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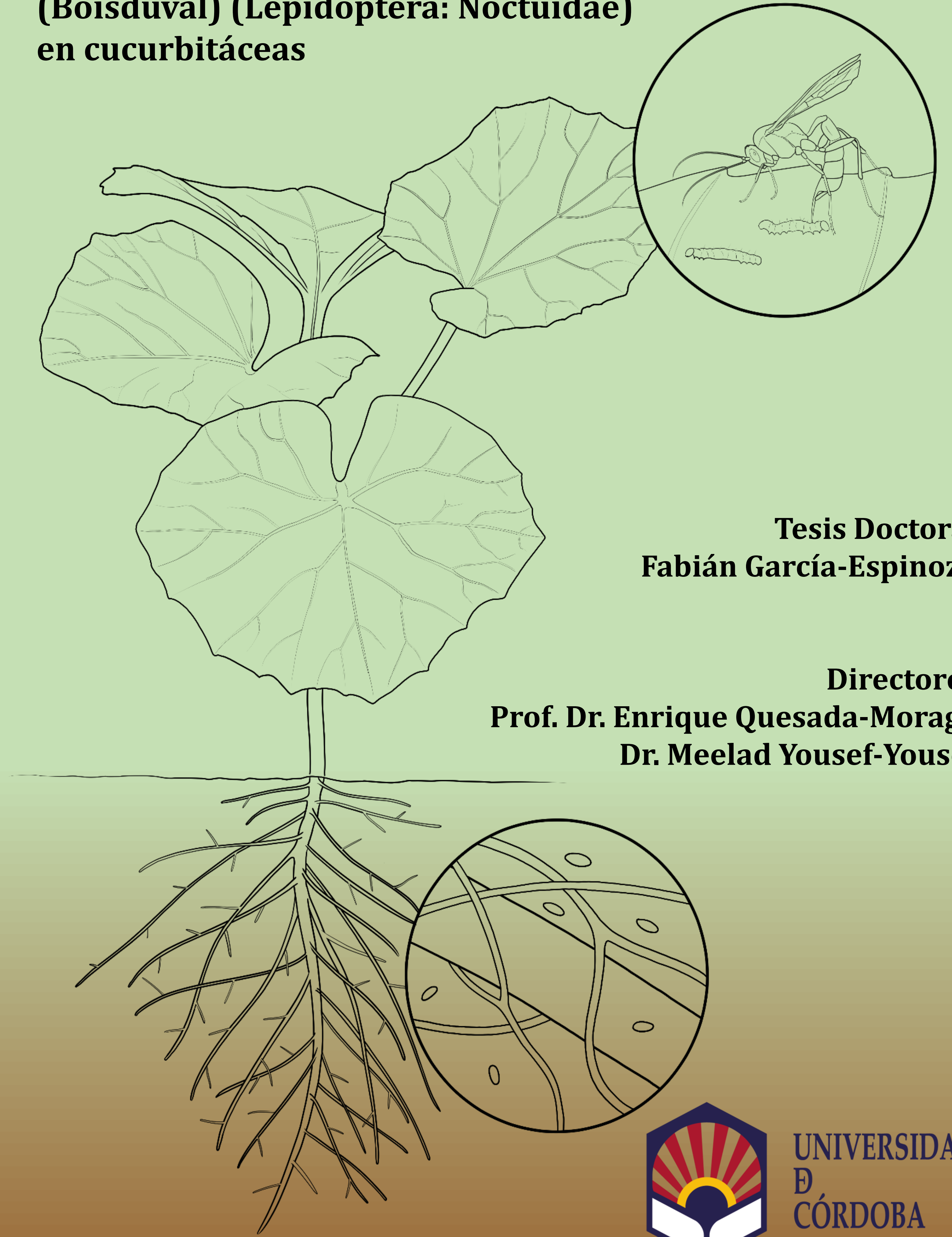

Tesis Doctoral

Fabián García-Espinoza

Directores
Prof. Dr. Enrique Quesada-Moraga
Dr. Meelad Yousef-Yousef

UNIVERSIDAD DE CÓRDOBA

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Tesis Doctoral

UNIVERSIDAD DE CÓRDOBA

Escuela Técnica Superior de
Ingeniería Agronómica y de Montes

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

TITULO: *Multifunctional entomopathogenic ascomycetes for "cotton leafworm" Spodoptera littoralis (Boisduval) (Lepidoptera: Noctuidae) control in cucurbits*

AUTOR: *Fabian García Espinoza*

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Córdoba, España, febrero de 2024

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INFORME RAZONADO

TÍTULO DE LA TESIS: Ascomicetos entomopatógenos multifuncionales para el control de la “rosquilla negra” *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) en cucurbitáceas / Multifunctional entomopathogenic ascomycetes for control of "cotton leafworm" *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) in cucurbits

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INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS: El **Prof. D. Enrique Quesada Moraga**, Doctor Ingeniero Agrónomo, Catedrático de Producción Vegetal del Departamento de Agronomía de la Universidad de Córdoba, y el **Dr. D. Meelad Yousef Yousef**, Doctor Ingeniero Agrónomo, Profesor Ayudante Doctor del Departamento de Agronomía de la Universidad de Córdoba.

INFORMAN: Que el trabajo titulado “Ascomicetos entomopatógenos multifuncionales para el control de la “rosquilla negra” *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) en cucurbitáceas / Multifunctional entomopathogenic ascomycetes for control of "cotton leafworm" *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) in cucurbits” realizado bajo su dirección por **D. Fabián García Espinoza**, se encuentra finalizado y cumple los requisitos para su presentación como Tesis Doctoral por compendio de publicaciones, las cuales se indican a continuación:

García-Espinoza, F., Yousef-Yousef, M., García del Rosal, M. J., Cuenca-Medina, M., and Quesada-Moraga, E. (2024). Greenhouse melon crop protection and production through the compatible use of a parasitoid with endophytic entomopathogenic ascomycetes. *Journal of Pest Science* 97(1). https://doi.org/10.1007/s10340-023-01735-0. Esta revista es Q1 (D1): 3/100

en la categoría de "Entomology" con un factor de impacto de 5.7, en el momento de la aceptación.

García-Espinoza, F., García, M. J., Quesada-Moraga, E., and Yousef-Yousef, M. (2023). Entomopathogenic fungus-related priming defense mechanisms in cucurbits impact *Spodoptera littoralis* (Boisduval) fitness. *Applied and Environmental Microbiology*, 89(8). https://doi.org/10.1128/aem.00940-23. Esta revista es Q1: 37/169 en la categoría de "Biotechnology & Applied Microbiology" con un factor de impacto de 4.4, en el momento de la aceptación.

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Yousef-Yousef, M., García-Espinoza, F., García del Rosal, M. J., and Quesada-Moraga, E. (2024). Guardians within: Entomopathogenic ascomycete-driven antibiosis and compensatory growth combine to protect melon plants from herbivore damage. *Submitted*.

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

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TESIS PRESENTADA POR COMPENDIO DE ARTÍCULOS

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

García-Espinoza, F., Yousef-Yousef, M., García del Rosal, M. J., Cuenca-Medina, M., and Quesada-Moraga, E. (2024). Greenhouse melon crop protection and production through the compatible use of a parasitoid with endophytic entomopathogenic ascomycetes. *Journal of Pest Science* 97(1). https://doi.org/10.1007/s10340-023-01735-0. Esta revista es Q1 (D1): 3/100 en la categoría de "Entomology" con un factor de impacto de 5.7, en el momento de la aceptación.

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De nacionalidad mexicana, nacido el 20 de enero de 1986 en Pascala del Oro, San Luis Acatlán, Guerrero. Ingeniero Agrónomo Parasitólogo y Maestro en Ciencias Agrarias por la Universidad Autónoma Agraria Antonio Narro (UAAAN).

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Los proyectos y trabajos de investigación, así como las tesis y trabajos de titulación que ha dirigido y codirigido, se han enfocado en áreas como la entomología agrícola y entomología forense.

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A mis abuelos.

A aquellos que nos dejaron

durante la época oscura de "la enfermedad".

A la memoria de mis Maestros y antepasados.

"Muchas cosas buenas vienen de nuestras raíces "

"A lot of good things come from our roots "

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RESUMEN

Durante el siglo XXI se ha acelerado el crecimiento de la población mundial, que superará los nueve mil millones en 2050, lo que plantea importantes desafíos para la agricultura en términos de seguridad e inocuidad alimentaria, en un contexto donde se ha incrementado la preocupación social y la presión regulatoria por los efectos negativos sobre el medio ambiente y los seres vivos de los insecticidas químicos de síntesis, de los que aun depende la reducción de las pérdidas de cosecha debidas a la acción de las plagas agrícolas. Así, la búsqueda de alternativas no químicas de control de plagas se ha convertido en un pilar de las políticas agrarias a nivel mundial, y muy en especial, en el contexto europeo, como refleja la controvertida Agenda 2030. Dentro de estas alternativas, el control microbiano de plagas por medio de ascomicetos entomopatógenos (AE), en especial especies de los géneros *Beauveria* sp. y *Metarhizium* sp., que tienen modo de acción por contacto, ha suscitado un interés creciente, no sólo por su virulencia, sino también por su carácter de microorganismos multifunción en agricultura, con asociaciones con las plantas como endófitos, epífitos o competentes en la rizosfera, de las que pueden derivarse nuevas estrategias sostenibles de protección y producción vegetal. Como resultado de estas interacciones, los AE proporcionan a la planta protección sistémica frente a estreses bióticos y abióticos por mecanismos directos e indirectos, estos últimos aún poco conocidos, pero asociados en muchos casos a reguladores comunes como el etileno (ET), vinculando respuestas a factores como la deficiencia de hierro y la Resistencia Sistémica Inducida (RSI). De hecho, estudios recientes ponen de manifiesto la multifuncionalidad de los AE, en particular las especies *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae) y *Metarhizium brunneum* Petch (Hypocreales: Clavicipitaceae), que no solo regulan numerosas relaciones multitróficas cultivo-insecto-fitófago-enemigo natural en distintos agroecosisemas, sino que además tienen un impacto positivo sobre la adquisición de nutrientes y sobre el crecimiento y la productividad de cultivos de importancia económica. En este contexto, la presente tesis doctoral ha abordado un sistema multitrófico donde cepas endófitas de las dos especies de AE mencionadas se han utilizado para el control de la plaga polífaga *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae), junto con el parasitoide koinbionte solitario *Hyposoter didymator* (Thunberg) (Hymenoptera: Ichneumonidae), en cultivo de melón, con

atención no solo a la compatibilidad directa de los agentes de control biológico, sino al impacto de los AE sobre el crecimiento del cultivo y el efecto indirecto sobre el fitófago.

El Capítulo II aborda el tipo de respuesta de plantas de melón inoculadas con AE al ataque de *S. littoralis*, así como los mecanismos moleculares que la regulan. Las plantas de melón se inocularon con tres cepas de AE, cepa EAMa 01/58-Su de *M. brunneum* y cepas EABb 04/01-Tip y EABb 01/33-Su de *B. bassiana*, mediante tratamiento de suelo, tratamiento de semilla y tratamiento foliar. Se evaluaron dos escenarios de infestación con larvas de *S. littoralis*, infestación secuencial de duración corta, así como infestación permanente. Las plantas inoculadas mostraron antibiosis frente al nóctuido (efectos letales y subletales), así como una compensación del crecimiento o tolerancia en respuesta a diversos escenarios de infestación de este. Se registró un aumento en el peso fresco y seco de la planta, contenido de clorofila, número de ramas secundarias y diámetro del tallo, al tiempo que inducía efectos subletales en *S. littoralis*. Además, se observó una sobreexpresión relativa de los genes relacionados con el ET (*ACO1*, *ACO3*, *EIN2*, *EIN3*) y el ácido jasmónico (AJ) (*LOX2*), siendo mayor la inducción de los genes relacionados con ET y AJ, por la presencia endofítica de *B. bassiana*, especialmente en plantas infestadas con *S. littoralis*. Estos resultados no solo confirman la multifuncionalidad de los AE, sino su implicación tanto en la inducción del sistema defensivo del melón, que se refleja no solo en la antibiosis frente al lepidóptero fitófago, sino también en un crecimiento compensatorio o tolerancia del cultivo al ataque del insecto.

Bajo la premisa de que los AE son microorganismos multifuncionales, el Capítulo III revela los mecanismos moleculares mediante los cuales estos ascomicetos brindan protección a través de respuestas de resistencia sistémica contra insectos fitófagos. En este trabajo, se aplicó un *priming* en las raíces de plántulas de cucurbitáceas, pepino y melón, con la cepa EAMa 01/58-Su de *M. brunneum*, y se estudió la expresión relativa de 18 genes relacionados con la síntesis de ET, AJ y ácido salicílico (AS), así como genes de proteínas relacionadas con patogénesis (PR) mediante qRT-PCR. Los efectos del *priming* en *S. littoralis* se estudiaron al exponer larvas del nóctuido a plantas que habían recibido un *priming* con el AE o bien plantas del testigo durante 15 días. Se observó la complejidad y el grado de solapamiento entre las rutas reguladoras, y aunque existió una sobreexpresión general de todos los genes estudiados en plantas con *priming* por AE, destacaron *EIN2* y *EIN3,* genes clave en la

vía de transducción de ET, que aumentaron sus niveles de expresión hasta ocho y cuatro veces, respectivamente. Además, los genes de síntesis de AJ, AS y PR mostraron una sobreexpresión significativa durante el período de observación (por ejemplo, *LOX1*, gen implicado en la síntesis de AJ, aumentó 506 veces). Se observó una mortalidad significativa de las larvas de *S. littoralis* alimentadas con plantas tratadas (con *priming* fúngico) en comparación con las plantas testigo, además de un efecto negativo del *priming* fúngico sobre el fitness del lepidóptero revelado por la duración de los estadíos larvarios, peso de pupas y porcentaje de pupas anormales, lo que pone de manifiesto el efecto beneficioso de la cepa EAMa 01/58-Su de *M. brunneum* sobre la inducción de resistencia en cucurbitáceas. La alta inducción de genes relacionados con la síntesis y señalización de ET detectada en este capítulo, que es un regulador común entre la RSI y la deficiencia de Fe, impulsó la investigación llevada a cabo en el Capítulo IV, en el que se investiga el efecto de las cepas de AE sobre la adquisición del hierro (Fe), tanto *in vitro* en cuanto a la exudación de sideróforos, como *in vivo* en lo que se refiere al contenido de Fe en plantas de melón y pepino, para las tres cepas de *B. bassiana* y *M. bruneum*. La cepa EAMa 01/58-Su de *M. brunneum* mostró una gran capacidad para producir sideróforos de hierro revelada por hasta un 58.4% de cambio en la coloración del medio (de azul a naranja) por acción de la exudación de sideróforos, porcentaje que alcanzó el 24.3% y 17.8% para las cepas EABb 04/01-Tip y EABb 01/33-Su de *B. bassiana*, respectivamente. Asimismo, la cepa EAMa 01/58-Su proporcionó un mayor contenido de Fe tanto en materia seca como en el sustrato en comparación con el control, por lo que fue seleccionada para dilucidar la posible inducción de respuestas de deficiencia de Fe, que incluyen la Actividad de la Reductasa Férrica (ARF), y la expresión relativa de genes de adquisición de Fe mediante qRT-PCR en plantas de melón y pepino. El *priming* de raíces con la cepa EAMa 01/58-Su de *M. brunneum* provocó respuestas a la deficiencia de Fe a nivel transcripcional, con una regulación positiva temprana (24, 48 o 72 h después de la inoculación) de los genes de adquisición de Fe como *FRO1*, *FRO2*, *IRT1*, *HA1* y *FIT*, así como de la ARF. Estos resultados destacan los mecanismos involucrados en la adquisición de Fe mediada por la cepa EAMa 01/58-Su de *M. brunneum*.

Finalmente, en el Capítulo V, el parasitoide *H. didymator* fue evaluado contra *S. littoralis* en un sistema multitrófico en invernadero con plantas de melón colonizadas endofíticamente por tres cepas de AE, cepa EAMa 01/58-Su de *M. brunneum* y cepas

EABb 04/01-Tip y EABb 01/33-Su de *B. bassiana*. En un primer escenario, se utilizaron tres métodos de aplicación para inocular las plantas de melón con los AE, tratamiento de suelo, tratamiento de semilla y tratamiento foliar, y tras su infestación con larvas de *S. littoralis*, se liberó el parasitoide en una proporción de 1:20. Los métodos de detección microbiológica y molecular permitieron detectar la colonización progresiva a lo largo de todo el ciclo fenológico del cultivo, e incluso para *B. bassiana*, alrededor del 20% de las semillas de nuevos frutos estaban colonizadas. Se demostró que el parasitoide es compatible con todas las cepas y métodos de aplicación, con tasas de mortalidad totales que oscilaban entre el 11.1% (EAMa 01/58-Su en inoculación por recubrimiento de semilla) y el 77.8% (EAMa 01/58-Su en inoculación por pulverización foliar). Además, para diferentes combinaciones de cepa y método de aplicación, se observó una disminución del peso de pupas, mortalidad pupal (tanto normales como con deformidades), así como una extensión de los tiempos de desarrollo larvario y pupal. Asimismo, los tratamientos con AE mejoraron el crecimiento del cultivo de melón, con un incremento significativo del peso de raíces, partes aéreas (hojas y tallos) y peso total de planta. En un segundo escenario, las plantas fueron inoculadas con estas cepas, mediante pulverización localizada de dos hojas basales, y después de ser infestadas con larvas del nóctuido, se liberó el parasitoide en una proporción de 1:10, lo que permitió revelar la traslocación del AE, así como su compatibilidad con el parasitoide. Estos hallazgos destacan el uso compatible de un parasitoide con cepas de AE multifuncionales, que permiten el control de *S. littoralis*, con impacto positivo sobre el cultivo de melón.

SUMMARY

During the 21st century, the world population growth has accelerated and it is expected to exceed 9 billion by 2050, which poses important challenges for agriculture in terms of food safety and security, in a context where social concern and regulatory pressure have increased due to risks to human health and to the environment of chemical insecticides, on which reduction of crop losses due to the action of agricultural pests still mostly depends. Thus, the search for non-chemical alternatives for pest control has become a pillar of agricultural policies worldwide, and especially in the European context, as reflected in the controversial Agenda 2030. Within these alternatives, microbial pest control by means of entomopathogenic ascomycetes (EA), especially species of the genera *Beauveria* sp. and *Metarhizium* sp., which have contact mode of action, has attracted increasing interest, not only for their virulence, but also for their multifunctionality in agriculture, with associations with plants as endophytes, epiphytes or competent in the rhizosphere, from which new sustainable strategies for plant protection and production can be derived. As a result of these interactions, EA provide the plant with systemic protection against biotic and abiotic stresses by direct and indirect mechanisms, the latter still poorly understood, but in many cases associated with common regulators such as ethylene (ET), linking responses to factors such as iron deficiency and Induced Systemic Resistance (ISR). Moreover, recent studies highlight the multifunctionality of EA, in particular the species *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae) and *Metarhizium brunneum* Petch (Hypocreales: Clavicipitaceae) that not only regulate numerous multitrophic interactions crop-insect pest-natural enemy, but also have a positive impact on nutrient acquisition and on the growth and productivity of economically important crops. In this context, the present PhD thesis has addressed a multitrophic system where endophytic strains of the two mentioned EA species have been used for the control of the polyphagous pest *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae), together with the solitary koinbiont parasitoid *Hyposoter didymator* (Thunberg) (Hymenoptera: Ichneumonidae), in melon crop, with attention not only on the direct compatibility of the biological control agents, but on the impact of the EA strains on crop growth and the indirect impact on the noctuid pest.

Chapter II addresses the type of response of melon plants inoculated with AE to *S. littoralis* attack, antibiosis and/or tolerance, as well as the molecular mechanisms regulating it. Two scenarios of infestation with *S. littoralis* larvae were evaluated: short-term sequential infestation and long-term infestation. Molecular techniques were used to study progressive endophytic colonization and the relative expression of plant defense genes. The inoculated plants showed antibiosis (lethal and sublethal effects) and growth compensation in response to different *S. littoralis* infestation scenarios. An increase in fresh and dry weight, chlorophyll content, number of secondary branches, and stem diameter was recorded, while causing sublethal effects in *S. littoralis*. In addition, up-regulation in the relative expression of ET (*ACO1*, *ACO3*, *EIN2*, *EIN3*) and jasmonic acid (JA) (*LOX2*)-related genes was observed, with higher induction of genes related to ET and JA, due the endophytism by *B. bassiana*, specially in *S. littoralis* infested plants. These results strongly confirm the EA multifunctionality and the involvement of the endophytic EA triggered melon defensive system induction in the antibiosis and compensatory growth to protect melon plants from pest damage*.*

Under the premise that EA are multifunctional microorganisms, Chapter III reveals the molecular mechanisms by which these EA provide protection through systemic resistance responses against phytophagous insects. In this work, the roots of cucurbits seedlings were primed with *M. brunneum* (EAMa 01/58-Su strain), and the relative expression of 18 genes related to ET, JA and salicylic acid (SA) synthesis, as well as pathogen related (PR) protein genes were studied by qRT-PCR. Effects of priming on *S. littoralis* were studied by feeding larvae for 15 days with primed and control plants. The complexity and degree of overlap between regulatory pathways was observed, and although there was a general overexpression of all genes studied in plants primed by AE, it was particularly important for *EIN2* and *EIN3* genes that are key in the ET transduction pathway, which increased their expression levels up to eightfold and fourfold, respectively. Also, genes related to JA, SA synthesis and PR showed significant up-regulation during the observation period (e.g. the JA gen *LOX1*, increased 506 times). Survivorship and fitness of *S. littoralis* were affected with significant effects on mortality of larvae fed on primed plants *vs.* controls. In addition, the length of the larval stage, the weight of the pupa, and the percentage of abnormal pupae were significantly affected. These results highlight the role of EAMa 01/58-Su strain in the induction of resistance, which would be translated into direct benefits for plant development. As

this chapter reveals that plants primed with *M. brunneum* EAMa 01/58-Su strain show high induction of genes related to ET synthesis and signaling, which is a common regulator between ISR and Fe deficiency, Chapter IV aims to investigate the effect of EA strains on iron (Fe) acquisition, both *in vitro* in terms of siderophore exudation and *in vivo* regarding Fe content in melon and cucumber plants, for the three strains of *B. bassiana* and *M. bruneum*. The *M. brunneum* EAMa 01/58-Su strain was revealed as a high iron siderophore producer, with a 58.4% change in the color of the medium (from blue to orange) due to the siderophores exudation, followed by the strains EABb 04/01-Tip and EABb 01/33-Su of *B. bassiana*, with exuded surface of 24.3% and 17.8%, respectively. Likewise, the *M. brunneum* EAMa 01/58-Su strain provided higher Fe content in both dry matter and substrate compared to the control, therefore, it was selected to elucidate the possible induction of Fe deficiency responses, including Ferric Reductase Activity (FRA), and the relative expression of Fe acquisition genes by qRT-PCR in melon and cucumber plants primed by root immersion. Root priming by *M. brunneum* EAMa 01/58-Su strain elicited Fe deficiency responses at the transcriptional level. Our results show an early up-regulation (24, 48 or 72 h post-inoculation) of the Fe acquisition genes *FRO1*, *FRO2*, *IRT1*, *HA1*, and *FIT* as well as an increase of the FRA. These results highlight the mechanisms involved in the Fe acquisition mediated by *M. brunneum* EAMa 01/58-Su strain.

Finally, Chapter II addresses, under greenhouse conditions, the control of *S. littoralis* with the parasitoid *H. didymator* in a multitrophic system with melon plants endophytically colonized by the three strains of AE, strain EAMa 01/58-Su of *M. brunneum* and EABb 04/01-Tip and EABb 01/33-Su of *B. bassiana*. In a first scenario, three application methods were used to inoculate melon plants: soil drenching, seed coating or entire plant spraying. After being infested with *S. littoralis* larvae, the parasitoid was released in a ratio of 1:20. Microbiological and molecular detection allowed detecting progressive colonization throughout the plant life cycle, and for *B. bassiana*, even about 20% of the seeds of new fruits were colonized. The parasitoid was shown to be compatible with all strains and application methods, with total mortality rates ranging from 11.1% (EAMa 01/58-Su in seed coating inoculation) to 77.8% (EAMa 01/58-Su in leaves spraying inoculation). In addition, significant sublethal effects were recorded, such as a decrease in the weight of pupae, increased pupal mortality (both normal and with deformities), as well as an extension of the larval and

pupal development times detected for different combinations of strain and application method. Likewise, EA treatments improved the growth of the melon crop, as revealed by the significant increases in root weight, aerial parts (leaves and stems) and total plant weight. In a second scenario, the plants were inoculated with the three EA strains by spraying only the two basal leaves, and after being infested with noctuid larvae, the parasitoid was released in a ratio of 1:10, which allowed to reveal the translocation of the fungus from the inoculation point and to confirm the compatibility of the parasitoid-EA-based strategy. These findings highlight the compatible use of a parasitoid with EA for the control of *S. littoralis*, which can also take advantage of its multifunctionality for the sustainable production of melon crop.

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ABBREVIATIONS

ACO1: 1-Aminocyclopropane-1-carboxylic acid oxidase 1 gene

ACO3: 1-Aminocyclopropane-1-carboxylic acid oxidase 2 gene

ACO5: 1-Aminocyclopropane-1-carboxylic acid oxidase 5 gene

ACS7: 1-aminocyclopropane-1-carboxylic acid synthases 7 gene

ANOVA: Analysis of Variance

CAS: Chrome Azurol Sulfonate

cDNA: Complementary Deoxyribonucleic Acid

CONAHCyT (formerly CONACyT): Consejo Nacional de Humanidades Ciencia y Tecnología

CsWRKY20: WRKY transcription factor of PR protein gene

Ct: Threshold Cycles

d: days

DAS: Days After Sowing

DAUCO: Department of Agronomy of The University of Córdoba

DNA: Deoxyribonucleic Acid

DPI: Days Post Inoculation

DPP: Days Post Priming

EA: Entomopathogenic Ascomycetes

EEUU or **USA**: United States of America

EIN2: Ethylene-insensitive protein 2 gene

EIN3: Ethylene-insensitive protein 3 gene

EIPF: Endophytic Insect Pathogenic Fungi

EPF: Entomopathogenic Fungus/Fungi

EPPO: European and Mediterranean Plant Protection Organization

ET: Athylene

EU: European Union

Fe: Iron

FIT: Induced Transcription Factor gene

FRA: Ferric Reductase Activity

FRO1: Ferric reductase oxidase gene

FRO1: Ferric reductase oxidase gene

FRO2: Ferric reductase oxidase gene

FRO3: Ferric reductase oxidase gene

FRO4: Ferric reductase oxidase gene

h: hours

HA1: ATPase gene

IPF: Insect Pathogenic Fungi

IPM: Integrated Pest Management

IRT1: Iron-regulated transporter1 gene

ISR: Induced Systemic Resistance

JA: Jasmonic acid

K: Potassium

L:D: Light: Darkness

L1, L2, L3, L4, L5 and L6: First to sixth larval instars of *Spodoptera littoralis*

LOX1: Lipoxygenase 1 gene

LOX2 (*Cm*): Lipoxygenase 2 *Cucumis melo* gene

LOX2 (*Cs*): Lipoxygenase 2 *Cucumis sativus* gene

LSpr: Leaves spraying

MAMPs: Microbe-Associated Molecular Patterns

MELO3C014222: Phenylalanine ammonia lyase gene

MELO3C014632: Linoleate 13S-lipoxygenase 2-1 gene

MELO3C019787: AP2-like ethylene-responsive transcription factor gene

N: Nitrogen

NBRIP: National Institute of Botanical Research

NCBI: The National Center for Biotechnology Information

ng: Nanograms

P: Phosphorus

PAL: Phenylalanine ammonia lyase gene

PDA: Potato Dextrose Agar

pg: Picograms

PGPFs: Plant growth-promoting fungi

PR proteins: Pathogenesis-related proteins

PR1: Pathogenesis-related protein 1 gene

PR*1-1a*: Pathogenesis-related protein 1-1a gene

PR3: Pathogenesis-related protein 3 gene

PR9: Pathogenesis-related protein 9 gene

PTI: Pathogen- or Pattern-Triggered Immunity

qPCR: Quantitative Polymerase Chain Reaction

qRT-PCR: Quantitative Reverse Transcription Polymerase Chain Reaction

RES: Relative efficiency of solubilization

RNA: Ribonucleic Acid

RNAi: RNA interference

SA: Salicylic acid

SAR: Systemic Acquired Resistance

SCo: Seed coating

SDAC: Sabouraud Dextrose Agar medium supplemented with 0.5 g l^{-1} chloramphenicol

SoD: Soil drenching

SPAD values: Index value displayed by Konica Minolta Chlorophyllmeters and having a correlation to chlorophyll density.

SWR: Systemic Wound Response

TFs: Transcription factors

UCO: University of Cordoba

CHAPTER I. INTRODUCTION

I.1. Present and future of arthropod pest control

The world population has reached 7.9 billion in 2022 and will reach 9-10 billion by 2050 (Reid and Greene 2012; Julot and Hiller 2021; United Nations 2023). The colossal challenge confronting agriculture is boosting crop yields to double production by 2050, rather than expanding cropland as far as the cultivated area per person has progressively declined since 1965 (Ray et al. 2013; Nishimoto 2019). Per contra, the crop yields of maize, rice, wheat, and soybean that produce nearly two-thirds of global agricultural calories are increasing at 1.0-1.6%, per year, which is far below than the 2.4% annual rate required to double global production by 2050 (Ray et al. 2013; Culliney 2014; Nishimoto 2019; Jacquet et al. 2022). In this scenario, where there is no contribution to improved food security through increased production and lower prices, it is noteworthy that between 20 and 40% of global crop production is lost due to pests (Oerke 2006; Savary et al. 2019; Mateos Fernández et al. 2022). Each year, arthropod pests, particularly insects, are responsible for destroying an estimated 10–26% of global crop production, amounting to over US$470 billion and, as if that weren't enough, plant diseases and invasive insects incur additional cost worldwide, amounting to approximately US$220 billion and US$70 billion per year, respectively (Oerke 2006; Culliney 2014; Julot and Hiller 2021; United Nations 2023).

Chemical pesticides are still the primary tool employed to reduce crop losses, decrease incidence of human vector-borne diseases, extend shelf life of agricultural commodities, increase livestock yields, minimize soil disruption, and enhance protection of timber structures (Smith et al. 2022). Hence, the use of chemical pesticides has increased over the last few decades and has become the cornerstone of the predominant crop production systems. As an example, in 2018, only in the European Union (EU), 370 million kilograms of pesticides were sold (Jacquet et al. 2022). However, despite their effectiveness, many of these pesticides have significant adverse consequences for human health and the environment and questions regarding the sustainability of the massive use of pesticides have frequently raised, particularly with the advent of the Agenda 2030 (Reid and Greene 2012; Deguine et al. 2021; Julot and Hiller 2021; Gupta et al. 2022a; Adeleke et al. 2022).

