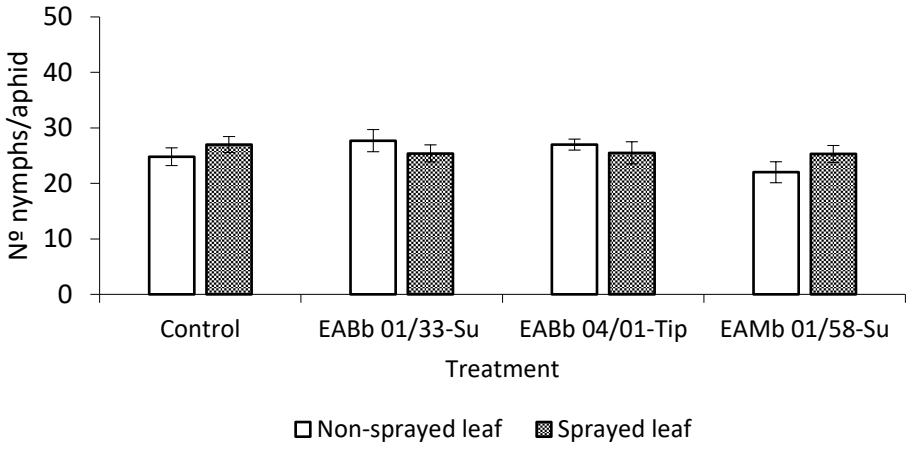

Figure 4. Total per capita aphid fecundity 5 days after applying the aphids to the plant (7 days after leaves had been sprayed). Fecundity data are pooled from the two occasions that the experiment was run.

3.4. Effect of endophytic-colonization of plants on aphid behavior

Endophytic colonization of leaves by entomopathogenic fungi did not influence host plant selection by aphids (Figure 5). There were no significant differences in the number of aphids on control and endophytically-colonized leaves when the aphids were offered a choice between the two, and this was consistent for all isolates either in the analyses by experiment ($\chi 2_{1df;}$ $p > 0.05$) or in the analyses with the experiments combined: EABb 01/33-Su (likelihood ratio test $\chi 2_{1df} = 0.19$; $p = 0.66$); EABb 04/01-Tip (likelihood ratio test $\chi 2_{1df} = 0.41$; $p = 0.52$); EAMb 01/58-Su (likelihood ratio test $\chi 2_{1df} = 0.27$; $p = 0.60$).

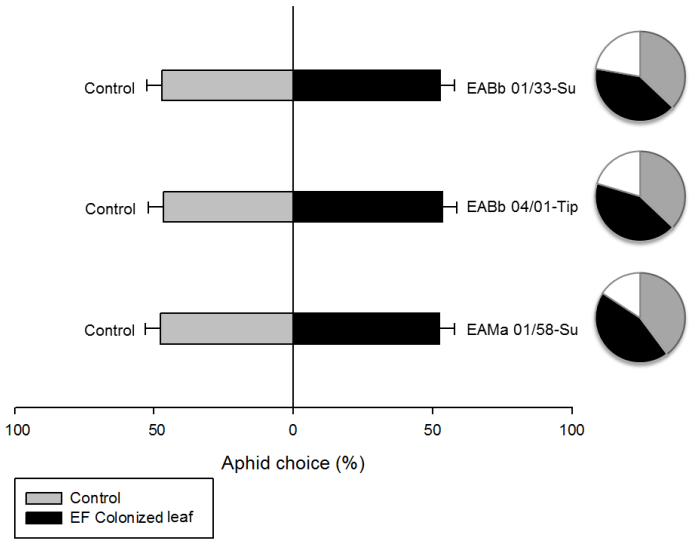

Figure 5. Percentage of *Aphis gossypii* adults choosing one of the two leaves offered in the choice experiment. The following choices were offered: (a) Unsprayed leaf from a fungus-sprayed plant (i.e. endophytically colonized only); (b) Unsprayed leaf from a control plant. Bars denote percentage of aphids choosing either the control or the endophytically-colonized leaf out of the total number of aphids making a choice. Circles denote the proportion of aphids orienting towards (a) the unsprayed endophytically-colonized leaf (black), (b) the unsprayed leaf from a control plant (grey) and (c) aphids showing no decision (white).

3.5. Volatile compounds from aphid-infested and endophytically-colonized leaves

A large number of volatile organic compounds (VOCs) were emitted from aphid-infested, endophytically-colonized leaves: terpenoids, phenylpropanoids/benzenoids, amino acid derivatives and fatty acid derivatives such as: aldehydes, alcohols, and ketones (Figure 6). Aldehydes were the main group of VOCs emitted by plants; E-2-hexenal and (E,Z)-2,6-nonadienal were emitted at the highest concentrations. In contrast, terpenoid and phenolic derivatives were

emitted in the lowest concentrations. The full listing of VOCs emitted are in Supplementary Tables 1–3.

These VOCs are biosynthesized as primary and secondary metabolites by complex enzyme pathways distributed across different organelles or compartments within the plant. In general, the vegetative tissues of melon only release small quantities of VOCs but can be induced by mechanical damage by herbivores. In this regard, the use of homogenized melon plants (i.e. complete destruction of plant tissue) was effective in establishing differences in the volatile profiles emitted from leaves that had been inoculated with different isolates of fungi compared with controls.

Finally, from Supplementary Tables 1-3 VOCs could largely be grouped into Σaldehydes, Σalcohols, and Σketones. Specifically: Σaldehydes = butanal, 3-methyl-butanal, pentanal, hexanal, 3-methyl-hexanal, (E)-2-pentenal, heptanal, 5-methyl-hexanal, (Z)-2-hexenal, (E)-2-hexenal, (Z)-4-heptenal, octanal, (Z)-2-heptenal, (E)-2-octenal, (E)-6-nonenal, (E,E)-2,4-hexadienal, nonanal, (E,E)-2,4-heptadienal, (E)-2-nonenal, (E,Z)-2,6-nonadienal, (E)-4-oxohex-2-enal; Σalcohols = ethanol, 1-penten-3-ol, 4-methyl-1-pentanol, (Z)-3-hexen-1-ol, (E)-2-hexen-1-ol, 1-octen-3-ol, 2-ethyl-1-hexanol, 2-propyl-1-pentanol; Σketones = 6-methyl-2-heptanone, 3-octanone, 6-methyl-5-hepten-2-one, (E,E)-3,5-octadien-2-one. There were also: terpenoic derivates = β-cyclocitral, eucarvone, 2-pinen-4-ol, β-citral, geranial, isomethyl-α-ionone, E-β-ionone, 2,2,6-trimethyl-cyclohexanone, β-ionone; and Σphenolic derivates = benzaldehyde, acetophenone, benzyl alcohol, 2-phenyl-isopranol, phenol, benzophenone, toluene.

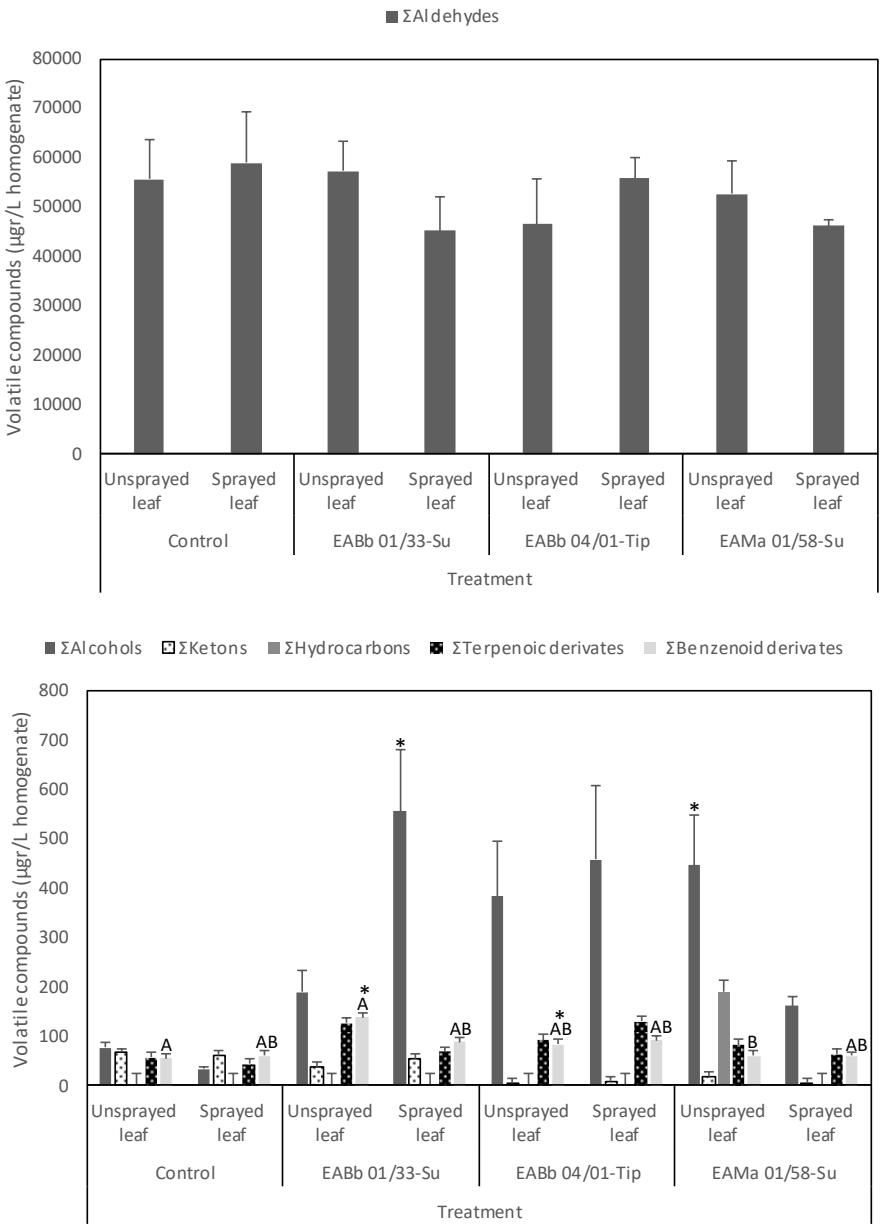

Figure 6. Aldehydes, alcohols, ketones, hydrocarbons, terpenoid and phenolic derivatives (Mean ± SE, from the two trials) emitted by melon leaves from different treatments (unsprayed leaves and sprayed leaves) in control and inoculated plants by different isolates: EABb 01/33-Su, EABb 04/01-Tip and EAMa 01/58-Su. Statistically significant differences were determined using the LSD ($\alpha = 0.05$).

4. Discussion

This study emphasizes the benefits of entomopathogenic fungi for crop protection that are in addition to the direct mortality that the fungus causes. Aphids may come into contact with conidia on the surface of sprayed leaves that are also endophytically colonized, or with leaves on the same plant that have not been sprayed so have no surface conidia, but are endophytically colonized.

Using microbiological techniques, high levels of endophytic fungal colonization were detected within fungus-sprayed leaves, while endophytic fungi were recovered from 20–40% of leaf sections from unsprayed leaves on the same plants as the sprayed leaves. The use of more sensitive endophytic colonisation-detection techniques may allow us to quantify the extent of colonisation more accurately and decrease the risk of false negatives.

Despite this, our results showed a significant decline in aphid survival on fungus-sprayed leaves that were also endophytically-colonized (leaves sprayed with the fungal suspension) compared with controls and this was consistent for all fungal isolates evaluated. However, when aphids fed on leaves that were only endophytically colonized there was only a significant difference in aphid mortality on leaves colonized with isolate EABb 01/33-Su compared with the control. Nevertheless, aphid mortality on leaves colonized by isolates EABb 04/01-Tip and EAMa 01/58-Su was significantly greater that the control at the 10% significance level. These results are consistent with the study of Garrido-Jurado et al. (2017), who also demonstrated that isolate EABb 01/33-Su had greater potential to control sap-sucking insects as an endophyte compared with the other isolates.

A reduction in aphid reproduction on endophytically-colonized plants has been demonstrated previously (Gurulingappa et al. 2010;

Akello and Sikora 2012; Castillo-Lopez et al. 2014; Clifton et al. 2018). In our study we did not observe any effect of endophytic colonization on total *per capita* aphid fecundity 6 days after exposure (8 days after leaf spraying). However, we did show, for the first time, a modification in the pattern of aphid fecundity during feeding on uninoculated but endophytically-colonized leaves; endophytic colonization accelerated aphid reproductive activity, revealing a different reproductive strategy than their control counterparts. This response ensured reproductive success achieved its full potential before the onset of fungal infection and death (Webb and Hurd 1996).

We observed significant qualitative and quantitative differences in the blend of volatiles released by endophytically-colonized and control leaves, which have potential to influence insect behavior. This is the first attempt to describe the total blend of volatiles released by endophytically-colonized melon leaves following herbivore damage. These VOCs are biosynthesized as primary and secondary metabolites by complex enzyme pathways distributed across different organelles or compartments within the plant. In general, the vegetative tissues of melon only release small quantities of VOCs but can be induced by mechanical damage by herbivores. In this regard, the use of homogenized melon plants (i.e. complete destruction of plant tissue) was effective in establishing differences in the volatile profiles emitted from leaves that had been inoculated with different isolates of fungi compared with controls.

When endophytically-colonized and control melon leaves were infested by *A. gossypii*, our volatile analysis revealed the presence in both treatments of a characteristic blend of melon plant volatiles that has been described previously (Kende et al. 2019); this blend is derived from five biosynthetic classes*:* aldehydes, alcohols, ketones, terpenoids

and phenolic compounds. A number of the compounds we recorded here were found in previous publications on melon volatiles (compounds 4, 7, 9, 17 25, 32, and 39 in Table 1, Supplementary material) (Kende et al. 2019). Furthermore, many volatile compounds we found have been reported in a wide range of plant species and are regarded as herbivore-induced plant volatiles (compounds 4, 9, 25, and 42 in Table 1, Supplementary material) (McCall et al. 1994; Aartsma et al. 2018; Turlings and Erb 2018). For example, the release of benzyl alcohol has been reported in *Camellia sinensis* and *Coffea canephora* under attack from different insect-feeding guilds (Cai et al.2014) and even when mechanically damaged (Cruz-López et al. 2016). In aphid species, perception of some of these plant-specific volatile component assists olfactory discrimination between host and non-host plants (Visser 1986).

Some of the compounds we identified were only detected in endophytically-colonized plants (compounds 8, 24, 29, 34, 38, 40, 41, 43, 46, and 47 Table 1, Supplementary material) or were only detected in control plants and suppressed in endophytically-colonized plants (compounds 2, 3, 5, 10, 12, 13, 22, 27, 29, 35, 36, 38, 41, and 47 Table 1, Supplementary material). These differences in production of particular VOCs could be as a result of endophytic colonization by entomopathogenic fungi. Many different alkyl benzenes have been retrieved previously from plants inoculated with entomopathogenic fungi and infested by *Delia radicum* (Diptera: Anthomyiidae), especially in the presence of high densities of entomopathogenic fungal inoculum (Cotes et al. 2015). For example, we only detected benzyl alcohol in endophytically-colonized plants. This compound, together with methyl benzoate and benzaldehyde, are known to elicit very strong responses in the important pollinators *Manduca sexta* and *Sphinx perelegans*

(Lepidoptera: Sphingidae) (Raguso and Light 1998; Hoballah et al. 2005). Benzaldehyde, which was found in high levels in leaves that had been sprayed with isolate EABb 04/01-Tip, is thought to be a biomarker for pathogenic infections in algae, together with 1-octen-3-one and E-2-nonenal (Sun et al. 2012).

We detected the terpenoids and carotenoid derivatives, beta-ionone and trans-beta-Ionone only in endophytically-colonized plants. It is reported that carotenoid derivatives affect insect behavior (Flath et al. 1994). For example, beta-ionone is highly attractive to beetles including *Anomala transvaalensis* (Coleoptera: Scarabaeidae) (Donaldson et al. 1990). However, this compound is repellent to *Cnaphalocrocis medinalis* (Lepidoptera: Pyralidae) (Duan et al. 2019). Beta-ionone is also present in rice extracts and attracts rice planthoppers (Obata et al. 1983).

Another fact that is immediately apparent from this study is the great increase in alcoholic compounds released by fungal sprayed and endophytically-colonized leaves (Figure 6), although there was no difference in the quantity of aldehydes, ketones, terpenoids and phenolic compounds when they were considered as a group.

In the present study endophytic colonization of leaves by entomopathogenic fungi did not influence host plant selection by aphids, which is likely to be the case for most insect-microbe associations (Davis et al. 2013). There have been a few studies evaluating the effect of endophytic colonization by entomopathogenic fungi on host plant selection behavior by herbivores (Kepler and Bruck 2006; Rondot and Reineke 2017; Sword et al. 2017). In some cases, phytophagous insects are able to detect and subsequently avoid plants treated with entomopathogenic fungi (Rashki and Shirvani 2013; Rondot and Reineke 2017; Sword et al. 2017) while others are attracted

by plants treated with entomopathogenic fungi (Kepler and Bruck 2006). A major difference between these experiments and our own was the phenological stage of the plant and how long the plant had been colonized by the entomopathogenic fungi, which was longer than in our study. It might be expected that, in older plants, the volatiles emitted would be different and could change the results. Furthermore, herbivore behavior was measured for 6 hours, and the deterrent effect was more evident at the beginning of the experiment than at the end, when final establishment of the herbivore was recorded. Results may have been different if a shorter response time had been used.

5. Conclusions

The results obtained in this study support the hypothesis that following a spray application of entomopathogenic fungi plant defense continues beyond the initial pest mortality caused, as a result of the endophytic capacity of some isolates. However, the effects described should now be confirmed under greenhouse and field conditions.

Further research on the volatile compounds released by plants entomopathogenic fungi endophytically colonizing would lead to a deeper understanding of the behavioral responses of the insect community associated with these plants. The benefits of endophytic colonization by entomopathogenic fungi for sustainable crop protection could be exploited more fully; these include direct and indirect defense, pathogen resistance and abiotic stress tolerance.

6. Acknowledgements

Funding: Funding was provided by the Spanish Ministry of Economy and Competitivity via grant AGL2016-80483-R 'Interacciones multitróficas reguladas por hongos entomopatógenos para una protección de cultivos sostenible'.