Whilst there are currently two primary strategies for reducing pesticide usage, namely, organic farming and Integrated Pest Management (IPM), the latter one is currently considered the main strategy for achieving this reduction (Jacquet et al. 2022). Indeed, low pesticide-input IPM systems are of obligatory implementation in the EU (Directive 2009/128/EC) and the United States (U.S. Code §136R–1). The Directive 2009/128/EC of the European parliament and of the council establishing a framework for community action to achieve the sustainable use of pesticides defines IPM as a “careful consideration of all available plant protection methods and subsequent integration of appropriate measures that discourage the development of populations of harmful organisms and keep the use of plant protection products and other forms of intervention to levels that are economically and ecologically justified and reduce or minimize risks to human health and the environment”. However, alternative interpretations of IPM define it either as “a holistic ‘approach’ or ‘strategy’ to combat plant pests and diseases using all available methods, while minimizing applications of chemical pesticides” (Stenberg 2017) or as the practice of “rotating chemicals from different mode of action groups to maintain pest control efficacy and reduce pesticide resistance with an emphasis on reducing pest damage” (Dara 2019a). Recently, the pesticide-free agriculture concept has been introduced as a third strategy in addition to the existing ones that avoids the use of both synthetic and natural pesticides that have negative impacts on the environment and human health (Jacquet et al. 2022).

The concept of pest control has evolved into pest management over the years, and it is now widely recognized that adopting a balanced approach to managing pest populations aiming to maintain them at levels that do not result in economic losses, is generally preferable to outright elimination or eradication (except for newly introduced invasive pests). Indeed, it is important to recognize the potential of biocontrol or biological control tactics as crucial component of IPM strategies (Deguine et al. 2021; Mateos Fernández et al. 2022; Jaiswal et al. 2022). In addition to biological pest control, which will be addressed latter in this chapter, there are several other strategies to consider within IPM programs. These strategies encompass cultural, behavioral, physical, mechanical, and chemical control, as well as the host plant resistance strategy (Stenberg 2017; Dara 2019b; Mateos Fernández et al. 2022; Jacquet et al. 2022).

In addition to the previously mentioned strategies, current pest management approaches offer some promising alternatives for the future:

- RNA interference (RNAi) is emerging as a powerful tool for pest control. Due to the molecular-level nature of this control mechanism, it holds the promise of safe and targeted management against problematic species (Horowitz et al. 2009; Baysal and Bastas 2022; Mateos Fernández et al. 2022).
- Genetic control methods such as the sterile male technique, have been implemented with excellent results in controlling certain pests of both agricultural and veterinary importance (Dara 2019a; Mateos Fernández et al. 2022).
- Biorational control along with the use of plant natural products as eco-friendly pesticides, are functional alternatives to consider in IPM. These methods aim to manage pests while minimizing adverse environmental impacts (Horowitz et al. 2009; Ishaaya and Horowitz 2009; Stenberg 2017; Mateos Fernández et al. 2022).
- Plant-mediated RNAi that enables the targeted suppression of specific genes in pests, leading to reduced survival and reproduction. It offers a precise and environmentally benign approach to pest control (Zhang et al. 2017; Kunte et al. 2020; Zhao et al. 2022; Li et al. 2023; Feng et al. 2023).

Another important topic to be addressed latter in this chapter is the concept of "priming", or what Stenberg (2017) referred to as “plant vaccination”. The use of endophytes to prime plants and boost their defense against pests, including phytopathogens and arthropods, is gaining interest and importance in IPM programs (Stenberg 2017; Bamisile et al. 2018a; Quesada-Moraga et al. 2020, 2022; Tiwari and Singh 2021; Segaran et al. 2022; Tiwari et al. 2022; Samanta et al. 2023). Furthermore, in modern pest control strategies, it is crucial to leverage technology including the use of informatics and robotics, as well as the integration of advanced communication networks tools to make more informed decisions about pest management (Deguine et al. 2021; Iost Filho et al. 2022; Kanwal et al. 2022). The concept of digital agriculture, an evolution of precision agriculture, enables monitoring and management of pests and precise and timely use pesticides by using technologies like remote sensing, the

internet of things, geographic information systems, artificial intelligence, radars, and automated insect monitoring (Willers et al. 2014; Iost Filho et al. 2022; Kanwal et al. 2022).

I.2. Biological pest control

Traditionally, biological control, as defined by Bale et al. (2008) and Barratt et al. (2018), involves the use of one organism to reduce the population density of another organism (either animals, weeds, or diseases). Whilst the International Organisation for Biological and Integrated Control (IOBC) defines biological control as the use of living organisms and their products to prevent or reduce the losses or harm caused by pests, it is nowadays accepted that biological control includes only the "exploitation of living agents" for pest control (Stenberg et al. 2021). The natural biological control is defined as the natural regulation of pest populations, without human intervention, by indigenous natural enemies or "naturally occurring beneficial organisms" (Bale et al. 2008; van Lenteren et al. 2018), apart from this, three main approaches of biological control have been defined (Bale et al. 2008; van Lenteren et al. 2018; Jeffers and Chong 2021; Stenberg et al. 2021):

- Conservation biological control: in conservation biological control, the human intervention maintains and promote the natural enemy populations in an ecosystem. This may involve practices like providing habitat or food sources for beneficial organisms.
- Augmentative biological control: this strategy is used to address immediate pest issues involving the periodic release of biocontrol agents to provide short term control of the pest population.
- Classical biological control: This approach involves gathering natural enemies in a study area, which is typically the pest original habitat, and releasing them into invasive areas. This strategy frequently leads to a long-term decrease in the pest population as well as significant financial gains (Kenis et al. 2017; van Lenteren et al. 2018).

In this sense, there are two groups of biological control agents, the entomophagous arthropods (macrobial control), including predators and parasitoids

and the entomopathogenic microorganism (microbial control) including viruses, fungi, bacteria and nematodes (Bale et al. 2008; Lacey 2017; Souza and Marucci 2021). However, it is also essential to consider the significant economic benefits derived from ecosystem services associated with biological control provided by naturally occurring control agents (Barratt et al. 2018, Quesada-Moraga et al. 2023).

I.2.1. Entomophagous arthropods

Predatory arthropods, insects and arachnids, present an important group among natural enemies in the context of IPM. Predatory species within the Class Insecta either polyphagous, oligophagous or monophagous, are mainly found in the orders Coleoptera, Hymenoptera, Neuroptera, Hemiptera, Thysanoptera, Diptera, and to a lesser extent in other orders Mantodea, Dermaptera and Odonata. Additionally, some genera and species of arachnids, predominantly belonging to the Class Acari, with emphasis in the Phytoseiidae are also important entomophagous (Nájera Rincón and Souza 2010; van Lenteren 2012; van Lenteren et al. 2018; Greco and Rocca 2020). These predators are free-living organisms that actively hunt and capture their prey, subsequently consuming them, often requiring a substantial quantity of prey to complete their development and life cycle. Most predatory arthropods maintain their predatory behavior consistently throughout their life cycle, seizing opportunities to attack when their prey is immobile or exhibits limited movement (Nájera Rincón and Souza 2010; Greco and Rocca 2020; Stenberg et al. 2021).

In addition to predators, there is another group of entomophagous insects, the parasitoids that could be found either parasitizing egg, larvae, pupae, or insect adult stages to complete their development, being typically monophagous and belonging to various holometabolous insect orders, with emphasis in Hymenoptera and Diptera (Nájera Rincón and Souza 2010; Koller et al. 2023).

One of the significant advantages of using predators and parasitoids, in contrast to synthetic pesticides, is the reduced likelihood of resistance development. Moreover, in many cases, the control provided by these natural enemies can be self-sustaining over extended periods, which makes them valuable tools in IPM, promoting sustainable and long-term solutions for controlling agricultural pests while minimizing the environmental impact associated with chemical pesticides (Bale et al. 2008).

I.2.2. Entomopathogenic microorganisms

Apart from predators and parasitoids, microbial biological control agents are among the most frequently employed organisms in augmentative biological control (Lacey 2017; van Lenteren et al. 2018). Arthropods, like all living organisms, are susceptible to diseases that are the focus of the invertebrate pathology science, encompassing the examination of the diseases, their symptoms, and manifestations, as well as methods for identifying pathogens and diagnosing infections, which are key for the effective use of microbial agents in the biological control of insect pests (Davidson 2012; Kaya and Vega 2012; Quesada-Moraga and Santiago-Álvarez 2023).

The history of insect pathology dates back millennia, with early observations of insect diseases recorded in ancient China more than 2000 years ago, particularly focusing on diseases affecting honeybees and silkworms (Davidson 2012; Kaya and Vega 2012; Quesada-Moraga and Santiago-Álvarez 2023). However, it was not until 1835 that Augusto Bassi, often referred to as the "Father of Insect Pathology" made a significant breakthrough successfully demonstrating the cause of insect diseases when reveling that the "calcinacci" disease was caused by the entomopathogenic fungus (EPF) *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae) (Davidson 2012; Solter et al. 2017; Lacey 2017; Quesada-Moraga and Santiago-Álvarez 2023).

Insect pathology includes a wide variety of insect pathogenic organisms, including viruses, bacteria, fungi, protists, and nematodes, whereas in the field of biological control, the most used agents are viruses, bacteria, nematodes and fungi (Lacey 2008, 2017; Solter et al. 2017; Koller et al. 2023). Among these pathogens, bacteria have been extensively studied as microbial control agents, and they represent a substantial volume and market share commercially (Lacey 2008, 2017; van Lenteren et al. 2018). One of the most well-known and widely used bacterial species for pest control is *Bacillus thuringiensis* (Berliner), including several subspecies. These bacteria are widely addressed and applied in agriculture, forestry, and public health to control various insect pests (Jurat-Fuentes and Jackson 2012; van Lenteren et al. 2018).

Viruses, particularly those belonging to the family Baculoviridae, are known for their specificity comprising the genera Alphabaculovirus (lepidopteran-specific nucleopolyhedroviruses), Betabaculovirus (lepidopteran-specific Granuloviruses),

Gammabaculovirus (hymenopteran-specific nucleopolyhedroviruses) and Deltabaculovirus (dipteran-specific nucleopolyhedroviruses) (Herniou et al. 2012; Harrison and Hoover 2012; Clem and Passarelli 2013; Grzywacz 2017). Baculoviruses can infect, replicate, spread both horizontally (among pests in the field) and vertically (from one generation to the next), and persist mainly in the soil (Harrison and Hoover 2012). The impact of baculoviruses on pest populations and their compatibility with sustainable agricultural practices make them an attractive option for pest management (Grzywacz 2017).

The entomoparasitic nematodes of the Steinernematidae and Heterorhabditidae families are also used for microbial pest control (Lewis and Clarke 2012). These nematodes are characterized by their symbiotic association with enterobacteria of the genus *Xenorhabdus* sp. and *Photorhabdus* sp. respectively. Their effectiveness as microbial control agents will depend both on the nematode, its ability to locate, recognize and invade the host, and on the virulence of the bacteria in the host. The nematode juvenile infectives invade the host, through the natural openings, spiracles, mouth, and anus, reaches the hemocoel, where the bacteria are released, which multiplies and provide the conditions for the nematode to complete its development, resulting in the death of the host and the release of a new juvenile infective generation (Lewis and Clarke 2012).

Entomopathogenic fungi encompass a broad spectrum of approximately 750 species belonging to 100 different genera that infect insects and other arthropods, such as mites, ticks, and spiders, resulting in observable disease symptoms (Chandler 2017). These fungi have been widely investigated for their potential in biological control of crop and forestry pests, as well as in the control of arthropod vectors of human and animal diseases (Chandler 2017; Quesada-Moraga et al. 2020).

Whilst microbial control of insect pests is among the most viable alternatives to synthetic chemical pesticides, not all entomopathogenic microorganisms invade susceptible hosts in the same manner. Viruses and bacteria must be ingested, while entomopathogenic fungi, with emphasis on hypocrealean ascomycetes, may enter their hosts by direct penetration through the cuticle, which, together with their natural incidence on the pest populations, and their symbiotic and mutualistic relationships with the plants put them at the forefront of the global development, innovation, and discovery of alternative control strategies and as a significant

component of any IPM program (Quesada-Moraga et al. 2020). Hence, fungal biocontrol agents are increasingly important as a permanent component in IPM programs not only of pest of agricultural and forestry importance, but also for pests of medical, veterinary, and urban significance (Quesada-Moraga et al. 2020; Sharma et al. 2020a; Sharma and Sharma 2021; Alfina and Haneda 2022; Sullivan et al. 2022).

I.3. Entomopathogenic fungi in pest control

The entomopathogenic fungi (EPF) represent a diverse group of naturally occurring microorganisms that have gained prominence as IPM biocontrol tools. These fungi infect a broad array of insect pests, offering a sustainable and environmentally friendly alternative to chemical pesticides (Vega et al. 2012; Skinner et al. 2014; Butt et al. 2016; Chandler 2017; Quesada-Moraga et al. 2022). Notably, some EPF have been described as endophytes, either maintaining transient associations with the plants or systemically colonizing them, with even vertical transmission described (Quesada-Moraga et al. 2014; Behie et al. 2015; Butt et al. 2016; Resquín-Romero et al. 2016a; Mattoo and Nonzom 2021).

Hence, EPF are gaining increased attention as sustainable crop protection and production tools (Varma et al. 2017; Zivanovic and Rodgers 2018; Quesada-Moraga et al. 2020, 2022; Quesada Moraga 2020).

I.3.1. Natural presence and diversity

Multiple estimates suggest that there are between 700 and 1000 species of EPF, which account for less than 1% of the total number of described fungal species (Butt et al. 2016; Chandler 2017; Lacey 2017). The EPF are mainly concentrated in four orders, namely Entomophthorales, Neozygitales, Onygenales, and Hypocreales (Charnley and Collins 2007; Vega et al. 2012; Boomsma et al. 2014; Chandler 2017) (Table I.1). Anyhow, most of them are members of the orders Entomophthorales and Hypocreales, the former consisting primarily of fungi with narrow host ranges and the latter having a broad host range within the classes Insecta and Acarina (Vega et al. 2012; Chandler 2017; Lacey 2017).

Table I.1. Classification of entomopathogenic fungi within the phyla Entomophthoromycota and Ascomycota, according to Charnley and Collins (2007), Vega et al. (2012) and Chandler (2017).

Phylum	Class	Order	Family	Genera
Entomophthoromycota	Basidiobolomycetes	Basidiobolales	Basidiobolaceae	*Basidiobolus*
	Neozygitomycetes	Neozygitales	Neozygitaceae	*Apterivorax, Neozygites,* and *Thaxterosporium*
	Entomophthoromycetes	Entomophthorales	Ancylistaceae	*Conidiobolus*
			Entomophthoraceae	Subfamily Erynioideae: *Erynia, Eryniopsis* (in part)*, Furia, Orthomyces, Pandora, Strongwellsea,* and *Zoophthora* Subfamily Entomophthoroideae*: Batkoa, Entomophaga, Entomophthora, Eryniopsis* (in part), and *Massospora*
Ascomycota	Sordariomycetes	Hypocreales	Clavicipitaceae	*Hypocrella, Metacordyceps, Regiocrella, Torrubiella*, *Aschersonia*, Metarhizium*, Nomuraea** and some *Paecilomyces**-like fungi excluded from *Isaria s.s*.
			Cordycipitaceae	*Cordyceps, Torrubiella*, *Beauveria*, Microhilum*, Engyodentium**, *Isaria**, *Mariannaea**-like species, *Akanthomyces* (formerly *Lecanicillium*)* and *Simplicillium**
			Ophiocordycipitaceae	*Ophiocordyceps, Elaphocordyceps*. Entomopathogenic anamorphs: *Haptocillium*, Harposporium*, Hirsutella**, *Hymenostilbe**, some *Paecilomyces**-like species, *Paraisaria**, *Sorosporella*, Syngliocladium** and *Tolypocladium**
	Eurotiomycetes	Onygenales	Ascosphaeraceae	*Ascosphaera*

*Anamorphs.

Although the primary reservoir of EPF is the soil, they also can be found infecting natural insect populations and even in different associations with the plants (Butt et al. 2016; Quesada Moraga 2020). Likewise, *Beauveria* and *Metarhizium* species have been reported as an important component of the soil and rhizosphere microbiota and even of the phylloplane (Hu and St. Leger 2002; Garrido-Jurado et al. 2015; Fernández-Bravo et al. 2016; Vega 2018; Quesada-Moraga et al. 2020; Quesada Moraga 2020).

Entomopathogenic fungi from the phylum Entomophthoromycota are obligate pathogens of arthropods (Hibbett et al. 2007; Vega et al. 2012), having significant importance due to their ability to cause epizootics in mite and insect populations (Vega et al. 2012; Chandler 2017). Cultivating Entomophthoromycota in a controlled environment requires complex media, typically enriched with natural products (Hajek et al. 2012), whereas despite these efforts, achieving typical growth and sporulation is challenging even under these specific conditions. For all that, these fungi are not frequently employed inundatively, as mycoinsecticides, but conservatively, to maintain or enhance native fungal populations in the environment (Chandler 2017; Mora et al. 2018). On the contrary, entomopathogenic ascomycetes can be produced in different artificial media that together with their natural presence, biocontrol potential and new ecological roles make them excellent candidates for IPM and sustainable agriculture (Quesada-Moraga et al. 2022).

I.3.2. Entomopathogenic ascomycetes

Ascomycota stands out as the phylum containing the highest number of EPF species (Vega et al. 2012; Zivanovic and Rodgers 2018). Entomopathogenic ascomycetes, hereinafter referred as EA, belong to the phylum Ascomycota, which is further divided into three subphyla, namely Taphrinomycotina, Saccharomycotina and Pezizomycotina. Among these, Pezizomycotina is the most numerous and exhibits both morphological and ecological complexity (Vega et al. 2012). The most widespread insect pathogenic fungal genera are found in the order Hypocreales (Pezizomycotina: Sordiaromycetes), divided into three families, namely, Clavicipitaceae, Cordycipitaceae, and Ophiocordycipitaceae (Charnley and Collins 2007; Vega et al. 2012; Chandler 2017; St. Leger and Wang 2020) (Table I.1). Several hypocrealean species from genera like *Beauveria, Metarhizium* and *Akanthomyces*

(formerly *Lecanicillium*) have been successfully used in plant protection and are more readily cultured *in vitro* (Charnley and Collins 2007; Quesada-Moraga et al. 2020; Chaudhary et al. 2022).

I.3.2.1. Mode of action

The typical route of host invasion involves the arthropod cuticle (Charnley and Collins 2007; Vega et al. 2009; Lacey 2017). The process of host invasion and development in EA generally unfolds through several stages, including conidial adhesion of specialized fungal structures to the cuticle, germination, the formation of infection structures, penetration, colonization of the hemocoel, and finally, sporulation (Boomsma et al. 2014; Butt et al. 2016; Aw and Hue 2017; Quesada-Moraga et al. 2020). In essence, the mode of action of EA integrates enzymatic precision, a diverse array of bioactive compounds, and strategic infection routes, offering a sophisticated and environmentally sustainable solution for insect pest control. EA exhibit a distinctive approach in their mode of action, strategically navigating these various steps to effectively colonize and kill insects. A pivotal element of their strategy involves the targeted release of enzymes, specifically designed to degrade the insect cuticle, a protective barrier that the fungi must overcome for successful invasion (Charnley and Collins 2007; Vega et al. 2012; Skinner et al. 2014; Boomsma et al. 2014; Mora et al. 2018). Enzymes such as proteases and chitinases are essential in breaking down the cuticle and enabling the fungi to enter the insect host (Butt et al. 2016; Chandler 2017).

Upon contact with the insect host, the fungal spores show a high affinity for the insect cuticle, starting the adhesion process. Once firmly attached, the fungi penetrate the insect cuticular barrier. Within the host, the hyphal bodies and/or hyphae rapidly multiply in the hemocoel, in which a diverse array of bioactive compounds are released, including secondary metabolites (Vega et al. 2012; Lacey 2017). These compounds play a pivotal role in disrupting the host physiological processes, ultimately leading to the insect death (Charnley and Collins 2007; Chandler 2017; Quesada-Moraga et al. 2020). Notably, EA, such as those belonging to the *Beauveria* and *Metarhizium* genera, employ advanced physiological mechanisms to facilitate host invasion (Butt et al. 2016; Dubovskiy et al. 2022). Apart for the typical integumentary mode of action, more recently there have been described other alternative infection

routes most of them with the fungal pathogen entering through the oral cavity (Mannino et al. 2019) (Figure I.1).

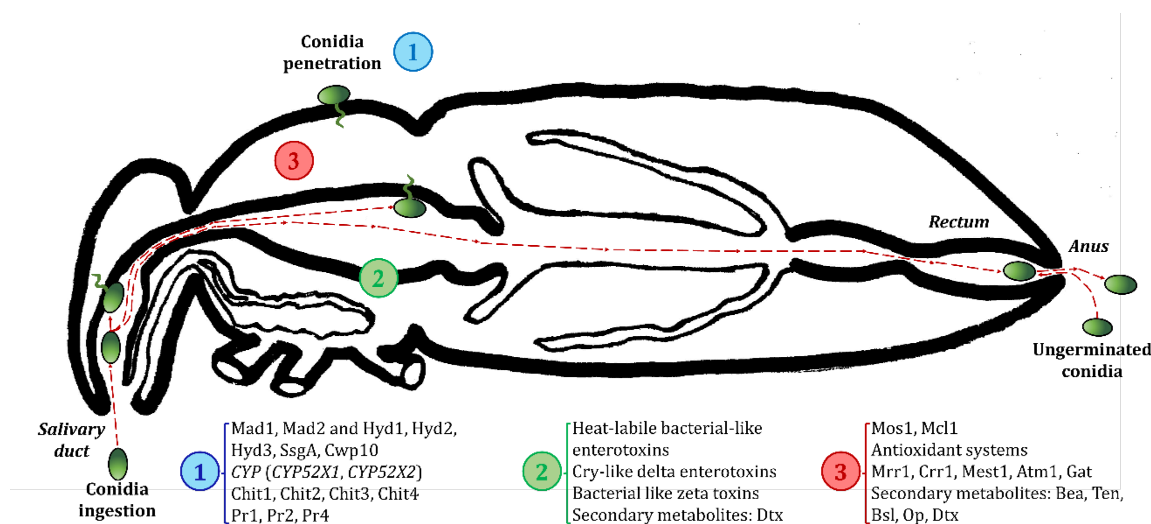

Figure I.1. Different pathways through which an entomopathogenic fungus can enter a host. The fungal genes involved in conidial penetration through the cuticle are shown in blue (1); the fungal genes proposed to participate in oral infection are shown in green (2); and the fungal genes expressed into the hemolymph are shown in red (3). Diagram adapted from Mannino et al. (2019). Original insect scheme based on Gullan and Cranston (2014).

I.3.2.2. Ecology

The fungal kingdom is extremely diverse, and it is challenging to find ecosystems in which fungi do not play a role (Vega et al. 2009; Araújo and Hughes 2016). The EA are no exception, they are integral components of complex ecological networks that are vital for maintaining ecosystems health and their interactions with plants and herbivores have particular significance. These organisms have coevolved over time, and their intertwined histories are essential for understanding the functioning of ecosystems (Ownley et al. 2010; Vega et al. 2012; Araújo and Hughes 2016; Naranjo-Ortiz and Gabaldón 2019; Quesada-Moraga et al. 2020). In this sense, the EA are important elements of the microbiota of agricultural and forest ecosystems where they contribute to the regulation of insect and mite populations (Meyling and Eilenberg 2007; Vega et al. 2012; Quesada Moraga 2020).

The EA show remarkable competence in the rhizosphere, with the soil serving as a primary reservoir and a center of ecological resilience, in which the effects of these fungi intersect with the complex web of subsurface interactions (Meyling and

Hajek 2010; Fernández-Bravo et al. 2016; Majchrowska-Safaryan and Tkaczuk 2021; Qayyum et al. 2021). The rhizosphere, which serves as the dynamic interface between soil and roots, functions as a strategic refuge for EA and fosters interactions that extend beyond the realm of insect (Meyling and Eilenberg 2007; Cachapa et al. 2021; Moustaka et al. 2022). As soil-dwelling microorganisms, EA establish complex relationships with plant roots, exhibiting dynamic ecological relationship. This adaptability within the rhizosphere not only makes EA effective biocontrol agents against insect pests but also highlights their potential contribution to plant health and soil ecology (Meyling and Eilenberg 2007; Garrido-Jurado et al. 2011; Cachapa et al. 2021; Moustaka et al. 2022).

Climatic factors, such as temperature and humidity, play a significant role in EA abundance and activity (Jaronski 2007, 2010; Quesada-Moraga et al. 2007, 2020; Bamisile et al. 2021). In adequate situations, these factors could facilitate initiating epizootics, highlighting the importance of suitable environmental conditions for the proliferation and persistence of EA (Jaronski 2010; Qayyum et al. 2021a). The pH of soil and organic matter content also determine the survival and persistence of fungi in the soil environment together with the microbiota (Quesada-Moraga et al. 2007). Likewise, EA distribution is broadly influenced by vegetation type and land use activities (Quesada-Moraga et al. 2007; Qayyum et al. 2021b). The EA *B. bassiana* and *M. anisopliae* are equally isolated in natural and cultivated soils (Quesada-Moraga et al. 2007), whereas orchards or cultivated lands seem to show higher diversity index for both fungal species than mountainous non-cultivated lands (Qayyum et al. 2021b). In this regard, it is critical to consider the general environmental variables mentioned above when EA are used as pest control agents.

I.3.2.3. Commercial development of entomopathogenic ascomycetes

Based on around twelve EA species, a total of 171 mycoinsecticides and mycoacaricides have been formulated and registered (Jiang and Wang 2023). Out of all these commercial products, 80% are *Metarhizium*- and *Beauveria*- based formulations (Vega et al. 2012; Butt et al. 2016; Jiang and Wang 2023). According to recent reports, mycoinsecticides primarily based upon species from the genera *Beauveria*, *Metarhizium*, *Isaria*, and *Akanthomyces* (formerly *Lecanicillium*), are being developed commercially and are becoming increasingly widely used worldwide (Vega

et al. 2012; Chandler 2017; Ruiu 2018; Quesada-Moraga et al. 2020, 2023; Baron and Rigobelo 2022; Jiang and Wang 2023) (Table I.2).

Table I.2. Hypocrealean fungi species with their respective strains which have been developed as mycoinsecticides. Adapted from Jiang and Wang (2023) and Quesada-Moraga et al. (2023).

Species	**Strains**	**Target pests**
Akanthomyces muscarius (formerly *Lecanicillium muscarium*)	Ve6 (ARSEF 5128)	Whiteflies, trips
Beauveria bassiana	ANT-03, ATCC 74040, Bb 10, Bb 9205, CFL-A, CG-716, ESALQ-PL63, GHA (ARSEF 6444), HF23, IBCB 66, IMI389521, K4B3, NCIM 1216, NPP111B005, PPRI 5339, R444, Strain 147, Strain 203, Strain 203, Strain 447, ZJU435	Ants, aphids, noctuids and other moths, scarab and other Coleoptera pests, foliar-feeding pests and certain grubs, houseflies, thrips, whiteflies and certain piercing, sucking, and chewing pests (insects and mites)
Cordyceps fumosorosea, formerly *Isaria fumosorosea*	Apopka 97 (PFR97, ATCC 20874), FE 9901	Aphids, whiteflies, spider mites, thrips, weevils
Cordyceps javanica, formerly *Isaria javanica*	ESALQ-1296, Ij01, JS001	Noctuids and whiteflies
Metarhizium acridum (syn. *Metarhizium anisopliae* var. *acridum*)	IMI 330189	Locusts
Metarhizium anisopliae	ESALQ E9 (ARSEF 925), ESF1, CQMa421	Thrips, locusts, noctuids and other moths
Metarhizium brunneum	ATCC 90448, BIPESCO 5 (F52, Met52, Ma 43, ARSEF 7711)	Scarab beetle pests, weevils, thrips, ticks, whiteflies and mites

The success of EA in microbial pest control, as highlighted by Quesada-Moraga et al. (2023), hinges on their environmental competence adaptability. Whilst factors such as UV radiation and humidity are key for fungal propagule depletion and inactivation in epigeal habitats, temperature is most critical for reducing the infectivity and virulence of EA in epigeal and hypogeal habitats (Quesada-Moraga et al. 2023). Additional factors such as geographical origin of the strain, other biotic and abiotic factors, and difficulties associated with application in the field, can have an important impact which may guarantee the environmental competence of selected entomopathogenic fungal strains and, therefore, farmer willingness to replace chemicals with mycoinsecticides (Quesada-Moraga et al. 2020, 2023, 2024; Baron and Rigobelo 2022).

Biopesticides capture a small portion of the market, constituting only 5–6% of the worldwide pesticide mark with a value of $3 billion worldwide (out of $50 billion) (McDougall 2018; Bremmer et al. 2021; Smit et al. 2021), whereas the biopesticide

market is steadily growing at a rate of 15-16% and there are expectations that by 2050, it will reach a similar position to that of the synthetic pesticides market (McDougall 2018; Quesada-Moraga et al. 2020; Smit et al. 2021). Mycoinsecticides currently hold a prominent position in sales of biopesticides (Jiang and Wang 2023) and they are gaining progressive farmers confidence, despite limitations such as cumbersome and risk-averse regulatory processes, increased bureaucratic obstacles, and insufficient commitment and communication among stakeholders (McDougall 2018; Buckwell et al. 2020; Bremmer et al. 2021).

It is increasingly important to promote the development of microbial control solutions adapted to relatively uniform climatic zones through more simplified, targeted, and less costly EA approval and authorization (Quesada-Moraga et al. 2023). Interestingly, the use of EA as plant endophytes, little explored to now, could overcome several barriers that have traditionally limited their widespread use in plants, with even greater benefits for those that can be transmitted vertically (Bamisile et al. 2021; Baron and Rigobelo 2022).

I.4. Relationships between entomopathogenic ascomycetes and plants

Entomopathogenic ascomycetes have been reported to have different associations with plants in the phylloplane, even as epiphytes, as plant endophytes and/or as rhizosphere competent microorganisms (Figure I.2) (Garrido-Jurado et al. 2015; Quesada Moraga 2020). Indeed, the natural endophytic capacity of EA, either transiens or systemic, which has been verified both under natural conditions and artificially through plant spray, soil treatment and seed dressing, has been successfully used in the systemic protection of crops against chewing, boring and sucking insect pests (Quesada-Moraga et al. 2019). Moreover, EA offer a variety of multifaceted indirect benefits to their crop plant hosts of enormous significance for crop production and protection (Quesada-Moraga et al. 2019; Quesada Moraga 2020; González-Guzmán et al. 2022) (Figure I.2). Thus, a new horizon opens in which elucidation of the effects of EA requires complete information about the benefits offered to the plant, not only in terms of its protection from pests, but also in terms of plant growth and response to others biotic and abiotic stresses (Quesada-Moraga et al. 2019; Quesada Moraga 2020).

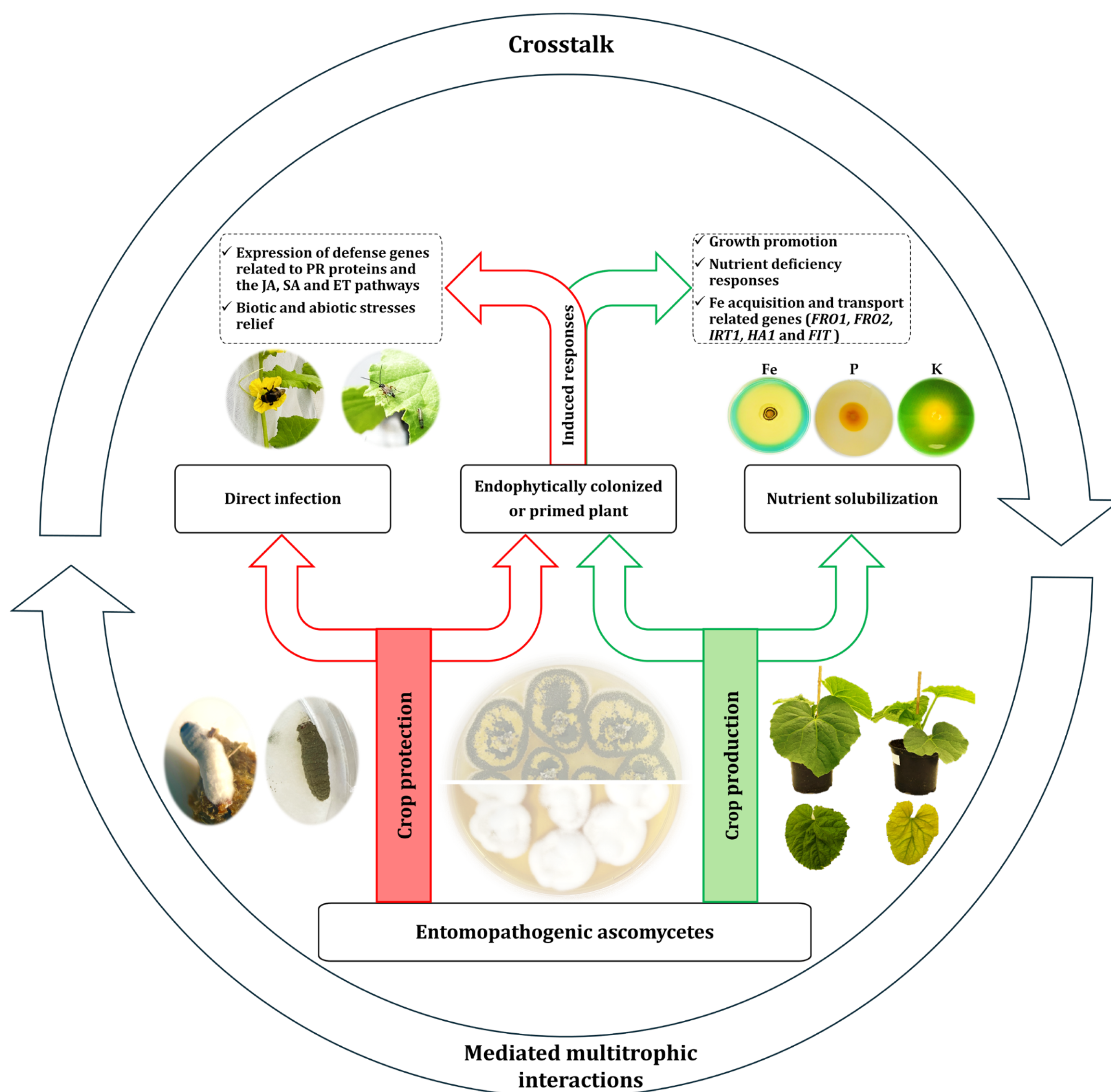

Figure I.2. The associations between entomopathogenic ascomycetes and plants and their impact on crop protection and production. Abbreviations: ET, ethylene; JA, jasmonic acid; SA, salicylic acid; PR pathogenesis related proteins; Fe, iron; P, phosphorus; K, potassium. *FRO1, FRO2, IRT1, HA1* and *FIT* are the main genes related to acquisition and transport of iron in dicot plants. Adapted from Quesada-Moraga (2020). Photos by Fabián García-Espinoza.

The signaling pathways involved in these interactions remain a focal point of investigation as researchers explore the potential for harnessing these relationships for sustainable pest management in agriculture. Understanding the multifaceted connections between EA and plants holds promise for developing novel, eco-friendly strategies that benefit both crop health and insect control in agroecosystems (Quesada Moraga 2020; Mattoo and Nonzom 2021; Kamran et al. 2022; Samanta et al. 2023; Suryanarayanan 2023).

I.4.1. Impact of entomopathogenic ascomycetes-plant relationships on arthropod pest control

The interactions and association between EA and plants have become a cornerstone in developing sustainable and effective approaches for arthropod pest control (Vega et al. 2012; Vega 2018; González-Mas et al. 2019c; Quesada Moraga 2020). It has been proposed that plants have evolved mechanisms to favor/retain the natural enemies of their herbivore pests and thereby protect themselves from damage (Vega et al. 2009). Hence, a recent study with a tritrophic system, which includes cabbage plants, the insect *Delia radicum* L. (Diptera: Anthomyiidae) and *M. brunneum* as root associated EA, demonstrate how the fungus significantly altered host plants traits used by *D. radicum* in their host plant selection (Cotes et al. 2020). Through these interactions, the EA have displayed the intricate and dynamic relationships with plants, exhibiting their potential as valuable tools in IPM strategies and sustainable agricultural practices.

Studies have demonstrated that certain EA show a positive impact on plant immunity and protection against generalist arthropod pests (Table I.3), as well as resistance to another biotic stresses including several diseases mainly caused by plant pathogenic fungi, nematodes and bacteria (Table I.4), showcasing the EA capability to control microorganisms that pose challenges to important crops, while protecting the plant against arthropod pests by the induction of systemic resistance (Jaber and Ownley 2018; Rondot and Reineke 2019; Kumari et al. 2021; Sinno et al. 2021; Gupta et al. 2022b; Posada-Vergara et al. 2022, 2023; Iida et al. 2023; Kinyungu et al. 2023).

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Table I.3. Indirect effects on arthropod pests and physiological responses in crop plants after entomopathogenic fungi application. Adapted from Quesada-Moraga et al. (2023).

Entomopathogenic fungal species	Plant	Physiological response and effects as result of the plant-fungi interaction	References
Beauveria bassiana	*Arachis hypogaea* L., *Cucumis melo L., Gossypium hirsutum* L., *Solanum lycopersicum* L., *Solanum tuberosum* L., *Triticum aestivum* L., *Vicia faba* L., *Phaseolus vulgaris* L. and *Zea mays* L.	Activation of enzymatic and nonenzymatic defense reactions; affects plant volatile emissions; defense response changed, similar to induced systemic resistance; enhance the expression of defense enzymes and pathogenesis-related proteins; increase production of terpenoids; promote systemic immunity and confer resistance; significantly enhanced the expression of genes involved in antioxidants production and JA biosynthesis cascade. Affecting the survival, development and reproduction of *Tetranychus urticae* Koch (Acari: Tetranychidae). Negatively affect survival of the cotton bollworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae). Negatively affect survival of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). Production of plant secondary metabolites is associated with a reduction of aphid population.	Shrivastava et al. 2015a; Lopez and Sword 2015; Dash et al. 2018; Jensen et al. 2020; Rasool et al. 2021; González-Mas et al. 2021; Tomilova et al. 2021; Gupta et al. 2022b; González-Guzmán et al. 2022; Batool et al. 2022
Cordyceps farinosa [formerly *Isaria farinosa*]	*P. vulgaris*	Affecting the survival, development and reproduction of *T. urticae*	Dash et al. 2018
Akanthomyces lecanii (formerly *Lecanicillium lecanii*)	*S. lycopersicum* and *P. vulgaris*	Strongly enhance the SA associated genes *PR1*, *BGL2*, *AOS*, *PAL*, *LOX* and *AOC*, indicating the enhancement of systemic resistance in plant. Stimulation of defense mechanisms. Lowest survival of green peach aphid. Affecting the survival, development and reproduction of *T. urticae.*	Dash et al. 2018; Hanan et al. 2020
Metarhizium brunneum	*Brassica oleracea* L., *Brassica napus* L., *G. hirsutum*, *S. lycopersicum* L., *T. aestivum* and *Sorghum bicolor* L.	Increase singlet oxygen ($^1O_2$), modulate the leaflets' response to herbivory, effect on the photosynthetic efficiency before herbivory. Priming the plant defense, increase in myrosinase activity upon herbivory. Promote Systemic Immunity and Confer Resistance. Production of plant secondary metabolites is associated with a reduction of aphid population. This species of fungus infects various life stages of the cabbage root fly and readily colonizes available plant roots as well as fungal colonization primed herbivore-induced JA and the expression of the JA-responsive plant defensin 1.2 (PDF1.2) gene in *B. napus.*	Cachapa et al. 2021; Rasool et al. 2021, 2023; Moustaka et al. 2022; Gupta et al. 2022b; González-Guzmán et al. 2022
Metarhizium robertsii	*Arabidopsis* sp., *S. tuberosum*, and *Z. mays*	Activation of enzymatic and nonenzymatic defense reactions. Altered defense gene expression in maize. Induces auxin-inducible gene expression. Production of plant secondary metabolites is associated with a reduction of aphid population.	Liao et al. 2017; Ahmad et al. 2020; Rasool et al. 2021; Tomilova et al. 2021
Purpureocillium lilacinum	*G. hirsutum*	Negatively affect survival of the cotton bollworm (*H. zea*).	Lopez and Sword 2015

Table I.4. Effects of entomopathogenic fungi and physiological response in crop plants against diseases and nematodes. Adapted from Quesada-Moraga et al. (2023).

Entomopathogenic fungal species	Plant	Physiological response and effects as result of the plant-fungi interaction	Reference
Beauveria bassiana	*S. lycopersicum, Cucumis sativus* L., *C. melo, Fragaria* × *ananassa* Duch., *Solanum melongena* L., *T. aestivum* and *Vitis vinifera* L.	Promote systemic immunity and confer resistance. Induced expression of SA–related genes. Improved plant protection against the phytopathogen. Reduced lesion sizes of *Botrytis cinerea* Whetzel on inoculated tomato leaves. Growth inhibition of *Rhizoctonia solani* Kühn by direct and indirect effects. Growth inhibition of *Podosphaera xanthii* (Castagne) U. Braun y Shishkoff. Disease severity caused by *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni and by *Fusarium culmorum* (Wm.G.Sm.) Sacc was significantly reduced in *B. bassiana*-treated plants. Effective biocontrol agents against *B. cinerea* and *Alternaria alternata*. *Beauveria bassiana* was efficient to control *Meloidogyne incognita* (Kofoid & White).	Jaber 2018; Rondot and Reineke 2019; Tomilova et al. 2020; Youssef et al. 2020; Sinno et al. 2021; Gupta et al. 2022b; Zitlalpopoca-Hernandez et al. 2022; Torkaman et al. 2023; Iida et al. 2023
Metacordyceps chlamydosporia	*S. lycopersicum*	Stimulation of defense mechanisms. Changes in the enzymatic activities of the polyphenol oxidase (PPO), peroxidase (POD), ascorbate peroxidase (APX), catalase (CAT), lipid peroxidation (MDA) phenols, and proteins content. Significantly reduced the gall index and female fecundity of root knot-nematode.	Hajji-Hedfi et al. 2022
Metarhizium anisopliae	*Vigna unguiculata* (L.) Walp.	*Metarhizium anisopliae* as antagonistic fungus proved to be efficient against *M. incognita.*	Youssef et al. 2020
Metarhizium brunneum	*B. napus, T. aestivum* and *S. lycopersicum*	Inhibited the in vitro growth of *Verticillium longisporum* (C. Stark) Karapapa, Bainbr. & Heale and changed the plant response to the pathogen by locally activating key defense hormones in the SA and abscisic acid (ABA) pathways. Improved plant protection against the phytopathogen. Reduced lesion sizes of *B. cinerea* on inoculated tomato leaves. Disease severity caused by *F. culmorum* was significantly reduced in *M. brunneum*-treated plants. Induces ISR.	Jaber 2018; Gupta et al. 2022b; Zitlalpopoca-Hernandez et al. 2022; Posada-Vergara et al. 2023
Metarhizium robertsii	*S. lycopersicum*	Improved plant protection against the phytopathogen. Growth inhibition of *R. solani* by direct and indirect effects. Reduced lesion sizes of *B. cinerea* on inoculated tomato leaves. Modulated the expression of defense genes and the phytohormone content in maize inoculated with *Cochliobolus heterostrophus* (Drechsler) (Pleosporales: Pleosporaceae) Drechsler. Growth inhibition of *R. solani* by direct and indirect effects.	Tomilova et al. 2020; Ahmad et al. 2022; Zitlalpopoca-Hernandez et al. 2022
Paecilomyces lilacinus	*V. unguiculata*	*Paecilomyces lilacinus* as antagonistic fungus proved to be efficient against *M. incognita.*	Youssef et al. 2020

I.4.2. Impact of entomopathogenic ascomycetes-plant relationships on the plant response to abiotic stresses

To now, there are few reports on the mutualistic interactions of EA, notably *Metarhizium* and *Beauveria* genera, with crops, enhancing plant nutrient acquisition and mitigating abiotic stresses such as drought and salinity (Khan et al. 2012, 2015; Ferus et al. 2019; Kumar and Nautiyal 2022; Chaudhary et al. 2023) (Table I.5).

Table I.5. Protective effect of endophytic and/or rhizosphere competent entomopathogenic ascomycetes against plant abiotic stresses. Adapted from Quesada-Moraga et al. (2023).

Abiotic stress	Entomopathogenic fungal species	Plants	References
Drought and salinity	*B. bassiana, Beauveria vermiconia, M. anisopliae, Metarhizium pinghaense Metarhizium* aff. l*epidiotae* and *Neotyphodium lolii*	*Quercus rubra* L., *Z. mays*, *Glycine max* (L). Merr., *S. lycopersicum* and *Lolium perenne* L.	Kunkel and Grewal 2003; Khan et al. 2012; Ferus et al. 2019; Kuzhuppillymyal-Prabhakarankutty et al. 2020; Vera et al. 2022; Chaudhary et al. 2023
Soil fertility and nutrients	*M. brunneum*	*Lupinus albus* L., *S. bicolor*, *T. aestivum, Triticum durum* L., *Helianthus annuus* L., *S. tuberosum*	Sánchez-Rodríguez et al. 2016; Krell et al. 2018; González-Guzmán et al. 2020

I.4.3. Impact of entomopathogenic ascomycetes-plant relationships on plant growth

Beyond their role as biological control agents, EA exhibit the capacity to enhance plant growth through the production of secondary metabolites, proteins, and various mechanisms to generate bioavailable forms of nutrients not naturally present (Moonjely et al. 2016; Barelli et al. 2016; Jaber and Enkerli 2017; Raya-Díaz et al. 2017b; Sánchez-Rodríguez et al. 2018; Stone and Bidochka 2020; Priyashantha et al. 2023; Zheng et al. 2023). Additionally, their contribution to plant development extend to indirect mechanisms, such as the production of phytohormones associated with plant defense and development (Jaber and Enkerli 2017; Baron et al. 2020; Sinno et al. 2021; Baron and Rigobelo 2022; Ahmad et al. 2022; González-Guzmán et al. 2022; Chaudhary et al. 2023; Zheng et al. 2023; Sui et al. 2023; Kinyungu et al. 2023) (Table I.6).

In this regard, iron chlorosis is a serious crop production problem in many calcareous soils of Southern Spain and taken into account that some reports indicate that *B. bassiana* is a good producer of siderophores (Barra-Bucarei et al. 2020), while

others suggest that some species of *Metarhizium* are not (Ghosh et al. 2017), it should be unraveled the possible role of EA on improving the solubility of Fe in soils and plant Fe nutrition, together with the direct and indirect mechanisms of the possible EA alleviation of Fe chlorosis in crop plants.

Whilst the possible positive impact of multifunctional EA in plant growth has been reported, it has not yet been evaluated under real greenhouse conditions including multitrophic systems with crop plants, EA, insect pests and natural enemies.

Table I.6. Entomopathogenic fungi-related growth promotion activities. Adapted from Quesada-Moraga et al. (2023).

Entomopathogenic fungal species	Plant	Growth promotion in terms of	References
Akanthomyces muscarius (formerly *Lecanicillium muscarium*)	*Capsicum annuum* L.	Plant height, stem diameter, number of leaves, canopy area, and plant weight	Wilberts et al. 2023a
Beauveria bassiana	*A. hypogaea*, *C. annuum*, *C. melo*, *Fragaria* sp., *G. max*, *G. hirsutum*, *Musa* spp., *P. vulgaris*, *S. lycopersicum*, *S. tuberosum*, *S. bicolor*, *Triticum* spp., *T. aestivum*, *T. durum*, *V. faba* and *Z. mays*	Aerial and root biomass, total surface area of leaves, blade width, canopy area, chlorophyll content, counts of stolons and leaves, dry biomass, early flowering fresh and dry shoot weight, fresh and dry weight, fresh root weight, fresh shoot weight, fresh weight, germinative capacity, ground fresh weight, leaf length and width, leaf length, leaf number, leaf surface area, leaf thickness, length of shoots and roots, number of branches per plant, number of leaf pairs, number of leaves, number of nodes, number of pods per branch, number of pods per plant, number of seeds per branch, number of seeds per pod, number of the pods per plant, nutrient concentrations, plant length, root architecture, root dry matter, root length, root surface area, seed weight per branch, seed weight per plant, seedling emergence, shoot biomass, shoot length, stem diameter, tillers, total dry matter, total fresh weight, underground fresh weight, vigor index, weight of the pods per plants, weight of the pods per branch, yield.	Akello et al. 2007; Senthilraja et al. 2013; Lopez and Sword 2015; Jaber and Enkerli 2016; Raya-Díaz et al. 2017b; Jaber 2018; Jaber and Araj 2018; Sánchez-Rodríguez et al. 2018; Tall and Meyling 2018; Dash et al. 2018; Russo et al. 2019; González-Guzmán et al. 2020; González-Guzmán et al. 2020; Barra-Bucarei et al. 2020; Tomilova et al. 2020, 2021; Silva et al. 2020; Kuzhuppillymyal et al. 2021; Gonzalez-Guzman et al. 2021; Sinno et al. 2021; Liu et al. 2022; González-Guzmán et al. 2022; Zitlalpopoca-Hernandez et al. 2022; Mantzoukas et al. 2022; Kramski et al. 2023; Torkaman et al. 2023; Wilberts et al. 2023a; Zheng et al. 2023; Sui et al. 2023; Kinyungu et al. 2023
Beauveria brongniartii	*V. faba*	Plant height, leaf pair number, fresh shoot and root weights	Jaber and Enkerli 2017

Table I.6. *Continued.*

Entomopathogenic fungal species	Plant	Growth promotion in terms of	References
Cordyceps fumosorosea	*S. lycopersicum* and *S. melongena*	Canopy area, seed germination, root length, shoot length, number of fresh leaves, the diameter of the stem, plant dry weight, root dry weight, shoot dry weight, and leaf dry weight	Sun et al. 2020; Wilberts et al. 2023a; Zheng et al. 2023
Cordyceps farinosa (formerly *Isaria farinosa)*	*S. bicolor*	Plant height, number of leaves and leaf chlorophyll concentration	Raya-Díaz et al. 2017b
Isaria fumosorosea	*G. hirsutum* and *P. vulgaris*	Leaf chlorophyll, plant height, fresh shoot weight, fresh root weight	Dash et al. 2018; González-Mendoza et al. 2019
Akanthomyces lecanii (formerly *Lecanicillium lecanii*)	*P. vulgaris*	Plant height, fresh shoot weight, fresh root weight	Dash et al. 2018
Metarhizium acridum	*P. vulgaris*, *Panicum virgatum*, *T. aestivum* and *G. max*	Leah weight, root weight, whole-plant weight. Transfer insect-derived nitrogen to plant	Behie and Bidochka 2014
Metarhizium anisopliae	*Arabidopsis thaliana* (L) Heynh., *G. max*, *Oryza sativa* L., *S. lycopersicum* and *Z. mays*	Blade width, chlorophyll contents, foliar growth, germination rate, ground fresh weight, leaf area, leaf collar, leaf length, leaf number, number of branches per plant, number of collar, number of corncobs, number of pods per branch, number of seeds per branch, number of seeds per pod, number of the pods per plant, photosynthesis rate, plant fresh weight, plant height, root dry weight root length, seed weight per branch, seed weight per plant, yield, shoot fresh and dry weight, shoot length, stalk fresh weight, stalk length, stand density, total fresh weight, transpiration rate, underground fresh weight, weight of corncobs, weight of the pods per plants, weight of the pots per branch, grain yield	Kabaluk and Ericsson 2007, Garcia et al. 2011, Khan et al. 2012, Liao et al. 2014, Russo et al. 2019, González-Pérez et al. 2022, Zheng et al. 2023

Table I.6. *Continued.*

Entomopathogenic fungal species	Plant	Growth promotion in terms of	References
Metarhizium brunneum	*C. annum*, *Coffea arabica* L., *G. max*, *H. annuus*, *P. virgatum*, *P. vulgaris*, *S. lycopersicum*, *S. tuberosum*, *S. bicolor*, *T. aestivum*, *T. durum*, *V. faba,* and *Z. mays*	Chlorophyll, foliar growth, fresh root weight fresh shoot weight, germination rate, grain yield, plant height, inflorescence production, chlorophyll, leaf collar, leaf number, leaf pair number, leaf surface area, leaf surface area, leaf thickness, leaf weight, number of collar, number of corncobs, nutrient concentrations, plant dry weight, plant height, root and shoot biomass, root architecture, root dry matter, root length, root length, root surface area, seedling emergence, shoot height, stalk fresh weight, stalk length, tillers, total dry matter, transfer insect-derived nitrogen to plant and weight of corncobs	Liao et al. 2014; Behie and Bidochka 2014; Jaber and Enkerli 2016, 2017; Raya-Díaz et al. 2017a, b; Jaber 2018; Jaber and Araj 2018; Krell et al. 2018; González-Guzmán et al. 2020; Gonzalez-Guzman et al. 2021; González-Guzmán et al. 2021, 2022; Zitlalpopoca-Hernandez et al. 2022; Franzin et al. 2022
Metarhizium flavoviride	*P. vulgaris*, *P. virgatum*, *S. lycopersicum T. aestivum* and *G. max*	Leah weight, root weight, whole-plant weight. Transfer insect-derived nitrogen to plant	Behie and Bidochka 2014; Zheng et al. 2023
Metarhizium guizhouense	*P. vulgaris*, *P. virgatum*, *T. aestivum* and *G. max*	Leah weight, root weight, whole-plant weight. Transfer insect-derived nitrogen to plant	Behie and Bidochka 2014
Metarhizium pinghaense	*S. lycopersicum*	Germination percentage, plant height, vigor index, fresh and dry seedling weight	Chaudhary et al. 2023

Table I.6. *Continued.*

Entomopathogenic fungal species	Plant	Growth promotion in terms of	References
Metarhizium robertsii	*Arabidopsis* sp., *C. arabica*, *G. max*, *P. vulgaris* L., *P. virgatum*, *T. aestivum*, *S. lycopersicum, S. tuberosum*, *S. bicolor* and *Z. mays*	Counts of stolons and leaves, foliar growth, fresh and dry weight, fresh weight, germination rate, lateral root growth, leaf chlorophyll, leaf collar, leaf weight, coffee leaf area, length of shoots and roots, number of branches per plant, number of collar, number of corncobs, number of lateral roots emerged, number of pods per branch, number of seeds per branch, number of seeds per pod, number of the pods per plant, plant length, root and shoot biomass, root hair density, root hair development, root hair length, root hair number, root length, seed weight per branch, seed weight per plant, stalk fresh weight, stalk length, total surface area of leaves, transfer insect-derived nitrogen to plant, weight of corncobs, weight of the pods per plants, weight of the pots per branch and yield	Sasan and Bidochka 2012; Behie et al. 2012; Liao et al. 2014, 2017; Behie and Bidochka 2014; Russo et al. 2019; Mantzoukas and Grammatikopoulos 2020; Tomilova et al. 2020, 2021; Zitlalpopoca-Hernandez et al. 2022; Franzin et al. 2022
Metarhizium rileyi	*S. lycopersicum*	Plant height, the diameter of the stem, aboveground and belowground biomass	Zheng et al. 2023
Purpureocillium lilacinum	*G. hirsutum*	Dry biomass, number of nodes	Lopez and Sword 2015
Akanthomyces lecanii (formerly *Verticillium lecanii*)	*G. hirsutum*	Leaf chlorophyll	González-Mendoza et al. 2019

I.4.4. Entomopathogenic ascomycetes as inducers of systemic resistance

Systemic Resistance (SR) is a mechanism by which susceptible plants, following a primary infection by mutualistic microorganisms, microbial pathogens or herbivory, develop enhanced resistance to further attacks (Conrath 2006; Newman et al. 2013; Pieterse et al. 2014; Wang et al. 2020a; Yu et al. 2022; Adeleke et al. 2022; Salwan et al. 2023). The SR can be categorized into Induced Systemic Resistance (ISR), triggered by root-colonizing mutualistic microbes (including mycorrhizal fungi and rhizobacteria), Systemic Acquired Resistance (SAR), triggered by plant pathogens; and Systemic Wound Response (SWR), induced by herbivore attack (Pieterse et al. 2014; Zivanovic and Rodgers 2018; Hilleary and Gilroy 2018; Romera et al. 2019; Zehra et al. 2021; Yu et al. 2022) (Figure I.3). ISR typically depends on ethylene (ET) and/or jasmonate (JA) (Verhagen et al. 2004; Pieterse et al. 2009). However, some ISR inducers can also activate a salicylic acid (SA)-dependent pathway, suggesting the operation of different signaling pathways during ISR elicitation (Ryu et al. 2003; Niu et al. 2011; Aswani and Radhakrishnan 2022; Aswani et al. 2022) (Figure I.3).

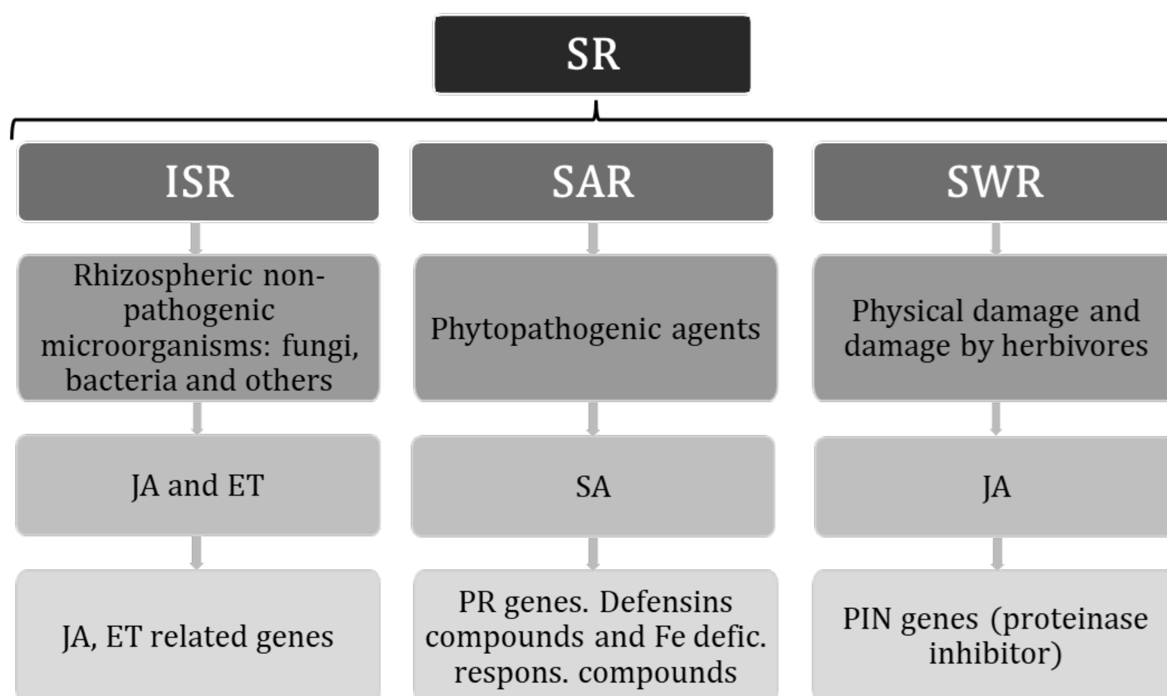

Figure I.3. The systemic resistance (SR) could be categorized into Induced Systemic Resistance (ISR), Systemic Acquired Resistance (SAR) and Systemic Wound Response (SWR), being each of them triggered by different elicitors and phytohormones, such jasmonic acid (JA), ethylene (ET), salicylic acid (SA) and the pathogenesis related proteins (PR).

It is known that endophytic colonization can induce systemic responses in the plant, whereas information on the possible activation of any SR by EA on crop plants together with its possible impact on the response of the plant towards insect pests is scarce (Rondot and Reineke 2019; Ahmad et al. 2020; Posada-Vergara et al. 2022; González-Guzmán et al. 2022). Understanding the possible existence of SR induced by EA together with its impact on the response of the plant to biotic and abiotic stresses will inform the development of new EA-based strategies for efficient biological pest control and sustainable agriculture (Jaber and Ownley 2018; St. Leger and Wang 2020).

To this end, it is important to investigate whether EA induce the expression of genes involved in both ISR and SAR responses in crop plants and to which extend such induced response impact the survival and fitness of key insect pests. Moreover, in order to optimize the impact of such EA-plant relationship, it is a key aim evaluating the effect of the fungal isolate and inoculation method on the possible EA-mediated plant defense against insect pests in the scenario of the above-mentioned iron chlorosis problem in many soils. For that, it is a key issue to unravel whether the ability of EA to induce defense responses in crop plants is influenced by possible iron nutritional deficiencies, and therefore, to highlight the cross talk among biotic and abiotic stresses.

I.4.4.1. Priming by entomopathogenic ascomycetes

Defense priming represents a physiological “state of readiness” that enhances the plant response to subsequent attacks, making it more effective compared to individuals that were not previously induced. This primed state allows plants to mitigate the defense associated costs (Karasov et al. 2017; Wilkinson et al. 2019; López Sánchez et al. 2021). As Tiwari and Singh (2021) assert, it can be likened to a "green vaccination", in sum, a strategy for crop protection that influences the plant immune capacity.

Primed plants exhibit the ability to induce defenses earlier, faster, and more efficiently in response to subsequent stress events (Hilker et al. 2016; Desmedt et al. 2021). Several studies have demonstrated the effects of EA on enhancing the plants defense system and their direct or indirect impact on pests. In many cases, the effects

on pests are attributed to the endophytic colonization by fungi like *B. bassiana* and *M. brunneum* (Jensen et al. 2020; Gupta et al. 2022b; Zheng et al. 2023; Posada-Vergara et al. 2023), leading to the subsequent enhancement of plant defenses through the up-regulation of ET, JA and SA pathway related genes (Ahmad et al. 2020; Kuzhuppillymyal et al. 2021; Gupta et al. 2022b) (Table I.7).

Table I.7. Recent studies about the Systemic Resistance induction by entomopathogenic ascomycetes on horticultural crops.

EA fungal species	Crop species	Pathway*	Study
B. bassiana	*V. vinifera*	SA	Rondot and Reineke 2019
B. bassiana	*S. lycopersicum*, *C. sativus*, *C. melo*, *F. × ananassa*, *S. melongena*	SA	Iida et al. 2023
B. bassiana	*Z. mays*	JA	Batool et al. 2022
B. bassiana	*V. faba*	ET, PR proteins	Jensen et al. 2020
B. bassiana and *M. brunneum*	*S. lycopersicum*	JA, SA	Gupta et al. 2022b
B. bassiana and *M. brunneum*	*T. aestivum*	Auxin, Cytokinin, Giberellin, ET, JA, SA	González-Guzmán et al. 2022
A. lecanii (formerly *L. lecanii*)	*S. lycopersicum*	JA, SA	Hanan et al. 2020
M. brunneum	*B. napus*	ABA, JA, SA	Posada-Vergara et al. 2022, 2023
M. robertsii	*Z. mays*	JA, SA	Ahmad et al. 2022

*SA: salicylic acid; JA: jasmonic acid; ET: ethylene; PR proteins: pathogenesis related proteins; ABA: abscisic acid.