7.Supplementary material

Table 1. *Mean value from three measurements in two different experiments (µg/L homogenate). Values for each volatile with different letters in the same row within each treatment (control and EABb 01/33-Su isolate leaves) are significantly different to each other ($P < 0.05$)

		Control						EABb 01/33-Su					
	Volatile compound*	Unprayed leaf			Sprayed leaf			Unsprayed leaf			Sprayed leaf		
	Aldehydes												
1	Butanal	33.46 ± 1.46			34.20 ± 1.27			32.72 ± 6.51					
2	3-methyl-Butanal	516.44 ± 64.81			460.33 ± 160.92			36.40 ± 16.99					
3	Pentanal	277.76 ± 51.60		A	100.53 ± 25.31		BC	132.58 ± 18.69		B			
4	Hexanal	2821.38 ± 490.76		A	2258.31 ± 587.30		A	2529.28 ± 358.45		AB	256.45 ± 84.93		B
5	3-Methyl-hexanal	29.94 ± 7.12			15.87 ± 2.86								
6	(E)-2-Pentenal	455.69 ± 59.25			603.18 ± 82.50			428.06 ± 86.53			493.13 ± 47.47		
7	Heptanal	42.99 ± 3.99			30.98 ± 7.79			54.63 ± 5.16					
8	5-Methyl-Hexanal												
9	(E)-2-Hexenal	1002.89 ± 154.60			1270.47 ± 218.95			976.03 ± 53.39			983.02 ± 197.96		
10	(Z)-2-Hexenal	40983.33 ± 5616.16			50015.83 ± 8148.49			41826.67 ± 2350.12			41781.88 ± 5975.75		
11	(Z)-4-Heptenal	34.68 ± 6.16		A	26.42 ± 5.31		A	42.08 ± 3.40		AB			
12	Octanal	68.40 ± 10.32		A	67.36 ± 11.83		A	54.92 ± 7.66		A			
13	(Z)-2-Heptenal	157.52 ± 31.36			31.96 ± 7.42			52.55 ± 14.04			28.87 ± 8.40		
14	(E)-2-Octenal	43.70 ± 3.23		AB	87.15 ± 9.23		A	88.85 ± 8.94		AB			
15	(E)-6-Nonenal	1266.36 ± 185.46		A	337.33 ± 42.74		BC	1734.36 ± 605.31		AB			
16	(E,E)-2,4-Hexadienal	421.24 ± 38.15		AB	585.81 ± 75.03		A	293.68 ± 22.73		B	587.13 ± 110.96		A
17	Nonanal	364.74 ± 78.17		A	309.59 ± 47.52		A	164.30 ± 17.51		B			
18	(E,E)-2,4-Heptadienal	1370.72 ± 198.33			1195.76 ± 176.80			1061.41 ± 77.27			955.62 ± 189.68		
19	(E)-2-Nonenal	294.45 ± 14.72			26.91 ± 3.61			740.13 ± 324.88			20.98 ± 3.47		
20	(E,Z)-2,6-Nonadienal	5396.60 ± 983.01			1520.04 ± 536.14			7068.75 ± 2008.75			144.49 ± 55.75		
21	(E)-4-Oxohex-2-enal	32.89 ± 5.73		A	41.30 ± 10.39		A	23.72 ± 1.56		B			
	Alcohols												
22	Ethanol												
23	1-Penten-3-ol	2.30 ± 0.57			3.35 ± 0.93								
24	4-Methyl-1-pentanol							36.51 ± 7.81		AB	151.41 ± 25.21		A
25	(Z)-3-Hexen-1-ol	4.23 ± 0.33		B	1.72 ± 0.41		B	9.28 ± 0.24		B	145.18 ± 33.15		A
26	(E)-2-Hexen-1-ol	12.76 ± 2.71		B				48.22 ± 16.91		B	204.22 ± 54.75		A
27	1-Octen-3-ol	41.15 ± 4.35		A	11.76 ± 1.03		BC	50.77 ± 8.20		A	11.01 ± 0.85		B
28	2-Ethyl-1-hexanol	15.91 ± 2.81		A	16.17 ± 1.20		A	18.56 ± 5.92		AB			
29	2-Propyl-1-pentanol							25.90 ± 6.03		AB	43.64 ± 9.30		A
	Ketones												
30	6-Methyl-2-heptanone	0.66 ± 0.02		A	0.46 ± 0.04		AB						
31	3-Octanone	2.07 ± 0.39		B	0.65 ± 0.13		B	8.31 ± 1.89		AB	17.80 ± 4.93		A
32	6-methyl-5-Hepten-2-one	1.31 ± 0.18		B	0.75 ± 0.20		B	23.09 ± 3.37		AB	31.97 ± 4.34		A
33	(E,E)-3,5-Octadien-2-one	61.86 ± 3.43			60.23 ± 7.04			5.39 ± 0.58			4.30 ± 0.16		
	Hydrocarbons												
34	3-ethyl-1,4-Hexadiene												
	Terpenoic derivates												
35	β-Cyclocitral	14.79 ± 1.89		B	16.37 ± 4.05		B	13.29 ± 1.35		AB	18.37 ± 3.78		A
36	Eucarvone	3.88 ± 1.04			5.49 ± 2.28								
37	2-Pinen-4-ol	1.77 ± 0.02			1.02 ± 0.15								
38	β-Citral							2.17 ± 0.60		AB	3.48 ± 0.44		A
39	Geranial	32.71 ± 0.94			17.89 ± 3.00			58.98 ± 5.33			26.76 ± 0.52		
40	Isomethyl-α-ionone							19.78 ± 1.44					
41	E-β-ionone							18.92 ± 1.57					
42	2,2,6-Trimethyl -cyclohexanone	3.54 ± 0.39			2.39 ± 0.32			2.62 ± 0.32			4.28 ± 1.02		
43	β-Ionone							9.82 ± 0.43		A	14.57 ± 1.77		B
	Phenolic derivates												
44	Benzaldehyde	27.00 ± 3.74		AB	32.57 ± 3.93		AB	47.44 ± 13.63		A	21.21 ± 9.72		A
45	Acetophenone	7.28 ± 0.54			7.11 ± 0.45			7.59 ± 0.85			7.02 ± 0.72		
46	Benzyl alcohol							16.85 ± 4.67		B	31.53 ± 4.05		A
47	2-Phenyl-isopranol							19.93 ± 7.85			±		
48	Phenol	4.86 ± 0.54			3.82 ± 0.61			17.56 ± 4.43			9.77 ± 1.99		
49	Benzophenone	10.48 ± 0.91			10.36 ± 0.88			11.42 ± 1.39			10.23 ± 1.49		
50	Toluene	5.62 ± 0.19		B	6.08 ± 0.57		B	17.03 ± 3.50		A	8.16 ± 0.71		B

Table 2. *Mean value from three measurements in two different experiments (μg/L homogenate). Values for each volatile with different letters in the same row within each treatment (control and EABb 04/01-Tip isolate leaves) are significantly different to each other ($P < 0.05$)

	Control						EABb 04/01-Tip					
Volatile compound*	UnSprayed leaf			Sprayed leaf			Unsprayed leaf			Sprayed leaf		
Aldehydes												
Butanal	33.46 ± 1.46			34.20 ± 1.27			11.33 ± 0.87			33.23 ± 1.89		
3-methyl-Butanal	516.44 ± 64.81			460.33 ± 160.92			301.46 ± 26.42			167.43 ± 47.06		
Pentanal	277.76 ± 51.60		A	100.53 ± 25.31		B	116.04 ± 10.67		B	135.82 ± 9.21		B
Hexanal	2821.38 ± 490.76			2258.31 ± 587.30			1695.53 ± 291.95			2186.75 ± 386.69		
3-Methyl-hexanal	29.94 ± 7.12			15.87 ± 2.86			15.92 ± 7.34			6.66 ± 1.52		
(E)-2-Pentenal	455.69 ± 59.25		AB	603.18 ± 82.50		A	288.91 ± 66.72		B	469.59 ± 108.89		AB
Heptanal	42.99 ± 3.99		B	30.98 ± 7.79		B	180.43 ± 8.61		A			B
5-Methyl-Hexanal							180.73 ± 8.31					
(E)-2-Hexenal	1002.89 ± 154.60			1270.47 ± 218.95			670.75 ± 16.23			1185.10 ± 77.78		
(Z)-2-Hexenal	40983.33 ± 5616.16		AB	50015.83 ± 8148.49		A	36584.38 ± 8388.05		B	46529.17 ± 2377.70		A
(Z)-4-Heptenal	34.68 ± 6.16			26.42 ± 5.31			34.24 ± 0.49			40.58 ± 4.50		
Octanal	68.40 ± 10.32			67.36 ± 11.83			47.66 ± 7.54			46.78 ± 11.66		
(Z)-2-Heptenal	157.52 ± 31.36		A	31.96 ± 7.42		AB	20.35 ± 5.26		AB			B
(E)-2-Octenal	43.70 ± 3.23			87.15 ± 9.23			29.51 ± 1.02			26.39 ± 7.67		
(E)-6-Nonenal	1266.36 ± 185.46		A	337.33 ± 42.74		B	596.94 ± 82.56		B	432.87 ± 123.05		B
(E,E)-2,4-Hexadienal	421.24 ± 38.15			585.81 ± 75.03			303.41 ± 47.30			568.58 ± 107.23		
Nonanal	364.74 ± 78.17		A	309.59 ± 47.52		A	153.98 ± 4.42		B	136.14 ± 21.49		AB
(E,E)-2,4-Heptadienal	1370.72 ± 198.33		A	1195.76 ± 176.80		A	690.03 ± 96.92		B	1296.20 ± 224.05		A
(E)-2-Nonenal	294.45 ± 14.72			26.91 ± 3.61			377.94 ± 43.69			158.75 ± 60.25		
(E,Z)-2,6-Nonadienal	5396.60 ± 983.01			1520.04 ± 536.14			4198.75 ± 171.10			2312.73 ± 677.61		
(E)-4-Oxohex-2-enal	32.89 ± 5.73		AB	41.30 ± 10.39		A	9.05 ± 0.03		C	28.82 ± 8.19		BC
Alcohols												
Ethanol							279.00 ± 95.31			322.05 ± 121.65		
1-Penten-3-ol	2.30 ± 0.57			3.35 ± 0.93								
(Z)-3-Hexen-1-ol	4.23 ± 0.33			1.72 ± 0.41			15.23 ± 3.09			28.67 ± 12.41		
(E)-2-Hexen-1-ol	12.76 ± 2.71						14.83 ± 2.34			17.54 ± 0.58		
1-Octen-3-ol	41.15 ± 4.35		A	11.76 ± 1.03		C	35.57 ± 7.96		AB	40.80 ± 4.89		BC
2-Ethyl-1-hexanol	15.91 ± 2.81		A	16.17 ± 1.20		A	7.79 ± 2.26		B	20.14 ± 4.83		AB
2-Propyl-1-pentanol							30.34 ± 1.61			28.08 ± 5.39		
Ketones												
6-Methyl-2-heptanone	0.66 ± 0.02			0.46 ± 0.04								
3-Octanone	2.07 ± 0.39			0.65 ± 0.13			1.31 ± 0.32			4.13 ± 1.30		
6-methyl-5-Hepten-2-one	1.31 ± 0.18		A	0.75 ± 0.20		AB	0.70 ± 0.04		B	0.88 ± 0.24		B
(E,E)-3,5-Octadien-2-one	61.86 ± 3.43			60.23 ± 7.04			4.04 ± 0.84			3.28 ± 0.49		
Hydrocarbons												
3-ethyl-1,4-Hexadiene												
Terpenoic derivates												
β-Cyclocitral	14.79 ± 1.89			16.37 ± 4.05			12.65 ± 3.94			13.89 ± 2.64		
Eucarvone	3.88 ± 1.04			5.49 ± 2.28								
2-Pinen-4-ol	1.77 ± 0.02			1.02 ± 0.15								
β-Citral												
Geranial	32.71 ± 0.94			17.89 ± 3.00			15.27 ± 4.94			30.55 ± 7.57		
Isomethyl-α-ionone							7.33 ± 2.04			10.63 ± 3.43		
E-β-ionone							53.14 ± 1.43		AB	69.71 ± 3.75		A
2,2,6-Trimethyl-	3.54 ± 0.39		A	2.39 ± 0.32		AB	1.37 ± 0.63		B	1.96 ± 0.92		AB
β-Ionone							1.86 ± 0.85		AB	2.92 ± 1.43		A
Phenolic derivates												
Benzaldehyde	27.00 ± 3.74			32.57 ± 3.93			32.92 ± 9.87			50.06 ± 15.58		
Acetophenone	7.28 ± 0.54			7.11 ± 0.45			6.77 ± 0.27			6.54 ± 0.55		
Benzyl alcohol							8.53 ± 1.27			10.38 ± 4.77		
2-Phenyl-isopranol							0.29 ± 0.02			0.28 ± 0.08		
Phenol	4.86 ± 0.54			3.82 ± 0.61			8.97 ± 2.56			6.69 ± 0.64		
Benzophenone	10.48 ± 0.91			10.36 ± 0.88			11.27 ± 0.89			12.62 ± 2.75		
Toluene	5.62 ± 0.19			6.08 ± 0.57			14.90 ± 9.91			5.36 ± 0.31		

Table 3: *Mean value from three mesurements in two different experiments (µg/L homogenate). Values for each volatile with different letters in the same row within each treatment (control and EAMa 01/58-Su isolate leaves) are significantly different to each other ($P < 0.05$)

	Control				EAMa 01/58-Su			
Volatile compound*	UnSprayed leaf		Sprayed leaf		unsprayed leaf		Sprayed leaf	
Aldehydes								
Butanal	33.46 ± 1.46		34.20 ± 1.27		13.57 ± 3.41			
3-methyl-Butanal	516.44 ± 64.81		460.33 ± 160.92					
Pentanal	277.76 ± 51.60	A	100.53 ± 25.31	B				
Hexanal	2821.38 ± 490.76		2258.31 ± 587.30		1665.06 ± 326.19		1630.25 ± 533.92	
3-Methyl-hexanal	29.94 ± 7.12		15.87 ± 2.86					
(E)-2-Pentenal	455.69 ± 59.25	A	603.18 ± 82.50	A	233.83 ± 19.87	B	551.29 ± 198.88	A
Heptanal	42.99 ± 3.99		30.98 ± 7.79		40.24 ± 8.68			
(E)-2-Hexenal	1002.89 ± 154.60		1270.47 ± 218.95		1119.68 ± 151.39		1026.88 ± 37.63	
(Z)-2-Hexenal	40983.33 ± 5616.16		50015.83 ± 8148.49		46673.96 ± 5916.26		40500.00 ± 0.00	
(Z)-4-Heptenal	34.68 ± 6.16	A	26.42 ± 5.31	A				
Octanal	68.40 ± 10.32	A	67.36 ± 11.83	A	50.61 ± 5.44	AB		
(Z)-2-Heptenal	157.52 ± 31.36		31.96 ± 7.42					
(E)-2-Octenal	43.70 ± 3.23		87.15 ± 9.23					
(E)-6-Nonenal	1266.36 ± 185.46	A	337.33 ± 42.74	B	313.90 ± 91.98	B	424.28 ± 77.72	B
(E,E)-2,4-Hexadienal	421.24 ± 38.15	A	585.81 ± 75.03	A	287.69 ± 10.14	B	547.54 ± 107.38	A
Nonanal	364.74 ± 78.17	A	309.59 ± 47.52	A	108.87 ± 23.56	B	287.96 ± 111.29	AB
(E,E)-2,4-Heptadienal	1370.72 ± 198.33	A	1195.76 ± 176.80	AB	1173.54 ± 41.46	B	891.58 ± 230.67	AB
(E)-2-Nonenal	294.45 ± 14.72		26.91 ± 3.61		64.13 ± 6.70		118.00 ± 1.93	
(E,Z)-2,6-Nonadienal	5396.60 ± 983.01		1520.04 ± 536.14		828.50 ± 40.50		181.80 ± 41.26	
(E)-4-Oxohex-2-enal	32.89 ± 5.73	AB	41.30 ± 10.39	A	22.36 ± 1.50	B	15.97 ± 3.31	B
Alcohols								
Ethanol					294.30 ± 66.80	A	123.66 ± 18.08	AB
1-Penten-3-ol	2.30 ± 0.57		3.35 ± 0.93					
4-Methyl-1-pentanol					30.11 ± 8.71			
(Z)-3-Hexen-1-ol	4.23 ± 0.33		1.72 ± 0.41		20.25 ± 1.27			
(E)-2-Hexen-1-ol	12.76 ± 2.71	B			58.45 ± 13.77	A	20.78 ± 1.58	AB
1-Octen-3-ol	41.15 ± 4.35	A	11.76 ± 1.03	B	42.31 ± 9.82	AB	15.82 ± 0.72	B
2-Ethyl-1-hexanol	15.91 ± 2.81	A	16.17 ± 1.20	A				
2-Propyl-1-pentanol								
Ketones								
6-Methyl-2-heptanone	0.66 ± 0.02		0.46 ± 0.04					
3-Octanone	2.07 ± 0.39		0.65 ± 0.13		9.33 ± 0.79			
6-methyl-5-Hepten-2-one	1.31 ± 0.18	A	0.75 ± 0.20	B	0.61 ± 0.05	B	1.24 ± 0.04	AB
(E,E)-3,5-Octadien-2-one	6.19 ± 3.43		6.02 ± 7.04		7.44 ± 1.06		4.00 ± 1.10	
Hydrocarbons								
3-ethyl-1,4-Hexadiene					189.40 ± 75.66			
Terpenoic derivates								
β-Cyclocitral	14.79 ± 1.89		16.37 ± 4.05		13.78 ± 3.01		18.76 ± 1.90	
Eucarvone	3.88 ± 1.04		5.49 ± 2.28					
2-Pinen-4-ol	1.77 ± 0.02		1.02 ± 0.15					
β-Citral					3.18 ± 0.38			
Geranial	32.71 ± 0.94		17.89 ± 3.00					
Isomethyl-α-ionone					3.88 ± 1.74			
E-β-ionone					60.64 ± 4.49		44.00 ± 30.59	
2,2,6-Trimethyl-cyclohexanone	3.54 ± 0.39	A	2.39 ± 0.32	B				
β-Ionone					0.54 ± 0.04			
Phenolic derivates								
Benzaldehyde	27.00 ± 3.74	A	32.57 ± 3.93	A	14.94 ± 0.90	B	19.89 ± 14.10	AB
Acetophenone	7.28 ± 0.54		7.11 ± 0.45		6.56 ± 0.40		6.29 ± 1.97	
Benzyl alcohol					15.44 ± 0.83			
2-Phenyl-isopranol					3.65 ± 0.71			
Phenol	4.86 ± 0.54		3.82 ± 0.61					
Benzophenone	10.48 ± 0.91		10.36 ± 0.88		14.55 ± 2.04		10.66 ± 4.77	
Toluene	5.62 ± 0.19	B	6.08 ± 0.57	B	5.20 ± 0.79	B	22.34 ± 18.46	A

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CAPÍTULO III. CHANGES IN FEEDING BEHAVIOUR ARE NOT RELATED TO THE REDUCTION IN THE TRANSMISSION RATE OF PLANT VIRUSES BY *APHIS GOSSYPII* (HOMOPTERA: APHIDIDAE) TO MELON PLANTS COLONIZED BY *BEAUVERIA BASSIANA* (ASCOMYCOTA: HYPOCREALES)

Este capítulo es una versión adaptada del artículo:

Biological Control 130 (2019) 95–103

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Changes in feeding behaviour are not related to the reduction in the transmission rate of plant viruses by *Aphis gossypii* (Homoptera: Aphididae) to melon plants colonized by *Beauveria bassiana* (Ascomycota: Hypocreales)

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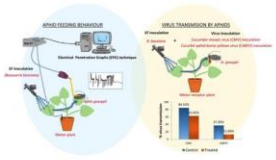
GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Endophytic colonization
Cucumber mosaic virus
Cucurbit aphid-borne yellows virus
Electrical penetration graph
Probing behaviour
Entomopathogenic fungi

ABSTRACT

Aphids are considered by far the most common and efficient vectors of non-persistent (NP) and persistent (P) plant viruses, with new, more environmentally friendly aphid control strategies urgently needed. Among these strategies, entomopathogenic fungi (EF) have successfully been employed against aphids mainly by foliar spray over infested crops. Plants benefit from not only their direct insecticidal activity on aphids but also a number of side outcomes of their application, such as sub-lethal effects on the target pest or their endophytic behaviour. Beyond aphid control, this endophytic quality has also been exploited to control diverse plant pathogenic microorganisms. In the present study, possible changes in cotton aphid (*Aphis gossypii*) probing and feeding behaviour mediated by the endophytic colonization of melon plants with *Beauveria bassiana* were studied using the electrical penetration graph (EPG) technique. Similarly, the effect of this endophytic colonization on P (*Cucurbit aphid-borne yellows virus*, *Polerovirus*) and NP (*Cucumber mosaic virus*, *Cucumovirus*) virus transmission by the cotton aphid was also studied. *B. bassiana* colonization did not alter the aphid feeding behaviour related to the NP or P virus inoculation process, but significant changes were detected in other variables that were not related to the virus inoculation process, such as the intracellular probe (pd) duration, which could suggest a possible effect of EF on the acquisition of CMV. Notably, a remarkable effect of *B. bassiana* endophytic colonization was found on CMV and CABYV transmission, with significant reductions of 21.9 and 24.4%, respectively, of the inoculation rates in *B. bassiana*-treated plants. These results suggest a possible systemic virus resistance in melon induced by fungal colonization.

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https://doi.org/10.1016/j.biocontrol.2018.11.001
Received 16 January 2018; Received in revised form 6 November 2018; Accepted 8 November 2018
Available online 09 November 2018

Changes in feeding behaviour are not related to the reduction in the transmission rate of plant viruses by *Aphis gossypii* (Homoptera: Aphididae) to melon plants colonized by *Beauveria bassiana* (Ascomycota: Hypocreales)

N. González-Mas, E. Quesada-Moraga, M. Plaza, A. Fereres, A. Moreno

Abstract

Aphids are considered by far the most common and efficient vectors of non-persistent (NP) and persistent (P) plant viruses, with new, more environmentally friendly aphid control strategies urgently needed. Among these strategies, entomopathogenic fungi (EF) have successfully been employed against aphids mainly by foliar spray over infested crops. Plants benefit from not only their direct insecticidal activity on aphids but also a number of side outcomes of their application, such as sub-lethal effects on the target pest or their endophytic behaviour. Beyond aphid control, this endophytic quality has also been exploited to control diverse plant pathogenic microorganisms. In the present study, possible changes in cotton aphid (*Aphis gossypii*) probing and feeding behaviour mediated by the endophytic colonization of melon plants with *Beauveria bassiana* were studied using the electrical penetration graph (EPG) technique. Similarly, the effect of this endophytic colonization on P (*Cucurbit aphid-borne yellows virus, Polerovirus*) and NP (*Cucumber mosaic virus, Cucumovirus*) virus transmission by the cotton aphid was also studied. *B. bassiana* colonization did not alter the aphid feeding behaviour related to the NP or P virus inoculation process, but significant changes were detected in other variables that were not related to the virus inoculation process, such as the intracellular probe (pd) duration, which could suggest a possible effect of EF on the acquisition of CMV. Notably, a remarkable

effect of *B. bassiana* endophytic colonization was found on CMV and CABYV transmission, with significant reductions of 21.9 and 24.4%, respectively, of the inoculation rates in *B. bassiana*-treated plants. These results suggest a possible systemic virus resistance in melon induced by fungal colonization.