The SR induced by priming may not provide the complete level of protection typically observed after application of pesticides. Nevertheless, when integrated into IPM programs alongside pesticides, microbes, biological control, or resistance breeding, it becomes a valuable component (Tiwari and Singh 2021; Tiwari et al. 2022). The elucidation of the mechanisms behind these diverse effects holds great potential for providing insightful perspectives and opportunities in sustainable agriculture (Tiwari et al. 2022).

It is reasonable to assert that plant defense priming, represents a thoughtful approach to individual plant health care and holds the potential to serve as a long-term strategy for crop protection with widespread effectiveness (Pieterse et al. 2014; Martinez-Medina et al. 2016; Tiwari and Singh 2021). Plant defense priming has been proposed to mark the commencement of an exciting new avenue of research where

the mechanisms, ecological significance, and potential applications of trans-generational plant defense are only beginning to be revealed (Tiwari et al. 2022).

In this context, it remains unknown whether there is an EA crop priming against insect pests after the plant tissue colonization by the fungus or even without fungal colonization, only by a temporary contact of the plant with the fungal inoculum.

I.5. The cotton leafworm *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae), a key polyphagous pest on cucurbits

The Noctuidae, the largest family in the order Lepidoptera, includes several species of moths whose larval stages are serious pests on various crops due to their feeding habits (Carter 1993; Pogue 2002; Triplehorn and Johnson 2005). Within this family, the genus *Spodoptera*, which includes several species of cutworms and armyworms, is particularly important and is classified under the subfamily Amphipyrinae (Tremblay 1982; Baccetti et al. 2000; Pogue 2002; Domínguez García-Tejero 2004; Triplehorn and Johnson 2005).

I.5.1. Biology and ecology

The cotton leafworm *S. littoralis*, is a polyphagous insect pest with a significant economic impact on agriculture, capable of causing extensive damage to various cultivated crops (EPPO 2023a, b). It is also commonly known as the Egyptian cotton worm or Mediterranean brocade moth (CABI 2023; EPPO 2023b), and referred to as "*rosquilla negra*" in Spanish due to the blackish color of the larvae and their characteristic spiraling posture when inactive (Domínguez García-Tejero 2004; El Shaadi 2015). This species is closely resembling *S. litura*, with early larval stages being indistinguishable, and only later instars allowing for identification (Brown and Dewhurst 1975; Pogue 2002; EPPO 2015).

The cotton leafworm undergoes complete metamorphosis (Figure I.4), with larval and adult stages exhibiting marked differences in form, function, and habits (Tremblay 1982; Baccetti et al. 2000; Alfaro-Moreno 2005). The life cycle duration ranges from 19 to 144 days, depending on temperature (EFSA-PLH 2015; EPPO 2023a; CABI 2023). In warm climates or greenhouse conditions, up to eight

continuous generations can occur, while in southern Europe, at least two generations occur outdoors, with additional generations possible in protected cultivation (EFSA-PLH 2015; EPPO 2023a).

The female begins the oviposition between 2 and 5 days after emergence, laying more than 3000 eggs in several masses, showing preference for the underside of young leaves in the upper parts of the plants. Eggs are spherical in shape (approximately 0.45 mm in width and 0.35 mm in height), laid in groups ranging from 20 to 500 per batch (Figure I.4). Hatching time varies with temperature, taking about 9 days at 17.5°C or 2 days at 32.5°C. The larvae undergo six instars over a period of 15-23 days at temperatures of 25-26°C (Figure I.4). Older larvae typically feature a Y-shaped pattern on the head/thorax shield, as depicted in the detailed cephalic capsule of the L6 instar in Figure I.4. During pupation, the fully grown larva burrows into the loose surface of the soil, descending approximately 3-5 cm deep to constructs a clay 'cell' or cocoon, completing this process within 5-6 hours. The pupa is brown, exhibiting a greenish tone when newly formed, and measures about 15–22 mm in length (Figure I.4). Adult moths have a lifespan of 5-10 days. These nocturnal moths display sexual dimorphism with forewings measuring 12–16 mm in males and 13–16 mm in females (Figure I.4) (De Liñán Vicente 1998; Pogue 2002; Domínguez García-Tejero 2004; Alfaro-Moreno 2005; Planes and Carrero 2008; EPPO 2015; CABI 2023). It is worth noting that reliable identification, despite color or shape, requires genitalia dissection (Carter 1993; Brambila 2013). Adults utilize pheromones for mate location, and females of *S. littoralis* are ready to mate shortly after emergence; these pheromones have been tested to combat this species for several decades (Neumark et al. 1975) and there are currently some available products on the market to be used in crops like pepper, tomato, and melon (AGROCHEM 2023; PROBODELT 2023).

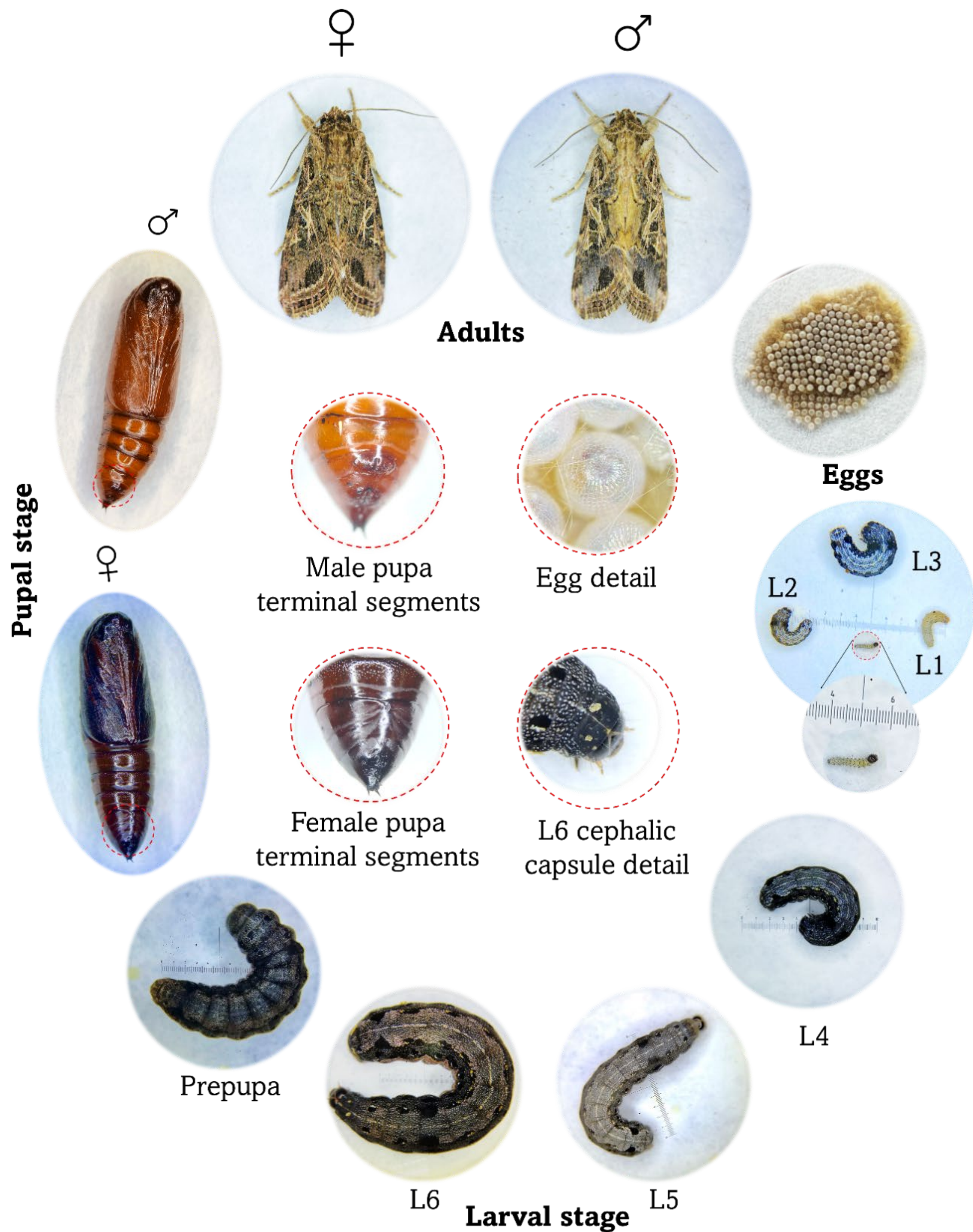

Figure I.4. Life cycle of *Spodoptera littoralis* Boisd. (Lepidoptera: Noctuidae). The eggs are laid in masses, typically covered with abdominal hairs from the female, being, initially, pale yellow, and get darken over time. This noctuid has eruciform larvae, characterized by a cylindrical body that is typically green or brown with lateral white stripes. As the larva develops, the color darkens, and the reticulation becomes more intense. The pupa, which is an obtecta, is characterized by a cremaster consisting of two spines, each approximately 0.5 mm long. Adult moths exhibit a light brown or grayish coloration and present sexual dimorphism, and females are slightly larger in size. Photos by Fabián García-Espinoza.

I.5.2. Distribution and economic impact

This noctuid is native to Sub-Saharan Africa (Brown and Dewhurst 1975; Wu et al. 2022; CISEH 2023), and it found widely distributed in the Mediterranean region, southern Europe, throughout Africa and Middle East (Toprak et al. 2006; EFSA-PLH 2015; EPPO 2023b). Additionally, recent reports indicate its presence as far as India and China (CABI, 2023) (Figure I.5). The distribution of *S. littoralis* in the northern regions is constraint by the climate conditions, with preference for zones where winter frosts are infrequent (EPPO 2023a; CABI 2023).

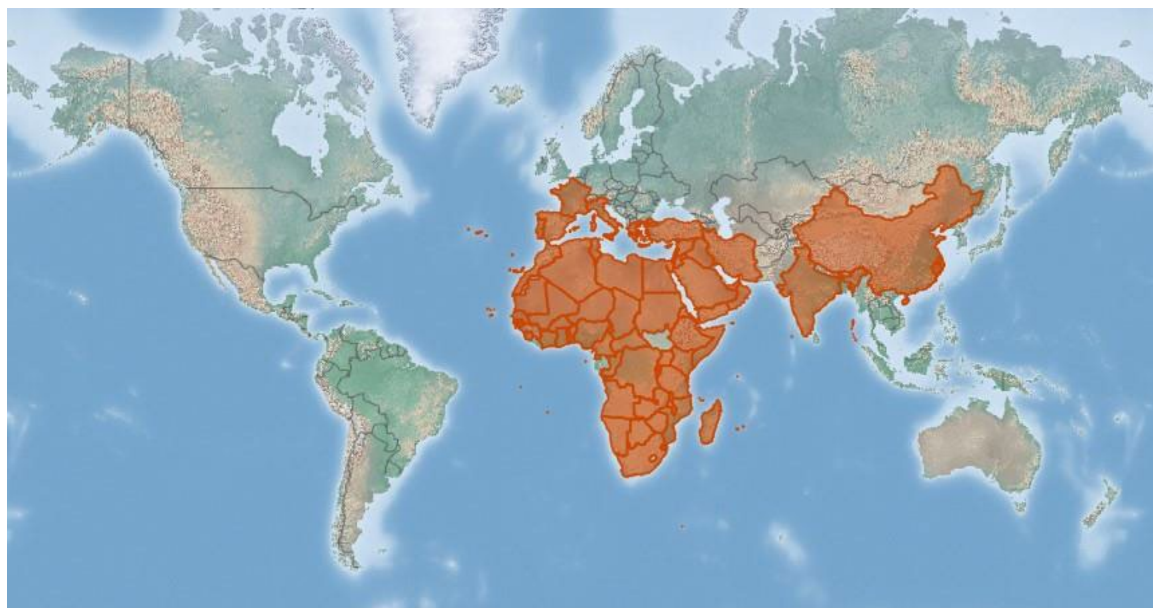

Figure I.5. Distribution map of *Spodoptera littoralis* according to CABI (2023).

The cotton leafworm is one of the most destructive agricultural pests within the order Lepidoptera, particularly in its subtropical and tropical range (Khan and Ahmad 2015; Wu et al. 2022; EPPO 2023a). It significantly affects economically important crops (Hosny et al. 1986; Jones et al. 1994; Toprak et al. 2006). The European and Mediterranean Plant Protection Organization (EPPO) has listed *S. littoralis* in the A2 category of pests recommended for regulation (EPPO, 2023a). This noctuid is a highly polyphagous defoliator that can be feed on a wide range of cultivated crops, with a host range that spans over 40 families (Salama et al. 1971; Noma et al. 2010; Khan and Ahmad 2015; Wu et al. 2022). It affects around 100 species of economic importance, including cucurbits, solanaceous, grasses, legumes, lucerne, corn, cotton, crucifers, and many other horticultural and floricultural crops,

as well as deciduous fruit trees (Salama et al. 1971; Brown and Dewhurst 1975; Noma et al. 2010; Khan and Ahmad 2015; EPPO 2015; CABI 2023). The major crops affected by *S. littoralis* include cucurbits, solanaceous plants, corn, and cotton (Figure I.6).

Figure I.6. Natural occurrence of an adult of *S. littoralis* on *Sorghum alepense* (L.) grass (A) and a L3 larva on *Medicago sativa* L. (B) by mid-October in Cordoba, Andalusia, Spain. Photos by Fabián García-Espinoza.

The cotton leafworm is known for its ability to feed on foliage, flowers, stems and fruits (Figure I.7) (EFSA-PLH 2015; Khan and Ahmad 2015; EPPO 2015, 2023a). The most significant economic damage caused by *S. littoralis* to crops resulted from the larval feeding habits. Young larvae display a preference for young tender leaves, which they may skeletonize through their feeding activity. As larvae mature, they have the capability to strip entire plants by consuming whole leaves. Additionally, they feed on young shoots, stalks, bolls, buds, and fruit, leaving characteristic feeding scars. The chewing action of larvae can result in large holes, and they may also mine shoots and stalks. In the early stages (first to third instar), larvae tend to feed in groups, leaving the opposite epidermis of the leaf intact. Subsequently (fourth to sixth instar), the larvae disperse and spend the day in the ground under the host plant, feeding during

the night and early morning hours. The highly voracious last larval instars (L5-L6) are consuming leaves, creating large, irregularly shaped holes, often leaving only the larger veins intact (EFSA-PLH 2015; EPPO 2023a; CABI 2023) (Figure I.8).

Figure I.7. Larvae of *S. littoralis* feeding on *Cucumis melo* flowers. A) Initial feeding and B) several damages caused on petals and stamens. Photos by Fabián García-Espinoza.

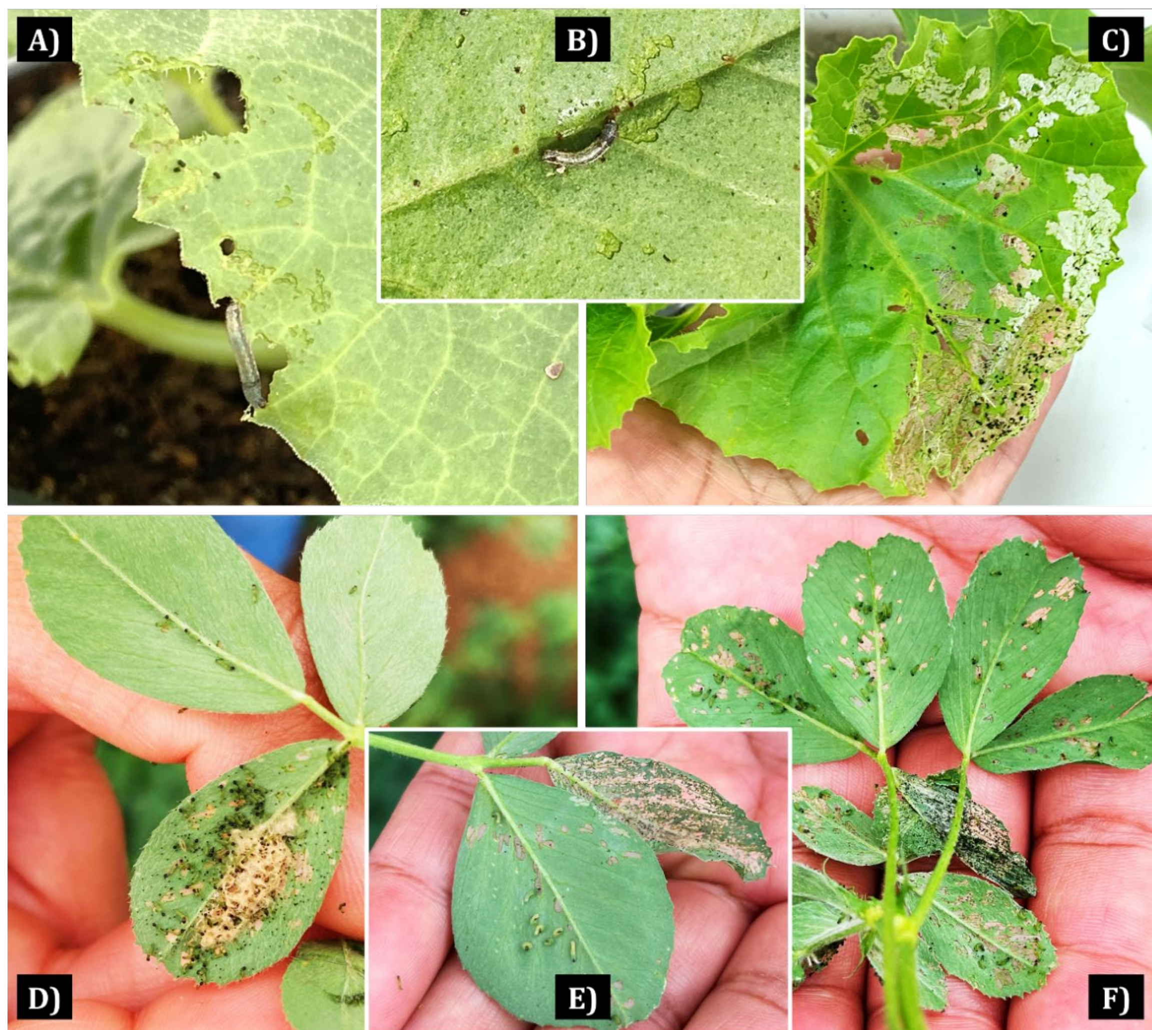

Figure I.8. Larvae of *S. littoralis* caused severe damages by feeding on foliage on economically important crops such as melon (A, B and C) and lucerne (D, E and F), in greenhouse and field conditions, respectively. Photos by Fabián García-Espinoza.

Due to its ability to have multiple generations throughout the year, *S. littoralis* can pose a continuous threat to several economically important crops, significantly affecting the Mediterranean region where it is considered a major pest (Hosny et al. 1986; Jones et al. 1994; Toprak et al. 2006). Currently, besides Spain (excluding the northern regions), *S. littoralis* has economic importance in Cyprus, France, Greece, Italy, Israel, Malta, and Morocco. It is particularly important in the cultivation of ornamental and vegetable crops in protected environments (EFSA-PLH, 2015).

I.5.3. Control

The control of cotton leafworm involves a range of measures, from agronomic-cultural practices to chemical control and legal methods, always considering local legislation and the approved list of pesticides, as well as their proper use. In this regard, it is essential to consult the relevant authority to determine the appropriate control strategies for this quarantine pest (CABI, 2023). While *S. littoralis* has a specific distribution as a pest, conventional control measures can be like those applied for other species of the *Spodoptera* genus (Ramasamy and Ravishankar 2018; Simmons et al. 2018). However, it is important to note that some natural enemies, including entomopathogens, predators and parasitoids of *S. littoralis* should be evaluated case by case, considering their compatibility or simultaneous use when are included in control strategies (Miranda-Fuentes et al. 2020; Miranda-Fuentes et al. 2021; Koller et al. 2023; EPPO 2023a; CABI 2023).

I.5.3.1. Legislation and recommended phytosanitary measures

The early and accurate identification of this A2 pest is crucial for its management to prevent it from spreading to new locations, with a series of recommendations being issued, including inspection measures, restrictions on the movement of plant products, and safety intervals for establishing new crops in areas with previous infestations (Directive 2000/29/EC 2000; CABI 2023; EPPO 2023b). Given the highly polyphagous nature of *S. littoralis*, the import or movement of any host plant product within the EU is prohibited (EFSA-PLH, 2015). It is worth highlighting that specific guidelines and requirements exist for certain ornamental plant species like *Dendranthema*, *Dianthus*, and *Pelargonium* when moving them within the EU zone (Directive 2000/29/EC 2000; EFSA-PLH 2015). In cases where the mobilization of plant material is necessary, one of the recommended phytosanitary measures for managing this pest is cold treatment, achieved by prolonged storage below the minimum threshold for *S. littoralis* development (9.9°C) (EFSA-PLH 2015; CABI 2023). Additionally, proper documentation certifying that products originate from *S. littoralis*-free areas is required (Directive 2000/29/EC 2000). These measures are important to prevent the spread of the pest to new locations and protect local agricultural and horticultural interests. To this end, the pest monitoring is a key goal by using colored sticky traps and sex pheromone traps

(Neumark et al. 1975; Salem and Salama 1985; Teich et al. 1985; Suckling et al. 2014; Ramasamy and Ravishankar 2018; Simmons et al. 2018).

Efforts to mitigate the economic impact of *S. littoralis* on agriculture involve a combination of strategies, including biological control, improved chemical management, and breeding for tolerance or resistance (Zavala 2010; Stout 2013). Researchers and farmers are working towards long-term solutions to address this issue (Mitchell et al. 2016).

I.5.3.2. Resistant varieties

In the context of crop protection, two primary plant defense strategies are recognized: resistance and tolerance (Douglas 2018). Resistance mechanism are antixenosis and antibiosis and occur when a plant physical characteristics or chemical composition discourage herbivores from consuming it (Stout 2013; Mitchell et al. 2016). Antixenosis (also known as no-preference) mechanisms adversely affect pest behavior by deterring the insect or, once the insect has arrived on the plant, preventing it from colonizing (Kloth et al. 2012; Stout 2013). Antibiosis had negative effects on the physiology or life history of pests by releasing toxins following tissue damage, feeding deterrents and other plant traits, and ultimately leading to a reduction in pest population (Kloth et al. 2012; Stout 2013). Tolerance, on the other hand, comes into play when plant traits mitigate the negative effects of herbivore damage on crop yield (Agrawal et al. 1999; Stout 2013). For example, cotton has developed a range of direct and indirect resistance mechanisms and hence, the development of cotton cultivars with increased resistance to *S. littoralis* has become a top priority (Hagenbucher et al. 2013a, 2016; Sjöstrand 2014). In addition to the successful use of genetically engineered cotton (Bt cotton, which produces Cry proteins derived from *B. thuringiensis*) (Naranjo 2011; Hagenbucher et al. 2013b). Some studies have explored the potential of plants producing phytohormones (Erb et al. 2009, 2012) and secondary metabolites such as terpenoids (Gale et al. 2022) to resist, tolerate, or repel *S. littoralis* attacks (Eisenring et al. 2017; Ferrero et al. 2020).

I.5.3.3. Chemical control

Since the 1950s, chemical management has been the primary method used to control *S. littoralis*. However, over the years, the pest has developed resistance to

chemical pesticides, posing a significant challenge to effective pest control. Various chemical classes, such as benzoylureas, oxadiazines, pyrethroids, pyrazoles, spinosins, carbamates, organophosphates, and insect growth regulators have been employed for *S. littoralis* management (Hosny et al. 1986; EFSA-PLH 2015; Ahmed et al. 2022; El-Sayed et al. 2023; EPPO 2023a). In a response to the challenges posed by *S. littoralis* resistance to conventional chemical pesticides, researchers and producers have been actively searching for alternative chemical solutions that are effective against the pest, safe for both humans and the environment, and align with IPM principles (EFSA-PLH 2015; Ahmed et al. 2022). One recent study explored innovative options, such as the application of Silver and Graphene Oxide nanocomposites to second instar (L2), with a significant impact on the fitness of treated larvae during both the larval and pupal stages, together with lower levels of total lipids, proteins, and carbohydrates reported (Abd El-Rahman et al. 2020). Several terpenoids and insect growth regulators have also been reported to show promise for *S. littoralis* IPM programs (Hamadah and Ghoneim 2018; Bassal, Shaaban, et al. 2018; Suárez-Lopez et al. 2022), together with combination of pesticides from different chemical groups or modes of action (Abd El-Mageed and Shalaby 2011; Bassal et al. 2018; Ahmed et al. 2022).

I.5.3.4. Biopesticides

Biopesticides are formulations for crop protection that contain living organisms, micro or macro, as well as molecules of plant, microbial or animal origin, which, because of their mode of action, must lack the adverse effects on the environment and living beings that characterize chemical pesticides (Quesada-Moraga 2023a, b). Regarding the molecules of plant, microbial or animal origin (pheromones already discussed), the most extensively used commercial plant extract is azadirachtin, extracted from the neem tree [*Azadirachta indica* A. Juss. (Meliaceae)], which is among the most effective compounds used to control pests and particularly cotton leafworm (Martinez and van Emden 2001). Furthermore, the use of crude leaf extracts from *Ajuga iva* (L.) Schrb. (Lamiaceae), which contains phytoecdysteroids and clerodanes, have demonstrated its effectiveness towards *S. littoralis* (Taha-Salaime et al. 2020). Hussein et al. (2023), evaluated extracts of *Salix babylonica* L. (Salicaceae), *Schinus terebinthifolius* Raddi (Anacardiaceae), and *Magnolia grandiflora* L. (Magnoliaceae), identifying that the wood methanol extract of *S. terebinthifolius*

dramatically decreased the percentages of pupation, adult emergence (reduced up to 56%), hatchability, and fecundity.

I.5.3.4.1. Biological control

Within the frame of the biopesticide definition, it is presently accepted that biological control is the use of living organisms, micro or macro, to maintain the pest populations below the economic threshold (Stenberg et al. 2021). In this context, biological control has gained prominence as alternative to chemical pesticides for controlling *S. littoralis*. These biocontrol methods encompass the use of predators, parasitoids, and entomopathogenic microorganisms, including bacteria, fungi, and nematodes (EFSA-PLH 2015; Stenberg et al. 2021; CABI 2023).

The egg parasitoid *Trichogrammatoidea bactrae* Nagaraja (Hymenoptera: Trichogrammatidae) has been proven to be effective under laboratory conditions (Mohamed 2021). The larval instars of the leafworm are targeted by predatory insects like ladybugs and lacewings, and parasitoids (Figure I.9). Larval parasitoids like *Cotesia icipe* Fernandez-Triana & Fiaboe and *Cotesia margiventris* (Cresson) (Hymenoptera: Braconidae), have been reported also as biological control agent of cotton leafworm (Vojtech et al. 2005; Agbodzavu et al. 2018). Another parasitoid, *H. didymator*, shares the same habitat as the cotton leafworm and targets its early larval instars. It has demonstrated its effectiveness in controlling the cotton leafworm under laboratory conditions, either on its own or in combination with EA, providing potential biological control solutions (Hatem et al. 2016; Miranda-Fuentes et al. 2020, 2021).

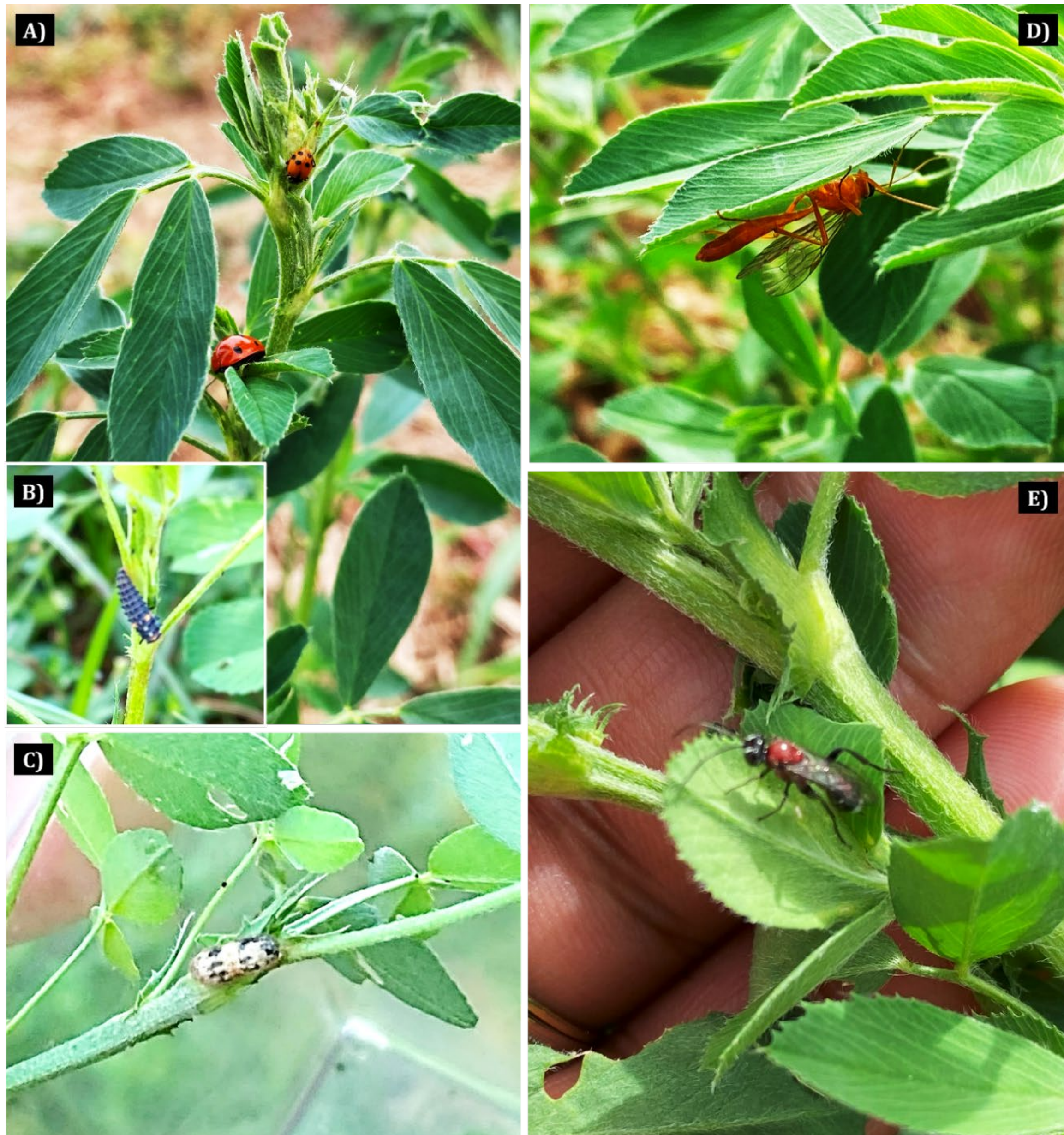

Figure I.9. Natural enemies of *S. littoralis* in a lucerne field by mid-October in Cordoba, Andalusia, Spain. Adults (A) and larva (B) of *Coccinella undecimpunctata* (Coccinellidae: Coleoptera) actively seeking for *S. littoralis* larvae; a cocoon of *H. didymator* (C) and other hymenopterans observed in the infested lucerne field (D and E). Photos by Fabián García-Espinoza.