Keywords: Endophytic colonization, *Cucumber mosaic virus, Cucurbit aphid-borne yellows virus,* Electrical penetration graph, Probing behaviour, Entomopathogenic fungi

1. Introduction

Aphids are phytophagous phloem-sucking insects that can be found in both herbaceous and woody crops worldwide. They cause significant damage and great economic losses in agricultural crop plants as a consequence of their direct feeding by removing plant sap, injecting saliva toxins and indirectly, but most importantly, transmitting plant viruses (Van Emden and Harrington 2007). They are considered by far the most common and efficient vectors of plant viruses, transmitting a great number of persistent (P) and non-persistent (NP) viruses (Nault 1997).

Plant virus control is frequently directed towards insect vectors and has been commonly based on the use of chemical insecticides, which has led to the emergence of resistance to currently available products (Nauen and Elbert 2003; Whalon et al. 2016; Sparks and Nauen 2015) that have been recently reduced with new regulations (Directive 2009/ 128/EC, 7 U.S.C. § 136r-1). Crop breeding for vector resistance or low vector preference for the host has also been performed. However, EU legislation on the use of GMOs (Bragard et al. 2013) limits further progress on transgenic approaches. Therefore, new and more environmentally friendly products are required for their control (OJEU 2009, 2013; FIFRA 2012).

In this respect, biopesticides offer a promising alternative to the use of chemical insecticides. Biopesticides may be better than synthetic insecticides in several ways, such as reducing pest resistance development and environmental persistence and improving compatibility with biological controls (Copping and Menn 2000; Chandler et al. 2011; Hubbard et al. 2014; Seiber et al. 2014; Czaja et al. 2015).

Among them, the use of entomopathogenic fungi (EF) has been highlighted as a successful method for aphid control (Lacey et al. 2015). In fact, different fungal species have been shown to be able to control cotton aphid (*Aphis gossypii* Glover) populations by spraying (Loureiro and Moino 2006; Vu et al. 2007; Kim and Roberts 2012; Jandricic et al., 2014). This management has been improved by the recently discovered endophytic behaviour of some EF strains (Gurulingappa et al. 2010; Castillo Lopez et al. 2014). These fungal biocontrol agents have been reported to be highly virulent towards aphids because they reduce aphid populations and, additionally, reduce aphid fecundity (Kim 2007; Rashki and Shirvani 2013; Gurulingappa et al. 2010; Gurulingappa et al. 2011; Castillo Lopez et al. 2014). Indeed, EF endophytic capability has also been exploited against different plant pathogenic microorganisms, serving as antagonist biological control agents in several plant hosts (Griffin 2007; Ownley et al. 2008; Jaber and Salem 2014; Jaber 2015; Jaber and Ownley 2018, Vega 2018).

However, it remains unknown whether endophytic colonization by EF may alter aphid probing and feeding behaviour and consequent virus transmission. Several abiotic and biotic factors, such as plant colonization by microorganisms, may trigger physiological and morphological plant changes that could alter insect host-plant selection and feeding (Pangesti et al. 2013). Therefore, endophytic colonization by EF could be considered a possible factor leading to the disruption or enhancement of virus spread.

Transmission of plant pathogens is associated with vector feeding behaviour, which can be monitored using the electrical penetration graph (EPG) technique (Fereres and Moreno 2009), which records signal waveforms reflecting different insect stylet penetration activities (Tjallingii 1988; Moreno et al. 2011; Antolinez et al. 2017).

Therefore, in the present study, we investigated the probing and feeding behaviour of *A. gossypii* by means of the EPG technique to understand the aphid response to non-colonized and colonized melon plants with a *Beauveria bassiana* strain, which has been reported to endophytically colonize melon plants. Moreover, transmission experiments with P (*Cucurbit aphid-borne yellow virus, Polerovirus*) and NP (*Cucumber mosaic virus, Cucumovirus*) viruses were conducted to assess whether endophytic colonization by EF can reduce the virus transmission rate by aphid vectors.

2. Materials and methods

2.1. Biological material: aphid colonies, plants, and fungal and virus isolates

Melon plants (*Cucumis melo* L. cv. Siglo) were germinated in 9×9 cm pots using a mixture of equal parts of vermiculite (No. 3, Asfaltex S.A., Barcelona, Spain) and soil substrate (Kekilla Iberica, Almería, Spain). They were watered three times a week, and a nutritional complex of 20–20–20 (N:P:K) Nutrichem 60 fertilizer (Miller Chemical & Fertilizer Corp., Hanover, USA) was added to the irrigation water in a proportion of 0.25 g/l dosage.

Aphis gossypii was used because it is an efficient vector of both NP (*Cucumber mosaic virus,* CMV) and P (*Cucurbit aphid-borne yellow virus,* CABYV) viruses tested in this study. A single virginiparous and apterous aphid collected from melon in Almeria, Spain, in 1998 was used for initiating a virus-free laboratory culture of *A. gossypii*. This colony was reared on melon plants for several generations in rearing cages inside environmental growth chambers at a 23:18 °C temperature (day:night), photoperiod of 16:8 h (light:dark) and 60–80% RH. For each experiment, newly emerged apterous adults (24–72 h after last

moult) were collected with a camel hairbrush from the rearing cages on the same day the experiments were started.

For transmission experiments, melon plants were inoculated with CMV and CABYV 13 days after sowing at the 1-true leaf stage and used 4 weeks post-inoculation as viral sources in the transmission experiments. Plants were mechanically inoculated with CMV isolate M06 obtained from a melon crop in 1996 in Tarragona (Spain) and kindly provided by Dr. E. Moriones (EELM-CSIC, Spain). For this, CMV-infected leaf material was homogenized with a mortar and pestle using a 0.03M Disodium phosphate, 0.2 diethyldithiocarbamate acid (DIECA) solution (1:10 (weight:volume)), and 20 µl of the solution were applied to the inoculated plants. As a non-infected control, melon plants of the same age were inoculated only with the homogenization solution.

The CABYV isolate kindly provided by H. Lecoq was obtained in Montfavet, France, from zucchini squash in 2003 and maintained in melon plants at ICA-CSIC by aphid serial transmission with *A. gossypii*. Melon plants were inoculated with viruliferous aphids at the 1-true leaf stage as described in Moreno et al. (2011): *A. gossypii* adults were allowed to feed for 48 h (Acquisition Access Period, AAP) on CABYV infected plants, and then, 20 viruliferous nymphs were transferred to healthy plants for an inoculation access period (IAP) of 72 h, when nymphs were removed. As a non-infected control, melon plants of the same growth stage were inoculated with 20 non-viruliferous nymphs.

All plants were grown in an insect-proof chamber at 23 °C:20 °C temperature (D:N), a photoperiod of 16:8 h (L:D) and 60–80% RH. Virus infection was assessed by symptom observation and confirmed by the double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Clark and Adams 1977) using specific commercial

antibodies against CMV (Agdia Inc., Indiana, USA) and CABYV (Sediag, France).

The *B. bassiana* strain EABb 01/33-Su, isolated from soil on a plot in El Bosque (Cádiz) and shown to behave as an endophytic strain when inoculated onto melon plants (Resquín-Romero et al. 2016; Garrido-Jurado et al. 2016), was used throughout the study. This strain is deposited at the C.R.A.F. The University of Córdoba Entomopathogenic Fungi Collection, Córdoba, Spain, and its nucleotide sequences for ITS and mtDNA intergenic regions can be found in the GenBank database [GenBank: EF115310 and GenBank: FJ972969 for the ITS region, GenBank: FJ973025 for intergenic region nad3-atp9 and GenBank: FJ972914 for the intergenic region atp6-rns].

To obtain spore suspensions, *B. bassiana* strain EABb 01/33- Su was grown on Petri plates on MA for 15 days at 25 °C in the dark. Then, conidial suspension was prepared by scraping them from Petri plates into an aqueous sterile solution of 0.01% Tween 20. The resulting conidial suspension was filtered through several layers of sterile cheesecloth to remove mycelium mats and sonicated for 10 min to homogenize the inoculum. The viability of conidia was assessed before preparation of suspensions by germinating tests in liquid Czapek-Dox broth plus 1% (w/v) yeast extract medium. In all experiments, germination rates were above 95% after 24 h at 25 °C.

The concentration of viable conidia used for inoculation was determined using a hemocytometer, and appropriate dilutions were carried out in 0.01% Tween 20 to obtain a suspension adjusted to 10^8 spores/ml.

2.2. Fungal inoculation of melon plants and confirmation of endophytic colonization of plants by *B. bassiana* EABb 01/33-Su strain

For the EPG experiments, 4-to-5-leaf stage melon plants were sprayed with the conidial suspensions of EABb 01/33-Su strain in 0.01% Tween 20 or with an aqueous sterile solution of 0.01% Tween 20 in the case of plants that served as control treatment, according to the following procedure.

The solution was sprayed over the two basal leaves preserving the apical leaves protected from the spray, where the aphids were going to be placed for the EPG assays. The non-target plant parts (non-target leaves, stems and roots) were covered with a transparent plastic sheet, and pulverization was carefully performed on the remaining leaves separating them from the rest of the plant with a piece of polystyrene covered with aluminium foil. Then, the complete plant was coated with a transparent plastic sheet to promote fungal growth for 24 h.

Spraying the target leaves was performed with 2 ml of the relevant solution using an aerograph 27,085 (piston compressor of 23 L/minute, 15–50 PSI and a 0.3mm nozzle diameter, Artesania Latina S.L., China). One millilitre of 10^8-conidial suspension was sprayed over each leaf, resulting in a spray deposition at the level of the target surface of approximately 0.1 µl/mm^2. Using the CFU method, we estimated that the suspension produced a deposition of 10,000 viable conidia/mm^2.

Plants were maintained individually for each treatment in two different environmental growth chambers at 25 °C temperature, a photoperiod of 16:8 h (L:D) and 60–80% RH. They were used 48 h after inoculation for EPG assays using different Faraday cages per treatment.

To confirm the endophytic colonization of the plants used in the EPG experiments, samples from inoculated and non-inoculated plants were collected once the EPG recording was finished 56 h after inoculation. For each independent plant, two different points were sampled: leaves not exposed to the treatment, where the aphid fed during the EPG assay, and leaves exposed to the treatment. Samples from each plant and leaf were surface-sterilized independently with 1% sodium hypochlorite for 2 min, rinsed twice in sterile distilled water and dried on sterile filter paper. Then, fragments of approximately 2 cm2 were cut with a sterile scalpel and immediately plated out, as well as the last rinse water, on plates containing selective culture medium [20 g of agar Sabouraud glucose chloramphenicol (Cultimed Panreac, Spain), 500 mg l^{-1} streptomycin sulfate (Sigma-Aldrich Chemie, China), 500 mg l^{-1} ampicillin (Intron Biotechnology, China) and 500 mg l^{-1} dodine 65 WP (BASF, Spain)] to assess the colonization percentage and the effectiveness of the surface-disinfested procedure, respectively. The resulting Petri plates were incubated at 25 °C in the dark until fungal growth was observed.

2.3. Electrical monitoring of aphid feeding behaviour

The probing and feeding behaviour of *A. gossypii* on *B. bassiana* colonized and control melon plants was monitored using the electrical penetration graph (EPG) technique (Tjallingii 1988). Apterous 7/9-day-old adult aphids were immobilized individually using a vacuumoperated plate (Eyela Aspirator A3S, Tokyo Rikakikai Co. Ltd., Tokyo, Japan). Then, a thin gold wire (20 μm diameter) was attached to thedorsum of the aphid with a small droplet of silver glue (Pelco Collodial Silver, Ted Pella Inc., Redding CA, USA). The opposite end of the gold wire was attached to a copper electrode (3 cm long–1mm

diameter), representing the input electrode. The output electrode was a copper post (10 cm long–2 mm diameter), which was inserted into the plant pot. Aphids with the attached gold wire were placed on the abaxial side of leaves not exposed to the spraying of a 4-to-5-leaf stage melon plant and connected to the DC-EPG device (Giga-4; EPG Systems, Wageningen, the Netherlands). The EPG acquisition procedure was performed inside a Faraday cage to prevent electrical noise. EPG data acquisition was conducted using Stylet+software for Windows (EPG Systems, Wageningen, The Netherlands). EPG signals were recorded for 8 h under laboratory conditions (22–24 °C) and started immediately after aphids had been placed on the melon leaf.

Twelve to fifteen replicates per treatment (fungus-colonized and control melon plants) were recorded using batches of 4 plants at a time. Each replicate was conducted using a different aphid for each EPG recording. EPG waveforms, previously described for aphids (Tjallingii, 1988), were identified as follows: non-probing (np), intercellular apoplastic stylet pathway (C), intracellular stylet puncture (pd), salivation into phloem sieve elements at the beginning of the phloem phase (E1), passive phloem sap uptake from the phloem sieve elements (E2), active intake of xylem sap (water+nutrients) from xylem elements (G) and mechanical work associated with stylet penetration difficulty (F).

All behavioural variables were processed using the MS Excel Workbook for automatic EPG data calculation, developed by Sarria et al. (2009). Data analysis was conducted for relevant EPG variables for the 8-hour recording period. Furthermore, the first 10 min of the data analysis was analysed separately to understand the first aphid response over time when exposed to *B. bassiana*-colonized and control melon plants and to relate the aphid behaviour to the transmission of NP,

which is transmitted mainly during the first phases of the probing in the host plants.

Selected EPG variables (mean ± SE) for analysis (Table 1) were calculated according to Backus et al. (2007): proportion of individuals who produced a specific waveform type, PPW; number of wave-form events per insect (that is the sum of the number of events of a particular waveform divided by the total number of insects under each treatment), NWEI; total waveform duration (minutes) per insect (that is the sum of durations of each event of a particular waveform divided by the total number of insects under each treatment), WDI; and total waveform duration (minutes) per event (that is the sum of the duration of the events for a particular waveform divided by the total number of events of that particular waveform under each treatment), WDE.

Table 1. Sequential and non-sequential EPG variables used to compare the probing and feeding behaviour of *A. gossypii* on *B. bassiana*-colonized and control melon plants.

Non-sequential variables	**Sequential variables**
Number of np [a,b]	Time to 1st probe from start of EPG [a,b]
Number of probes [a,b]	Time from 1st probe to 1st E [a]
Number of pd [a,b]	Time from the beginning of that probe to 1st E [a]
Number of C [a,b]	Duration of 1st probe [a,b]
Number of E1 [a]	Duration of 2nd probe [a,b]
Number of E2 [a]	Duration of np just after the probe of the first sustained E2 [a]
Number of sustained E2 (10 min) [a]	Duration of the longest E2 [a]
Total duration of np [a,b]	Total duration of E1 followed by sustained E2 (>10 min) [a]
Total probing time [a,b]	Total duration of E1 followed by E2 [a]
Total duration of pd [a,b]	Number of short probes (C<3 minutes) [a,b]
Total duration of C [a,b]	Number of probes to the 1st E1 [a]
Total duration of E1 [a]	Number of probes after 1st E [a]
Total duration of E2 [a]	Number of probes (shorter than 3 minutes) after 1st E [a]
Total duration of sustained E2 (10 min) [a]	Time from the beginning of the 1st probe to first pd [b]
Total duration of E [a]	Time from the end of the last pd to the end of the probe [b]
	Duration of the second non probe period [b]
	Average number of pd per probe [b]

[a] During 8 hours of EPG recording; [b] During the first 10 minutes of EPG recording

2.4. Effect of *B. bassiana* plant colonization on virus transmission

Transmission experiments with P (CABYV) and NP (CMV) viruses were conducted to assess the effect of *B. bassiana* plant colonization on virus inoculation *by A. gossypii*. Receptor plants at the three-leaf growth stage were sprayed either with conidial or with control solution in each case before virus inoculation. Suspension was inoculated on all the leaves except one, the apical leaf, where the aphids were going to be placed for virus inoculation. Fungal inoculation was conducted as indicated above. Transmission experiments were conducted two days after the treatment, when the fungal colonization in the receptor plants was settled.

Plants used for the experiments were maintained before, and during transmission, experiments were conducted in different environmental growth chambers according to the treatment at 25°C temperature, photoperiod of 16:8 h (L:D) and 60–80% RH.

CMV transmission tests were essentially performed as previously described by Fereres et al. (1993). After a 1-h pre-acquisition starving period, groups of 20 young-adult apterae aphids were released on the upper side of a CMV-infected leaf for virus acquisition. Following a 10-min acquisition access period, aphids were transferred into groups of five aphids to each receptor plant and confined by a clip-cage attached to a non-sprayed leaf of the plant. After a 24-h inoculation access period, aphids were removed from the receptor plants.

For CABYV transmission assays, viruliferous apterae adult aphids were used. To obtain these viruliferous aphids, *A. gossypii* adults were allowed to feed and reproduce for 24 h on CABYV-infected plants. Nymphs produced during this period, which fed on these CABYV-infected plants, were used for the assays when reaching the adult stage

(acquisition access period (AAP) of 8 days). After the AAP, they were transferred into groups of 5 individuals to a non-treated leaf of the receptor plant and confined for a 2-day inoculation access period (IAP), and then, they were removed.

In both cases, melon receptor plants were finally sprayed with imidacloprid (Confidor, Bayer AG, Leverkusen, Germany) at a 1.5 ml/l dilution and transferred to an aphid-free greenhouse where they were grown under facilities at 20:16 °C (day:night), a photoperiod of 16:8 h (light:dark) and 60% HR until symptom observation. Plants were checked regularly for the appearance of virus symptoms during a period of 3–5 weeks. Then, the viral infection was confirmed by ELISA using specific antibodies. Thirty plants were used in each virus treatment, and the entire experiment was repeated three times using new plants, insects, and viral and fungal inocula.

2.5. Statistical analysis

All the behavioural variables obtained by EPG recording were tested for normality using the Shapiro-Wilk test. When they did not show a normal distribution, they were transformed prior to analysis by either sqrt (x+1) or ln (x+1) and tested for normality using the Shapiro–Wilk W test. Comparisons between treatments were carried out by Student's t-test (Gaussian variables) or the Mann–Whitney U test (for non-Gaussian variables). This statistical analysis of the data was conducted using SPSS 24.0 software package.

The transmission of each virus was analysed with a generalized linear model with the factors treatment and experiment (distribution= binomial and link=logit) (JMP-pro v.12.2). Pairs of virus transmission rates were compared using a Pearson's chi-squared test with Yates' continuity correction. The differences in virus transmission rates were

simultaneously tested using the Marascuilo procedure, with significant differences observed when the value (pj-pi) exceeded the critical range value. This statistical analysis was performed with the software R 3.

3. Results

3.1. Electrical monitoring of aphid probing and feeding behaviour

The endophytic colonization of melon plants by EABb 01/33-Su strain was verified in all the plants that were previously sprayed with the fungal suspension. Nevertheless, the presence of *B. bassiana* was not detected in all of the leaves on which the aphid was placed and connected to the EPG device. Only records obtained from aphids that fed on colonized leaves were considered for the analysis, resulting in a total of 12 valid EPG recordings for fungal treated plants and 15 valid EPG recordings for the control treatment.

EPG variable values obtained for the feeding activities of *A. gossypii* on *B. bassiana*-colonized and non-colonized melon plants were first analysed during the 8 h of EPG recording (0–480 min) (Tables 2 and 3; time was expressed in minutes, except the duration of pd, which was expressed in seconds). The analysis of non-sequential EPG variables showed that the duration of pd was significantly shorter in colonized plants (WDE: 5.68 ± 0.03 s) than in control plants (WDE: 6.00 ± 0.03 s) [$T=-7.61$, $p < 0.01$] when it was considered per event. Both inoculation and acquisition of NP viruses occur in intracellular stylet punctures (pd) in plant superficial tissues. However, their duration is not related to the inoculation of NP viruses but to their acquisition by aphids. Therefore, differences observed in the pd duration could suggest a possible effect of EF on the acquisition of CMV but not on its inoculation. Moreover, significant differences were observed in the duration of the first probe, which was significantly shorter in colonized

plants (WDE: 7.34 ± 5.89 min) than in control plants (WDE: 36.38 ± 11.47 min) [U=42.00, P=0.02].

When the sequential EPG variables were analysed for an 8-hour recording period, significant differences were perceived on the "Number of probes after 1st E" and "Number of probes (shorter than 3 min) after 1st E". The number of probes developed by aphids after they had reached the phloem ("Number of probes after the first E") was significantly higher in colonized plants (NWEI: 16.67 ± 6.24 times) than in control plants (NWEI: 5.15 ± 2.41 times) [U=14.50, P=0.03]. As above, the number of short probes developed by aphids after they had reached the phloem ("Number of probes (shorter than 3 min) after 1st E") was also significantly higher in colonized plants (NWEI: 12.80 ± 5.11 times) than in control plants (NWEI: 2.31 ± 1.29 times) [U=8.00, P=0.01].

When EPG variables were analysed considering only the first 10 min of the recording, significant differences among treatments were observed for EPG variables related to intercellular pathway phase (C) (Table 4; time expressed in seconds). Aphids developed more intercellular probes (C) on colonized plants (NWEI: 1.83 ± 0.34 times) than on control plants (NWEI: 1.00 ± 0.20 times) [U=49.50, P=0.04], and the number of short probes was also higher for aphids probing on colonized plants (NWEI: 1.58 ± 0.29 times) than on control plants (NWEI: 0.47 ± 0.17 times) [U=34.00, P=0.00]. Moreover, the number of non-probes (np) was higher when they were fed on colonized plants (NWEI: 2.50 ± 0.29 times) than on control plants (NWEI: 1.60 ± 0.19 times) [U=44.00, P=0.01]. Related to the duration of the probing events, significant differences were observed in the probe and intercellular pathway phase that were longer for aphids on control (WDE: 255.47 ± 51.10 s) than on colonized plants (WDE: 83.07 ± 19.52 s) (U=79.00; P <

0.01). Moreover, significant differences by insect were found in the second probe, which was longer for aphids probing on colonized plants (WDI: 51.93 ± 18.11 s) than on control plants (WDI: 44.50 ± 29.27 s) [U=53.00, P=0.04] and in the "duration of the second non probe period"; aphids took longer without a probe in colonized plants (WDI: 122.84 ± 44.18 s) than in control plants after the first probe (WDI: 10.10 ± 5.75 s) [U=43.00, P=0.01]. No significant differences were observed for the other EPG variables analysed.

3.2. Effect of *B. bassiana* plant colonization on virus transmission

The experimental effect was not statistically significant for any of the viruses (χ^2_{2df} test, $p > 0.05$). Therefore, the results are presented combined in a single graphic (Figure 1). The treatment effect was significant on virus transmission, both for CMV and CABYV virus.

The CMV transmission rate was significantly lower when receptor plants had previously been treated with *B. bassiana* conidial suspension (62.65%) compared to that observed in the non-treated plants (84.52%) (n=167; $\chi2=9.20$). The results showed a reduction in CMV incidence of 21.87% because of *B. bassiana* treatments (Figure 1).

In the case of the P virus CABYV, significant differences in the virus transmission rate were also discerned between control (37.35%) and *B. bassiana*-treated plants (12.94%) (n=168; $\chi2=12.07$), which represents a 24.41% reduction (Figure 1).

Table 2. Non-sequential (mean ± standard error) EPG variable values (ranges in parenthesis) during the probing behaviour of *A. gossypii* on *B. bassiana*-colonized and control melon plants during an 8-hour recording.

Non-sequential variables	Treatment	PPW	NWEI	P[a]	WDI	P[a]	WDE	P[a]
Non-probe	Colonized	12/12	18.75±3.73 (4.00-51.00)	0.84	82.01±14.00 (20.90-194.40)	0.90	4.37±0.35 (0.03-32.53)	0.38
	Control	15/15	17.40±2.19 (2.00-34.00)		86.20±15.16 (9.28-252.34)		4.95±0.53 (0.00-79.70)	
Probe	Colonized	12/12	18.75±3.73 (4.00-51.00)	0.82	388.07±11.98 (285.60-450.98)	0.54	20.70±3.55 (0.17-359.67)	0.72
	Control	15/15	17.33±2.16 (2.00-33.00)		389.57±14.19 (227.66-453.41)		22.48±3.48 (0.17-418.95)	
C	Colonized	12/12	20.42±3.96 (5.00-56.00)	0.91	222.38±24.17 (129.42-373.49)	0.35	10.89±1.19 (0.17-105.17)	0.43
	Control	15/15	18.40±2.35 (2.00-34.00)		232.82±28.77 (54.11-415.54)		12.56±1.30 (0.05-143.11)	
pd[b]	Colonized	12/12	148.67±21.88 (71.00-307.00)	0.76	14.08±2.14 (6.07-26.51)	0.97	5.68±0.03 (2.95-19.70)	**0.00**
	Control	15/15	145.73±18.04 (35.00-279.00)		14.57±1.93 (3.07-28.74)		6.00±0.03 (3.36-19.58)	
G	Colonized	12/12					18.09±9.51 (8.58-27.60)	0.67
	Control	15/15					23.85±6.59 (12.13-52.80)	
E1	Colonized	12/12	2.58±0.56 (1.00-6.00)	0.14	9.72±4.52 (0.44-53.39)	0.12	3.76±1.04 (0.34-27.34)	0.94
	Control	13/15	1.53±0.34 (0.00-5.00)		5.52±3.51(0.00-52.74)		3.60±2.18 (0.18-49.33)	
E2	Colonized	12/12	1.58±0.26 (1.00-4.00)	0.28	152.96±30.01 (22.89-269.20)	0.25	96.60±25.30 (0.58-269.20)	0.57
	Control	13/15	1.33±0.30 (0.00-5.00)		141.69±34.47 (0.00-384.78)		106.26±28.78 (0.30-384.78)	
sE2	Colonized	12/12	1.00±0.12 (0.00-2.00)	0.18			149.92±30.94 (12.18-269.20)	0.38
	Control	13/15	0.73±0.15 (0.00-2.00)				191.50±35.30 (28.30-384.78)	
E	Colonized	12/12			162.67±26.89 (43.05-270.18)	0.15		
	Control	13/15			147.21±33.67 (0.00-386.39)			
Duration of 1st probe	Colonized	12/12					7.34±5.89 (0.28-71.94)	**0.02**
	Control	15/15					36.38±11.47 (0.57-143.11)	
Duration of 2nd probe	Colonized	12/12					27.09±14.48 (0.37-161.01)	0.98
	Control	15/15					38.00±26.89 (0.29-401.91)	
Number of probes (shorter than 3 minutes)	Colonized	12/12	11.33±2.90 (0.00-34.00)	0.61				
	Control	15/15	8.60±1.51 (0.00-18.00)					

PPW. proportion of individuals that produced the waveform type; **NWEI**. number of waveform events per insect; **WDI**. waveform duration (min) per insect; **WDE**. waveform duration (min) per event (Backus et al.. 2007). **Non-probe**. non-probe activity; **Probe**. probe activity. Waveforms: **C**. intercellular stylet pathway; **pd**. short intracellular punctures; **G**. xylem ingestion; **E** shows phloem-related activities: **E1**. correlates with salivation into phloem sieve elements; **E2**. ingestion from phloem (Prado and Tjallingii. 1994); **sE2**. sustained E2 (longer than 10 minutes).

[a] Bold-type indicates significant differences ($p<0.05$) according to *t*-Student test (for Gaussian variables) or Mann–Whitney *U* test (for non-Gaussian variables).

[b] Potential drop (pd) duration WDE is expressed in seconds.

Table 3. Sequential (mean ± standard error) EPG variable values (ranges in parenthesis) for the probing behaviour of *Aphis gossypii* on *B. bassiana*-colonized and control melon plants during an 8-hour recording

Sequential variables	Treatment	PPW	NWEI	P[a]	WDI	P[a]
Time to 1st probe from start of EPG	Colonized	12/12			4.26±1.79 (0.08-20.10)	0.85
	Control	15/15			5.03±1.40 (0.00-17.01)	
Time from 1st probe to 1st E	Colonized	12/12			181.96±27.21 (40.65-353.30)	0.19
	Control	15/15			234.46±36.34 (10.36-478.48)	
Time from the beginning of that probe to 1st E	Colonized	12/12			45.74±8.28 (14.42-105.17)	0.28
	Control	13/15			36.47±7.42 (7.72-83.54)	
Duration of np just after the probe of the first sustained E2	Colonized	2/12			6.28±0.19 (6.09-6.47)	-
	Control	1/15			14.20±0.00 (14.20-14.20)	
Total duration of E1 followed by sustained E2 (>10 min)	Colonized	11/12			3.27±2.41 (0.44-27.39)	0.94
	Control	10/15			2.57±1.43 (0.36-15.03)	
Total duration of E1 followed by E2	Colonized	12/12			4.46±2.47 (0.44-28.00)	0.36
	Control	13/15			2.31±1.11 (0.23-15.03)	
Number of probes to the 1st E1	Colonized	12/12	10.42±1.69 (2.00-20.00)	0.33		
	Control	13/15	12.87±1.99 (2.00-27.00)			
Number of probes after 1st E	Colonized	6/12	16.67±6.24 (1.00-40.00)	**0.03**		
	Control	13/15	5.15±2.41 (0.00-30.00)			
Number of probes (shorter than 3 minutes) after 1st E	Colonized	5/12	12.80±5.11 (1.00-26.00)	**0.01**		
	Control	13/15	2.31±1.29 (0.00-16.00)			

PPW. proportion of individuals that produced the waveform type; **NWEI**. number of waveform events per insect; **WDI**. waveform duration (min) per insect; **WDE**. waveform duration (min) per event (Backus et al.. 2007). **Non-probe**. non-probe activity; **Probe**. probe activity. Waveforms: **C**. intercellular stylet pathway; **pd**. short intracellular punctures; **G**. xylem ingestion; **E** shows phloem-related activities: **E1**. correlates with salivation into phloem sieve elements; **E2**. ingestion from phloem (Prado and Tjallingii. 1994); **sE2**: sustained E2 (longer than 10 minutes).

[a] Bold-type indicates significant differences ($p<0.05$) according to *t*-Student test (for Gaussian variables) or Mann–Whitney *U* test (for non-Gaussian variables).

Table 4. Sequential and non-sequential (mean ± standard error) EPG variable values (ranges in parenthesis) for the probing behaviour of *Aphis gossypii* on *B. bassiana*-colonized and control melon plants during the first 10 minutes

	Treatment	PPW	NWEI	P[a]	WDI	P[a]	WDE	P[a]
Non-probe	Colonized	11/12	2.5±0.29 (0.00-4.00)	**0.012**	447.71±47.01 (94.99-600.00)	0.098	179.08±33.55 (4.73-600.00)	0.93
	Control	15/15	1.6±0.19 (1.00-3.00)		309.70±61.10 (0.08-600.00)		193.56±41.72 (0.02-600.00)	
Probe	Colonized	10/12	1.83±0.34 (0.00-4.00)	0.058	152.29±47.01 (0.00-505.01)	0.129	83.07±19.52 (13.62-354.80)	**0.004**
	Control	12/15	1.07±0.21 (0.00-3.00)		272.51±57.23 (0.00-599.92)		255.47±51.10 (22.45-599.92)	
C	Colonized	10/12	1.83±0.34 (0.00-4.00)	**0.037**	152.29±47.01 (0.00-505.01)	0.129	83.07±19.52 (13.62-354.80)	**0.004**
	Control	12/15	1.00±0.20 (0.00-3.00)		272.51±57.23 (0.00-599.92)		255.47±51.10 (22.45-599.92)	
pd	Colonized	6/12	1.75±0.83 (0.00-10.00)	0.639	15.17±5.68 (0.00-54.53)	0.834	8.67±0.96 (4.56-16.87)	0.86
	Control	9/15	2.00±0.59 (0.00-6.00)		16.69±4.47 (6.25-42.56)		8.34±0.74 (3.89-600.00)	
Time to 1st probe from start of EPG	Colonized	11/12			193.22±66.29 (4.73-600.00)	0.545		
	Control	15/15			247.39±57.25 (0.02-600.00)			
Time from the beginning of the 1st probe to 1st pd	Colonized	6/12			21.44±4.03 (5.11-35.35)	0.22		
	Control	9/15			97.51±6.43 (13.79-67.23)			
Time from the end of the last pd to the end of the probe	Colonized	6/12			26.61±11.76 (0.65-70.55)	0.186		
	Control	9/15			829.383±73.52 (0.00-526.44)			
Duration of 1st probe	Colonized	10/12			71.62±28.07 (0.00-354.80)	0.156		
	Control	12/15			214.05±59.86 (0.00-599.92)			
Duration of 2nd probe	Colonized	8/12			51.93±18.11 (0.00-183.89)	**0.043**		
	Control	3/15			44.50±29.27 (0.00-344.80)			
Duration of the second non probe period	Colonized	8/12			122.84±44.18 (0.00-364.26)	**0.01**		
	Control	3/15			10.10±5.75 (0.00-73.61)			
Average number of pd per probe	Colonized	6/12	0.75±0.26 (0.00-2.50)	0.293				
	Control	9/15	1.44±0.43 (0.00-6.00)					
Number of short probes (C<3 min)	Colonized	11/12	1.58±0.29 (0.00-3.00)	**0.004**				
	Control	15/15	0.47±0.17 (0.00-2.00)					

PPW. proportion of individuals that produced the waveform type; **NWEI**. number of waveform events per insect; **WDI**. waveform duration (s) per insect; **WDE**. waveform duration (s) per event (Backus et al.. 2007). **Non-probe**. non-probe activity; **Probe**. probe activity. Waveforms: **C**. intercellular stylet pathway; **pd**. short intracellular punctures; **G**. xylem ingestion; **E** shows phloem-related activities: **E1**. correlates with salivation into phloem sieve elements; **E2**. ingestion from phloem (Prado and Tjallingii. 1994); **sE2**: sustained E2 (longer than 10 minutes).

[a] Bold-type indicates significant differences ($p<0.05$) according to *t*-Student test (for Gaussian variables) or Mann–Whitney *U* test (for non-Gaussian variables).

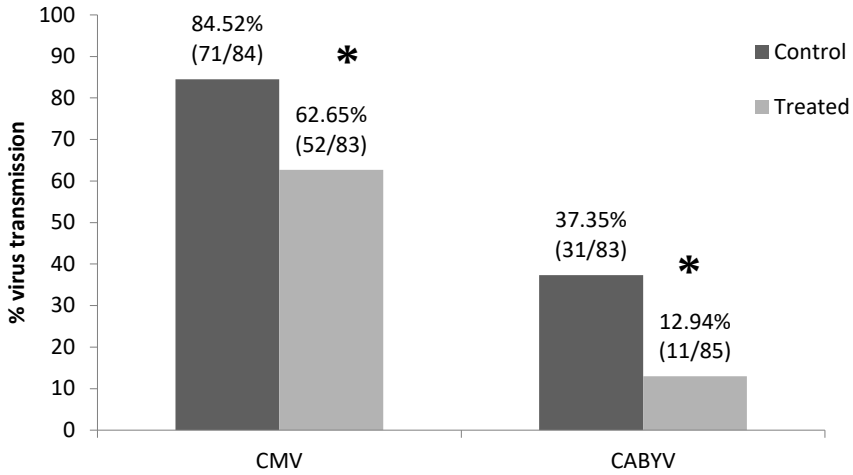

Figure 1. Virus transmission by *Aphis gossypii* to melon plants endophytically colonized by the entomopathogenic fungus *B. bassiana* (dark grey bars) and control plants (light grey bars). *Significant differences were observed for CMV (n=167; χ^2=9.20) and CABYV (n=168; χ^2=12.07).

4. Discussion

The use of entomopathogenic fungi (EF) has been proposed in the past decade as a new alternative to control plant pathogenic microorganisms and viruses (Ownley et al. 2010). Here, we show for the first time that the presence of endophytic EF can affect the probing and feeding behaviour of aphids, as well as the transmission of NP and P viruses.

Using the electrical penetration graph (EPG) technique (Tjallingii 1988), it was possible to characterize the insect stylet activities associated with the transmission of plant viruses. It is well known that the transmission of NP viruses is related to the intracellular stylet punctures visualized as potential drops (pd) in brief probes in epidermal and mesophyll cells (Powell 1991, 2005; Martín et al. 1997). Conversely, the P plant viruses, which have a more specific and intimate

relationship with their aphid vectors, are acquired and inoculated when aphids ingest and salivate in the phloem tissues (Gray and Gildow 2003; Hogenhout et al. 2008).

The EPG study indicates that *B. bassiana* colonization does not alter the feeding behaviour of *A. gossypii* associated with the inoculation of NP viruses but could modify the virus acquisition process. Despite the fact that no EPG variable related to the inoculation of NP viruses was altered by *B. bassiana* colonization, EF seems to have some effects on other EPG variables related to NP virus acquisition. For example, EPGs showed a shorter duration of pd by aphids exposed to plants treated with *B. bassiana* than those exposed to untreated plants. It is known that the acquisition of NP viruses is related to sub phase II-3 of the pd but not to their inoculation (Martín et al. 1997). Moreover, Collar and Fereres (1998) suggest that a "time threshold" in the duration of sub phase II-3 or in the number of II-3 pulses is required for the occurrence of an ingestion event, and therefore, the probability of acquisition of NP viruses is higher with an increased duration of the pd. According to the results obtained, the endophytic colonization by EF reduces the pd duration, reducing the likelihood of acquisition of an NP virus such as CMV.

Other EPG variables that have been previously correlated with the acquisition of NP viruses are the number of short probes and the number of intracellular stylet punctures (pd) (Collar et al. 1997; Collar and Fereres 1998). In the present paper, no significant differences were detected in the number of pds between the control and the *B. bassiana* endophytically colonized melon plants when both the 8-hour recordings and the first 10-minute ones were analysed. However, when the first 10-minute EPG recordings were analysed, the number of short probes was significantly lower in the control than in the EF-treated

melon plants. Therefore, further work is needed to elucidate the impact of the increase in the number of short probes recorded during the first 10 min of EPGs on endophytically colonized plants and the possible EF effect on the acquisition of NP viruses. No modification of the aphid probing behaviour related to the transmission of P viruses was observed after the endophytic colonization by EF.

Regarding the EPG variables, where significant differences were found, even if they had not been involved in processes of virus transmission, similar results to our results were found by Cao et al. (2014) when aphid probing behaviour was studied using the EPG technique. The first probe duration was shorter, and the number of probes and short probes was approximately twice as high in plants challenged by methyl-jasmonate as in control plants due to feeding deterrents in the mesophyll. Furthermore, endophytic colonization of different crops by several microorganisms has been reported to cause physiological changes and induction of jasmonic acid (JA)- and salicylic acid (SA)-mediated defences, finally repelling enemies and attracting mutualistic organisms (Arnold 2008; Jallow et al. 2008). Indeed, a secondary metabolite concentration has been observed in shoots and roots regulating insect-plant or vector behaviour, finally diminishing virus transmission (Stinson et al. 2003; Bruinsma et al. 2009; Ingwell et al. 2012; Pangesti et al. 2013; Cotes et al. 2015; Rostás et al. 2015; De Sassi et al. 2006; Zhang et al. 2016).

The reduction in *A. gossypii* probe duration detected in the endophytically colonized melon plants, when the first 10 min were analysed, could be related to an EF effect on the aphid feeding behaviour mediated either by fungal-related metabolites of propagules in the mesophyll (Landa et al. 2013; Resquín-Romero et al. 2016)

and/or by the activation of the abovementioned defensive pathways in the plant.

Although the main EPG variables related to the inoculation of NP and P viruses were not modified due to the colonization by EF *B. bassiana*, according to our results from virus transmission assays, NP and P virus incidence was significantly reduced when melon plants were inoculated and endophytically colonized by EF *B. bassiana* before they had been infested with viruliferous aphids. Moreover, a reduction in the CMV incidence in *B. bassiana*-treated plants was observed both when inoculation took place using aphids and mechanically, whereas CABYV incidence in such plants was observed when the inoculation took place using aphids.

The potential of EF *B. bassiana* to confer protection against mechanical inoculation of NP viruses was probed previously with ZYMV on squash by Jaber and Salem (2014). In this paper, disease incidence and severity were significantly lower in *B. bassiana*-inoculated plants compared to the non-inoculated control plants, irrespective of the strain inoculated. In our study, we obtained similar results for NP mechanical inoculation, and it has been demonstrated that a reduction in virus transmission since endophytic colonization by EF also occurs when aphid vectors inoculate NP and P viruses.

Taking all our results together and considering that no effect due to endophytic colonization by EF was observed on the site where aphids were probing, it is possible to suggest that the mechanism by which *B. bassiana* reduces the transmission rate of NP and P viruses is a systemic resistance to viruses induced by the fungal biocontrol agent. Similarly, several studies previously showed that endophytic microorganisms, such as leaf-colonizing bacteria *Bacillus amyloliquefaciens* or endophytic fungi (Ascomycota: Hypocreales), such

as *Trichoderma harzianum*, serve in biocontrol management as microbial disease control agents of plant viruses (Lee and Ryu 2016; Vitti et al. 2016). Indeed, previous studies have shown the potential of the entomopathogenic fungus *B. bassiana* to act as a biocontrol agent against several plant pathogens, such as plant pathogenic bacteria (Griffin et al. 2006; Ownley et al. 2008) and soilborne (Griffin 2007; Ownley et al. 2008) and airborne phytopathogenic fungi (Jaber 2015), and the EF have also been successfully conferred protection by endophytic plant colonization against the mechanically inoculated NP virus (Jaber and Salem 2014).