Among entomopathogens, bacterial species such as *B. thuringiensis* and *B. subtilis* have a long history of successfully suppressing the cotton leafworm (Vojtech et al. 2005; Suarez-Lopez et al. 2022). Entomopathogenic nematodes, specifically *Steinernema carpocapsae* (Weiser) and *Heterorhabditis bacteriophora* Poinar have proven effective at controlling the last larval instars in the soil (Sobhy et al. 2020). Regarding Baculovirus, among the various polyhedrovirus species used for

lepidopterans control, *S. littoralis* nucleopolyhedrovirus (SpliNPV) stands out as one of the most common and well-studied (Toprak et al. 2006; Ali 2018; El Sayed et al. 2022), whereas variable results have been provided for other baculoviruses such as *S. exigua* multiple nucleopolyhedrovirus (SeMNPV) (Carballo et al. 2017), *Lymantria dispar* nucleopolyhedrovirus (LdNPV) (Barber et al. 1993; Hajek and Tobin 2011), and *Euproctis chrysorrhoea* nucleopolyhedrovirus (EcNPV) (Cory et al. 2000). In particular, SpliNPV is known for its narrow host range, showing high specificity to control only species from *Spodoptera* genus (Martins et al. 2005; Ali 2018; El Sayed et al. 2022)

Undoubtedly, in the last years, there have been an increasing research activity on the use of entomopathogenic fungi for the control of *S. littoralis*, probably due to both, their contact mode of action and they relationships with the plants that may allow developing new tools for its control and for promoting sustainable crop protection and production.

I.5.4.1.1. Multifunctional entomopathogenic ascomycetes for *Spodoptera littoralis* control

The potential of EA such *B. bassiana* and *M. brunneum* as biological control agents for *S. littoralis*, have been show, either against the immature larval or pupal stages, as well as in the adult stage. At laboratory conditions, it was found that crude extracts and fungal exotoxins from the EAMa 01/58-Su strain of *M. brunneum* had a deterrence and toxic effect on *S. littoralis* larvae (Quesada-Moraga et al. 2006). Injection of *Metarhizium rileyi* (Farl.) Kepler, Rehner & Humber (Hypocreales: Clavicipitaceae) (formerly *Nomuraea rileyi*) and *B. bassiana* blastospores suspensions reduced lipid and protein content along with a robust humoral immune response, supporting the idea that infection with EA had a significant impact on cotton leafworm larvae, particularly in terms of reduced hemolymph nutrients (Meshrif et al. 2010). Apart from these experimental injections, direct EA larval inoculation by immersion, spraying, or even soil/substrate drenching targeting preimaginals and offering inoculated artificial diet or vegetal material, especially foliage, have shown potential for *S. littoralis* control (Resquín-Romero et al. 2016b, a; Sánchez-Rodríguez et al. 2018; Garrido-Jurado et al. 2019b, 2020a; El Husseini 2019; Miranda-Fuentes et al. 2021; Şahin and Yanar 2021). Researchers have also explored the combined use of different

EA strains (Resquín-Romero et al. 2016b), and their combination with insect hormones (Gautam et al. 2020), as well as their co-application with other entomopathogens such as bacteria and nematodes (Suarez-Lopez et al. 2022; Spescha et al. 2023). Additionally, studies have investigated the synergistic effects of ascomycetes in combinations with diatomaceous earth (Abdou et al. 2022).

Experiments using EA for cotton leafworm control under controlled conditions have demonstrated their high effectiveness, with varying mortality rates and sublethal effects based on the specific inoculation methods, strains, and dosages applied. Notably, the strain EAMa 01/58-Su of *M. brunneum* has exhibited substantial efficacy against cotton leafworm larvae in several studies. This strain has induced high mortality rates when directly applied to the insect larvae (up to 80%), when larvae fed on treated plants (up to 50%), and when larvae consumed plants endophytically colonized by the fungus (up to 20%). However, it is worth noting that despite the high mortality rates observed, some studies reported a lack of fungal outgrowth in dead larvae.

This suggests that the precise mechanisms by which EA are causing death in *S. littoralis* are not yet fully understood. Further research is needed to unravel the intricate mechanisms involved in the plant-fungus-insect relationship, which may ultimately contribute to more effective pest control strategies. Very recently, it has been shown that endophytic EA strains of *B. bassiana* and *M. brunneum* can induce defensive responses in the plants with negative impact on the fitness of several lepidopteran pests highlighting the complex interplay between plants, fungi, and insects (Ahmad et al. 2020; Kuzhuppillymyal et al. 2021; Gupta et al. 2022b).

In conclusion, the effectiveness of biological control agents depends on various factors, including the diversity of natural enemies, the timing of their application, and the precision of these tactics. Further research and field assessment are required to optimize the use of biological control agents and effortlessly integrate them into sustainable pest management systems. The utilization of biological management techniques offers several advantages, such as reduced reliance on chemical pesticides, minimized impact on non-target pest species, and the potential for long-term pest suppression.

To successfully control the pest and minimize harm to the ecosystem and ecological systems, it is essential to adhere to the principles of IPM. This approach involves monitoring, accurate pest identification, and the combined use of selective pesticides in appropriate quantities and at the right times. IPM practices are critical for effective and environmentally responsible pest management (Willers et al. 2014; Deguine et al. 2021; Kanwal et al. 2022).

I.5.5. Compatibility of multifunctional entomopathogenic ascomycetes with predators and parasitoids for *Spodoptera littoralis* control

The use of entomophagous and entomopathogenic microorganisms in biological control significantly reduces the reliance on conventional pesticides, consequently minimizing their environmental impact. However, it is crucial to assess compatibility between these methods (Quesada-Moraga et al. 2022; Koller et al. 2023) and their compatibility with other pest control techniques when incorporating biological control into an IPM program (Skinner et al. 2014).

The EA can mediate different types of trophic interactions, both tritrophic (e.g., EA and plants or EA, phytophagous, and its natural enemies) as well as multitrophic interactions (e.g., EA, plants, phytophagous, and its natural enemies) (Wilberts et al. 2022, 2023b; Quesada-Moraga et al. 2022). Research has shown that the combined use of natural enemies, such as predators and parasitoids, with entomopathogenic microorganisms in biological control can reduce the detrimental effects of more conventional pests control methods (Roy et al. 2010).

In the last years, there has been extensive research into the compatibility between EA and parasitoids when used simultaneously to control pests under laboratory conditions. Miranda-Fuentes et al. (2020) demonstrated compatibility, with additive effects, between the use of EAMa 01/58-Su *M. brunneum* isolate and the endoparasitoid *H. didymator* under different joint attack scenarios for *S. littoralis* control. In another work, Miranda-Fuentes et al. (2021) observed that simultaneous invasion by the fungus (EAMa 01/58-Su strain) and the parasitoid (*H. didymator*) can occur in *S. littoralis* larvae. In this context, González-Mas et al. (2019) studied the effect of *B. bassiana* endophytic colonization of melon plants on the generalist predator *Chrysoperla carnea* Stephens, and on the parasitoid *A. colemani* Viereck. They

demonstrated compatibility with the EA against the phytophagous *Aphis gossypii* Glover (Hemiptera: Aphididae).

In an extensive and recent review conducted by Koller et al. (2023), it was found that out of 49 combinations between entomopathogens and parasitoids, 38 exhibited compatibilities, while only 6 were reported as incompatible. Incompatible combinations often resulted in a lower emergence rate, which could be attributed to issues such as poor timing, direct infections, or to the use of high doses of the entomopathogen. The study emphasized the increasing importance of investigating the combined use of entomopathogenic fungi and parasitic wasps, particularly in the fight against economically significant pests. However, the findings of Jensen et al. (2020) revealed that the emergence of the aphid parasitoid, *A. colemani*, was significantly affected when fava bean seeds were inoculated with *B. bassiana*. They reported a 67% vs. 76% adult emergence from parasitized aphids on inoculated plants compared to control plants, suggesting that the combined use of these two types of biological control agents should be assessed on a case-by-case basis. Weber et al. (2020) reported that primary and secondary metabolites related to plant resistance did not necessarily correlate with parasitoid survival. In contrast, Jensen et al. (2020) suggested that the plant inoculation with an EA may have an impact on beneficial insects. This underscores the need for caution when dealing with tritrophic or multitrophic systems, as the interactions can be complex and context-dependent.

Indeed, as emphasized by Koller et al. (2023), the efficacy of biological pest control can vary, and combining different biocontrol agents has the potential to enhance success rates. Successful biological control also hinges on the ability of the control agents to establish themselves rapidly in their ecological niches and adapt to the specific conditions of the crop microclimate, as noted by Varma et al. (2017).

In this context, it has been shown the compatibility of EA with parasitoids for controlling noctuid pests under laboratory conditions (Miranda-Fuentes et al. 2020; Miranda-Fuentes et al. 2021), whereas the compatibility of parasitoids and endophytic EA for pest control under real greenhouse conditions remains unknown.

I.6. Justification and objectives of this Doctoral Thesis

Agricultural sustainability has emerged in the last years in all the national and international agricultural policies, taking a highlighted place nowadays the requirement of apply during all the production chain friendly environmental, sustainable and economically viable alternatives. In the case of insect pest management, microbial control is considered the best option, particularly entomopathogenic ascomycetes since they are naturally found in the environment and present a unique contact mode of action, which can allow an effective control of a great variety of insect pest; hence these fungi have been adopted in recent years and are now gaining more popularity and are being explored for crop production in both small- and large-scale farming. Nonetheless, there are several challenges that must be addressed to provide mycoinsecticides for commercial success and grower adoption, with emphasis the exploitation of their role as valuable multipurpose plant beneficial microorganism. The present Doctoral Thesis focuses on the intricacy of the EA crop plant association by investigating EA capacity as plant growth promoters and their attribute to prime defense responses against biotic and abiotic stresses. The results of the present research are expected to provide key information to incorporate mycoinsecticides as part of successful pest management programs and to exploit the new pest control and crop production strategies emerging from their multiple lifestyles.

The present Doctoral Thesis focuses in a multitrophic systems including the melon crop, a pest, *S. littoralis*, EA such *M. brunneum* and *B. bassiana* and the parasitoid *H. didymator*. As described throughout the introduction, our previous research highlights the virulence of *M. brunneum* EAMa 01/58-Su strain, and *B. bassiana* EABb 04/01-Tip and EABb 01/33-Su strains against *S. littoralis* either targeting the larvae in the plant or the preimaginals in the soil, and even the compatibility of EAMa 01/58-Su strain with *H. didymator*, revealing that this *M. brunneum* strain can be exploit in multitrophic systems in a compatible way with natural enemies as part of IPM programs. Notwithstanding, our previous research reveal that *S. littoralis* larval mortality in larvae exposed to crop plants challenged with EA is not related to fungal outgrowth in the cadavers, suggesting that there must be not yet known indirect mechanism of EA related insect pest mortality. Thus, more

research is still required to fully understand the mechanisms underlying the plant-fungus-insect relationship and which may eventually help in the efficient management of pests in a real scenario, either in field or greenhouse conditions.

Firstly, it has been investigated whether there is an EA melon priming against *S. littoralis* after the plant tissue colonization by the fungus or even without necessarily colonizing the plants. In this context, it has been investigated whether the selected EA strains induce the expression of genes involved in both ISR and SAR responses in melon and to which extend such induced response impact the survival and fitness of *S. littoralis*. Moreover, to better exploit the EA multifunctionality, it has been elucidated the effect of the EA fungal isolate and inoculation method on the possible EA-mediated melon crop defense against *S. littoralis*.

Considering that iron chlorosis is a serious crop production problem in many calcareous soils of Southern Spain, it has been unraveled the possible role of the selected EA strains on improving the solubility of Fe in soils and cucurbit plant, together with the direct and indirect mechanisms of the possible EA alleviation of Fe chlorosis, and to which extend, the ability of EA to induce defense responses in this crop is influenced by possible iron nutritional deficiencies, and therefore, to highlight the cross talk among biotic and abiotic stresses.

Finally, it has been evaluated the compatibility of the selected EA strains with parasitoids for controlling *S. littoralis* under real greenhouse conditions, together with the possible growth promotion activity of the EA on the melon crop. Additionally,

For all that, the following goals were established for this work:

1. To evaluate the progressive endophytic behaviors of *M. brunneum* EAMa 01/58-Su strain, and *B. bassiana* EABb 04/01-Tip and EABb 01/33-Su strains in melon plants and their impact on *S. littoralis* fitness. **Chapters II** and **V.**
2. To investigate the molecular basis of the induced resistance response in melon plants inoculated with *M. brunneum* EAMa 01/58-Su strain, and *B. bassiana* EABb 04/01-Tip and EABb 01/33-Su strains in response to attack by *S. littoralis*. **Chapters II** and **III.**
3. To evaluate the plant growth promotion capacity of *M. brunneum* EAMa 01/58-Su strain, and *B. bassiana* EABb 04/01-Tip and EABb 01/33-Su strains in melon plants. **Chapters II** and **V.**

4. To evaluate the direct and indirect mechanisms of *M. brunneum* EAMa 01/58-Su strain, and *B. bassiana* EABb 04/01-Tip and EABb 01/33-Su strains to induce iron deficiency responses in cucumber and melon plants. **Chapter IV**.
5. To evaluate the compatibility of endophytic *M. brunneum* EAMa 01/58-Su strain, and *B. bassiana* EABb 04/01-Tip and EABb 01/33-Su strains with the parasitoid *H. didymator* under greenhouse conditions for *S. littoralis* control. **Chapter V.**

CHAPTER II. GUARDIANS WITHIN: ENTOMOPATHOGENIC ASCOMYCETE-DRIVEN ANTIBIOSIS AND COMPENSATORY GROWTH COMBINE TO PROTECT MELON PLANTS FROM HERBIVORE DAMAGE

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Abstract

Endophytic and rhizosphere competent entomopathogenic ascomycetes (EA) are important plant bodyguards, although the mechanisms underlying this phenomenon are poorly understood. Therefore, we aimed to elucidate the roles of antibiosis (lethal and sublethal effects), and potential growth compensation (in response to leaf injury) in melon plants exposed to cotton leafworm. Plants were inoculated with one of three EA strains (*Metarhizium brunneum* [one] or *Beauveria bassiana* [two]) by either foliar spray, seed dressing or soil drenching and then challenged with either multiple short-term, or single long-term *Spodoptera littoralis* larval infestation. Endophytic colonization and relative expression of plant defense genes were tracked using molecular techniques alongside evaluation of antibiosis effects on *S. littoralis* and plant tolerance to larval-induced leaf injury. Inoculated plants exhibited antibiosis and potential growth compensation in response to various *S. littoralis* challenges, which resulted in increased fresh and dry weight, chlorophyll content, number of secondary branches and stem diameter. Furthermore, up-regulation in the relative expression of ethylene (ET) (*ACO1*, *ACO3*, *EIN2*, *EIN3*) and jasmonic acid (JA) (*LOX2*)-related genes was observed, with the endophytic *B. bassiana*-induction of ET and JA production being higher in *S. littoralis* infested plants. Our findings strongly confirm the EA multifunctionality and the involvement of the endophytic EA triggered melon defensive system induction in the antibiosis and compensatory growth to protect melon plants from pest damage.

Keywords: *Metarhizium*, *Beauveria*, tolerance, antibiosis, resistance, sublethal effects

II.1. Introduction

Entomopathogenic ascomycetes (EA) are ubiquitous microorganisms that were originally saprophytes and evolved into insect pathogens via a plant symbiont lifestyle (Barelli et al. 2016; Quesada-Moraga et al. 2020; Quesada Moraga 2020). EA interact with crops and establish mutualistic relationships that benefit plants in several ways (Gange et al. 2019). This includes promoting plant growth, improving plant nutrient acquisition, and eliciting the plant defense system, which enhances plant tolerance to biotic and abiotic stresses, particularly herbivory (Barelli et al. 2016; Sánchez-

Rodríguez et al. 2016, 2018; Raya-Díaz et al. 2017b; Kuzhuppillymyal-Prabhakarankutty et al. 2020; García-Espinoza et al. 2023b, 2024). Thus, EA induce expression of specific subsets of Induced Systemic Resistance (ISR), a class of Systemic Resistance (SR) in plants (Ahmad et al. 2020; Posada-Vergara et al. 2022; González-Guzmán et al. 2022; García-Espinoza et al. 2023a), which is typically dependent on ethylene (ET) and/or jasmonate (JA) (Verhagen et al. 2004; Pieterse et al. 2009; García-Espinoza et al. 2023a). Beside these, SA-dependent pathway is activated by some ISR inducers too, suggesting that ISR can be elicited trough different signaling routes (Ryu et al. 2003; Niu et al. 2011; García-Espinoza et al. 2023a).

Whilst EA parasitize susceptible hosts via direct penetration of the cuticle, insects feeding on EA-challenged plants also suffer other lethal and sublethal effects (Gange et al. 2019). Sublethal effects occur when individuals or populations survive exposure to any killing agent that occur as a result of various biological, physiological, demographic, or behavioral changes. These changes can persist even after the exposure ends, regardless of whether the initial dose was lethal or sublethal (de Franca et al. 2017) and can be highly significant, particularly in the context of killing agents, like EA, with slower modes of action where both sublethal and acute effects occur together (Quesada-Moraga et al. 2004, 2006b; Ortiz-Urquiza et al. 2010; Garrido-Jurado et al. 2011; Yousef et al. 2013; González-Mas et al. 2019c; García-Espinoza et al. 2024). Sublethal effects on herbivores can have significant implications for life table parameters, including acceleration or deceleration of development, and reductions in longevity and fecundity as supported by both our own previous studies and those of others (Quesada-Moraga et al. 2006a; Resquín-Romero et al. 2016a; González-Mas et al. 2019b; Liu et al. 2020; Garrido-Jurado et al. 2020; de Souza et al. 2020; Mousavi et al. 2022).

Our mechanistic understanding of the sublethal effects exerted by EA against several chewing and sap-sucking insect pests is incomplete, especially effects related to EA endophytic colonization (Quesada Moraga 2020). Previous studies have demonstrated that insecticidal compounds produced within the colonized host plant are one of the possible mechanisms involved in sublethal effects (Ríos-Moreno et al. 2016). However, a recent study revealed that plant priming through root immersion in a fungal suspension induced systemic resistance without fungal colonization, resulting in sublethal effects on *S. littoralis* due to multiple other mechanisms (García-Espinoza

et al. 2023a); this indicated that endophytic establishment of EA in plant tissues may not be the primary cause of all sublethal effects. In this regard, Rasool et al. (2021), showed that seed inoculation with EA modulated plant secondary volatiles that resulted in sublethal effects on aphids. Nonetheless, as proposed by Simmonds (2001), there is an indication that plant secondary volatiles may not consistently impact insect herbivores. This observation further reinforces the idea that other mechanisms are likely to be involved in the tritrophic relationship amongst fungus, plant, and herbivore. The current study aimed to investigate the hypothesis that EA plant inoculation could not only cause mortality in the insect feeding on it (antibiosis) but also confer a level of compensatory growth (tolerance) in response to insect damage. In this study, we conducted a novel investigation into the possible tolerance of melon plants to *S. littoralis*, taking into consideration application of several EA strains (*M. brunneum* EAMa 01/58-Su, *B. bassiana* EABb 04/01-Tip, *B. bassiana* EABb 01/33-Su), and different application methods (foliar spray, soil drenching, seed coating). Evaluation was done within the context of two distinct pest attack scenarios: multiple short-term infestations and a single long-term infestation. We also used molecular techniques to monitor endophytic colonization patterns and uncover potential mechanisms responsible for inducing resistance in this tritrophic system, which encompasses interactions amongst the fungus, the plant, and the insect.

II.2. Material and methods

II.2.1. Biological material

Individuals of *S. littoralis* used in this study came from an insectary colony of the Agricultural and Forestry Entomology Laboratory, University of Cordoba, Spain (Miranda-Fuentes et al. 2020; Garrido-Jurado et al. 2020; García-Espinoza et al. 2023a); the rearing chamber was maintained at 26±2 ºC, 70±5% RH and a photoperiod of 16:8 (L:D) h (Resquín-Romero et al. 2016b; Miranda-Fuentes et al. 2020; Garrido-Jurado et al. 2020).

Three EA strains obtained from the culture collection of the Agronomy Department, University of Córdoba, Spain, namely EABb 04/01-Tip, EABb 01/33-Su and EAMa 01/58-Su, were used in this study (Table II.1).

Table II.1. Origin and data on *M. brunneum* and *B. bassiana* strains used in this study.

Fungal Species	Strain	Origin	Agroecosystem	Habitat	GenBank Accession Number	CECT* Accession Number
B. bassiana	EABb 04/01-Tip	Ecija (Sevilla, Spain)	Opium poppy crop	Insect (*Iraella luteipes*)	FJ972963	20744
B. bassiana	EABb 01/33-Su	El Bosque (Cadiz, Spain)	Traditional olive orchard	Soil	FJ972969	21149
M. brunneum	EAMa 01/58-Su	Hinojosa del Duque (Córdoba, Spain)	Wheat crop	Soil	JN900390	20764

* The Spanish Type Culture Collection

Previous studies by our group showed that endophytic colonization of melon plants was transient and temporary when strains were applied via foliar application (Resquín-Romero et al. 2016a; Garrido-Jurado et al. 2017); and that EAMa 01/58-Su primed defense mechanisms in cucurbits after root immersion (García-Espinoza et al. 2023b, a).

All strains were obtained by subculturing from stored slant cultures on Potato Dextrose Agar (PDA) in Petri dishes (García-Espinoza et al. 2023b). Cultures were incubated in darkness at 25°C for 15 days. Subsequently, inoculum suspensions were prepared by scraping conidia from mycelia in the Petri plates into a sterile aqueous solution of 0.1% Tween 80. Suspensions were sonicated for 5 minutes and filtered through cheesecloth to remove mycelia ensuring purified conidial suspensions for further experiments. According to Miranda-Fuentes et al. (2020), a haemocytometer was used to estimate conidial concentration which was adjusted to $1x10^8$ conidia/ml by adding sterile aqueous 0.1% Tween 80.

II.2.2. Effects of EA inoculation on melon plant growth and its resistance to infestation by *S. littoralis*

To quantify tolerance against *S. littoralis* infestation conferred by EA inoculation of melon plants, and subsequent effects on plant and insect development, two experiments were done. In the first experiment, we investigated tolerance of melon plants to multiple short-term *S. littoralis* infestations, examining the influence of EA strain and application method. In the second experiment, we studied the response of melon plants to long-term *S. littoralis* infestation after inoculation with EA.

II.2.2.1. Experiment 1

II.2.2.1.1. Plant inoculation and growth conditions

Three EA strains (EABb 04/01-Tip, EABb 01/33-Su and EAMa 01/58-Su) and three application methods (seed coating, soil drenching, leaf spraying) were evaluated for each of three fungal strains. We used a completely randomized design with nine combinations comprised of three strains and three inoculation methods, each with its corresponding control group.

The growing substrate (Floragard, Germany) was sterilized twice (121 °C for 30 minutes), with an interval of 24 h (González-Mas et al. 2019a). Washed and sterilized 500 ml pots were filled with sterile substrate. We used certified endophyte-free melon (*Cucumis melo* L. cv. Galia) as the crop plant in all experiments (Resquín-Romero et al. 2016a; García-Espinoza et al. 2023b). Seeds were surface sterilized as described previously (Garrido-Jurado et al. 2017).

For seed coating, seeds were submerged in $1x10^8$ conidia/ml suspension in Falcon tubes on a rotary shaker at 12 rpm for 4 h. Then, seeds were sown in pots prepared previously. Soil drenching and leaf spraying were done when the melon plants reached the 'four true leaves' stage (equivalent to 30 d after seed coating treatments were made in the appropriate treatments). For soil drenching, 5 ml of the suspension was pipetted onto the substrate surface. Leaf spraying was carried out according to González-Mas et al. (2019), briefly, two basal leaves of each plant were sprayed (adaxial and abaxial leaf surfaces) with 2 ml of suspension using an aerograph. To avoid contamination by run-off, soil and uninoculated plant parts were protected by aluminum foil and plastic bags, respectively. Control plants were sprayed with a sterile aqueous solution of 0.1% Tween 80. To ensure humidity and facilitate conidial germination and subsequent plant colonization, treated and control plants were covered with plastic bags for 48 h in the soil drenching and leaf spraying treatments (and their respective controls), and maintained in the growth chamber.

Pots were placed in a growth chamber at 24 ± 2 °C, 70 ± 5% RH in a 16: 8 light: dark regime. Plants were watered three times a week and fertilized with Nutrichem 60 (N: 20, P: 20, K:20) (Miller Chemical & Fertilizer Corporation, Hanover, Pennsylvania, EEUU) twice a month in the irrigation water at a ratio of 1 g/L (González-Mas et al. 2019c).

Systemic progression of endophytic colonization in melon plants was evaluated by leaf sampling 2, 7, 14, 21 and 28 DPI. Plant material was sampled for both microbiological and molecular assessment of endophytic colonization.

II.2.2.1.2. Assessment of endophytic colonization by microbiological techniques

Sampled leaves were surface sterilized in 1% sodium hypochlorite for 2 min, rinsed twice in sterile deionized water (2 min each), and dried on sterile filter paper. Subsequently, ten fragments of each leaf were cut with a sterile scalpel and plated out independently in Petri dishes containing Sabouraud Dextrose Agar supplemented with 0.5 g l^{-1} chloramphenicol (SDAC) (Scharlab, S. L., Spain) (González-Mas et al. 2019c). Dishes were sealed and incubated for 7 d at 25º C in darkness. Ten µl of water were taken from the second rinse and cultured in Petri dishes of the same medium to verify the efficiency of the leaf surface disinfection process (González-Mas et al. 2019b; Miranda-Fuentes et al. 2021b). Endophytic colonization was represented as a percentage based on the number of fragments that presented fungal growth out of the original ten sampled per leaf.

II.2.2.1.3. Assessment of the progression of endophytic colonization using qPCR

Assessment of endophytic colonization using qPCR was conducted according to García-Espinoza et al. (2023a). Total DNA was isolated using HigherPurity™ Plant DNA Purification Kit (Canvax Biotech S.L., Córdoba, Spain) according to the manufacturer's instructions. DNA concentration and quality were determined by assessment of absorbance at 260 nm and 280 nm in a NanoDrop™ 2000 (Thermo Fisher Scientific Inc.).

To identify and quantify *M. brunneum* strain EAMa 01/58-Su, a primer pair from the *nrr* gene (F: TCA GGC GAT CTC GTG GTA AG, R: GGG GTG TAC TTG AGG AAT GGG) was used (Barelli et al. 2018), while for the two strains of *B. bassiana* (EABb 04/01-Tip and EABb 01/33-Su), primers for the ITSII rRNA gene pair (F: GCC GGC CCT GAA ATG G, R: GAT TCG AGG TCA ACG TTC AGA AG) were used (Bell et al. 2009). Real-time PCR was done in a qRT-PCR Bio-Rad CFX connect thermal cycler set to the following amplification profiles: i) for *M. brunneum,* initial denaturation and polymerase activation (95 ºC for 3 min), amplification and quantification repeated 40 times (94 ºC for 10 s, 65 ºC for 15 s and 72 ºC for 30 s); ii) for *B. bassiana,* initial denaturation and

polymerase activation (95 ºC for 3 min), amplification and quantification repeated 40 times (95 ºC for 10 s, 60 ºC for 30 s and 72 ºC for 30 s). For all strains there was a final melting curve stage of 65 ºC to 95 ºC with increments of 0.5 ºC for 5 s to ensure the absence of primer dimer or non-specific amplification products. All PCR reactions were set up with a total of 40 ng DNA in a final volume of 20 µL with SYBR Green Bio-Rad PCR Master Mix, following the manufacturer's instructions (García-Espinoza et al. 2023a).

Following the methods of Barelli et al. (2018) and Bell et al. (2009), absolute quantification was conducted. Briefly, templates (DNA from samples) were standardized at at 30 ng/µl and a gradient of 1:4 from 40 ng to 0.16 pg of fungal and plant genomic DNA was used to establish standard curves (Bell et al. 2009; Barelli et al. 2018).

II.2.2.1.4. Infestation of melon plants by *S. littoralis* and evaluation of plant tolerance

At 2 DPI, melon plants with four true leaves that were fully grown were initially divided into two groups: the uninoculated group and the group of plants that had received either seed coating, soil drenching or leaf spraying inoculations with EAMa 01/58-Su, EABb 04/01-Tip or EABb 01/33-Su strains. Within each group, half of the plants were subjected to multiple short-term infestations by *S. littoralis* at 2, 14, and 28 DPI. Each infestation involved six larvae (L3) of *S. littoralis* per replicate, with three replicates (plants) per treatment. After introduction to plants, the *S. littoralis* larvae were enclosed within textile organza bags and left for 72 hours. In the case of the leaf spraying treatment, larvae were confined to non-sprayed leaves. Subsequently, larvae were individually collected and placed in methacrylate boxes for recording of mortality and larval development. During this period, they were fed with artificial diet. We monitored larval mortality and development daily, recording the length of each larval instar, pupal abnormality, pupal weight, and successful development to adulthood.

All plants were maintained under controlled conditions as described in the 'Plant inoculation and growth conditions' section, and two controls groups of plants were established, namely, an absolute control and a control with *S. littoralis* larvae only.

II.2.2.1.5. Study of ISR-SAR-related genes by qRT-PCR

Samples of leaves from the two groups of plants, one infested with *S. littoralis* and the other non-infested, were collected 2, 7, 14, 21 and 28 DPI to evaluate gene expression related to the synthesis pathways of ethylene (ET) and jasmonic acid (JA).

RNA isolation, cDNA synthesis and qRT-PCR analysis were conducted according to García et al. (2021) and García-Espinoza et al. (2023a). Real-time PCR analysis was done as described by García et al. (2021), briefly, leaves were ground to a fine powder and total RNA extracted using Tri Reagent solution (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. cDNA synthesis was achieved using the iScript™ cDNA Synthesis Kit (Bio-Rad laboratories, Inc, Hercules, CA, USA) from 3 µg of DNase-treated RNA as the template.

18S cDNA was amplified as an internal control using the QuantumRNA Universal 18S Standards primer set (Ambion, Austin, TX, USA) (Lucena et al. 2006; García et al. 2013).

Relative gene expression was quantified in a qRT-PCR Bio-Rad CFX connect thermal cycler following the methods of García et al. (2021) and García-Espinoza et al. (2023a), briefly, PCR reactions were set up with 2 µL of cDNA in 23 µL of SYBR Green Bio-Rad PCR Master Mix, following the manufacturer's instructions and the following amplification profile was used: initial denaturation and polymerase activation (95 ºC for 3 min), amplification and quantification repeated 40 times (94 ºC for 10 s, 57 ºC for 15 s and 72 ºC for 30 s), and a final melting curve stage of 65 - 95 ºC with gradual increases of 0.5 ºC, each for 5 s.

The expression of ET biosynthesis (*ACO1* and *ACO3*) and transduction (*EIN2* and *EIN3*) pathway genes as well as genes related to JA (*LOX2*) biosynthesis were studied at 2 DPI and before *S. littoralis* L3 larvae were introduced to determine initial expression levels. The relative expression levels of all genes were studied at 7, 14, 21 and 28 DPI in both non-infested and infested *S. littoralis*-plants. The Pfaffl method (Pfaffl, 2001) was used to calculate relative expression levels. The relative expression levels of genes were determined by comparing all treatments with an absolute control group, which consisted of uninoculated plants without any *S. littoralis*. Each PCR analysis was conducted on three biological replicates and each PCR reaction repeated twice (García-Espinoza et al. 2023a). Table II.2 shows the primers used in this study.

Table II.2 Name and primer sequences of genes studied on melon shoot samples.