However, to our knowledge, the present study is the first where endophytic *B. bassiana* colonization confers protection against NP and P plant virus transmission by aphids. Different mechanisms may be involved in plant disease suppression or tolerance mediated by *B. bassiana*, including the production of fungal metabolites that may act as antibiosis substances, competition for space and resources, mycoparasitism, triggering plant defence induction resistance or enhancing plant growth (Ownley et al. 2008, 2010; Vega et al. 2009; Gómez-Vidal et al. 2009; Porras-Alfaro and Bayman 2011; Jaber and Enkerli 2017; Jaber and Ownley 2018; Vega 2018).

These antagonistic mechanisms may directly affect virus replication and accumulation inside plants (Raupach et al. 1996) or virus movement (Loebenstein 1972; Martelli 1980). Indeed, it has been found that application of JA followed by SA inhibits CMV replication (Luo et al. 2010). Moreover, Jaber and Salem found in 2014 that the DAS-ELISA assay detected low ZYMV concentrations in *B. bassiana*-inoculated plants that apparently had no symptoms, indicating a low rate of viral multiplication and thus accumulation in those plants.

Our results show that *B. bassiana* could be used to reduce virus transmission by aphids as part of an integrated pest management programme in combination with other control tactics. Further studies should be conducted in the laboratory and in the field to determine whether endophytic *B. bassiana*-colonized melon plants could reduce the acquisition rate of plant viruses and therefore reduce their secondary spread in natural conditions.

5. Acknowledgements

The authors thank the regional government of Andalusia (Junta de Andalucía) for granting P11-AGR-7681, Sustainable strategies for pest control based on the establishment of rhizosphere competent and endophyte entomopathogenic fungi and the Spanish Ministry of Economy and Competitivity grant AGL2013-47603-C2-1-R, Desarrollo de estrategias novedosas y sostenibles para el control integrado de insectos vectores y virosis asociadas en cultivos hortícolas.

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CAPÍTULO IV. BOTTOM-UP EFFECTS OF ENDOPHYTIC *BEAUVERIA BASSIANA* ON MULTITROPHIC INTERACTIONS BETWEEN THE COTTON APHID, *APHIS GOSSYPII*, AND ITS NATURAL ENEMIES IN MELON

Este capítulo es una versión adaptada del artículo:

Journal of Pest Science (2019) 92:1271–1281
https://doi.org/10.1007/s10340-019-01098-5

ORIGINAL PAPER

Bottom-up effects of endophytic *Beauveria bassiana* on multitrophic interactions between the cotton aphid, *Aphis gossypii*, and its natural enemies in melon

N. González-Mas[1] · M. Cuenca-Medina[1] · F. Gutiérrez-Sánchez[1] · E. Quesada-Moraga[1]

Received: 2 December 2018 / Accepted: 18 February 2019 / Published online: 25 February 2019

Abstract
Entomopathogenic fungi are now known to have an endophytic capacity that induces a wide range of changes in the composition of plant nutrients and/or defensive compounds. These changes could influence interactions between the plant and higher trophic levels. In this study, we evaluated the predation/parasitism efficacy of larvae of the lacewing, *Chrysoperla carnea*, and the braconid parasitoid, *Aphidius colemani*, when offered aphids that had been challenged by the entomopathogenic fungus *Beauveria bassiana*. Aphids were either inoculated directly with a fungal suspension (lacewing bioassay only) or had been feeding on melon plants endophytically colonized by *B. bassiana*. Our results indicate that *B. bassiana* application did not significantly influence the number of aphid prey consumed by lacewings, or the time took them to consume each aphid. In a choice bioassay, *C. carnea* larvae preferred to feed on aphids reared on *B. bassiana*-colonized plants compared with control plants. In another choice assay, the number of aphids parasitized by *A. colemani* and their sex ratio were not influenced by whether the aphids had been feeding on *B. bassiana*-colonized plants or not. Our findings support the hypothesis that endophytic entomopathogenic fungi can be used in combination with other natural enemies, such as predators and parasitoids, in Integrated Pest Management programmes.

Keywords *Chrysoperla carnea* · *Aphidius colemani* · Predator · Parasitoid · Entomopathogenic fungus · Endophyte · IPM

Key message

- The predatory and parasitic efficacy of lacewings and parasitoids on aphids were unaffected when these aphids had been feeding on plants endophytically colonized by the entomopathogenic fungus *Beauveria bassiana*.
- When given a choice, lacewings did show a preference for predating aphids that were feeding on *B. bassiana*-colonized plants compared with control plants.
- These findings suggest that predators and parasitoids can be used together in Integrated Pest Management programmes with endophytic entomopathogenic fungi.

Communicated by M. Traugott.

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Introduction

Integrated Pest Management (IPM) is an interdisciplinary approach for control of agricultural pest populations that is regulated in the European Union Member States (Directive 2009/128/EC) and in the United States (U.S. Code § 136r–1). In principal, IPM uses optimal combinations of the most suitable techniques and methods available for controlling a given pest. The aim is to retain pest populations below damage thresholds while simultaneously reducing reliance on chemical pesticides. In this way, the development of insecticide resistance is minimized and hazards to non-targets, including humans and the environment, are reduced (Stern et al. 1959; Smith 1978; Metcalf and Luckmann 1994; FAO 2018). Biological control agents, such as entomophagous arthropods (predators and parasitoids) and

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Entomopathogenic fungi are a diverse assemblage of species that infect insect and mite hosts. They are present in almost all terrestrial ecosystems and habitats and play many different ecological roles (Lovett and St. Leger 2017). They have been found in the soil, the phylloplane and most recently as plant endophytes and as rhizosphere-competent microorganisms (Quesada-Moraga et al. 2006; Meyling and Eilenberg 2007; Vega et al. 2009; Vega 2018).

The combined use of entomopathogenic fungi and entomophagous arthropods within IPM strategies has been explored. This takes advantage of the contact action of the fungi and the ability of predators and parasitoids to search for their prey which, together, could ensure that fewer pests escape treatment (Baverstock et al. 2010; Ren et al. 2010). It is likely that combinations of various biological control agents could be additive with

respect to suppression of pest populations (Wraight 2003). However, intraguild and multitrophic interactions should be considered when multiple biological control agents are applied simultaneously or sequentially after planned time intervals (Martins et al. 2014). Indeed, several studies have examined the safety and effectiveness of combinations of entomopathogenic fungi and other components of biological control such as predators, parasitoids and nematodes (Roy and Pell 2000; Acevedo et al. 2007; Labbéet al. 2009; Ansari et al. 2010; Martins et al. 2014).

The recent discovery that entomopathogenic fungi can be endophytic with a new systemic biological control role raises concerns about previously unconsidered interactions with beneficial and non-target organisms, including other biological control agents (Jaber and Ownley 2018; Vega 2018). Endophytic plant colonization by entomopathogenic fungi induces a wide range of changes in the composition and quantity of plant nutrients and/or defensive compounds within the plant; this has implications for the quality of the plant as a food source for herbivores (Gualandi et al. 2014; Shrivastava et al. 2015; Sánchez-Rodríguez et al. 2015; Liao et al. 2017; Raya-Díaz et al. 2017; Krell et al. 2018). These changes may lead to a cascade of positive or negative changes in the life history characteristics and behaviour of herbivores and their interactions with natural enemies. Effects on third trophic level (i.e. natural enemies) have been reported in other microorganism-plant associations, such as those with mycorrhizal fungi (Gange et al. 2003; Guerrieri et al. 2004; Hempel et al. 2009), plant growth-promoting rhizobacteria (PGPR) (D'Alessandro et al. 2014; Gadhave et al. 2016) and other fungal endophytes in grass (Bultman et al. 1997, 2012; de Sassi et al. 2006; Härri et al. 2008).

Likewise, it has been reported that endophytic colonization by entomopathogenic fungi induces analogous effects in members of the third trophic level such as parasitoids (Akutse et al. 2014; Gathage et al. 2016;

Jaber and Araj 2018). However, it remains unknown whether such colonization caused any effect on predators. Moreover, there is only one study on the combined use of endophytic entomopathogenic fungi and entomophagous arthropods for aphid control and only considered parasitoids (Jaber and Araj 2018).

In the current study, we evaluated whether the efficacy of aphid control using plants endophytically colonized by an entomopathogenic fungus would be affected if combined with predators and parasitoids. Specifically, we used the endophytic *Beauveria bassiana* isolate EABb 01/33-Su along with the most widespread and important natural enemies of the cotton aphid, *Aphis gossypii* (Homoptera: Aphididae), i.e. the generalist predator *Chrysoperla carnea* (Neuroptera: Chrysopidae) and the braconid parasitoid *Aphidius colemani* (Hymenoptera: Braconidae). We used predator–prey bioassays and choice tests to compare the efficacy and behaviour of *C. carnea* larvae when offered aphids that had either been sprayed directly with a suspension of *B. bassiana* conidia or had been feeding on melon plants endophytically colonized by *B. bassiana*. We also evaluated the behaviour and reproductive success of the aphid parasitoid, *A. colemani*, when offered a choice between aphids that had been feeding on melon plants endophytically colonized with *B. bassiana* and control aphids.

2. Materials and methods

2.1. Study organisms

Melon seeds (*Cucumis melo* L. var. Galia) were surfacesterilized in 2% sodium hypochlorite (Sigma-Aldrich, MO, USA) for 2 min, rinsed twice with sterile Mili-Q water and dried in a laminar-flow hood under sterile conditions. The soil substrate (Floragard, Germany) was also sterilized twice in an autoclave for 20 min at 121 °C with a 24-h interval between each sterilization process. Surface-sterilized seeds were germinated in 9 × 9 cm

pots containing a mixture of equal parts of vermiculite (No. 3, Asfaltex S.A., Barcelona, Spain) and the sterilized soil substrate. Germinated seeds were maintained in an environmental chamber under controlled conditions: 25 ± 2 °C and a 16-h light: 8-h dark regime. A nutritional complex of 20:20:20 (N:P:K) Nutrichem 60 fertilizer (Miller Chemical & Fertilizer Corp., PE, USA) was added to the irrigation water at a rate of 1 g l−1 three times a week.

Aphis gossypii population was provided by the Institute of Agricultural Sciences (ICA) CSIC (Madrid, Spain) and then reared for several generations in the laboratory. Aphids were reared in cages on melon plants in an environmental growth chamber under controlled conditions of 25 ± 2 °C, a 16-h light: 8-h dark regime and 70% RH.

Beauveria bassiana isolate EABb 01/33-Su was used in all the bioassays. The endophytic ability of this isolate when inoculated onto melon plants had been demonstrated previously (Resquín-Romero et al. 2016), as had its ability to cause mortality in sap-sucking insects when endophytic (Garrido-Jurado et al. 2016; González-Mas and Quesada-Moraga 2017). EABb 01/33-Su was originally isolated from soil from El Bosque (Cádiz) and deposited in the University of Córdoba Entomopathogenic Fungi Collection, Córdoba, Spain. Nucleotide sequences for the ITS and mtDNA intergenic regions of EABb 01/33-Su can be found in the Gen-Bank database (EF115310 and FJ972969 for the ITS region; FJ973025 for intergenic region nad3-atp9; and FJ972914 for the intergenic region atp6-rns).

For all bioassays, isolate EABb 01/33-Su was grown on potato dextrose agar (PDA) in Petri dishes for 15 days at 25 °C in darkness. A cellophane film was placed on the agar prior to inoculation to prevent nutrients transferring to the conidial suspension at the time of harvest. Conidial suspensions were prepared by scraping conidia from the dishes into an aqueous sterile solution of 0.01% Tween 80. The resulting conidial suspension was filtered through several layers of sterile cheesecloth to

remove mycelia, and sonicated for 5 min to homogenize the inoculum. Conidial concentrations were determined using a haemocytometer and appropriate dilutions made in 0.01% Tween 80 to achieve a concentration of 10^8 conidia ml^{-1} for experiments. Prior to experimentation conidial viability was determined on liquid Czapek-Dox broth plus 1% (w/v) yeast extract medium and only suspensions with > 97.0% germination after 24 h were used.

Chrysoperla carnea larvae and *A. colemani* mummies (pupae) were supplied by Koppert Biological Systems (Almeria, Spain).

2.2. Predation efficacy of *C. carnea* offered healthy or *B.bassiana*-infected aphids

Ten groups of newly emerged apterous adult aphids (24–72 h since last moult), each containing ten individuals, were collected from the rearing cages into Petri dishes (60 mm Ø) with a hole sealed with fine-mesh netting to provide air-circulation, using a camel-hair brush. Each group was immersed in separate 5 ml aliquots of either a 1×10^8 conidia ml−1 suspension of *B. bassiana* (*n* = five groups), or a 0.01% sterile solution of Tween 80 in the case of the control (*n* = five groups) and then removed with the aid of a camel-hair brush. Each group of aphids was incubated in the absence of food in Petri dishes (60 mm Ø) for 24 h under the same controlled conditions as described previously for aphid rearing. At the same time, ten lacewingswere placed individually in Petri dishes (60 mm Ø) and incubated under the same aphid controlled conditions in the absence of food for 24 h prior to the assay.

Aphids (24 h after being treated) were offered ad libitum to the lacewings one by one. Once an aphid was completely consumed, it was automatically replaced with a new one. The number of aphids consumed and the time required for consumption of each one were recorded over a

period of 4 h. The entire experiment was done twice, each time using fresh fungal inoculum, aphids and lacewings.

2.3. Predation efficacy of *C. carnea* offered control aphids or aphids reared on *B. bassiana*-colonized melon plants

Replicate melon plants were grown, as described previously, to the four-leaf stage. Leaves of these plants were sprayed either with conidial suspensions of *B. bassiana* (*n* = ten) or with a sterile solution of 0.01% Tween 80 (control; *n* = ten). Specifically, two leaves per treatment plant were each sprayed with 1 ml of fungal suspension using an aerograph 27085 (piston compressor of 23 l min−1, 15–50 PSI and a 0.3 mm nozzle diameter, China). The remaining plant leaves were covered with a transparent plastic sheet prior to spraying to prevent them from being inoculated. After spraying, the entire plant was enclosed in clean plastic sheeting for 24 h to promote fungal growth. Control plants were treated similarly. Plants were incubated in an environmental chamber at 25 ± 2 °C, 16-h light: 8-h dark regime and 70% RH for 48 h; unsprayed leaves were covered with plastic bags to avoid any contamination between sprayed and unsprayed leaves. Ten newly emerged apterous adult aphids (24–72 h after last moult) were transferred, using a camelhair brush, to a non-sprayed leaf in each of the *B. bassiana* treated plants, and to a non-sprayed leaf in each of the control plants. Aphids were confined to the leaves in clip cages and incubated at 25 ± 2 °C, 16-h light: 8-h dark regime and 70% RH for 6 days. Each clip cage had a hole sealed with fine-mesh netting to provide air-circulation. Aphids were then removed from the leaves and offered to the lacewings as described in the previous section. The number of aphids consumed and the time required for consumption of each one were recorded over a period of 4 h. The entire experiment was done twice, each time using fresh fungal inoculum, plants, aphids and lacewings.

2.4. Predatory behaviour of *C. carnea* offered a choice of aphids reared on *B. bassiana*-colonized or control melon plants

Lacewing predatory behaviour was observed in a choice assay based on ten replicates of endophytically colonized and control leaves. To achieve this, endophytically colonizedand control plants were produced as described previously. However, in this case, only five apterous adult aphids were transferred to each non-sprayed leaf in both *B. bassiana*colonized and control melon plants 48 h after spraying. Aphids were confined in a clip cage for 6 days under the same abiotic conditions as described previously. Three of the adult aphids were transferred to another aphid-free *B. bassiana*-colonized (not directly sprayed) or control leaf on the same plant, and these aphids were used in the experiment. This was done to ensure that the number of aphids presented to the predator on each leaf was the same in each replicate and thus prey density would not affect predator choice. Without detaching leaves from mother plants, one non-sprayed leaf (bearing three aphids) from a *B. bassiana* colonized plant and one from a control plant (also bearing three aphids) were placed through two holes made in a Petri dish arena (150 mm × 15 mm); the arena was designed such that the leaves were 7 cm apart from each other. One lacewing was introduced into each of the ten arenas at a central position (Figure 1) and its behaviour observed over a period of 3 h using a point sampling procedure, with a final observation 24 h after initiation. At each sampling interval (every hour), the activity of the lacewings was recorded as follows: walking on the control leaf, feeding on an aphid on the control leaf, walking on the *B. bassiana*-colonized leaf, feeding on an aphid on the *B. bassiana*-colonized leaf, or exploring throughout the arena (i.e. walking but on neither of the leaves). Final position of the lacewing after 24 h was also recorded. The entire experiment was done on two occasions, each time using fresh fungal inoculum, plants, aphids and lacewings.

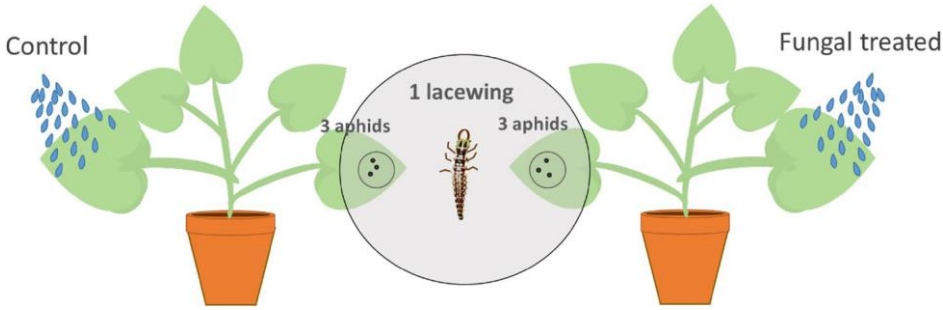

Figure 1. Design of an arena for the lacewing choice bioassay. The setup consists of one large Petri dish (150 mm × 15 mm) with two holes (diameter 25 mm each) in the bottom and covered by the lid. The area in contact with the plant was lined with foam rubber to avoid damaging the leaves. Lacewings could choose between an unsprayed leaf from a *B. bassiana*-colonized plant and a leaf from the control plant. Position on control/treatment plant or off the plant, feeding activity and final position at the end of the experiment were recorded

2.5. Oviposition preference and development of *A. colemani* offered a choice of aphids reared on *B. bassiana*–colonized or control melon plants

Control and endophytically colonized melon plants were established as described previously (n = 10). Once endophytic colonization was established (48 h after spraying) (Resquín-Romero et al. 2016), a group of ten second instar *A. gossypii* aphids (a stage suitable for parasitization by *A. colemani*; Sampaio et al. 2008) was transferred onto one of the non-sprayed leaves on each replicate plant using a fine camel-hair brush, and enclosed within a clip cage (2 cm in diameter; 1 cm high). Aphids were allowed to feed on the plants for a further 72 h before the experiment was started. After this period, some of them had become 3rd instars but were still suitable hosts for the parasitoid. While the aphids were developing, the *A. colemani* mummies were divided into 20 groups of 100 in lidless Petri dishes (60 mm Ø) and each group placed into a mesh cage (350 mm × 250 mm × 200 mm). Cages were incubated in an environmental chamber at 25

°C and 16-h light: 8-h dark regime until the parasitoids emerged (24–48 h). Cotton wool soaked with a 50% honey: water solution was provided as a food resource in each cage for the emerging adults. Five days after the parasitoids began emerging, unsprayed leaves bearing aphids (clip cages removed) from one endophytically colonized plant and one control plant, were introduced to each cage of parasitoids. Each leaf remained attached to the mother plant (which remained outside the cage) and was introduce through sealable access holes in the cage sides. The areas of cage in contact with the plant were lined with foam rubber to avoid damaging the leaves (Figure 2). Parasitoid females present (4–5 days old and mated) were allowed to parasitize aphids for 24 h; they choose between aphids on endophytically colonized or control leaves. After this time the leaves, still attached to the mother plant, were removed from the cages and entire plants incubated in an environmental chamber under suitable conditions for parasitoid development. Endophytically colonized plants were held separately from the control plants to ensure there was no transfer of aphids between endophytically colonized and control plants. The number of parasitoid mummies that developed were counted and removed from each plant daily until no more were produced. The mummies removed were incubated inPetri dishes at 25 ± 2 °C, 16-h light: 8-h dark regime and 70% RH and the sex ratio of the emerging adults determined. The entire experiment was done on two occasions, each time using fresh fungal inoculum, plants, aphids and parasitoids.

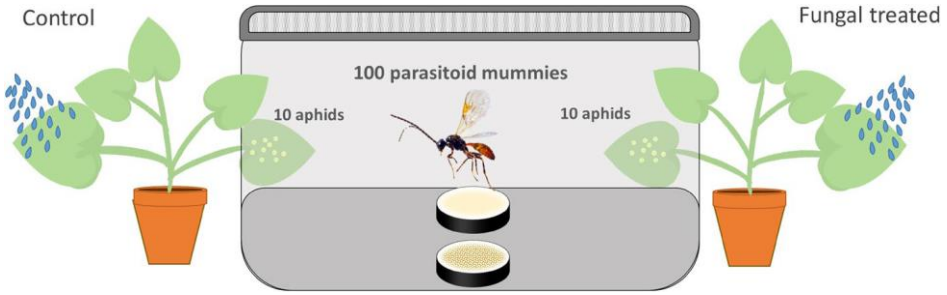

Figure 2. Design of the cage used for the parasitoid choice bioassay. The set-up consists of one large cage (350 mm × 250 mm × 200 mm) with two holes at the sides and covered by a mesh lid. The area in contact with the plant was lined with foam rubber to avoid leaf damage. Parasitoids chose between aphids feeding on a non-sprayed leaf endophytically colonized by *B. bassiana* or aphids feeding on a control leaf. Final number of mummies was recorded

2.6. Confirmation of endophytic colonization of melon plants by *B. bassiana*

At the end of each assay, plant samples were taken from each replicate plant to evaluate endophytic colonization. Specifically, sprayed and non-sprayed leaves from each treatment and control plant were collected, surface-sterilized with 2% sodium hypochlorite for 2 min, rinsed twice in sterile distilled water and dried on sterile filter paper. From each sample, 10 fragments of a 2 cm^2 area were excised using a sterile scalpel and plated out on selective medium. The selective medium used was made up of 20 g of Sabouraud Glucose

Agar with Chloramphenicol (Cultimed Panreac, Spain), 500 mg l^{-1} streptomycin sulfate (Sigma-Aldrich Chemie, China), 500 mg l^{-1} ampicillin (Intron biotechnology, China) and 500 mg l^{-1} dodine 65 WP (Barcelona, Spain). In addition, a water sample [100 µl] from the last rinse of each leaf section sample was plated on to the same selective culture medium. In this way, we could assess the per cent colonization of the leaves and also the effectiveness of the surfacesterilization procedure. Plates were incubated at 25 °C in darkness until fungal growth was observed. In all bioassays, only

data from treatment replicates with confirmed endophytic colonization were considered for data analysis.

2.7. Statistical analysis

Data of the number of aphids offered and consumed by the lacewings were analysed using a generalized linear model with a Poisson distribution link function. A Pearson's correlation and Chi-square ($p \leq 0.05$) analysis were performed to evaluate the predatory behaviour of *C. carnea* in the choice of aphid prey bioassay (endophytically colonized leaves vs control leaves).

Aphid prey mortality data based on lacewing consumption times were subjected to Kaplan–Meier survival analysis (Kaplan and Meier 1958) to calculate Average Survival Time (AST; i.e. average consumption time) values in minutes and compared by the Log-rank test using SPSS 19.0® software for Windows.

The percentage of mummified aphids, parasitoid emergence, and parasitoids that emerged as either female or male were subjected to the Kruskal–Wallis nonparametric test using SPSS 19.0® software for Windows.

When there was no statistically significant difference in the results from the two occasions on which each experiment was done the data were pooled, analysed together, and presented as a single graph.

3. Results

3.1. Predation efficacy of *C. carnea* offered healthy aphids or aphids infected with *B. bassiana* as prey

Predation efficacy of *C. carnea* was affected by fungal treatment of the aphids neither in the number of prey consumed nor in the number of prey offered during the observation period ($\chi^2_{1df} = 0.20$, $p = 0.66$ and $\chi^2_{1df} = 0.35$, $p = 0.56$, respectively) (Figure 3). In addition, there was no difference between treatment and control in the mean time employed by each lacewing to consume its aphid prey (Control AST = 130.39 ± 15.07 min; Treatment AST = 126.12 ± 12.34 min) (Figure 4).

3.2. Predation efficacy of *C. carnea* on control aphids or aphids that had been reared on melon plants endophytically colonized by *B. bassiana*

No *B. bassiana* outgrowth was observed in any of the control samples examined or from the plated rinse water. Incontrast, all the leaves from inoculated plants had been colonized endophytically by *B. bassiana*. They were observed higher levels of colonization in sprayed leaves (93.33 ± 3.10 colonies per leaf fragment) compared with non-sprayed leaves on the same plant (24.00 ± 9.81 colonies per leaf fragment). Predation efficacy of *C. carnea* was not affected when feeding on aphids reared on *B. bassiana*-colonized plants compared with the control, either in the number of prey consumed or the number of prey offered during the observation period ($\chi^2_{1df} = 0.53$, $p = 0.47$ and $\chi^2_{1df} = 0.22$, $p = 0.66$, respectively) (Figure 5). However, there was an increase (though statistically insignificant) in the mean time employed by each lacewing to consume its aphid prey when the aphid was reared on *B. bassiana*-colonized plants compared with the

control (Control AST = 113.08 ± 10.99 min; Treatment AST = 127.80 ± 13.72 min) (Figure 6).

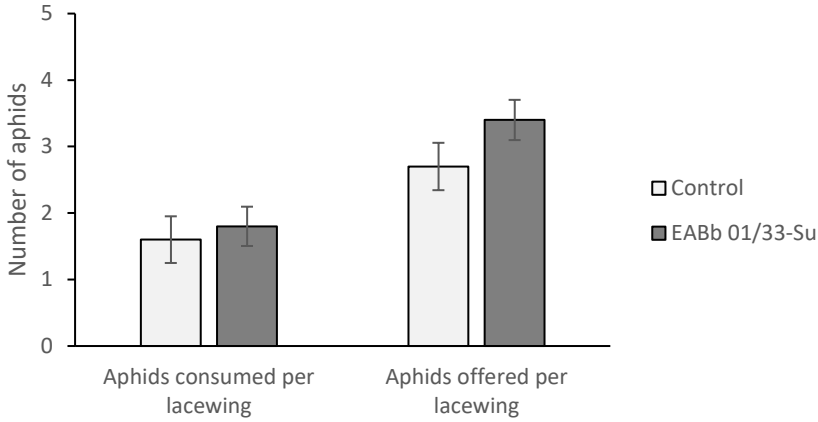

Figure 3. Number of aphid prey (mean ± SE) consumed and number offered during the observation period. Prey were either aphids immersed in *B. bassiana* suspension (1 × 10^8 conidia ml−1) or sterile 0.01% Tween 80 (controls) 24 h prior to experiment. Asterisks indicate significant differences between control and treatment (χ^2_{1df}, $p < 0.05$)

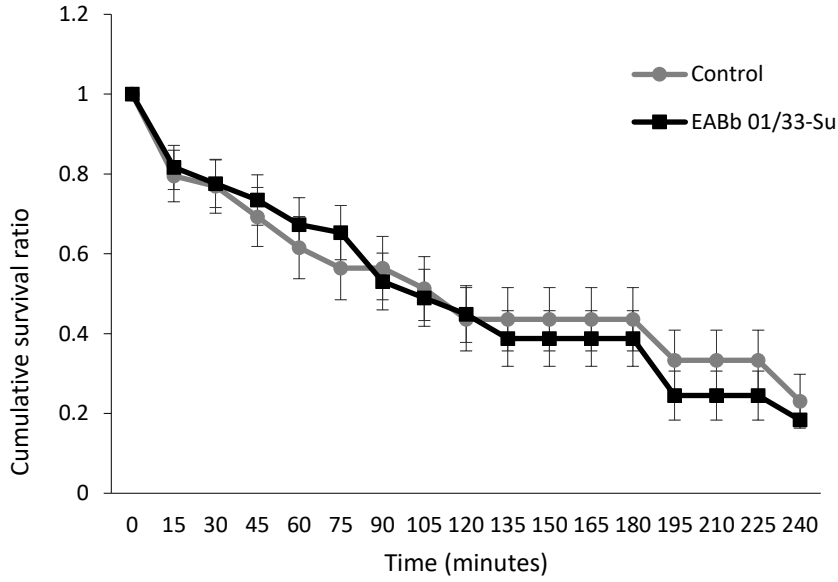

Figure 4. Cumulative proportion of *A. gossypii* prey (either immersed in *B. bassiana* suspension [1 × 10^8 conidia ml^{-1}] or sterile 0.01% Tween 80 (controls) 24 h prior to experiment) that were offered to lacewingsand survived until the end of the observation period. Data are expressed as the mean ± SE

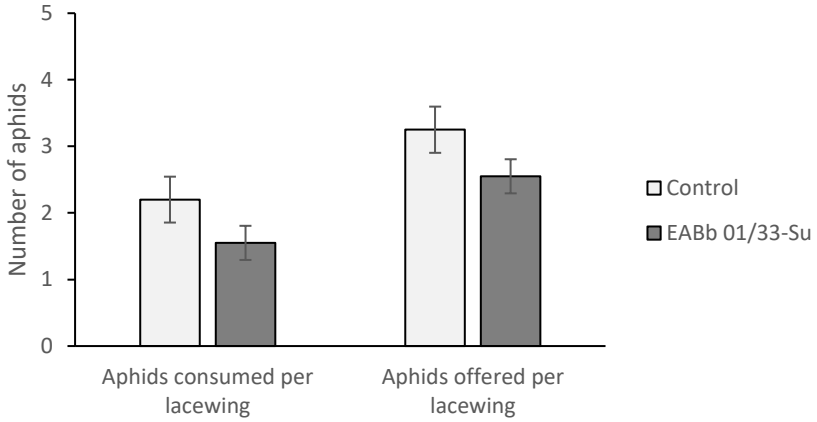

Figure5. Number of aphid prey (mean ± SE) consumed and number offered during the observation period. Prey were aphids that were either fed for 140 h on *B. bassiana*-colonized melon plants, or fed for 140 h on control plants. Asterisks indicate significant differences between control and treatment (χ^2_{1df}, $p < 0.05$)

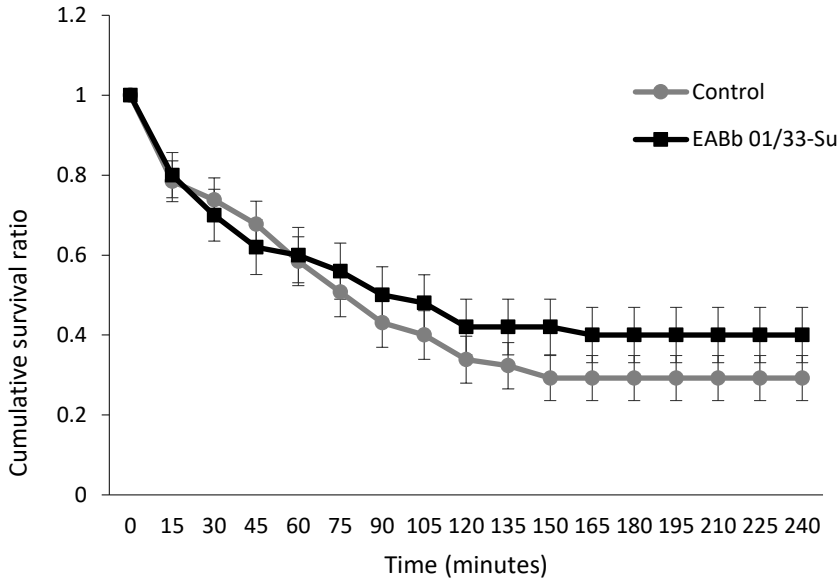

Figure 6. Cumulative proportion of *A. gossypii* prey (either fed on *B. bassiana*-colonized plants or control plants) that were offered to lacewings and survived until the end of the observation period. Data are expressed as the mean ± SE

3.3. Predatory behaviour of *C. carnea* when offered a choice of aphid prey feeding either on leaves endophytically colonized by *B. bassiana* or control leaves

No *B. bassiana* outgrowth was observed in any of the control samples examined or from the plated rinse water. In contrast, all the leaves from inoculated plants had been colonized endophytically by *B. bassiana*. The level of endophytic colonization of non-sprayed leaves was 36.11 ± 4.86 colonies per leaf fragment compared with 95.50 ± 2.11 colonies per leaf fragment for sprayed leaves.

The choice assays revealed a significant preference of the predators for aphids feeding on *B. bassiana*endophytically colonized plants compared with control plants ($\chi^2_{1df} = 9.29$; $p = 0.00$) (chi² test, $\alpha = 0.05$). Initially, we observed that the lacewings were mainly exploring throughout the experimental arena and not on the leaves. However, the longer the exposure time, the higher the number of lacewings preferring endophytically colonized leaves to prey on aphids (Figure 7).

3.4. Oviposition preference and development of *A. colemani* when offered a choice of aphid prey feeding either on leaves endophytically colonized by *B. bassiana* or control leaves

No *B. bassiana* outgrowth was observed in any of the control samples examined or from the plated rinse water. In contrast, all the leaves from inoculated plants had been colonized endophytically by *B. bassiana*. The level of endophytic colonization of unsprayed leaves was 43.62 ± 2.86 colonies per leaf fragment compared with 93.50 ± 2.04 colonies per leaf fragment for sprayed leaves.

No significant differences were detected in parasitoid preference for aphids feeding on *B. bassiana*-colonized plants compared to the controls. There was no significant difference in the number of mummies recovered

from aphids reared on *B. bassiana*-colonized plants compared with controls (H = 0.40; p = 0.53), mummy development time (H = 2.17; p = 0.15) or the sex ratio (females H = 0.00; p = 0.96; males H = 0.82; p = 0.37) (Figure 8).

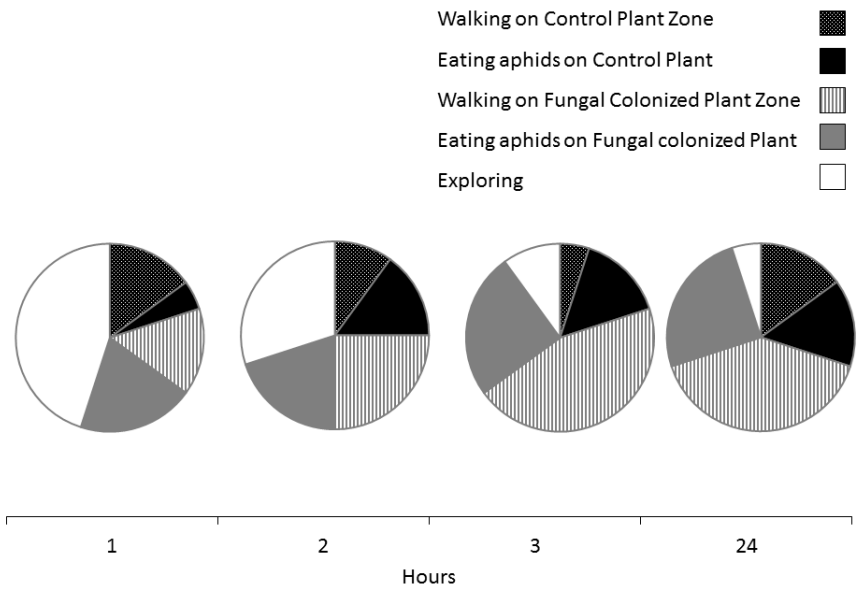

Figure 7. Percentage of *C. carnea* larvae choosing aphids feeding either on a *B. bassiana*-colonized plant or a control plant. Circles denote the proportion of lacewings (a) walking on the *B. bassiana*-colonized leaf, (b) feeding on aphids on the *B. bassiana*-colonized leaf, (c) walking on the control leaf, (d) feeding on aphids on the control leaf, and (e) exploring the arena but not walking on either of the leaves. Lacewing behaviour was observed once every hour for three hours and the final establishment was recorded at 24 h

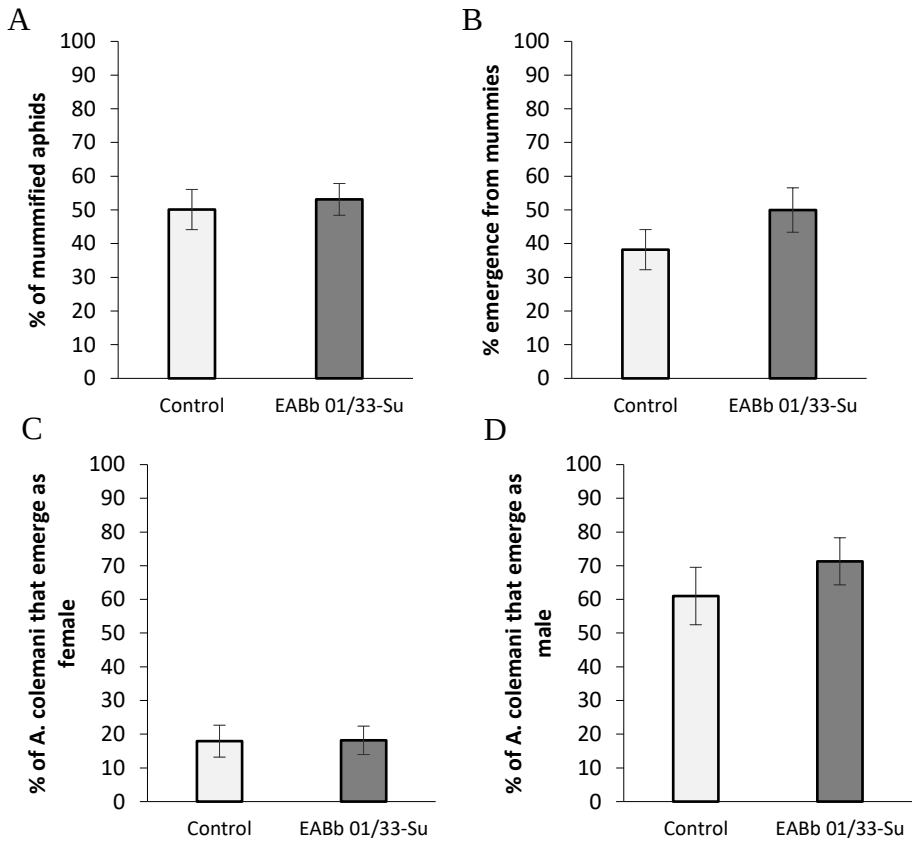

Figure 8. Parameters of parasitoid fitness when offered a choice of parasitizing aphids feeding on *B. bassiana*-colonized leaves or aphids feeding on control leaves. a Percentage of mummified aphids, b percentage of F1 *A. colemani* emerging as adults, c percentage of F1 *A. colemani* that were female and d percentage of F1 *A. colemani* that were male. Asterisks indicate significant differences between control and treatment (χ^2_{1df}, $p < 0.05$)

4. Discussion

Several studies have evaluated the lethal and sub-lethal effects of entomopathogenic fungi on predators and parasitoids when applied directly as fungal suspensions. In general, these effects are strongly related to fungal dosage and the sequence of treatments. Lower fungal dosages and application of the arthropod natural enemy first reduced negative effects of entomopathogenic fungi on ladybird beetles (James et al. 1995; Pingel and

Lewis 1996; Todorova et al. 1996; Roy and Pell 2000; Smith and Krischik 2000; Pell and Vandenberg 2002; Roy et al. 2008), lacewings (*C. carnea*) (Portilla et al. 2017), and several aphid parasitoid species (Brodeur and Rosenheim 2000; Mesquita and Lacey 2001; Kim et al. 2005; Aqueel and Leather 2013; Oreste et al. 2016; Shrestha et al. 2017). Likewise, infection of prey and the time interval between fungal application and predator/parasitoid release may also affect the number of aphids consumed by the predator or the number of parasitoid mummies that may develop (Mesquita and Lacey 2001; Aqueel and Leather 2013; Ibarra-Cortés et al. 2018).

In our study, lacewing prey consumption rate and the time spent to consume each aphid were not significantly affected by whether the aphid prey had been directly exposed to *B. bassiana* or not. There was a trend for prey consumption rate to decrease and consumption time to increase on endophytically colonized plants compared with controls. Fungal infection could reduce the nutritional quality of aphids leading to 'poor quality prey' which could account for a slight increase in prey consumption rate (Pell et al. 1997).