Hormone	Gen	Gen name / function	Reference	Sequence
Ethylene	*ACO1*	1-Aminocyclopropane-1-carboxylic acid oxidase 1	(Aparicio et al. 2023)	F: TTTGGTGGCGGAGGAGAAAA R: ATGGCTTCAAACCTCGGCTC
	ACO3	1-Aminocyclopropane-1-carboxylic acid oxidase 2	(Aparicio et al. 2023)	F: ACTCAAAACAGTGGAACTGGA R: GGGGTACACTTCCTTCTTCTCC
	EIN2	Ethylene-insensitive protein 2	(Aparicio et al. 2023)	F: TGCCGACAAGGTTAAATGGG R: TGCTGCTGCACAATAGAAGA
	EIN3	Ethylene-insensitive protein 3	(Aparicio et al. 2023)	F: GCTTTCTGGGGTTGCGATTT R: CCGAACAGTCTCCCAAAGCA
Jasmonic acid	*LOX2*	Lipoxygenase 2	(García-Gutiérrez et al. 2013)	F: GCGTAAGGAATGGGATAGAATATATGA R: CGACGAGGATAAGGGAATTGG
Constitutive genes	*Actin*	Actin	(Aparicio et al. 2023)	F: AACCCAAAGGCAAACAGGGA R: TCCGACCACTGGCATAGAGA
	Cyclo	Cyclophilin	(Aparicio et al. 2023)	F: ATTTCCTATTTGCGTGTGTTGTT R: GTAGCATAAACCATGACCCATAATA

F, forward; R, reverse.

II.2.2.2. Experiment 2

Strain EAMa 01/58-Su was used to inoculate melon plants by soil drenching to evaluate plant responses to long-term *S. littoralis* infestation after inoculation with EA. For that, three treatments were employed, namely an absolute control (both uninoculated and uninfested with *S. littoralis*), a control (uninoculated plants) and EA-inoculated plants, with five plants per treatment grown and inoculated as described in the 'Plant inoculation and growth conditions' section.

At 21 DPI, each replicate from both control and inoculated plants were infested with 12 L5 *S. littoralis* larvae. Each treatment had five replicates. Larvae were released on plants, confining them by covering the entire plant with an organza bag (40x55 cm). For the absolute control, 60 larvae for subsequent evaluation were reared individually on artificial diet. Larvae were recollected when they reached the prepupal stage and placed individually in methacrylate cages.

II.2.2.3. Assessment of potential growth promotion on melon plants

At the end of both experiments, plant fresh and dry matter were weighed. In the second experiment SPAD values were also taken as a proxy for chlorophyll concentration in leaves (SPAD 502 Minolta Camera Co., Osaka, Japan), and the number of secondary branches and stem diameter recorded. Growth promotion measures were taken at 31 DPI in the first experiment and at 37 DPI in the second experiment.

II.2.3. Statistical analysis

Successful development to adulthood, expressed as percentages, were analyzed using a generalized linear mixed model with binomial distribution and logit link function. Significance of treatments were determined using F-tests and Tukey's multiple comparisons ($\alpha < 0.05$) (JMP 8.0, SAS Institute Inc.). Data for the weight of pupae and larval instar duration were analyzed by analysis of variance (ANOVA) followed by a Tukey multiple range test; different letters over the bars indicate significant differences ($p < 0.05$) amongst treatments (Statistix 9.0®, Analytical Software, Tallahassee, FL, USA). The values of qRT-PCR represent the mean ± SE of three independent technical replicates. Results of relative gene expression were analyzed by one-way analysis of variance (ANOVA) followed by a Dunnett's test; * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$) over the bars indicate significant differences in relation to the

control treatment (GraphPad Prism 9.4.0, GraphPad Software, LLC, 2365 Northside Dr., Suite 560, San Diego, CA 92108 USA). Pearson's coefficient correlations were done to detect relationships between endophytic colonization, gene expression and effects on *S. littoralis*.

II.3. Results

II.3.1. Experiment 1

II.3.1.1 Microbiological and qPCR assessment of progression of endophytic colonization

At 2 DPI, and prior to *S. littoralis* infestation, all strains successfully colonized melon plants following inoculation by leaf spraying (Supplementary material Figure II.S1). Colonization rates were higher than those observed in plants inoculated by seed coating or soil drenching. Molecular detection revealed fungal traces in samples from plants inoculated with EABb 04/01-Tip and EABb 01/33-Su strains via seed coating and soil drenching. However, microbiological assessment did not show any colonization except in plants inoculated with EABb 04/01-Tip via soil drenching (Supplementary material Figure II.S1).

After the time at which *S. littoralis* infestation was initiated, there were no significant differences in endophytic colonization between plants infested with *S. littoralis* larvae and non-infested plants as revealed by microbiological techniques ($F_{1,269}$=0.07, p=0.7943) (Figure II.1A, B, C upper graphs) and molecular quantification ($F_{1,322}$=0.0, p=0.9741) (Figure II.1A, B, C lower graphs).

Similarly, no significant differences were recorded by either detection method (microbiological or molecular) when comparisons were made at the strain level [microbiological assessment: EAMa 01/58-Su ($F_{1,89}$=0.10, p=0.7551), EABb 04/01-Tip ($F_{1,89}$=0.01, p=0.9125), EABb 01/33-Su ($F_{1,89}$=0.00, p =0.9440); and molecular quantification: EAMa 01/58-Su ($F_{1,95}$ =0.0, p=0.9898), EABb 04/01-Tip ($F_{1,108}$=0.0, p=0.9514), EABb 01/33-Su ($F_{1,117}$=0.05, p=0.8320).

Molecular techniques provided a clearer depiction of the progression of systemic colonization of melon plants by the three fungal strains, revealing significant differences between strains in the samples collected at 2, 7, 14, 21 and 28 DPI ($F_{2,238}$=74.62, p=0.001). Notably, strain EABb 01/33-Su consistently colonized the foliar

tissues of melon plants to a greater extent than the other strains. Furthermore, in a comprehensive analysis spanning from 2 to 28 DPI, the inoculation method ($F_{2,269}$=28.12, p=0.001) and the sampling time ($F_{4,269}$=17.82, p=0.001) exhibited significant differences, with the samples collected at 2 DPI from plants inoculated via leaf spraying showing the highest rates of colonization.

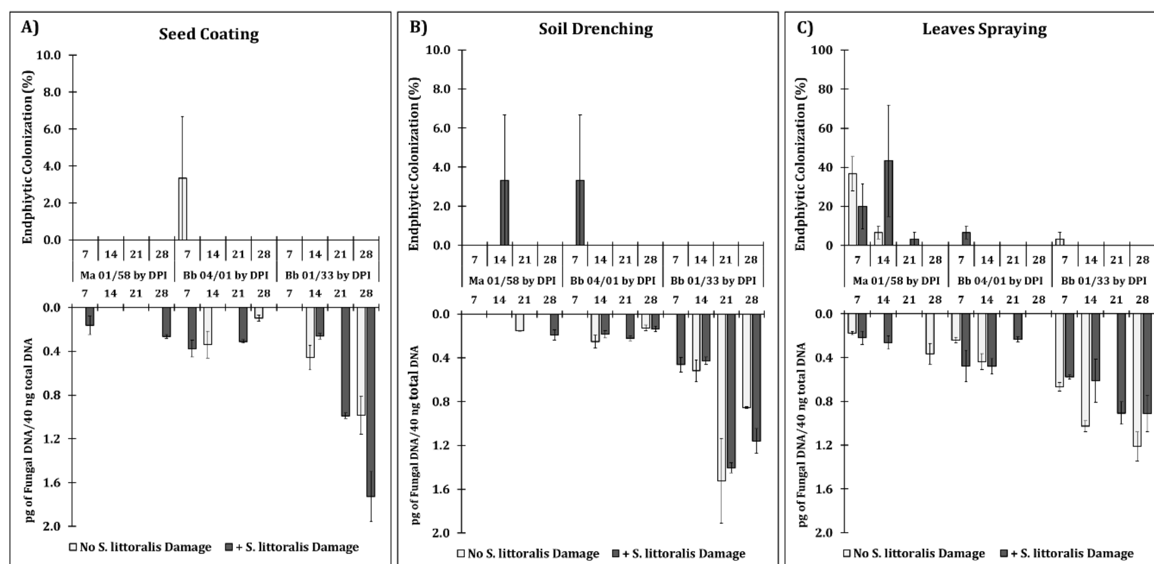

Figure II.1. Progressive endophytic colonization detected in melon leaves inoculated with EAMa 01/58-Su, EABb 04/01-Tip or EABb 01/33-Su strains using three application methods: seed coating (A), soil drenching (B) and leaf spraying (C). Endophytic colonization was assessed by microbiological (upper graph) and by molecular techniques (lower graph) over time (7, 14, 21 and 28 DPI); endophytic presence of fungi was evaluated in plants without (white bars) or with (black bars) *S. littoralis* infestation. Endophytic colonization assessed by microbiological technique is expressed as a percentage of melon leaf fragments in which fungal growth was observed; molecular assessment and quantification is expressed in picograms (pg) of fungal DNA in 40 nanograms (ng) of total DNA per reaction (mean ± SE). Evaluation in this figure began 7 days after leaf spraying or soil application, with samples collected from non-sprayed leaves throughout the plant. In the case of seed coating, plants were inoculated at phenological stage 0 (germination); for soil application and leaf spraying, plants were inoculated at the four-leaf stage.

II.3.1.2. Experiment 1: Tolerance of EA inoculated melon plants to multiple short-term *S. littoralis* infestation

II.3.1.2.1. Successful development to adulthood by *S. littoralis* fed on EA-inoculated plants

In the first experiment, the effects of EA were reflected mainly in whether larvae successfully reached the imaginal stage, with a significant reduction detected in the percentage achieving adulthood at 14 DPI when strains EAMa 01/58-Su strain ($\chi^2_{(3)}$=8.79, *p*=0.0322) and EABb 04/01-Tip ($\chi^2_{(3)}$=16.65, *p*=0.0008) were applied by seed coating and leaf spraying, respectively (Table II.3); and at 28 DPI, in plants inoculated with strain EAMa 01/58-Su through soil drenching ($\chi^2_{(3)}$= 16.04, *p*=0.0011) (Table II.3). In the case of strain EABb 04/01-Tip, there were significant differences between the soil drenching and leaf spraying treatments compared with the control ($\chi^2_{(3)}$= 15.08, *p*=0.0018), where only 61% (soil drenching and leaf spraying treatments) and 83% (in control) successfully achieved adulthood (Table II.3).

Table II.3. Proportion of *S. littoralis* larvae achieving adulthood when fed on melon plants for 3 d. Melon plants were inoculated with EAMa 01/58-Su, EABb 04/01-Tip and EABb 01/33-Su strains by seed coating, soil drenching and leaf spraying.

	Adulthood Succes (%)											
Treatment	**EAMa 01/58-Su**				**EABb 04/01-Tip**				**EABb 01/33-Su**			
2 DPI	**($\chi^2_{(3)}$=1.40, *p*=0.7051)**				**($\chi^2_{(3)}$=1.37, *p*=0.7116)**				**($\chi^2_{(3)}$= 0.53, *p*<0.9129)**			
Control	94.43	±	5.57	a	94.43	±	5.57	a	94.43	±	5.57	a
Seed coating	100.00	±	0.00	a	100.00	±	0.00	a	94.43	±	5.57	a
Soil drenching	88.87	±	5.57	a	88.87	±	5.57	a	88.87	±	5.57	a
Leaf spraying	88.90	±	11.10	a	100.00	±	0.00	a	88.87	±	5.57	a
14 DPI	**($\chi^2_{(3)}$=8.79, *p*=0.0322)**				**($\chi^2_{(3)}$=3.36, *p*=0.3385)**				**($\chi^2_{(3)}$=16.65, *p*=0.0008)**			
Control	72.23	±	5.53	b	72.23	±	5.53	a	72.23	±	5.53	b
Seed coating	55.57	±	5.57	a	72.23	±	5.53	a	66.67	±	19.26	b
Soil drenching	77.77	±	5.53	b	66.67	±	9.61	a	72.23	±	5.53	b
Leaf spraying	77.80	±	11.10	b	83.30	±	0.00	a	44.43	±	5.57	a
28 DPI	**($\chi^2_{(3)}$= 16.04, *p*=0.0011)**				**($\chi^2_{(3)}$= 15.08, *p*=0.0018)**				**($\chi^2_{(3)}$= 4.17, *p*=0.2433)**			
Control	83.33	±	9.61	b	83.33	±	9.61	ab	83.33	±	9.61	a
Seed coating	88.87	±	5.57	b	88.87	±	5.57	b	77.77	±	5.53	a
Soil drenching	55.57	±	5.57	a	61.13	±	5.57	a	83.30	±	0.00	a
Leaf spraying	77.77	±	5.53	b	61.13	±	5.57	a	66.70	±	0.00	a

Means ± SE within columns, for each strain and control, with the same lowercase letter are not significantly different from each other according to the Tukey HSD test (*p*<0.05).

II.3.1.2.2. Growth response of EA-inoculated and *S. littoralis*-infested melon plants

A significant difference in fresh and dry matter weight was observed in plants inoculated with strain EAMa 01/58-Su, compared with the control, in both *S. littoralis*-infested ($F_{3,11}$=5.42, p=0.0250, fresh weight; $F_{3,11}$=6.60, p=0.0148, dry weight) and non-infested plants ($F_{3,11}$=9.80, p = 0.0047, fresh weigh; $F_{3,11}$=7.97, p=0.0087, dry weight) (Figure II.2A and B).

Plants inoculated with strain EABb 04/01-Tip and infested with *S. littoralis* larvae exhibited a significant increase in their fresh ($F_{3,11}$=6.60, p=0.0148) and dry ($F_{3,11}$=9.60, p=0.0050) matter production (Figure II.2C and D). In the case of non-infested plants, a significant difference was only observed in the dry matter weight ($F_{3,11}$=9.37, p=0.0054) (Figure II.2D). In contrast, when plants were inoculated with strain EABb 01/33-Su no significant difference was recorded in *S. littoralis*-infested ($F_{3,11}$ = 3.59, p=0.0657, fresh weight; $F_{3,11}$=1.87, p=0.2113, dry weight) or non-infested plants ($F_{3,11}$=1.52, p=0.2819, fresh weigh; $F_{3,11}$=0.75, p=0.5533, dry weight) (Figure II.2E and F).

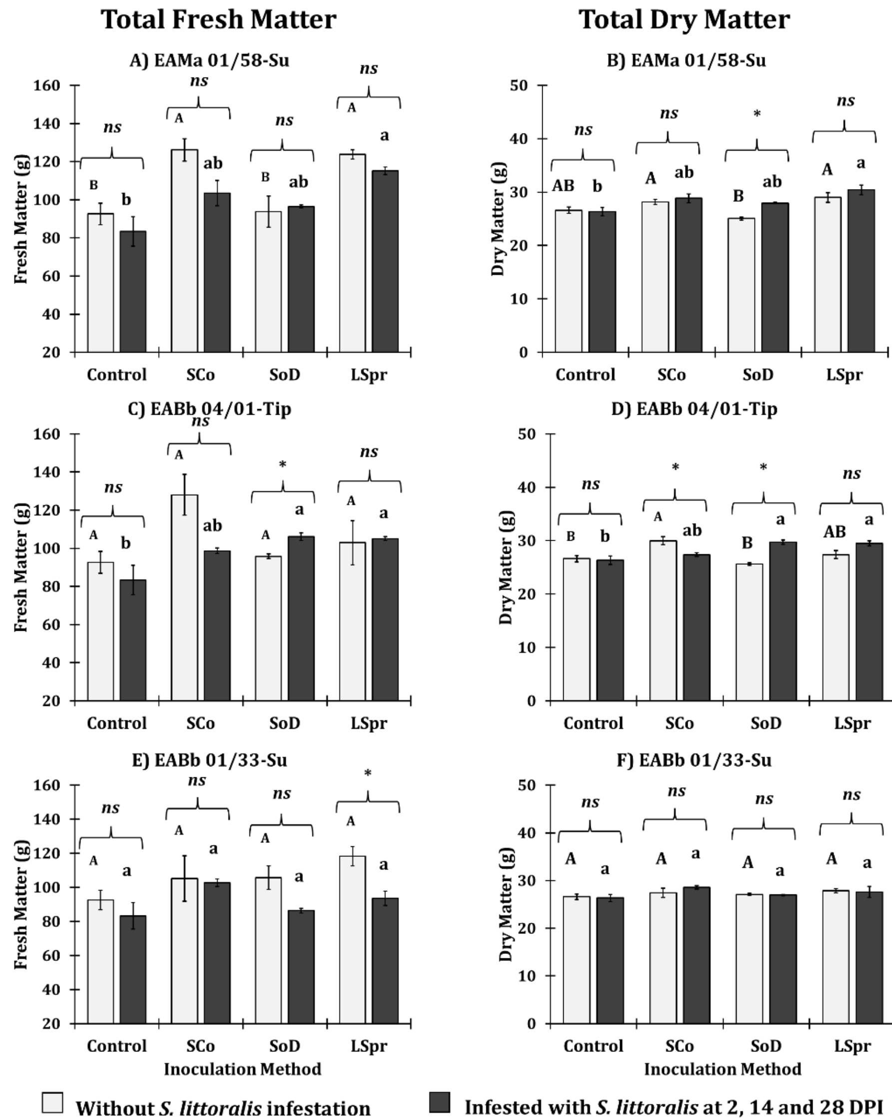

Figure II.2. Fresh (A, C and E) and dry (B, D and F) matter from melon plants without *S. littoralis* infestation (clear bars) and plants that were exposed to three successive short infestations (2, 14 and 28 DPI) of *S. littoralis* (dark bars). Melon plants were inoculated with a suspension containing 1x10^{8} conidia/ml of EAMa 01/58-Su, EABb 04/01-Tip or EABb 01/33-Su strains by three methods of inoculation: seed coating (SCo), soil drenching (SoD) and leaf spraying (LSpr). Infestation was comprised of six L3 *S. littoralis* larvae confined per plant with organza textile bags for 3 days at 2, 14 and 28 DPI. Samples were taken at the end of the experiment (31 DPI). Capital letters over the bars denotes significant differences between inoculated plants and their respective controls, and in the absence of *S. littoralis* infestation. Lower case letters over the bars denote significant differences between inoculated plants and their respective controls, and in the presence of *S. littoralis* infestation at 2, 14 and 28 DPI. Asterisks denote significant differences between non-infested plants and infested plants in the presence of *S. littoralis* larvae. Data were analyzed by completely randomized ANOVA followed by a Tukey test (p<0.05).

In general, no significant differences ($p \geq 0.05$) were observed when comparing non-infested and infested plants based on the inoculation method for each strain. However, a notable exception was seen for dry weight, where plants inoculated by soil drenching with strains EAMa 01/58-Su ($F_{1,5}$=105.51, p=0.0005) and EABb 04/01-Tip ($F_{1,5}$=79.27, p=0.0009) displayed significant variation (Figure II.2B and D). Specifically, 25.05 (non-infested) and 27.97 g (infested) of dry matter were recorded in plants inoculated by soil drenching with strain EAMa 01/58-Su; in the case of plants inoculated by soil drenching with strain EABb 04/01-Tip, 25.60 (non-infested) and 29.69 g (infested) of dry matter were recorded.

II.3.1.2.3. Mechanisms of phytohormone involvement in the fungus-driven plant defense

At 2 DPI, before *S. littoralis* infestation, it was only observed a significant increase in the relative expression of two genes, *ACO3* in plants inoculated by soil drenching with EABb 04/01-Tip *B. bassiana* strain (Figure II.3B) and *LOX2* in plants inoculated with EAMa 01/58-Su *M. brunneum* strain by leaf spraying (Figure II.3E). Beside this, *LOX2* experimented an increase, no significant, in those plants inoculated by leaves spraying with EABb 04/01-Tip and EABb 01/33-Su strains (Figure II.3E). However, in the remaining studied genes, it could be observed a significant downregulation of relative expression in all treatments (Figure II.3).

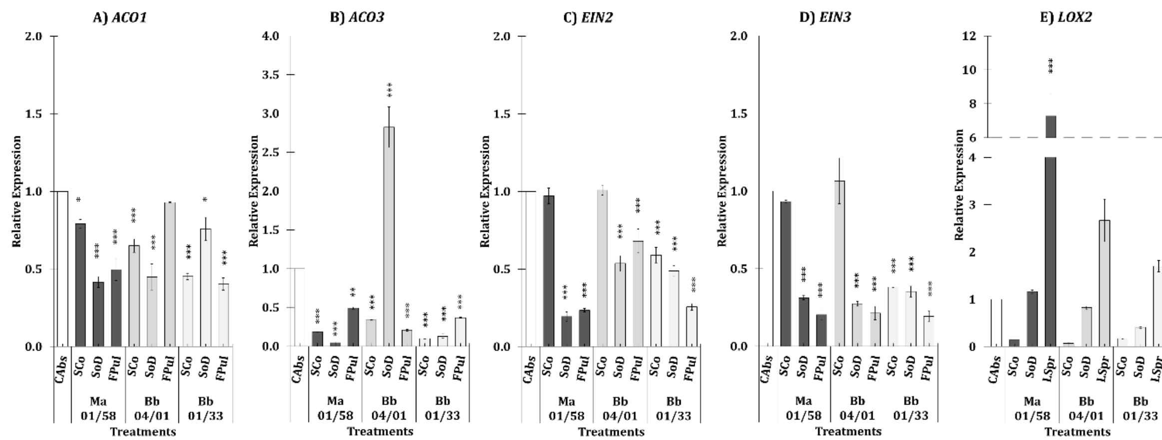

Figure II.3. Relative expression of genes related to ET synthesis (*ACO1* and *ACO3*) and transduction (*EIN2* and *EIN3*) pathway and JA synthesis (*LOX2*) were analyzed in melon leaves at 2 d after inoculation with a suspension containing $1x10^8$ conidia/ml of EAMa 01/58-Su, EABb 04/01-Tip or EABb 01/33-Su strains using three methods of inoculation: seed coating (SCo), soil drenching (SoD) or leaf spraying (LSpr). Plants were maintained in a culture chamber under controlled conditions. Relative expression data represent the mean of three independent biological replicates ± SE. Relative expression was calculated in relation to the absolute control. Bars with *, ** or *** indicate significant differences ($p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively) in relation to the absolute controls (CAbs) according to the Dunnett's test.

After the first *S. littoralis* infestation, broadly, across all strains used, the relative expression of studied genes significantly increased at specific time points assessed (by some methods used), regardless of the presence or absence of *S. littoralis*. Out of all the genes studied, the jasmonate synthesis related *LOX2*, the most closely associated with plant defense against chewing herbivores, was the one that reached the highest relative expression levels in colonized plants independently of the EA strain used.

In many cases, it could be observed a time gap between the induction of the ET synthesis related genes (*ACO1* and *ACO3*) and the ET transduction pathway related genes (*EIN2* and *EIN3*), e.g., in plants inoculated with EAMa 01/58-Su *M. brunneum* strain by soil drenching, *ACO1* and *ACO3* increased their expression level at 14 DPI while *EIN2* did it at 21 DPI (Figure II.4A, B and C) or in plants inoculated with EABb 04/01-Tip *B. bassiana* strain, *ACO1* increased its expression level at 14 DPI while *EIN2* did it at 21 DPI (Figure II.5A and C)

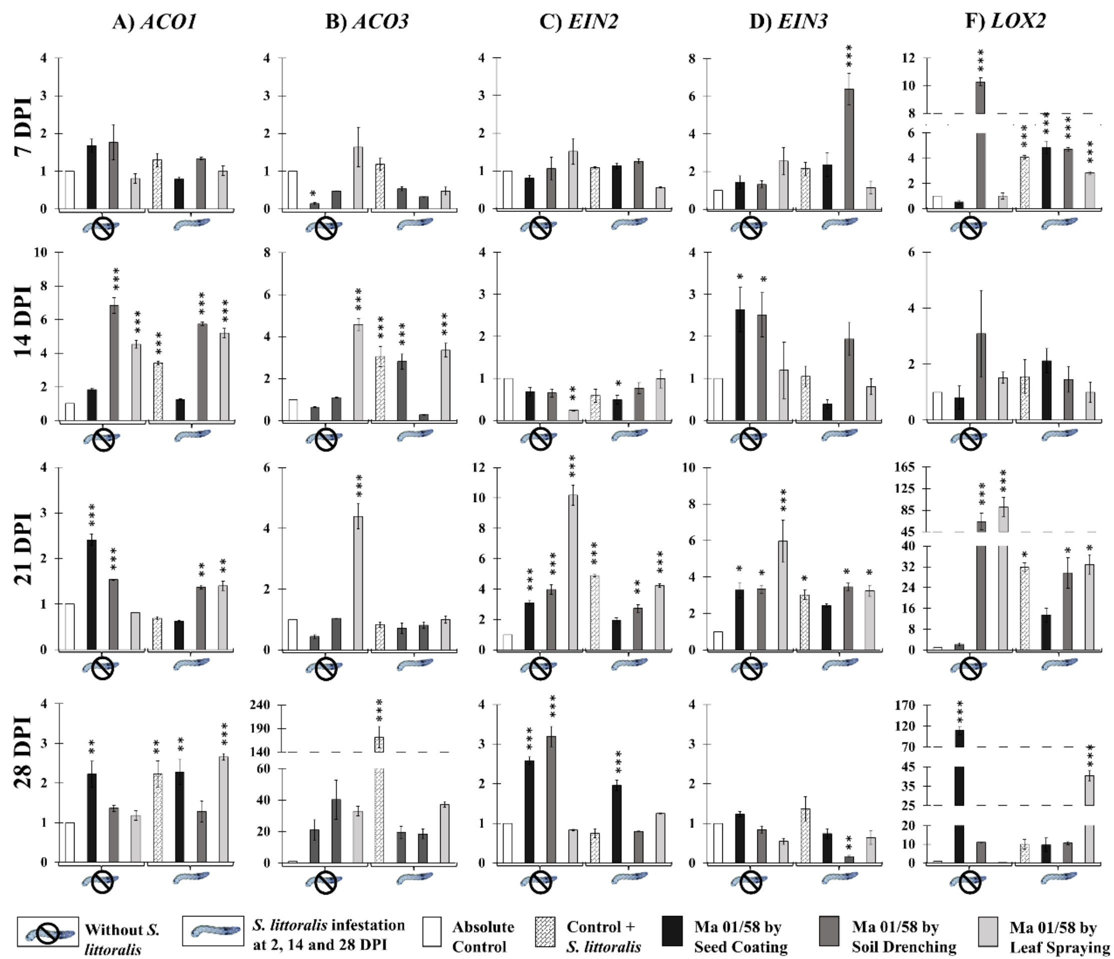

Figure II.4. Relative expression of ET biosynthesis (*ACO1* and *ACO3*), ET transduction (*EIN2* and *EIN3*) and JA (*LOX2*) biosynthesis pathway related genes analyzed in melon leaves after inoculation with a suspension containing $1x10^8$ conidia/ml of strain EAMa 01/58-Su by three methods of inoculation: seed coating, soil drenching or leaf spraying. Two controls were used, namely, an absolute control and a control with *S. littoralis* larvae; plants were maintained under controlled conditions in a culture chamber. Samples were collected at 7, 14, 21 and 28 DPI for qRT-PCR gene expression analysis. Relative expression data represent the mean of three independent biological replicates ± SE. Relative expression was calculated in relation to the absolute control. Bars with *, ** or *** indicate significant differences ($p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively) in relation to the absolute controls according to the Dunnett's test.

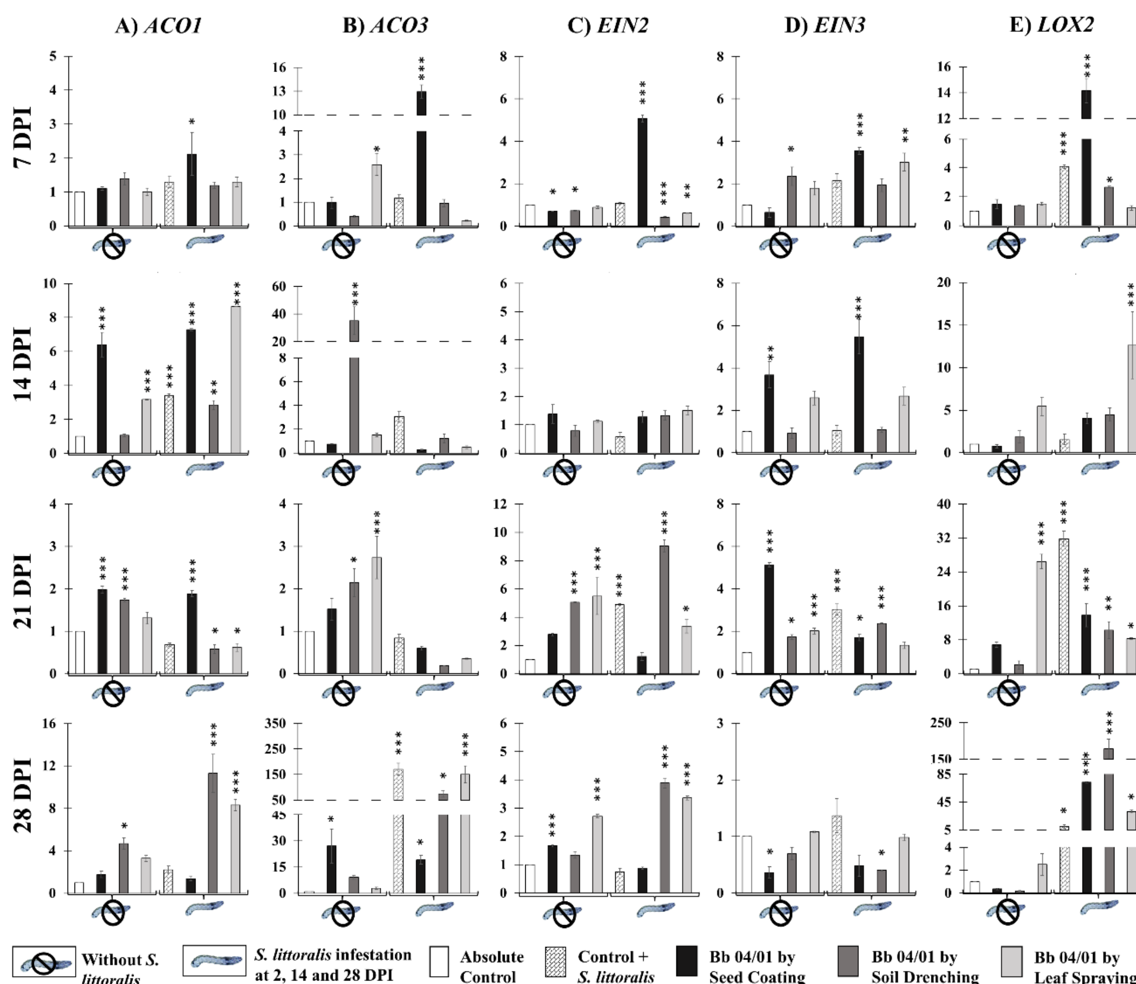

Figure II.5. Relative expression of ET biosynthesis (*ACO1* and *ACO3*), ET transduction (*EIN2* and *EIN3*) and JA (*LOX2*) biosynthesis pathway related genes analyzed in melon leaves after inoculation with a suspension containing 1x10^8 conidia/ml of strain EABb 04/01-Tip by three methods of inoculation: seed coating, soil drenching or leaf spraying. Two controls were used, namely, an absolute control and a control with *S. littoralis* larvae; plants were maintained under controlled conditions in a culture chamber. Samples were collected at 7, 14, 21 and 28 DPI for qRT-PCR gene expression analysis. Relative expression data represent the mean of three independent biological replicates ± SE. Relative expression was calculated in relation to the absolute control. Bars with *, ** or *** indicate significant differences ($p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively) in relation to the absolute controls according to the Dunnett's test.

LOX2 relative expression experienced a large and significant increase at any of the studied time points by any combination of inoculation method and strain used, reaching its maximum relative expression level at 21 DPI in those plants inoculated with EAMa 01/58-Su *M. brunneum* strain (Figure II.4E), while in plants inoculated with both *B. bassiana* strains this occurred at 28 DPI (Figure II.5E and Figure II.6E).

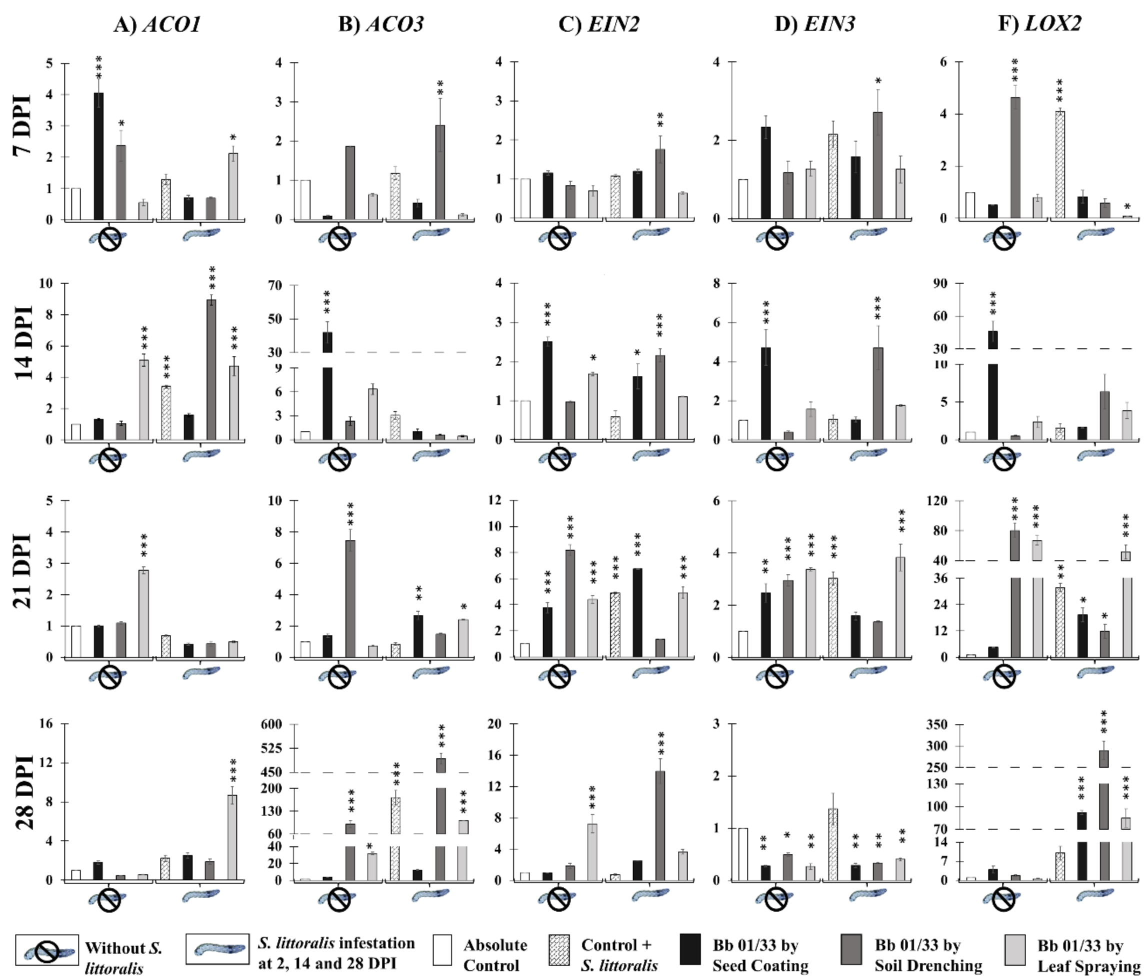

Figure II.6. Relative expression of ET biosynthesis (*ACO1* and *ACO3*), ET transduction (*EIN2* and *EIN3*) and JA (*LOX2*) biosynthesis pathway related genes analyzed in melon leaves after inoculation with a solution containing 1x10^{8} conidia/ml of strain EABb 01/33-Su by three methods of inoculation: seed coating, soil drenching or leaf spraying. Two controls were used, namely, an absolute control and a control with *S. littoralis* larvae; plants were maintained under controlled conditions in a culture chamber. Samples were collected at 7, 14, 21 and 28 DPI for qRT-PCR gene expression analysis. Relative expression data represent the mean of three independent biological replicates ± SE. Relative expression was calculated in relation to the absolute control. Bars with *, ** or *** indicate significant differences (p < 0.05, p < 0.01 or p < 0.001, respectively) in relation to the absolute controls according to the Dunnett's test.

Despite of the great diversity of scenarios we have in our experimental set up, due to the number of strains and inoculation methods used, our results showed a differential behavior between *M. brunneum* and the two *B. bassiana* strains respect to the relative expression observed. In the case of *M. brunneum* it could be observed that the highest genes relative expression levels were observed in those treatments without

S. littoralis (Figure II.4), while in the case of both *B. bassiana* strains, those plants challenged with *S. littoralis* larvae showed highest relative expression levels of most of studied genes. These results suggest that the gene expression levels reached with *M. brunneum* treatment are enough for plants defense against *S. littoralis* and that the infestation with the noctuid does not represent an additional stress to plants (Figure II.4). In contrast, in *B. bassiana* treatments, the subsequent infestation of *S. littoralis* would represent to plants an additional challenge that trigger a relative expression increase up to those levels reached without the noctuid (Figure II.5 and 6). Hence, a model of melon plant defense signaling network in response to endophytic entomopathogenic fungus colonization and *S. littoralis* herbivory is proposed (Figure II.7). At the first observation point (2 DPI), it could be observed a general downregulation of the relative expression of all gene studied. The first induction occurred at 7 DPI when a relative expression increase of *LOX2* was observed. Then, it is evidenced a crosstalk between JA and ET synthesis related genes in which JA would induce ET synthesis and vice versa. In this way, at 21 DPI it could be observed an increase of the relative expression of both hormones related genes, JA and ET. As consequence of this crosstalk, an increase of ET and JA related genes is observed at 28 DPI (Figure II.7A). In the case of inoculated plants infested with *S. littoralis* (Figure II.7B), basically occurred something like that observed in uninfested plants (Figure II.7A), but the relative expression levels reached, mainly of *LOX2*, were higher.

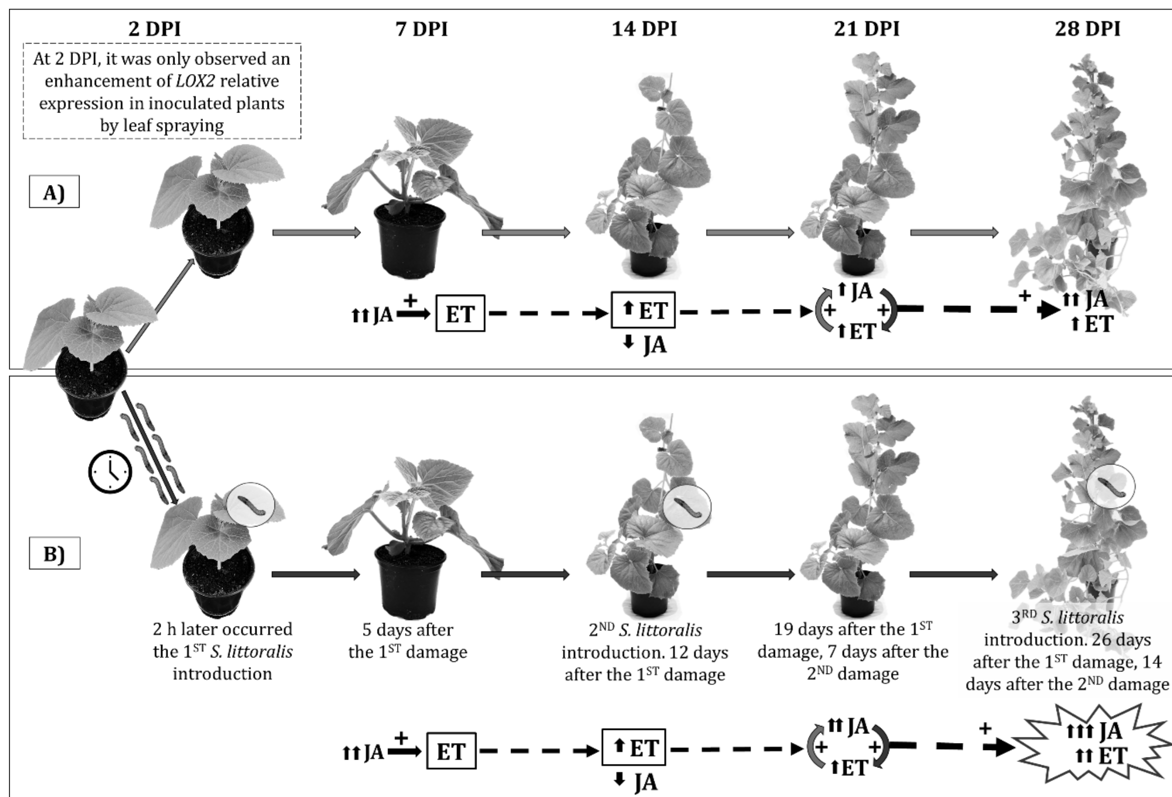

Figure II.7. Model of melon plant defense signaling network in response to endophytic entomopathogenic fungus colonization and *S. littoralis* herbivory. Melon plants were inoculated with a suspension containing 1x10^8 conidia/ml of EAMa 01/58-Su, EABb 04/01-Tip or EABb 01/33-Su strains using three methods of inoculation: seed coating, soil drenching or leaf spraying. Plants were maintained in a culture chamber under controlled conditions. Subsequently, in a set of plants *S. littoralis* larvae were introduced in unsprayed leaves for three days at 2, 14 and 28 DPI, whereas another set of plants remained free of armyworm infestation throughout the experiment. Genes relative expression levels were analyzed in melon leaves, before *S. littoralis* introduction, at 2 DPI, and after the noctuid larvae introduction samples were taken at 7, 14, 21 and 28 DPI. A and B represent inoculated melon plants uninfested or infested with *S. littoralis* larvae, respectively. In each case, A and B, bellow the plants, it is represented the crosstalk between JA and ET. Up or down arrows indicate up or down-regulation of related genes, respectively; plus sign indicates an activating signal; arrow with dotted line represents the hormone remaining signal between different observation time points.

II.3.2. Experiment 2. Tolerance of EA-inoculated melon plants to one long-term *S. littoralis* infestation

II.3.2.1. Successful development to adulthood of *S. littoralis* fed on inoculated plants

In the second experiment, plants were exposed to long-term infestation by L5 *S. littoralis* larvae that were able to feed on the melon plants until they reached prepupal stage. When strain EAMa 01/58-Su was applied by soil drenching it significantly

reduced the likelihood of *S. littoralis* larvae successfully achieving adulthood ($\chi^2_{(2)}$=53.57, *p*=0.0001). Indeed, numbers reaching adulthood were lower in both cases, compared with the absolute control (supplemented with artificial diet only) ($\chi^2_{(1)}$= 49.95, *p*=0.0001) and the control (fed on non-inoculated plants) ($\chi^2_{(1)}$= 5.52, *p*=0.0188); specifically the percentages successfully achieving adulthood were 100%, 63.64 and 49.67% in the absolute control, control and inoculated treatment, respectively (Figure II.8A).

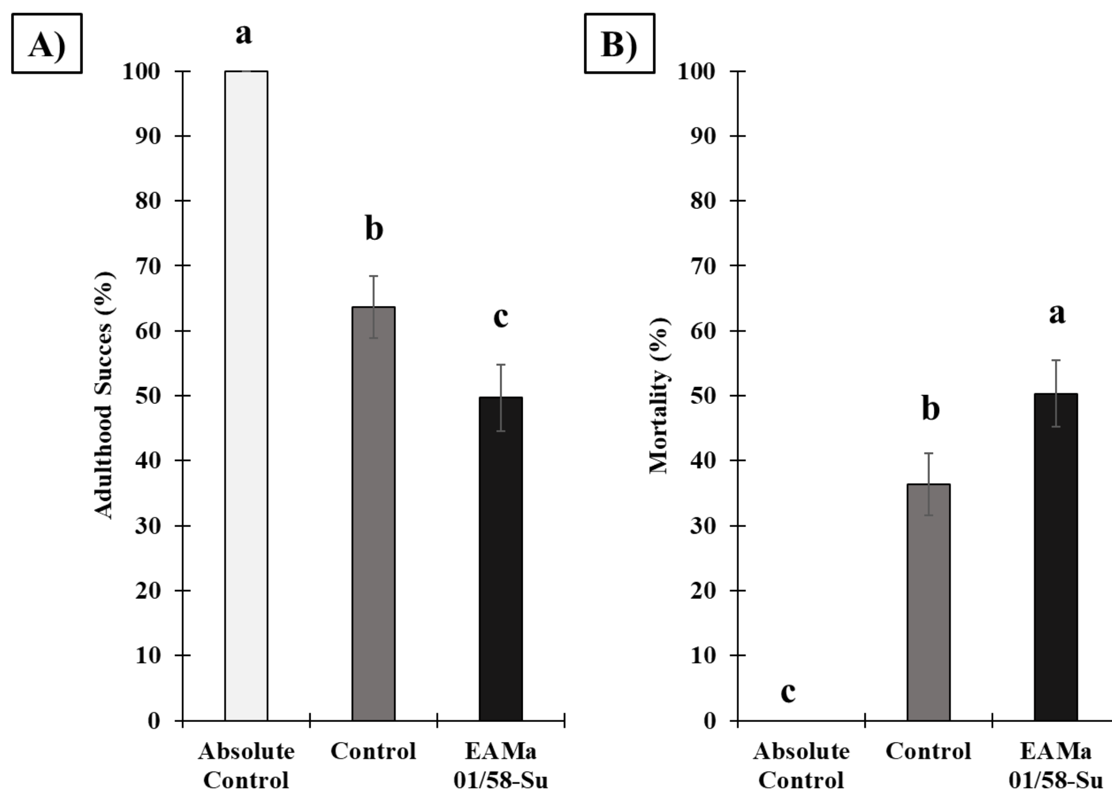

Figure II.8. Achieving successful adulthood from L5 instar to the prepupal stage (A) and Total mortality (B) of *S. littoralis* on melon plants. Sixty L5 (six per plant) larvae were introduced 21 DPI to inoculated *Cucumis melo* plants, a group of 12 larvae constituted a replicate in each treatment; an absolute control was included in this part of the study, which was comprised of larvae supplemented with artificial diet. Plants were inoculated by soil drenching with 5 ml of a suspension containing 1x108 conidia/ml of EAMa 01/58-Su strain. Data presented are means ± SE for each strain and control; columns with different letters are significantly different from each other according to the Tukey HSD test (p<0.05).

Total mortality was also significantly different amongst treatments ($\chi^2_{(2)}$=104.94, *p*=0.0001). Mortality was significantly higher on inoculated plants than in the absolute control ($\chi^2_{(1)}$=48.29, *p*=0.0001) and the control ($\chi^2_{(1)}$=7.91, *p*=0.0049); mortality was 0%, 36.36 and 50.33% in larvae supplemented with artificial diet, fed on non-inoculated plants or fed on plants inoculated with strain EAMa 01/58-Su, respectively (Figure II.8B).

II.3.2.2. Effects of EAMa 01/58-Su colonization and *S. littoralis* infestation on biomass production by melon plants

There were significant differences between treatments in fresh weight ($F_{3,29}$=9.05, *p*=0.0003), dry matter weight ($F_{3,29}$=173.33, *p*=0.0001), chlorophyll content ($F_{3, 29}$=3.53, *p=0.0287*), number of secondary branches ($F_{3,29}$=3.84, *p=0.0212*) and stem diameter ($F_{3,29}$=4.46, *p=0.0118*) (Figure II.9A-E). Soil inoculation with strain EAMa 01/58-Su increased all studied growth parameters. Indeed, dry matter was significantly higher (*p*<0.05) in inoculated and infested plants compared with non-inoculated and infested plants (Figure II.9B).

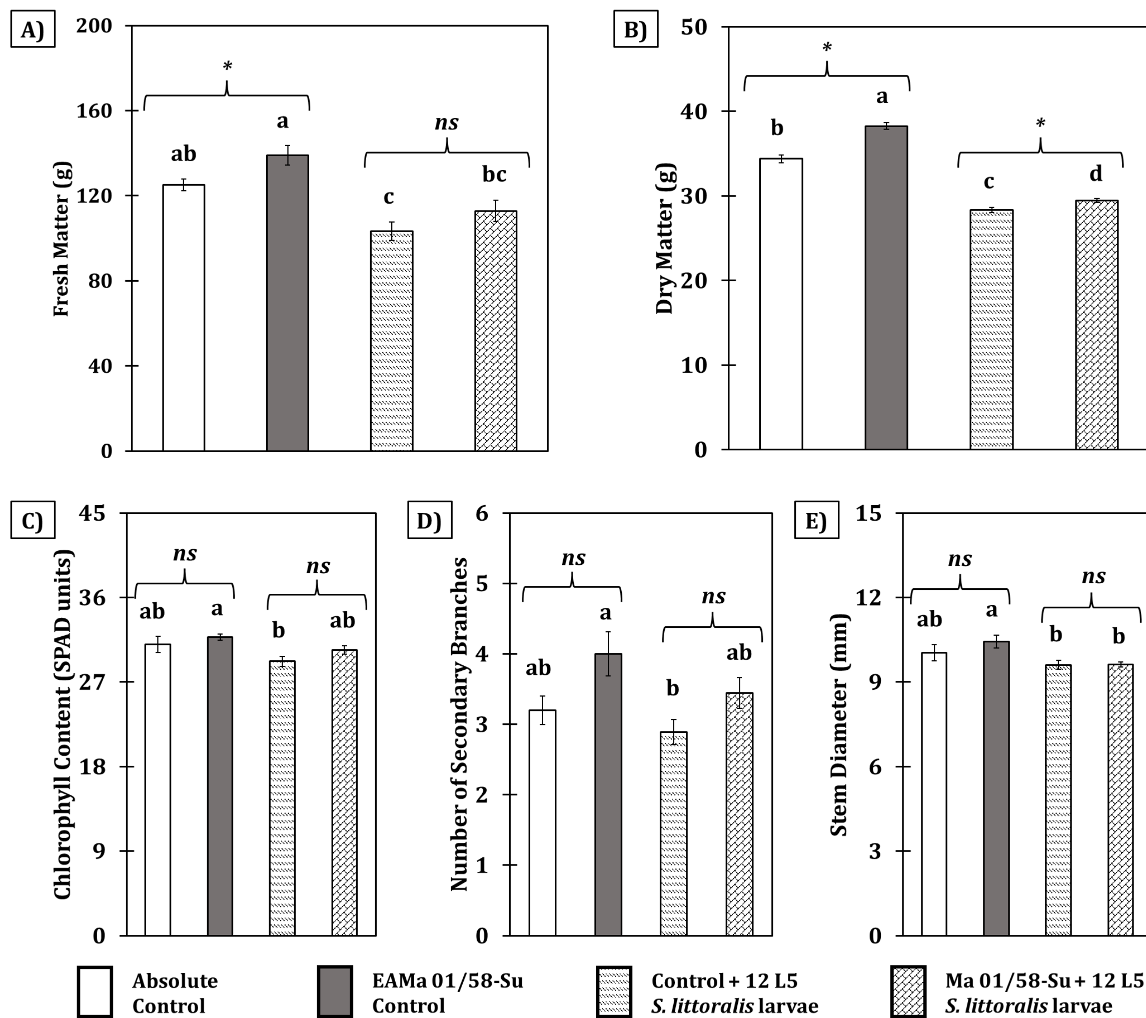

Figure II.9. Mean ± SE of fresh (A) and dry (B) matter, chlorophyll content (C), number of secondary branches (D) and stem diameter (E) at the end of the experiment (37 DPI) of melon plants in the presence and absence of *S. littoralis* infestation. Melon plants were inoculated by soil drenching with 5 ml of a suspension containing $1x10^8$ conidia/ml of EAMa 01/58-Su strain. Sixty L5 (a group of 12 larvae constituted a replicate for each treatment) were introduced at 21 DPI in inoculated *Cucumis melo* plants. Samples were taken at the end of the experiment (37 DPI). Letters over bars indicate significant differences between treatments. Asterisks denote significant difference between non-inoculated and inoculated plants or between non-inoculated and inoculated infested plants. Data were analyzed using a completely randomized ANOVA followed by a Tukey test (p<0.05).

II.4. Discussion

In this study, we have demonstrated, for the first time, that an EA inoculation mediated both an antibiosis response and a tolerance response in melon plants. EA-inoculated melon plants responded positively to various scenarios of *S. littoralis* infestation, which resulted in increases in fresh and dry weight while also inducing sublethal effects on *S. littoralis* larvae feeding on fungus-challenged plants. Furthermore, we elucidated the molecular mechanisms governing regulation of the observed effects. We also examined progression of endophytic colonization at the molecular level, and our findings suggest that it may not necessarily be a single unique factor mediating interactions amongst plants, insects, fungi, and their subsequent effects on *S. littoralis* fitness.

Significant sublethal effects were noted in terms of *S. littoralis* larval development time and pupal weight. These effects were observed when larvae were introduced 2, 14, and 28 DPI to inoculated plants and fed exclusively on them for a 3-day period. In general, it was evident that larvae introduced to inoculated plants at 2 DPI, regardless of the fungal strain and inoculation method, exhibited more pronounced sublethal effects. These sublethal effects may be attributed to the notably high relative expression levels observed in most of the studied genes related to ET and JA regulation at various time points, even though, in many instances, induction of these genes did not align in time with the observed sublethal effects. It should be noted that larvae fed on the plant for three days, and that gene expression has an 'undulating' (increasing and decreasing) profile that may have varied during this time (Rubio et al. 2014; Jensen et al. 2020; García-Espinoza et al. 2023a). Sublethal effects caused by EA on noctuid larvae and pupae have been reported previously, but the underlying mechanisms were not explained in earlier studies (Resquín-Romero et al. 2016a; Kalvnadi et al. 2018).

Results from molecular quantification of fungi showed low rates of progression of endophytic colonization by strains EAMa 01/58-Su, EABb 04/01-Tip and EABb 01/33-Su when applied using the three inoculation methods; foliar inoculation led to the greatest colonization as reported previously by García-Espinoza et al. (2024). However, the presence of *S. littoralis* had no effect on the endophytic behavior of any strain. Interestingly, subsequent effects on *S. littoralis* fitness were not correlated with

internal presence of the fungi. Soil drenching and seed coating were as effective as leaf spraying for initiating sublethal effects on *S. littoralis*. Our previous studies demonstrated that these strains achieved temporal and transient endophytic colonization of melon plants (Resquín-Romero et al. 2016a; Ríos-Moreno et al. 2016; Garrido-Jurado et al. 2017). Additionally, Rasool et al. (2021), suggested that the frequency of endophytic colonization by EA did not determine subsequent effects on aphid populations. While EA strains were capable of colonizing melon plants, the observed increase in relative expression of studied genes was mainly due to priming rather than EA presence in the plant, since only minimal traces of strains were detected in plant tissues over time. Comparable results were obtained in a recent study in *C. sativus* and *C. melo* plants primed with EAMa 01/58-Su strain (García-Espinoza et al. 2023a).

In general, establishment of endophytic microorganisms implies early down-regulation in expression of most defense genes (González-Guzmán et al. 2022). This is consistent with the results obtained in the current study, which showed downregulation of most studied genes, except for *ACO3* and *LOX2* at 2 DPI. Furthermore, there was upregulation in the relative expression of all genes at subsequent time points after all inoculation methods, demonstrating an 'undulating' relative expression profile, as described in previous studies (Rubio et al. 2014; García-Espinoza et al. 2023a). Additionally, in most cases, relative expression levels of the studied genes reached similar values in plants inoculated with strain EAMa 01/58-Su (by any inoculation method) in presence or absence of *S. littoralis*. These results suggest that *S. littoralis* infestation did not represent an additional stress to inoculated plants and that the relative expression level achieved by inoculation with strain EAMa 01/58-Su, was enough to defend plants against herbivore attack. However, in the case of inoculated plants with both *B. bassiana* strains the relative expression of most of studied genes increase drastically after the two *S. littoralis* infestation in relation to uninfested plants. Interestingly, the gene expression increase in inoculated plants with EAMa 01/58-Su *M. brunneum* strain is not correlated with the endophytic presence in the studied tissue, this mean that the relative expression enhancement is mainly due to SR induction. However, in inoculated plants with EABb 01/33-Su *B. bassiana* strain, the higher relative expression levels are observed at 28 DPI, coinciding with the highest endophytic colonization. The fact that *B. bassiana* has been found naturally as an

endophyte in several plant species and has been artificially introduced into many others and even its vertical transmission as a real endophyte reported, and that *Metarhizium* species are less well known as endophytes and more as rhizosphere competent fungi could be behind this differential response (Vega 2018; Quesada Moraga 2020).

Traditionally, the expression of plant defense traits in response to increased JA levels is accompanied by potent inhibition of plant growth (Havko et al. 2016), mainly due to antagonistic crosstalk between plant hormonal signaling pathways (Züst and Agrawal 2017; Karasov et al. 2017; Ha et al. 2021). In the last decade, much progress has been made towards understanding the core components of JA signaling and how they are integrated into the wider hormone response network; despite this, our understanding of how JA signaling controls plant growth is still poorly understood (Havko et al. 2016). However, in the present study, in any case this relative expression increase had a detrimental effect on plant growth. It can be observed similar growth rates both in uninfested and infested inoculated plants. According to our results, in a very recent study, it was reported that *Trichoderma harzianum* and *B. bassiana* are not only potential growth promoters but also elicit stronger defense responses (mainly SA and JA-dependent) and reduce damage due to herbivory (Van Hee et al. 2023).

Although the potential uses of EA beyond their traditional role in insect pest control, has been noted previously (Quesada-Moraga et al. 2022), there have been few studies on: the mechanisms underlying plant antibiosis and tolerance to herbivore attack; plant responses to priming by EA strains; effects of inoculation method; and effects of the presence of insect herbivores. Our research represents a significant advancement in understanding these mechanisms and the indirect effects of EA on tritrophic relationships. It demonstrates the remarkable ability of EAMa 01/58-Su, EABb 04/01-Tip and EABb 01/33-Su strains to induce a ‘state of readiness’ in plants, priming them to respond to future attacks.

Author contributions

Conceptualization, EQM, MYY and FGE; methodology, FGE, MYY and MJG; formal analysis, FGE, MYY and MJG; writing, review, and editing, FGE, MYY, MJG and EQM. All authors have read and agreed to the published version of the manuscript.

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Data Availability. Data are in the possession of the authors; for any additional information contact the corresponding author. Supporting information is available as additional files in the web version of this article.

Conflict of interest. The authors declare that there is not competing interest.

Supplementary material

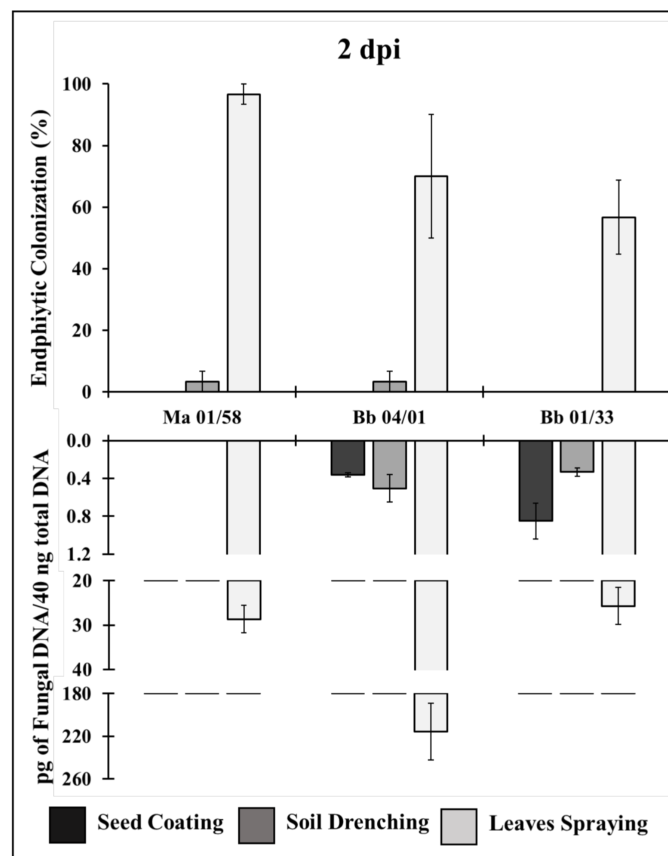

Figure II.S1. Endophytic colonization detected in melon leaves at 2 DPI after inoculation with EAMa 01/58-Su, EABb 04/01-Tip and EABb 01/33-Su strains following three application methods: seed coating, soil drenching and leaf spraying. Endophytic colonization was assessed by microbiological (upper graph) and by molecular techniques (lower graph) in samples collected prior to the first *S. littoralis* infestation. Endophytic colonization assessed by microbiological technique is expressed as a percentage of melon leaf fragments in which fungal growth was observed; molecular assessment and quantification is expressed in picograms (pg) of fungal DNA in 40 nanograms (ng) of total DNA per reaction (mean ± SE). Evaluation of endophytic colonization was done 2 days after leaf spraying or soil application in samples of basal true leaves; in the case of leaf spraying, samples came from sprayed leaves; in the case of seed coating, plants were inoculated at phenological stage 0 (germination); for soil application and foliar spray, plants were inoculated at the four-leaf stage.

Relationship between endophytic colonization, gene expression and effects on *S. littoralis*

Although Pearson's Coefficient indicated that, overall, there was no significant correlation between gene expression and endophytic presence of any of the strains or any of the inoculation methods used, it was apparent that, in some specific cases there was a direct correlation, particularly when strains EAMa 01/58-Su and EABb 01/33-Su

were applied as seed coatings (Table II.S1). The strongest direct correlations were observed between expression of *ACO3* and endophytic colonization of strain EAMa 01/58-Su applied by soil drenching. In most instances, neither soil drenching or leaf spraying (after which more endophytic colonization was observed for all strains) resulted in significant correlation between gene level expression and endophytic presence of strains (Table II.S1).

Table II.S1. Correlation coefficients (Pearson) between endophytic colonization and relative gene expression observed at 2, 7, 14, 21 and 28 DPI in plants inoculated with strains EAMa 01/58-Su, EABb 04/01-Tip and EABb 01/33-Su by seed coating (SCo), soil drenching (SoD) and leaf spraying (LSpr).

Strain	**EAMa 01/58-Su**			**EABb 04/01-Tip**			**EABb 01/33-Su**		
Gene	**SCo**	**SoD**	**LSpr**	**SCo**	**SoD**	**LSpr**	**SCo**	**SoD**	**LSpr**
ACO1	0.63 *	-0.15	-0.47	-0.50	-0.22	-0.40	0.52 *	-0.36	-0.44
ACO3	0.70 **	0.99 ***	-0.27	-0.27	-0.14	-0.23	0.82 **	0.45	-0.24
EIN2	0.37	-0.19	-0.42	0.48	-0.02	-0.48	0.33	0.47	-0.47
EIN3	-0.13	-0.44	-0.44	-0.18	-0.38	-0.62 *	-0.54 *	-0.39	-0.47
LOX2	0.22	0.03	-0.30	-0.54 *	-0.17	-0.41	0.83 **	0.50	-0.34

*$p<0.05$; **$p<0.01$; ***$p<0.001$

Correlation coefficients between endophytic colonization and sublethal effects on *S. littoralis* (Table II.S2), and between relative gene expression and sublethal effects on *S. littoralis* (Table II.S3) were, overall, not significant. These results should be analyzed case by case since the values for each sublethal effect have relationships with both endophytic colonization and relative gene expression. Together, these results suggest that the most effective inoculation method to improve *S. littoralis* control by indirect mechanisms, would be soil drenching.