In general, previous studies have shown that treatments with entomopathogenic fungi should be considered as a low risk to predators and parasitoids. However, there are contradictory reports concerning the effect of entomopathogenic fungal treatments on the efficacy of predators and parasitoids. Avoidance of fungal-infected hosts and fungus-contaminated leaf surfaces by predators has been demonstrated (Pell and Vandenberg 2002; Meyling and Pell 2006), but also reports of compatibility between ladybirds (*Coccinella septempunctata* and *Harmonia axyridis*) consuming aphids at a late stage of *Pandora neoaphidis* and *Neozygites fresenii* infection (Pell et al. 1997; Roy et al. 1998, 2008; Simelane et al. 2008); between *C. carnea* feeding on *Metarhizium brunneum*-treated

Spodoptera littoralis larvae (Ríos-Moreno et al. 2018); and between the parasitoid *Aphidius ervi* ovipositing in aphids infected with *Pandora neoaphidis* (Baverstock et al. 2005). In the last example, overall aphid control was enhanced, but the number of parasitoid mummies recovered was reduced in the presence of fungal infection in the aphid hosts (Baverstock et al. 2005). In our study, lacewings did not always entirely consume living aphids infected with *B. bassiana* after direct spraying, which suggests avoidance of the infected parts of the aphid by the lacewing, which would increase lacewing safety. This has been reported previously in *C. carnea* feeding on living *M. brunneum* infected *S. littoralis* larvae (Ríos-Moreno et al. 2018). Recently, it has been demonstrated that spraying of entomopathogenic fungal conidia onto plants can lead to transient endophytic colonization of the plant that enhanced treatment efficacy (Garrido-Jurado et al. 2016; Resquín-Romero et al. 2016). In these scenarios, the fungal propagules were not in direct contact with entomophagous insects and they did not consider that natural enemies may feed on endophytically exposed prey or hosts (Garrido-Jurado et al. 2016). Furthermore, endophytic colonization by entomopathogenic fungi could influence plant signaling pathways in an analogous way to arbuscular mycorrhizal symbiosis, plant growth-promoting microorganisms like the rhizobacteria, and fungal endophytes of grasses, which may also alter insect population dynamics at higher trophic levels (Bultman et al. 1997, 2012; Barker and Addison 1996; Gange et al. 2003, 2012; Guerrieri et al. 2004; Hempel et al. 2009; Gadhave et al. 2016).

The present study indicated no significant effect of endophytically colonized plants on *C. carnea* predatory behaviour, although there was a trend for a decrease in aphid consumption rate and an increase in consumption time for aphids from *B. bassiana*-colonized plants compared with controls. However, the choice assay revealed a significantpreference of

the predators for aphids feeding on *B. bassiana* endophytically colonized plants. Feeding site location, plant foraging activity, mate location, oviposition site selection and detection of natural enemies are mediated by chemical cues (Dicke and Grostal 2001; Bruce et al. 2005; Sigsgaard 2005; Xu and Turlings 2018). Endophytic colonization by *B. bassiana* may not only alter the characteristics of plantemitted volatiles, but it may also improve the nutritional quality of the host plant for herbivores by reducing the aphid immune response (i.e. altering aphid symbionts), which may lead to higher predator attraction to aphids feeding on endophytically colonized plants (de Sassi et al. 2006; Polin et al. 2015; Shrivastava et al. 2015; Sánchez-Rodríguez et al. 2015; Liao et al. 2017; Krell et al. 2018). However, further research is needed to unravel the potential role of endophytic entomopathogenic fungi in governing trophic interactions between plants, pests and their natural enemies.

Our results agree with previous studies that have investigated the effect of endophytic entomopathogenic fungi on parasitoids; there was no significant effect on developmental time and percentage female (Akutse et al. 2014; Jaber and Araj 2018; Gathage et al. 2016). Likewise, Akutse et al. (2014) found no significant differences in terms of parasitism rates of the parasitoids *Phaedrotoma scabriventris* and *Diglyphus isaea,* when they parasitized 2nd and 3rd instar larvae of the pea leafminer *Liriomyza huidobrensis*, which had been reared on control and *B. bassiana*-colonized broad bean plants.

Equally, Gathage et al. (2016) carried out field trials to study the effect of seed inoculation with *B. bassiana* on six associated parasitoid species (*Opius dissitus*, *Phaedrotoma scabriventris*, *Diglyphus isaea* Walker, *Neochrysocharis formosa*, *Hemiptarsenus varicornis* and *Halticoptera arduine*) of the pea leafminer under field conditions. They found no

significant differences in parasitoid emergence from pupae formed in *B. bassiana*-colonized and control plants.

Apart from that, Jaber and Araj (2018) investigated the effect of the endophytic colonization of sweet pepper plants by the fungal entomopathogens *B. bassiana* and *M brunneum* on the parasitism of the green peach aphid *Myzus persicae* by *Aphidius colemani* under greenhouse conditions. They observed no differences in development time, percentage female, and adult longevity of the aphid parasitoid *Aphidius colemani* progenies among inoculated and control plants.

Therefore, all of these findings support the strategy that entomopathogenic fungal sprays can be used in combination with other natural enemies such as predators and parasitoids in IPM programmes. In particular, our results support the combined use of endophytic entomopathogenic fungi and entomophagous insects in aphid IPM programmes. The bottom-up effects of endophytically colonized plants are a low risk for predators and parasitoids. However, further researchis needed to unravel the possible mechanisms involved in changes in prey or host selection.

5. Acknowledgements

Funding was provided by the Spanish Ministry of Economy and Competitivity via grant AGL2016-80483-R 'Interacciones multitróficas reguladas por hongos entomopatógenos para una protección de cultivos sostenible'. We also thank Pablo Valverde-García for his assistance with the statistical analysis.

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CAPÍTULO V. DISCUSIÓN GENERAL

El comportamiento endofítico de los ascomicetos mitospóricos entomopatógenos (AMEs) extiende las posibilidades y consecuencias de su empleo en protección y producción vegetal. En los últimos 15 años, las investigaciones del Grupo de Investigación PAIDI AGR 163 "Entomología Agrícola" han aportado novedades sobre la repercusión de los AMEs endófitos para la protección sistémica de cultivos frente a insectos barrenadores, sobre el efecto de la colonización endofítica temporal en insectos masticadores y fitomizos, así como su uso para proteger la planta frente a hongos fitopatógenos, e incluso, promover el crecimiento vegetal y la respuesta a estreses nutricionales (Quesada-Moraga et al. 2019).

La presente tesis doctoral explora el impacto de la colonización de la planta por cepas endófitas de AMEs sobre la biología y capacidad como vectores de pulgones, insectos fitomizos de gran importancia agrícola, pero, además, responde por primera vez en las posibles implicaciones de esta asociación de los AMEs con las plantas, sobre las relaciones tritróficas de los pulgones con estas y con sus enemigos naturales entomófagos.

El capítulo II refleja que la cepa EABb 01/33-Su de *B. bassiana* (Ascomycota: Hypocreales) reduce significativamente la población de *A. gossypii* (Homoptera: Aphididae) tanto cuando los especímenes se alimentan a expensas de hojas pulverizadas y colonizadas, como cuando lo hacen a expensas de hojas colonizadas distantes de las pulverizadas, mientras que las cepas EABb 04/01-Tip de *B. bassiana* y EAMa 01/58-Su de *M. brunneum* (Ascomycota: Hypocreales), solo redujeron la población en la primera circunstancia (aunque a un nivel de significación del 10% también en la segunda). La eficacia como endófita de la cepa EABb 01/33-Su para manifestar su máxima virulencia en tejidos distantes a los tratados ya fue revelada en experimentos llevados a cabo con *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae) en plantas de melón (Garrido-Jurado et al. 2017),

aunque la movilidad de los pulgones en contraposición con el carácter inmóvil de las ninfas de mosca blanca, realza la capacidad endofítica de esta cepa y su impacto sobre la población de insectos fitomizos, y justifica su protagonismo en los capítulos III y IV tesis doctoral.

El capitulo II pone de manifiesto la ausencia de efecto de la colonización endófitica de plantas de melón con las tres cepas seleccionadas sobre fecundidad total *per capita* de los pulgones 5 días después de la exposición (7 días después de que las hojas hubieran sido pulverizadas con la suspensión fúngica), lo que contrasta con trabajos previos en otros cultivos y con otras cepas fúngicas, como con *Aphis glycines* Matsumura (Homoptera: Aphididae) en soja (Clifton et al. 2018). Sin embargo, se detecta una modificación en el comportamiento reproductivo de los áfidos que se alimentan a expensas de plantas colonizadas, que, con respecto a los especímenes sanos, aceleran su actividad reproductiva para alcanzar con anterioridad su máximo potencial biótico. Este resultado inédito podría apuntar a un acortamiento de todo el proceso fisiológico conducente a la reproducción vivípara como consecuencia del estrés asociado a la patogénesis fúngica, que se convierte en un reto científico de primer nivel (Klowden 2009).

En este capitulo también se describen en primicia diferencias cualitativas y cuantitativas en el perfil de compuestos volátiles liberados por las plantas colonizadas endofíticamente e infestadas posteriormente por *A. gossypii* en comparación con los compuestos que emiten las plantas testigo. Aunque muchos de los compuestos descritos coinciden con los detectados en plantas de melón en condiciones normales de cultivo (Kende et al. 2019), otros han sido previamente identificados en el perfil de volátiles que emiten un amplio rango de especies de plantas y se relacionan con la fitofagia (McCall et al. 1994; Aartsma et al. 2018; Turlings y Erb 2018). Sin embargo, es muy importante señalar que algunos de los

compuestos identificados han sido detectados únicamente en las plantas que se encontraban colonizadas endofíticamente por las cepas de *B. bassiana* y *M. brunneum* utilizadas o, por el contrario, se han detectado únicamente en las plantas control, habiendo sido suprimida su producción en las plantas colonizadas. A pesar de que la detección de compuestos específicos de las plantas ayuda a los pulgones a discriminar entre plantas hospedantes (Visser 1986), nuestro trabajo refleja que la colonización endofítica de plantas por AMEs no influye en su elección por parte de los pulgones, si bien, la modificación de los volátiles en plantas de melón colonizadas por estas cepas fúngicas podría alcanzar niveles tróficos superiores, como revela el capítulo IV.

Otro valor añadido del empleo de los AMEs endófitos es la protección de la planta frente a microorganimos fitopatógenos y virus de plantas (Ownley et al. 2010). El capítulo III aporta resultados muy originales sobre la influencia de la colonización endofítica sobre el comportamiento alimenticio de los pulgones y a la transmisión de virus persistentes y no persistentes. Así, mediante el empleo de la técnica de gráficos de penetración eléctrica (EPGs), que se aplica por primera vez a pulgones que se alimentan a expensas de plantas colonizadas con HEs, ha sido posible detectar la actividad del estilete del insecto y relacionarla con los procesos de transmisión de virus en plantas de melón colonizadas con la cepa EABb 01/33-Su de *B. bassiana*. La técnica empleada revela que la colonización endofítica de plantas de melón con *B. bassiana* no afecta al comportamiento alimenticio de *A. gossypii* asociado a la inoculación del Cucumovirus no persistente, *Cucumber mosaic virus* (CMV), y al Polerovirus persistente *Cucurbit aphid-borne yellows virus*, pero modifica algunas relacionadas con el proceso de adquisición de virus no persistentes, posiblemente asociadas con alteraciones en el comportamiento alimenticio del pulgón en un mesófilo con estructuras fúngicas o los metabolitos secretados por ellas

(Landa et al. 2013; Resquín-Romero et al. 2016), o incluso por la activación de las defensas de la planta (Cao et al. 2014). Hay que destacar que los ensayos de transmisión llevados a cabo revelan como novedad que la incidencia de los dos virus estudiados se ve significativamente reducida cuando las plantas se encuentran colonizadas endofíticamente con la cepa EABb 01/33-Su de *B. bassiana* antes de producirse su infestación con pulgones virulíferos, a lo que debe unirse la reducción en la incidencia de CMV en las plantas tratadas con *B. bassiana,* tanto cuando la inoculación tuvo lugar utilizando pulgones virulíferos como cuando se realizó mecánicamente. A este respecto, se ha descrito recientemente que una cepa endófita de *B. bassiana* confería protección contra la inoculación mecánica de un virus no persistente como el *Zucchini yellow mosaic virus* (ZYMV) en plantas de calabacín (Jaber y Salem 2014).

La existencia de una reducción de la transmisión de virus por pulgones en plantas colonizadas por la cepa EABb 01/33-Su, que no esta asociada a una modificación de su comportamiento de alimentación, apuntaría a la posible inducción de resistencia sistémica inducida por la colonización de la planta por el hongo, tal y como se ha observado en otros microorganismos endófitos (Lee y Ryu 2016; Vitti et al. 2016), hipótesis que se convierte en reto científico de primer nivel dada su repercusión práctica.

En su capítulo IV, la tesis doctoral explora por primera vez las posibles implicaciones de la capacidad endofítica de los AMEs sobre las relaciones tritróficas de los pulgones, no solo con las plantas colonizadas, sino con los enemigos naturales entomófagos, el depredador generalista *C. carnea* (Neuroptera: Chrysopidae) y el parasitoide de pulgones *Aphidius colemani* Viereck (Hymenoptera: Aphidiidae), al atacar a pulgones que se alimentan a expensas de ellas.

La cantidad de hempras ápteras de *A. gossypii* consumidas por *C. carnea*, así como el tiempo de consumo, no se vieron afectadas

significativamente tras su exposición directa a una suspensión de conidios de la cepa EABb 01/33-Su, en comparación con lo observado con los pulgones testigo. Sin embargo, las crisopas no consumen por completo los pulgones que presentan indicios de infección fúngica, como posible mecanismo de escape, tal y como se ha descrito cuando *C. carnea* consume larvas de *S. littoralis* Boisduval (Lepidoptera: Noctuidae) infectadas por *M. brunneum* (Ríos-Moreno et al. 2018), en lo que sería un mecanismo de seguridad por parte del neuróptero. Más allá, se ha demostrado que no existe un efecto sobre la eficacia depredadora de *C. carnea* cuando se alimenta de pulgones que se habían alimentado previamente de plantas de melón colonizadas endofíticamente con *B. bassiana*, aunque se detecta una reducción en el consumo de presas y un incremento en el tiempo de consumo en comparación con el control.

Sin embargo, los resultados obtenidos en los ensayos de elección muestran una preferencia significativa de las crisopas por los pulgones de *A. gossypii* que se alimentan de plantas de melón colonizadas con la cepa EABb 01/33-Su, con respecto a las plantas testigo, lo que podría estar relacionado con los resultados del capitulo II, ya que algunos de los compuestos detectados en las plantas que se encontraban colonizadas endofíticamente podrían afectar al comportamiento de los insectos actuando como atrayentes (p.e. beta-ionona) (Obata et al. 1983; Donaldson et al. 1990; Flath et al. 1994). La colonización endofítica por *B. bassiana* podría alterar el perfil de volátiles que emiten las plantas, pero además las vías de señalización de estas, de igual manera que lo hacen las micorrizas arbusculares, rizobacterias, y los hongos endófitos de pastos, los cuales alteran las dinámicas de población de los insectos en niveles tróficos más altos (Barker y Addison 1996; Bultman et al. 1997, 2012; Gange et al. 2003, 2012; Guerrieri et al. 2004; Hempel et al. 2009; Gadhave et al. 2016), hipótesis que hace necesarios experimentos adicionales.

En cuanto al efecto sobre parasitoides, no se han observado diferencias significativas en cuanto al número de momias de *A. colemani* formadas, porcentaje de emergencia y ratio sexual de los adultos emergidos cuando las hembras ápteras de su insecto hospedante *A. gossypii* se encontraban alimentándose en hojas de melón colonizadas endofíticamente con la cepa EABb 01/33-Su en comparación con el testigo, de forma análoga a lo detectado en los únicos trabajos realizados hasta la fecha con sistemas planta colonizada por AMEs-insecto fitófago- parasitoide (Akutse et al. 2014; Gathage et al. 2016; Jaber y Araj 2018), lo que finalmente refuerza el empleo de HEs en combinación con otros enemigos naturales como depredadores y parasitoides en programas de control integrado de pulgones.

Esta tesis sienta las bases del empleo de HEs endófitos para el control de pulgones, no solo por el efecto directo de los propágulos fúngicos sobre los áfidos, sino también por los efectos directos e indirectos sobre los mismos que acarrea la colonización endofítica de las plantas por las cepas fúngicas. Además, la tesis ilustra las ventajas de la estrategia, no solo por extender su eficacia a la capacidad de los pulgones para transmitir virus, sino por su compatibilidad en control integrado con entomófagos, parasitoides y depredadores.

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CAPÍTULO VI. CONCLUSIONES

A lo largo de los distintos capítulos de la presente Tesis Doctoral se han obtenido una serie de conclusiones que se enumeran de forma resumida a continuación. Las conclusiones número 1, 2, 3 y 4 y 10 corresponden al capítulo II, "Effects of endophytic entomopathogenic ascomycetes on *Aphis gossypii* Glover Life-History Traits and interaction with melon plants", publicado en la revista Insects (2019), https://doi.org/10.3390/insects10060165. Factor de Impacto: 1.848, Q1 (21/96) en "Entomology". Las conclusiones 5, 6 y 10 corresponden al capítulo III, que incluye el artículo "Changes in feeding behaviour are not related to the reduction in the transmission rate of plant viruses by *Aphis gossypii* (Homoptera: Aphididae) to melon plants colonized by *Beauveria bassiana* (Ascomycota: Hypocreales)" publicado en la revista Biological Control 130 (2019) 95-103, https://doi.org/10.1016/j.biocontrol.2018.11.001. Factor de Impacto: 2.112, Q1 (13/96) en "Entomology". Finalmente, las conclusiones 7, 8, 9 y 10 corresponden al capítulo IV, "Bottom-up effects of endophytic *Beauveria bassiana* on multitrophic interactions between the cotton aphid, *Aphis gossypii*, and its natural enemies in melon" publicado en la revista Journal of Pest Science (2019), https://doi.org/10.1007/s10340-019-01098-5. Factor de Impacto: 4.402, Q1 (2/96) en "Entomology".

1. La pulverización de plantas de melón con suspensiones de conidios de las cepas de *B. bassiana* y *M. brunneum* utilizadas tiene un efecto directo sobre hembras ápteras de *A. gossypii* como consecuencia de su contacto con el inoculo presente en el filoplano, pero además, un efecto adicional asociado a la capacidad de colonización endófita de la cepa utilizada, que alcanza a la población de pulgones lejos de la zona pulverizada.
2. La colonización endofitica de plantas de melón infestadas con hembras ápteras de *A. gossypii* por las cepas de *B. bassiana* y *M. brunneum*

utilizadas modifica el comportamiento reproductivo de las mismas, que tienden a alcanzar su potencial biótico máximo con anterioridad a las del testigo.

3. La colonización endofitica de hojas de plantas de melón por las cepas de *B. bassiana* y *M. brunneum* utilizadas modifica su perfil de volátiles, resultado inédito que abre nuevos interrogantes sobre el impacto de asociación de los hongos entomopatógenos con las plantas sobre las relaciones planta-insecto-enemigo natural entomófago.
4. La colonización endofitica de hojas de plantas de melón por las cepas de *B. bassiana* y *M. brunneum* utilizadas no influye sobre la elección por hembras ápteras de *A. gossypii*.
5. El empleo de la técnica de gráficos de penetración eléctrica (EPGs), que se aplica por primera vez a pulgones que se alimentan a expensas de plantas colonizadas con hongos entomopatógenos, ha permitido detectar que las plantas de melón colonizadas con la cepa EABb 01/33-Su de *B. bassiana* no afecta a las actividades de comportamiento alimenticio relacionadas con la transmisión del Polerovirus persistente *Cucurbit aphid-borne yellows virus*, así como la inoculación del Cucumovirus no persistente, Cucumber mosaic virus, pero modifica algunas relacionadas con el proceso de adquisición de estos virus, asociadas probablemente a la presencia de propágulos fúngicos en el mesófilo de la hoja y a la activación de las defensas de la planta.
6. Los ensayos de transmisión de virus llevados a cabo revelan que la incidencia de estos dos virus se ve significativamente reducida cuando las plantas se encuentran colonizadas endofíticamente con la cepa EABb 01/33-Su de *B. bassiana* antes de producirse su infestación con pulgones virulíferos, resultado inédito hasta la fecha.
7. El depredador *C. carnea* no distingue entre hembras ápteras de *A. gossypii* del testigo y aquellas tratadas por contacto directo con una

suspensión de conidios de la cepa EABb 01/33-Su de *B. bassiana* en términos de número de pulgones consumidos y tiempo de consumo, aunque se ha observado una tendencia general a la reducción en el numero de pulgones consumidos y un aumento en el tiempo de consumo por parte de las crisopas que se alimentan a expensas de pulgones que se habían alimentado previamente de plantas de melón colonizadas endofíticamente con la cepa fúngica.

8. En ensayos de elección, se ha observado una preferencia de las crisopas por hembras ápteras de *A. gossypii* que se alimentan de plantas de melón colonizadas con la cepa EABb 01/33-Su de *B. bassiana* con respecto a las plantas testigo, lo que podría estar relacionado con la alteración del perfil de volátiles emitido por las plantas colonizadas por esta cepa fúngica ya descrito en la tercera conclusión.
9. No se han observado diferencias significativas en cuanto al número de momias del parasitoide *A. colemani* formadas, porcentaje de emergencia y ratio sexual de los adultos emergidos cuando las hembras ápteras de su insecto hospedante *A. gossypii* se encontraban alimentándose en hojas de melón colonizadas endofíticamente con la cepa EABb 01/33-Su de *B. bassiana* en comparación con cuando se encontraban en una hoja de una planta testigo.
10. En su globalidad, la presente tesis doctoral pone de manifiesto el potencial de cepas endófitas de hongos entomopatógenos para el control biológico de pulgones no solo en el corto plazo, por la actividad directa de las suspensiones sobre los especímenes tratados, sino en el medio y largo plazo, por los efectos indirectos asociados a la colonización endofítica de la planta, con énfasis en la reducción de la transmisión de virus, además de ser compatibles con depredadores y parasitoides para el desarrollo de estrategias de control integrado de plagas

ANEXO II PRODUCCIÓN CIENTÍFICA DERIVADA DE LA TESIS DOCTORAL

Contribución a revistas internacionales de carácter científico (SCI):

• **González-Mas, N**., Quesada-Moraga, E., Plaza, M., Fereres, A., Moreno, A., 2019. Changes in feeding behaviour are not related to the reduction in the transmission rate of plant viruses by *Aphis gossypii* (Homoptera: Aphididae) to melon plants colonized by *Beauveria bassiana* (Ascomycota: Hypocreales). Biological Control 130: 95-103, https://doi.org/10.1016/j.biocontrol.2018.11.001. Factor de Impacto: 2.112, Q1 (13/96) en "Entomology".

• **González-Mas, N**., Cuenca-Medina, M., Gutiérrez-Sánchez, F. Quesada-Moraga, E., 2019.Bottom-up effects of endophytic *Beauveria bassiana* on multitrophic interactions between the cotton aphid, *Aphis gossypii*, and its natural enemies in melon. Journal of Pest Science, https://doi.org/10.1007/s10340-019-01098-5. Factor de Impacto: 4.402, Q1 (2/96) en "Entomology".

• **González-Mas, N**., Sánchez-Ortiz, A., Valverde-Garcia, P., Quesada-Moraga, E. "Effects of endophytic entomopathogenic ascomycetes on *Aphis gossypii* Glover Life-History Traits and interaction with melon plants". Insects, https://doi.org/10.3390/insects10060165. Factor de Impacto: 1.848, Q1 (21/96) en "Entomology".

Por otro lado, aunque no se trata de un trabajo derivado directamente de la tesis doctoral, ha sido publicado recientemente el artículo:

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Alumna: Dña. María Cuenca Medina. Universidad de Córdoba. E.T.S.I.A.M. Septiembre de 2018. Calificación: 10.

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Alumno: D. Antonio Ayllón González. Universidad de Córdoba.

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Calificación: Pendiente de evaluación

ANEXO II ESTANCIA EN EL GRUPO DE INVESTIGACIÓN "FUNDAMENTAL AND APPLIED RESEARCH IN CHEMICAL ECOLOGY" DE LA UNIVERSIDAD DE NEUCHÂTEL, EN NEUCHÂTEL, SUIZA

Objetivos

Como actividad complementaria a la Tesis Doctoral se realizó una estancia de 3 meses en Neuchâtel, Suiza, en el grupo de investigación "Fundamental and applied research in chemical ecology" de la Universidad de Neuchâtel bajo la supervisión del Profesor Ted Turlings, director del Centro de Competencia de Ecología Química (Centre of Competence In Chemical Ecology) de la Universidad de Neuchâtel

Durante los tres meses que duró la estancia en la Universidad de Neuchâtel, se realizó una investigación sobre las señales químicas que emiten las plantas, en especial volátiles, y cómo pueden interactuar tanto con el fitófago como con depredadores y parasitoides.

El objetivo principal de esta tesis es el estudio del efecto que puede tener la colonización endofítica de los hongos entomopatógenos sobre los insectos fitófagos y otros insectos no objetivo como depredadores y parasitoides.

La síntesis y emisión de compuestos orgánicos volátiles (*Volatile Organic Compounds*, VOCs) desde las hojas, flores y frutos de las plantas hacia la atmósfera, y de sus raíces en el suelo, supone un importante mecanismo de interacción de las plantas con el ecosistema. Los VOCs se encuentran altamente relacionados con la defensa de las plantas contra herbívoros y patógenos. Se ha demostrado que pueden tener un efecto sobre los microorganismos mediante la inhibición o promoción de su crecimiento, pero también ser capaces de provocar un efecto disuasorio sobre los insectos fitófagos que se alimentan de ellas (De Moraes et al. 2001; Junker y Tholl 2013), un efecto atrayente sobre polinizadores y dispersores

de semillas, o servir como señales químicas en las interacciones planta-planta y con otros microorganismos beneficiosos, como los hongos endófitos (Delory et al. 2016).

El control de patógenos de plantas mediante hongos endófitos se encuentra actualmente muy estudiado debido a que se ha demostrado que son capaces de producir un sinfín de metabolitos secundarios, como los VOCs, que han resultado ser importantes compuestos antagonistas de patógenos de plantas incluyendo a insectos, hongos y nematodos. De hecho, se conoce que varias especies de endófitos producen metabolitos con efecto deterrente que afectan a la alimentación de los insectos (Daisy et al. 2002), lo que sugiere que la producción de estos compuestos *in planta* puede afectar a las actividades de los insectos y provocar una respuesta de atracción o de repulsión sobre ellos (Vega et al. 2009). La respuesta olfativa de los insectos a los compuestos volátiles emitidos por los microorganismos de su entorno son mucho más comunes de lo que parece, de hecho, las emisiones de estos microorganismos pueden tener un efecto sobre la gregarización, acoplamiento y oviposición de los insectos (Davis et al. 2013).

Además, se han utilizado VOCs procedentes de hongos como un método de control biológico para evitar el desarrollo de patógenos de plantas. Así, los VOCs emitidos por hongos endófitos pueden beneficiar a la planta hospedante facilitándole mecanismos de defensa adicionales contra patógenos que afectan a la planta hospedante (Morath et al. 2012). De hecho, varios trabajos relatan en la actualidad la efectividad de compuestos volátiles emitidos por hongos como agentes de control biológico de patógenos de plantas y de productos almacenados, además de considerarse importantes contribuyentes en el desarrollo de una agricultura sostenible (Lacey et al. 2009; Menjivar et al. 2012; Suwannaracha et al. 2013).

Los compuestos volátiles fúngicos se alzan como una estrategia de control respetuosa con el medio ambiente, inocua para las plantas y rápidamente biodegradable en el ecosistema.

Sin embargo, a pesar de este potencial, es necesaria una mayor investigación en cuanto a la producción de volátiles fúngicos en nuevas cepas, descripción de nuevos compuestos volátiles, optimización de las técnicas analíticas para caracterizar los compuestos volátiles de los hongos y la evaluación de la respuesta de la planta a la colonización por HE.

Por ello, nos preguntamos si los hongos entomopatógenos endófitos pueden inducir una respuesta en la planta hospedante que modifique el perfil de volátiles emitido por las plantas colonizadas, o si son también capaces de secretar compuestos insecticidas o deterrentes que puedan afectar a la preferencia de la planta y aceptación por parte del insecto fitófago.

Plan de trabajo realizado y resultados obtenidos

-Primera etapa: aprendizaje de técnicas utilizadas por el grupo de investigación "Fundamental and applied research in chemical ecology" de la Universidad de Neuchâtel (Neuchâtel, Suiza), para la detección y análisis de compuestos volátiles y su importancia en las relaciones tritróficas entre la planta y los agentes de control microbiano y macrobiano, así como diseño experimental de ensayos de comportamiento con estos elementos de la cadena trófica.

-Segunda etapa: evaluar el efecto de la colonización endofítica de la planta en la emisión de volátiles y su efecto en el comportamiento de fitófagos, depredadores y parasitoides.

Durante el desarrollo de la presente tesis, se han realizado diferentes experimentos en los que se ha evaluado la mortalidad, desarrollo biológico y comportamiento de pulgones que se desarrollan en plantas de melón colonizadas endofíticamente por hongos entomopatógenos y la emisión de volátiles por parte de estas plantas tras su infestación por estos insectos fitomizos (González-Mas et al. 2019a), así como en el efecto de esta colonización sobre la función de estos insectos como vectores de virus (González-Mas et al. 2019b). Además, se ha estudiado la respuesta depredadores y parasitoides en la elección de presa cuando ésta se encuentra alimentándose en una planta colonizada por hongos entomopatógenos (González-Mas et al. 2019c). Mediante esta estancia se ha continuado con el aprendizaje de técnicas de estudio del comportamiento de insectos iniciada durante el año 2014 mediante la colaboración con el ICA-CSIC (Madrid), profundizando así en este conocimiento.

Como complemento a estos trabajos, se ha estudiado durante la estancia si dicha colonización puede inducir la liberación de compuestos volátiles por parte de las plantas y provocar una respuesta en los insectos que interaccionan con ella, afectando a su comportamiento.

Para ello se ha estudiado la liberación de compuestos volátiles por parte de plantas colonizadas por hongos entomopatógenos, identificando y cuantificando los volátiles emitidos por las mismas en diferentes etapas de la colonización y tras la infestación de las plantas por herbívoros.

Se han tomado como modelo plantas de algodón silvestre (*Gossypium davidsonii* Kellogg), la cepa EABb 01/33-Su perteneciente a la especie *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) la cual ha demostrado previamente su comportamiento endófito en diferentes especies de plantas (Resquín-Romero et al. 2016), y la larva del

lepidóptero *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae), con aparato bucal masticador.

En una primera etapa se ha preparado el material biológico para los ensayos a realizar: siembra de semillas, cultivo de hongos y cría de insectos.

Hasta que las plantas adquirieron el estado fenológico adecuado, se trabajó paralelamente para adquirir conocimientos de las técnicas utilizadas por el grupo de investigación "Fundamental and applied research in chemical ecology" del centro "Faculté des sciences. Institute of biology. Université de Neuchâtel" de Suiza, para la detección y análisis de compuestos volátiles así como el funcionamiento y el aprendizaje de técnicas de estudio del comportamiento de insectos como el uso de olfatómetros.

Una vez las plantas de algodón alcanzaron el estado fenológico de 4 hojas verdaderas, se preparó una suspensión de $1x\ 10^8$ conidios/ml en agua con Tween 80.

Mediante la utilización de un aerógrafo se pulverizaron dos hojas con 1ml de la suspensión cada una, se dejaron secar y se cubrieron con papel de aluminio. Éste también se colocó en la base de la planta, para evitar la extracción de volátiles del sustrato. En el caso de las plantas control, fueron pulverizadas únicamente con agua con Tween 80.

Además, tanto plantas control como plantas pulverizadas con la suspensión fúngica fueron infestadas 72 h después de la pulverización con 10 larvas L2 de *S. frugiperda* / planta, de las que se les permitió alimentarse durante 24 h, produciendo un daño mecánico en la planta debido a la alimentación de la misma mediante su aparato bucal masticador.

Las plantas se colocaron en el equipo de recolección de volátiles y se efectuó la recolección durante 2h en diferentes etapas: 1) inmediatamente tras la pulverización; 2) 48 horas después de pulverizar; 3)24 h después de la infestación con larvas de *S. frugiperda* (96 h después de pulverizar).

Los volátiles se recogieron utilizando filtros de captura que contenían 25 mg de absorbente HayesepQ de 80-100 mallas. Antes del uso, los filtros de captura se limpiaron con 1000 µl de diclorometano. Después de la recolección, los volátiles se extrajeron de los filtros con 150 µl de diclorometano. A continuación, las muestras se almacenaron a -80 ° C hasta su análisis. Con el fin de cuantificar los volátiles, se añadieron 10 µL de estándar interno (IS) mezcla (n-octano y nonyl acetato, cada 200 ng en 10 µL de cloruro de metileno) a 20 µL de cada muestra. Los volátiles se analizaron con un cromatógrafo de gases Agilent 6890 acoplado a un detector selectivo de masa de la red 5973 (línea de transferencia 230°C, fuente 230°C, potencial de ionización 70 eV). Se inyectó una alícuota de 2 µL de cada muestra en modo sin división pulsada sobre una columna no polar (HP-1 ms, 30 m, 0,25 mm ID, 0,25 µm de espesor de película, Agilent J & W Scientific, EE.UU.). Se utilizó helio a flujo constante (1,9 ml / min) como gas portador. Las identidades de los compuestos se confirmaron por análisis de espectrometría de masas. Los compuestos se identificaron comparando los espectros obtenidos de las muestras con los de una base de datos de referencia (biblioteca espectral de masa NIST).

Una vez finalizada la última recolección, se analizó la respuesta del parasitoide *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae). mediante la utilización de un olfatómetro de cuatro brazos, sin embargo los resultados no fueron concluyentes.

Se realizaron seis repeticiones por tratamiento simultáneamente y fue repetido tres veces en el tiempo.

Tabla 1. Compuestos detectados en plantas colonizadas por *Beauveria bassiana* y plantas control inmediantamente después de haber aplicado el tratamiento

Compounds	Unsprayed plants (mean±SE)	Sprayed plants (mean±SE)
Aldehydes		
3-methyl-2-butenal	64.71 ± 11.9	35.29 ± 11.95
decanal	29.41 ± 11.39	58.82 ± 12.30
E-2-hexenal	0.00 ± 0.00	23.53 ± 10.60
nonanal	58.82 ± 12.30	70.59 ± 11.39
Alcohols		
(Z)-3-hexen-1-ol	11.76 ± 8.05	23.53 ± 10.60
Hydrocarbons		
1,1,3,4-tetrachloro-1,2,2,3,4,4-hexafluoro-butane	58.82 ± 12.30	58.82 ± 12.30
1,2,4-trichloro-heptafluoro-butane	23.53 ± 10.60	11.76 ± 8.05
1,2,3,4-tetrachloro-1,1,2,3,4,4-hexafluoro-butane	70.59 ± 11.39	70.59 ± 11.39
1,1-dichloro-1,2,2,2-tetrafluoro-ethane	5.88 ± 5.88	23.53 ± 10.60
2,3,3-trimethyl-1-hexene	0.00 ± 0.00	23.53 ± 10.60
2,4-dimethyl-heptane	29.41 ± 11.39	47.06 ± 12.48
n-octane	100.00 ± 0.00	94.12 ± 5.88
octane	52.94 ± 12.48	64.71 ± 11.95
4-methyl-octane	11.76 ± 8.05	23.53 ± 10.60
2-chloro-2-methyl-pentane	35.29 ± 11.95	23.53 ± 10.60
3-chloro-3-methyl-pentane	35.29 ± 11.95	29.41 ± 11.39
2,4-diphenyl-4-methyl-2(E)-pentene	52.94 ± 12.48	35.29 ± 11.95
1-methyl-7-(1-methylethyl)-phenanthrene	23.53 ± 10.60	23.53 ± 10.60
Esters		
butanoic acid, butyl ester	11.76 ± 8.05	23.53 ± 10.60
Z-3-hexenyl acetate	11.76 ± 8.05	35.29 ± 11.95
nonyl acetate	100.00 ± 0.00	100.00 ± 0.00
sulfurous acid, cyclohexylmethyl nonyl ester	58.82 ± 12.30	52.94 ± 12.48
propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester	41.18 ± 12.30	23.53 ± 10.60
Terpenoic derivates		
α-caryophyllene	82.35 ± 9.53	94.12 ± 5.88
β-caryophyllene	100.00 ± 0.00	100.00 ± 0.00
limonene	5.88 ± 5.88	29.41 ± 11.39
β-myrcene	41.18 ± 12.30	64.71 ± 11.95
trans-ocimene	29.41 ± 11.39	23.53 ± 10.60
α-pinene	64.71 ± 11.95	64.71 ± 11.95
Phenolic derivates		
1,2,3,3,4-pentamethyl-cyclopentene	11.76 ± 8.05	23.53 ± 10.60
1,2,3,4,5-pentamethyl-cyclopentene	47.06 ± 12.48	47.06 ± 12.48
Pyrrolidine	23.53 ± 10.60	17.65 ± 9.53

Tabla 2. Compuestos detectados en plantas colonizadas por *Beauveria bassiana* y plantas control 48h después de haber aplicado el tratamiento

Compounds	Unsprayed plants (mean±SE)	Sprayed plants (mean±SE)
Aldehydes		
3-methyl-2-butenal	23.53 ± 10.60	13.33 ± 8.53
Hydrocarbons		
1,1,3,4-tetrachloro-1,2,2,3,4,4-hexafluoro-butane	47.06 ± 12.48	80.00 ± 10.04
1,2,3,4-tetrachloro-1,1,2,3,4,4-hexafluoro-butane	76.47 ± 10.60	100.00 ± 0.00
3,7-dimethyl-decane	17.65 ± 9.53	40.00 ± 12.30
1,1,2-trichloro-1,2,2-trifluoro-ethane	35.29 ± 11.95	26.67 ± 11.10
2,4-dimethyl-heptane	29.41 ± 11.39	60.00 ± 12.30
2,4-dimethyl-1-heptene	17.65 ± 9.53	26.67 ± 11.10
n-octane	100.0 ± 0.00	100.00 ± 0.00
2,4-diphenyl-4-methyl-2(E)-pentene	47.06 ± 12.48	40.00 ± 12.30
5-methyl-undecane	0.00 ± 0.00	26.67 ± 11.10
1-methyl-7-(1-methylethyl)-phenanthrene	35.29 ± 11.95	40.00 ± 12.30
Esters		
nonyl acetate	100.0 ± 0.00	100.00 ± 0.00
sulfurous acid, cyclohexylmethyl nonyl ester	17.65 ± 9.53	26.67 ± 11.10
Terpenoic derivates		
(E)-4,8-Dimethyl-1,3,7-nonatriene	11.76 ± 8.05	26.67 ± 11.10
tridecatetraene	35.29 ± 11.95	26.67 ± 11.10
Phenolic derivates		
1,2,3,4,5-pentamethyl-cyclopentene	11.76 ± 8.05	33.33 ± 11.83

Tabla 3. Compuestos detectados en plantas colonizadas por *Beauveria bassiana* y plantas control 72h después de haber aplicado el tratamiento (24h después de la exposición a larvas de *Spodoptera frugiperda*)

Compounds	Unsprayed plants (mean±SE)	Sprayed plants (mean±SE)
Hydrocarbons		
1,1,3,4-tetrachloro-1,2,2,3,4,4-hexafluoro-	25.00 ± 10.85	29.41 ± 11.39
1,2,3,4-tetrachloro-1,1,2,3,4,4-hexafluoro-	31.25 ± 11.61	76.47 ± 10.60
1-decyne	6.25 ± 6.06	29.41 ± 11.39
2,4-dimethyl-heptane	6.25 ± 6.06	23.53 ± 10.60
n-octane	100.00 ± 0.00	100.00 ± 0.00
2,4-diphenyl-4-methyl-2(E)-pentene	12.50 ± 8.28	23.53 ± 10.60
Esters		
Z-3-hexenyl acetate	12.50 ± 8.28	23.53 ± 10.60
nonyl acetate	100.00 ± 0.00	100.00 ± 0.00
Terpenoic derivates		
α-caryophyllene	31.25 ± 11.61	64.71 ± 11.95
β-caryophyllene	68.75 ± 11.61	94.12 ± 5.88
indole	37.50 ± 12.13	58.82 ± 12.30
β-myrcene	18.75 ± 9.78	41.18 ± 12.30
(E)-4,8-Dimethyl-1,3,7-nonatriene	62.50 ± 12.13	64.71 ± 11.95
trans-ocimene	62.50 ± 12.13	70.59 ± 11.39
α-pinene	62.50 ± 12.13	64.71 ± 11.95
tridecatetraene	25.00 ± 10.85	17.65 ± 9.53
Phenolic derivates		
1,2,3,4,5-pentamethyl-cyclopentane	25.00 ± 10.85	23.53 ± 10.60
1-methyl-7-(1-methylethyl)-phenanthrene	12.50 ± 8.28	29.41 ± 11.39

Los resultados están siendo analizados y se espera que el trabajo realizado durante la estancia, junto con los ensayos realizados en colaboración con el IFAPA de Venta del Llano (Jaén), bajo la supervisión de la doctora Araceli Sánchez Ortiz sobre técnicas de estudio de los volátiles que emiten las plantas enteras colonizadas por la misma cepa de *B. bassiana* y posteriormente infestadas con pulgones, insectos fitomizos, de lugar a una cuarta publicación.

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