Table II.S2. Correlation coefficients (Pearson) between endophytic colonization and effects on *S. littoralis* fed at 2, 14 and 28 DPI during 3 days on plants inoculated with EAMa 01/58-Su, EABb 04/01-Tip and EABb 01/33-Su by seed coating, soil drenching and leaf spraying.

Isolate	**Ma 01/58**			**Bb 04/01**			**Bb 01/33**		
Effect/Gene	**SCo**	**SoD**	**LSpr**	**SCo**	**SoD**	**LSpr**	**SCo**	**SoD**	**LSpr**
Mort	-0.26	0.09	0.23	-0.50	-0.44	-0.45	-0.23	-0.13	-0.62
LDev	-0.71 *	0.14	0.20	-0.07	-0.33	-0.10	-0.12	-0.10	-0.30
PWe	-0.11	-0.65	0.23	-0.17	0.30	0.21	-0.79 *	-0.45	-0.01
Ad. Succ.	0.26	-0.86 **	0.22	0.70 *	0.77 *	0.77 *	0.11	0.09	0.85 **

*$p<0.05$; **$p<0.01$; ***$p<0.001$

Table II.S3. Correlation coefficients (Pearson) between relative gene expression and effects on *S. littoralis* fed at 2, 14 and 28 DPI during 3 days on plants inoculated with EAMa 01/58-Su, EABb 04/01-Tip and EABb 01/33-Su by seed coating, soil drenching and leaf spraying.

	Isolate	**Ma 01/58**			**Bb 04/01**			**Bb 01/33**		
Gene	**Effect**	**SCo**	**SoD**	**LSpr**	**SCo**	**SoD**	**LSpr**	**SCo**	**SoD**	**LSpr**
ACO1	**L. Mort**	-0.05	0.01	0.08	0.56	0.31	0.52	0.33	0.49	0.29
	LDev	-0.60	0.22	-0.30	0.26	0.30	0.11	0.01	0.49	-0.07
	PWe	-0.18	0.65	-0.45	0.51	-0.72 *	-0.24	-0.57	0.55	-0.42
	Ad. Succ.	-0.02	0.00	-0.30	-0.85 **	-0.57	-0.74 *	-0.43	-0.68 *	-0.45
ACO3	**L. Mort**	-0.17	0.23	-0.17	0.11	0.20	0.78 *	0.10	-0.25	0.00
	LDev	-0.67 *	0.11	-0.03	-0.13	0.18	-0.27	-0.09	0.02	-0.14
	PWe	-0.11	-0.63	0.08	-0.32	-0.77 *	-0.90 **	-0.81 **	-0.66	-0.60
	Ad. Succ.	0.13	-0.86 **	-0.20	0.04	-0.45	-0.73 *	-0.13	0.14	0.00
EIN2	**L. Mort**	-0.43	0.21	0.10	-0.01	0.32	0.61	0.25	-0.20	0.10
	LDev	-0.78 *	0.31	-0.08	0.02	0.19	-0.19	0.03	-0.06	-0.15
	PWe	0.00	-0.12	-0.22	0.32	-0.79 *	-0.71 *	-0.55	-0.48	-0.53
	Ad. Succ.	0.52	-0.75 *	-0.35	-0.29	-0.58	-0.96 ***	-0.43	0.06	-0.22
EIN3	**L. Mort**	-0.59	-0.18	0.36	0.34	0.34	0.21	0.29	0.41	0.72 *
	LDev	-0.29	0.06	-0.20	0.24	0.44	0.60	0.20	0.58	0.30
	PWe	0.37	0.59	-0.33	0.52	0.46	0.46	0.62	0.59	0.44
	Ad. Succ.	0.80 *	0.11	-0.28	-0.66	-0.28	-0.20	-0.51	-0.45	-0.86 **
LOX2	**Mort**	-0.07	0.37	-0.14	0.01	0.25	0.58	0.10	-0.24	-0.11
	LDev	-0.57	0.28	0.02	-0.12	0.19	0.21	-0.09	-0.07	-0.23
	PWe	-0.04	-0.72 *	0.18	-0.29	-0.80 **	-0.31	-0.82 **	-0.56	-0.59
	Ad. Succ.	-0.03	-0.80 *	-0.13	-0.06	-0.51	-0.86 **	-0.09	0.13	0.00

*$p<0.05$; **$p<0.01$; ***$p<0.001$

CHAPTER III. ENTOMOPATHOGENIC FUNGUS-RELATED PRIMING DEFENSE MECHANISMS IN CUCURBITS IMPACT *Spodoptera littoralis* (BOISDUVAL) FITNESS

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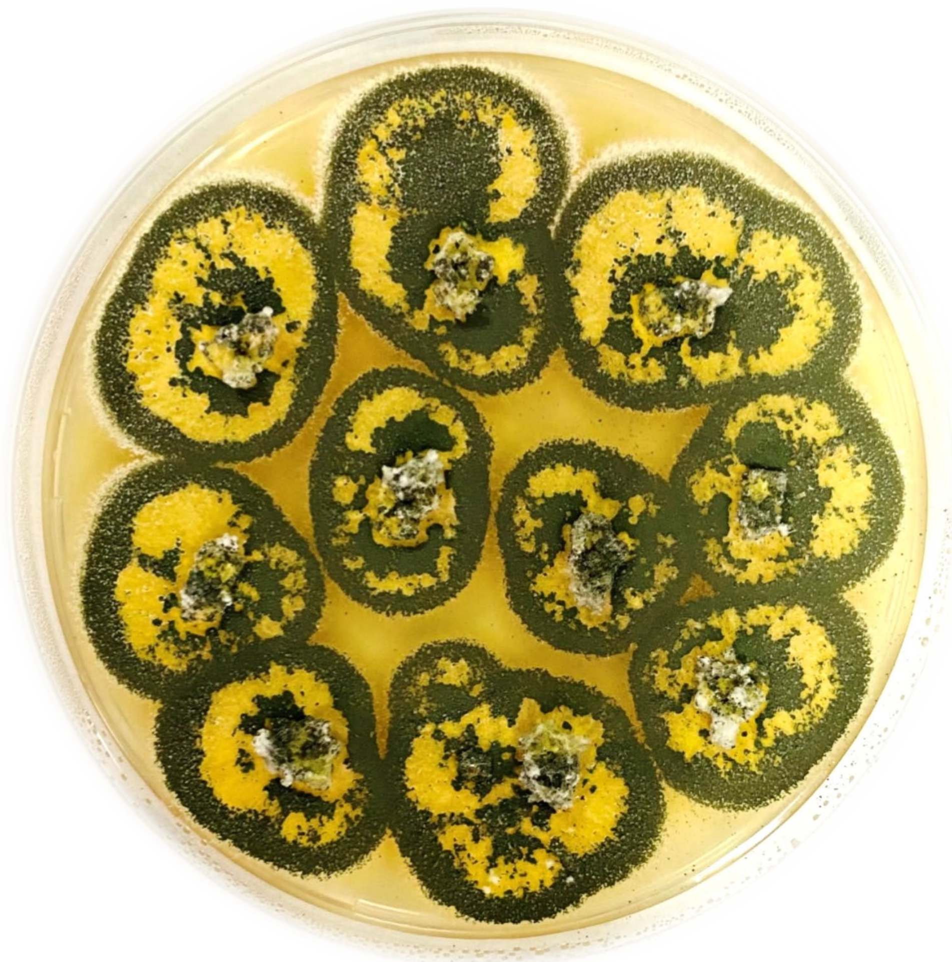

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Abstract

Entomopathogenic fungi (EPF) exhibit direct and indirect mechanisms to increase plant resistance against biotic and abiotic stresses. Plant responses to these stresses are interconnected by common regulators such as ethylene, which is involved in both iron (Fe) deficiency and Induced Systemic Resistance responses. In this work, the roots of cucurbits seedlings were primed with *Metarhizium brunneum* (EAMa 01/58-Su strain), relative expression of 18 genes related to ethylene (ET), jasmonic acid (JA) and salicylic acid (SA) synthesis, as well as pathogen related (PR) protein genes were studied by qRT-PCR. Effects of priming on *Spodoptera littoralis* was studied by feeding larvae for 15 days with primed and control plants. Genes showed up-regulation in studied species, however, highest relative expression were observed in roots and shoots of plants with Fe deficiency, demonstrating the complexity and the overlapping degree of the regulatory network. *EIN2* and *EIN3* should be highlighted, both key genes of the ET transduction pathway that enhanced their expression levels up to eight and four times respectively in shoots of primed cucumber. Also, JA, SA synthesis and PR genes showed significant up-regulation during observation period (e.g. the JA gen *LOX1*, increased 506 times). Survivorship and fitness of *S. littoralis* were affected with significant effects on mortality of larvae fed on primed plants vs. controls, length of the larval stage, pupal weight and the percentage of abnormal pupae. These results highlight the role of EAMa 01/58-Su strain in the induction of resistance, which would be translated into direct benefits for plant development.

Importance

Entomopathogenic fungi are multipurpose microorganisms with direct and indirect effects on insect pests. Also, EPF provide multiple benefits to plants by solubilizing minerals and facilitating nutrient acquisition. A very interesting and novel effect of this fungi is the enhancement of plant defense systems by inducing systematic and acquired resistance. However, little is known about this function. This study sheds light on the molecular mechanisms involved in cucurbits plants' defense activation after being primed by the EPF *M. brunneum*. Furthermore, the subsequent effects on the fitness of the lepidopteran pest *S. littoralis* are shown. In this regard, a significant up-regulation was recorded for the genes that regulate JA, SA and ET pathways. This

increased expression of defense genes caused lethal and sublethal effects on *S. littoralis*. This could be considered as an added value for the implementation of EPF in integrated pest management programs.

Keywords *Metarhizium brunneum*, EAMa 01/58-Su strain, cucumber, melon, ISR, Fe deficiency.

III.1. Introduction

Entomopathogenic fungi (EPF) are recognized as excellent biocontrol agents to form part of any Integrated Pest Management program due to their capacity to infect wide range of arthropod pests in a unique way of action, by contact (Resquín-Romero et al. 2016b, a; Yousef et al. 2017; Garrido-Jurado et al. 2017; Sánchez-Rodríguez et al. 2018; Quesada-Moraga et al. 2020; Miranda-Fuentes et al. 2021b). Also, they can interact with crops and establish mutualistic interactions that not only protect it against arthropod pests but also that could bring benefits to the plant such as plant nutrient acquisition improvement, enhancement of growth and development, immunity, and resistance to other biotic and abiotic stresses (Kogel et al. 2006; Barelli et al. 2016; Bamisile et al. 2018a, b; Hu and Bidochka 2019; Branine et al. 2019; Dara 2019b; Hossain and Sultana 2020; Quesada Moraga 2020; Kumari et al. 2021; Nosheen et al. 2021; García-Espinoza et al. 2023b). These functions of EPF led to several multitrophic interactions with important roles in biocontrol (Quesada-Moraga et al. 2022). Indeed, most EPF species are an important component of the soil microbiota and widely used to control soil-dwelling insect pests, and even well-known rhizosphere competent microorganism, especially *Metarhizium* spp. (Hu and St. Leger 2002; St. Leger 2008; Jaronski 2010; Yousef et al. 2017, 2018; Garrido-Jurado et al. 2019). In addition to the ability of most of EPF to endophytically colonize plant tissues, several species have been shown to provide a systemic protection to the plant by the activation of induced resistance (Jensen et al. 2020; Tiwari and Singh 2021; Tiwari et al. 2022). This indirect effect of EPF has been poorly studied compared to other non-entomopathogenic microorganisms with a proven ability to confer resistance to plants, in which several references can be found in the literature such as bacteria (van Loon et al. 1998; García-Gutiérrez et al. 2013; Djami-Tchatchou et al. 2020; Kong et al. 2020),

mycorrhizae (Pozo and Azcón-Aguilar 2007; Shrivastava et al. 2015; Zitlalpopoca-Hernandez et al. 2022) and specially with the genus *Trichoderma* (Alkooranee et al. 2015; Di Lelio et al. 2021, 2023; Morán-Diez et al. 2021; Monte 2023; Woo et al. 2023), where this indirect effect has been widely studied. In the case of EPF, although it has been described some cases of induction of the expression of several genes related to induced resistance, the lethal and sublethal effects showed in these works have been referred to the fungus presence in the plant tissues (Ahmad et al. 2020; Jensen et al. 2020), in *Metarhizium* genus case, the works that can be found in the literature about the induction of systemic resistance are too scarce (Ahmad et al. 2020; González-Guzmán et al. 2022).

The induced resistance is referred to the phenomenon that occurs when susceptible plants, as the result of a primary infection by a microbial pathogen, or attack by herbivores or by the interaction with parasitic or non-pathogenic microorganisms, develop defense responses or enhanced genetically programmed resistance to further attack (Heil 2001; Heil and Bostock 2002; Paré et al. 2005; Walters and Heil 2007). Some studies reported up-regulation of ET, JA, SA and PR genes as endogenous responses of resistant genotypes against phytopathogens such as *Phytophthora capsici* and *P. melonis* (Wang et al. 2020b; Hashemi et al. 2020) or as a result of inoculation/interaction with other microorganisms like bacteria or mycorrhizal fungi (García-Gutiérrez et al. 2013; Fiorilli et al. 2018). Recently, it has been published several works that show the effects of EPF on the enhancement of plants defense system and their lethal and sublethal effects on some pests by direct contact with fungus strain or by feeding on endophytically colonized tissues (Ahmad et al. 2020; Jensen et al. 2020; Miranda-Fuentes et al. 2021b).

Induced resistance is classified into two types, namely; Induced Systemic Resistance (ISR) and Systemic Acquired Resistance (SAR); SAR is triggered by plant pathogens, and ISR, triggered by root-colonizing mutualistic microbes, generally inhabitants of the rhizosphere (Romera et al. 2019; Dara 2019a; Aswani et al. 2022; Yu et al. 2022; Ding et al. 2022), likewise, when plants are exposed to nonpathogenic microbes, SAR could be also induced (Dara 2019a). Pathogen infection is sensed by innate immune receptors. The binding of conserved microbial molecules (pathogen-associated molecular patterns, PAMPs) by immune receptors induces PAMP-triggered immunity (PTI) which provides early protection. As consequence of the coevolution of

host and pathogen, PTI is suppressed by pathogen-derived virulence factors (effectors) which are released to host cells to facilitate infection. The recognition of specific pathogen effectors by intracellular nucleotide-binding/leucine-rich-repeat (NLR) receptors, activates the effector-triggered immunity (ETI). ETI induce PTI-associated defense pathways including the production of reactive oxygen species (ROS), mobilization of Ca^{2+}-dependent protein kinase and mitogen-activated protein kinase (MAPK) signaling cascades, generation of the phenolic hormone salicylic acid (SA), and transcriptional reprogramming (Cui et al. 2017).

ISR responses are mainly regulated by ethylene (ET) and jasmonic acid (JA) and typically independent of salicylic acid (SA) and functions without PR gene activation (Heil and Bostock 2002; Grant and Lamb 2006; Walters and Heil 2007; Beckers and Conrath 2007; Doornbos et al. 2011; Romera et al. 2019; Wang et al. 2020b; Aswani et al. 2022; Rajan et al. 2022). On the contrary, SAR is associated with pathogen infection, and it is characterized by increased SA levels which, through the redox-regulated protein NON-EXPRESSOR OF PR GENES1 (NPR1), activates the expression of a large set of PR genes, involved in defense responses (Pieterse and Van Loon 2004; Grant and Lamb 2006; Walters and Heil 2007; Doornbos et al. 2011; Romera et al. 2019; Aswani et al. 2022; Adeleke et al. 2022). SA accumulation can be controlled by some protein regulators, such as enhanced disease susceptibility 1 (EDS1), phytoalexin-deficient 4 (PAD4), EDS4, EDS5, and non-race-specific disease resistance 1 (NDR1), likewise, SA can enhance the expression of EDS1/PAD4/SAG101 through a positive feedback loop (Ding et al. 2022).

The cross-communication between these hormones signaling permits the plant to finely balance the defense response (Segaran et al. 2022). ET and JA act in an antagonistic way to regulate plant responses against cold, drought, and salinity stress (Yang et al. 2019; Wang et al. 2020a), however, against necrotrophic fungi and wounds, ET and JA act synergistically to coordinate plant defense responses (Liu and Timko 2021; Ding et al. 2022). SA inhibits the JA/ET pathway by the activation of NPRl. The cross-point between JA and ET signaling pathway occurs at ERF1 level, an ET response factor. JA can promote the activation of MYC2 transcription factor to induce JA responses signal through the interaction between JAZ, a repressor of JA signaling, and the SCFCOI1 ubiquitin ligase, which result in the ubiquitination of the JAZ protein and its degradation by the 26S proteasome (Ding et al. 2022). JAZ1 can also interacts with

DELLA proteins which results in increased JA signaling and decreased SA (Gilroy and Breen 2022). The DELLA family proteins are key regulators of GA signaling that repress transcription of GA-responsive genes (Yang et al. 2012). Low gibberellins (GA) hormone levels and high ET levels result in a high abundance of DELLA proteins. An increase in GA hormone levels results in gibberellins binding its receptor GID1 that induces interaction with DELLA proteins. The complex GA–GID1–DELLA interacts with SCFSLY1/GID2, a E3 ubiquitin ligase, targeting it for proteasomal degradation which results in a decrease of DELLAs abundance. This reduction in DELLAs initiates transcription of gibberellin response genes and release JAZ1, which results in an increase of SA signaling (Gilroy and Breen 2022).

Induced resistance, including ISR and SAR, is associated with an enhanced ability to resist pathogen attack by stronger activation of cellular defense responses (Conrath et al. 2001, 2015; Vallad and Goodman 2004; Conrath 2006, 2009), this enhanced ability or activation of defense is known as "*priming*" (Conrath et al. 2001; Paré et al. 2005; Conrath 2006, 2009; Gaupels and Vlot 2012; Cachapa et al. 2021) . Originally, priming was described as an enhanced resistance in response to natural or synthetic chemicals agents (Conrath et al. 2006, 2015; Beckers and Conrath 2007). Nowadays priming has been described in response to rhizosphere microbes, EIPF or pathogens (Conrath et al. 2001; Conrath 2006, 2009; Kumari et al. 2021; Martínez-Arias et al. 2021; Aswani et al. 2022; Segaran et al. 2022), in this sense, plant defense priming could be used as an Integrated Pest Management strategy for crop protection (Tiwari and Singh 2021; Tiwari et al. 2022).

Some of these ISR inducer microorganisms also promote growth plant and development (Pieterse et al. 2014) and favor Fe acquisition in plants (Pii et al. 2015, 2016; Zamioudis et al. 2015; Zhou et al. 2016; Marastoni et al. 2019; Aparicio et al. 2023; García-Espinoza et al. 2023b). This is in part due to the common involvement of ET and nitric oxide in the regulation of both processes and because of the crosstalk among ET and JA signaling pathways (Romera et al. 2019). The effect of these microorganisms on the improvement of Fe nutrition is related to their ability to up-regulate key genes related to Fe acquisition, such as *FIT, MYB72, IRT1* and *FRO2* (Pieterse et al. 2014; Romera et al. 2019; Aparicio et al. 2023; García-Espinoza et al. 2023b). On the other hand, MYB72, a key transcription factor (TF) in ISR activation also

participates in the regulation of the Fe deficiency responses through its interaction with FIT TF, a key regulator of Fe deficiency responses.

Due to this crosstalk among Fe deficiency responses and ISR in the present work we aimed to evaluate the ability of *Metarhizium brunneum* Petch (Hypocreales: Clavicipitaceae) strain EAMa 01/58-Su to induce defense responses in cucumber and melon plants under two nutritional conditions, Fe sufficient and deficient, to highlight the crosstalk among biotic and abiotic stresses. Relative expression of several genes involved in the JA, SA and ET synthesis/signaling pathways besides to the induction of PR protein genes was studied. Furthermore, it was evaluated the effect of root priming by EAMa 01/58-Su strain on the survival and development of larvae of the cotton leafworm *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae), a widely distributed very dangerous polyphagous insect pest that has been previously demonstrated to be susceptible to this fungal strain either by contact or by feeding on endophytically colonized tissues (Miranda-Fuentes et al. 2021b).

III.2. Results

Genes related to ET biosynthesis and transduction pathway. In cucumber roots and shoots, three genes related to ET biosynthesis were studied, namely *ACO1*, *ACO3* and *ACS7* (Figure III.1). In roots we found significant differences in relative expression levels of the three genes studied in both nutritional conditions at different time points in each case (Figure III.1).

It could be observed higher relative expression level of primed plants shoots under Fe sufficient conditions, *ACO1* and *ACS7* (Figure III.1A and C), and under Fe deficient conditions in the case of *ACO3* (Figure III.1F).

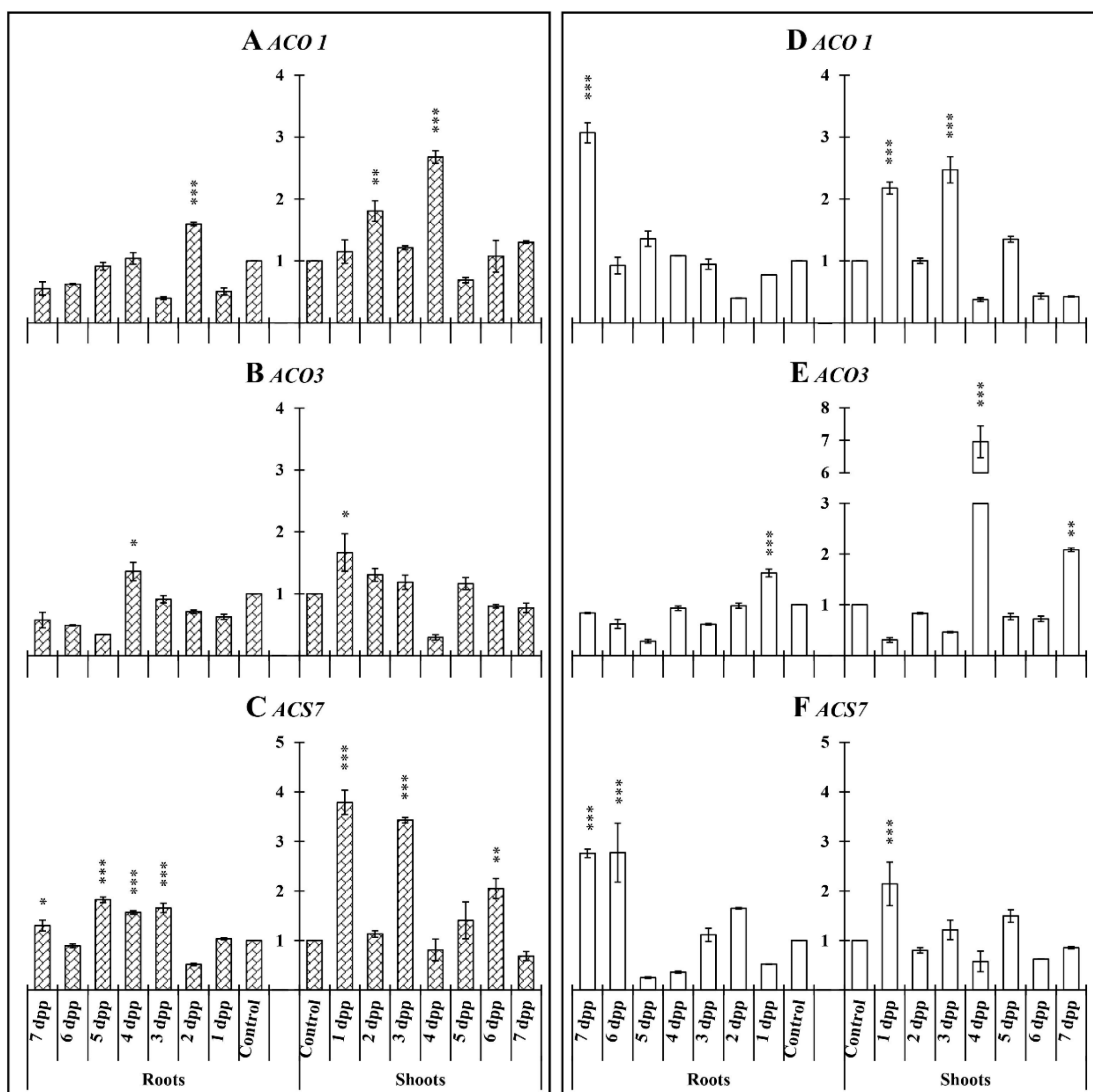

Figure III.1. Time-course evolution of the relative expression of genes involved in ET biosynthesis on roots and shoots of *C. sativus*. Plants were primed by root immersion during 30 minutes in EAMa 01/58-Su solution with 1x10^{7} conidia/ml; plants were maintained in a hydroponic system. Samples were collected during 7 d post-priming for qRT-PCR gene expression study. Left and right bars represent gene expression of plants grown under Fe sufficient and Fe deficient conditions, respectively. Data of *ACO1*, *ACO3* and *ACS7* expression represent the mean of three independent biological replicates ± S.E. The relative expression is based on the expression ratio of a target gene versus a reference gene. Bars with *, ** or *** indicate significant differences ($p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively) in relation to their respective control (+Fe40 or -Fe) according to the Dunnett's test.

In melon, the genes studied related to ET biosynthesis in roots and shoots were the following: *ACO1*, *ACO3, ACO5* and *ACS7.* In roots, only *ACO3* and *ACO5* significantly increased their relative expression in both conditions (Figure III.2B, C, F and G), it also could be observed significant differences in relative expression level of *ACO1* in roots of primed plants under Fe deficient conditions (Figure III.2E). *ACS7* was not detected in roots.

In shoots of primed plants all 4 genes studied showed a significant increment of their relative expression in both conditions at different time points (Figure III.2). *ACS7* showed an important increase of its relative expression at different times under both nutritional conditions (Figure III.2D and H).

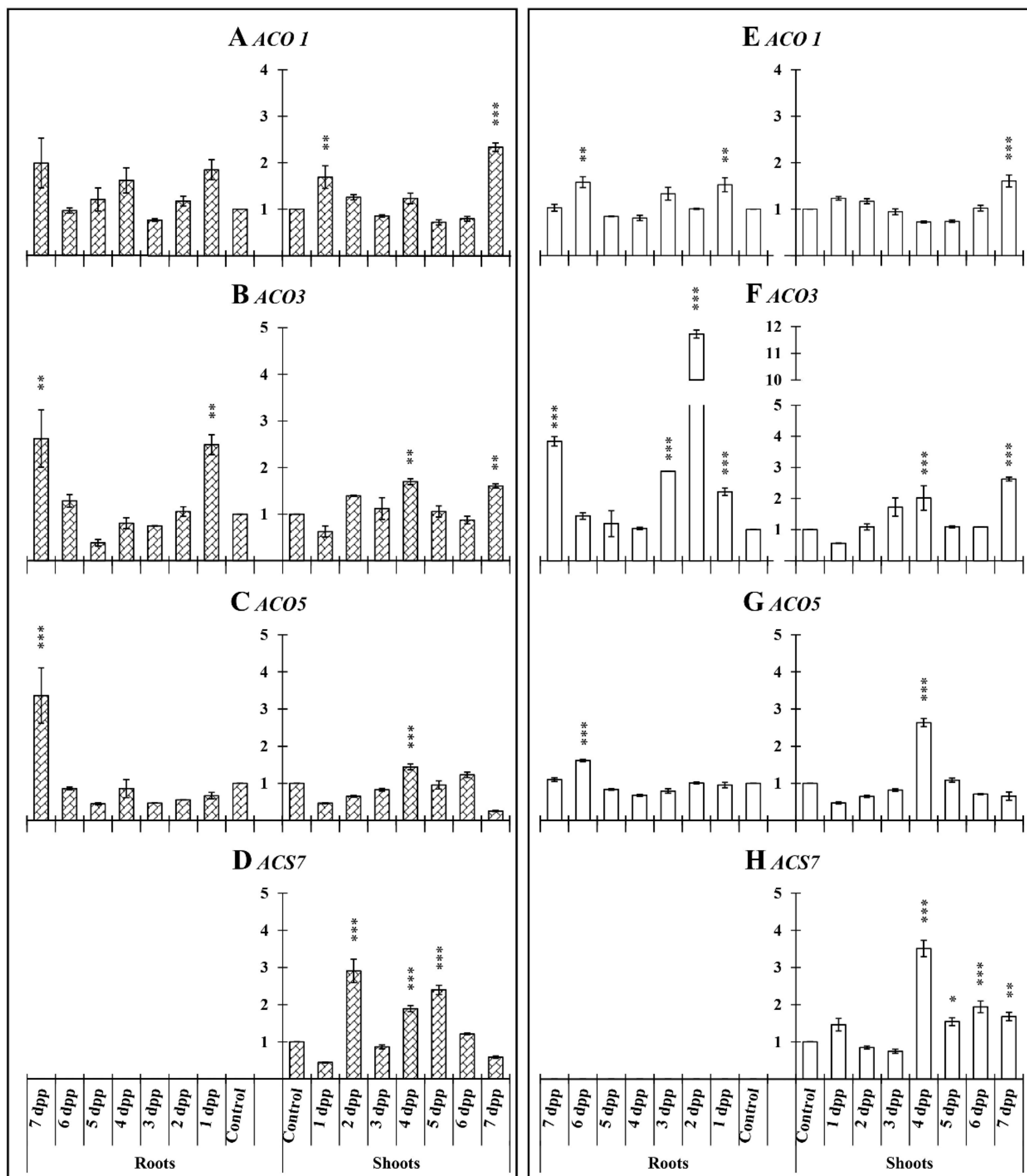

Figure III.2. Time-course evolution of the relative expression of genes involved in ET biosynthesis on roots and shoots of *C. melo*. Plants were primed by root immersion during 30 minutes in EAMa 01/58-Su solution with $1x10^7$ conidia/ml; plants were maintained in a hydroponic system. Samples were collected during 7 d post-priming for qRT-PCR gene expression study. Patterned bars (left) and white bars (right) represent gene expression of plants grown under Fe sufficient and Fe deficient conditions, respectively. Data of *ACO1*, *ACO3*, *ACO5* and *ACS7* expression represent the mean of three independent biological replicates ± S.E. The relative expression is based on the expression ratio of a target gene versus a reference gene. Bars with *, ** or *** indicate significant differences ($p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively) in relation to their respective control (+Fe40 or -Fe) according to the Dunnett's test.

Besides ET biosynthesis genes, the relative expression of three key genes in the ET transduction pathway were studied in roots and shoots of cucumber (*EIN2* and *EIN3*) and melon (*EIN2*, *EIN3* and the AP2-like ethylene-responsive transcription factor *MELO3C019787*).

In cucumber *EIN2* and *EIN3* relative expression significantly increased at several time points in roots and shoots in both nutritional conditions, (Figure III.3). In shoots of cucumber, the relative expression values reached were higher in both nutritional conditions than the ones observed in roots. In both *EIN2* and *EIN3* genes, it could be observed an induction peak at 2 dpp in Fe sufficient conditions (Figure III.3A and B) and at 4 dpp in Fe deficient conditions (Figure III.3C and D).

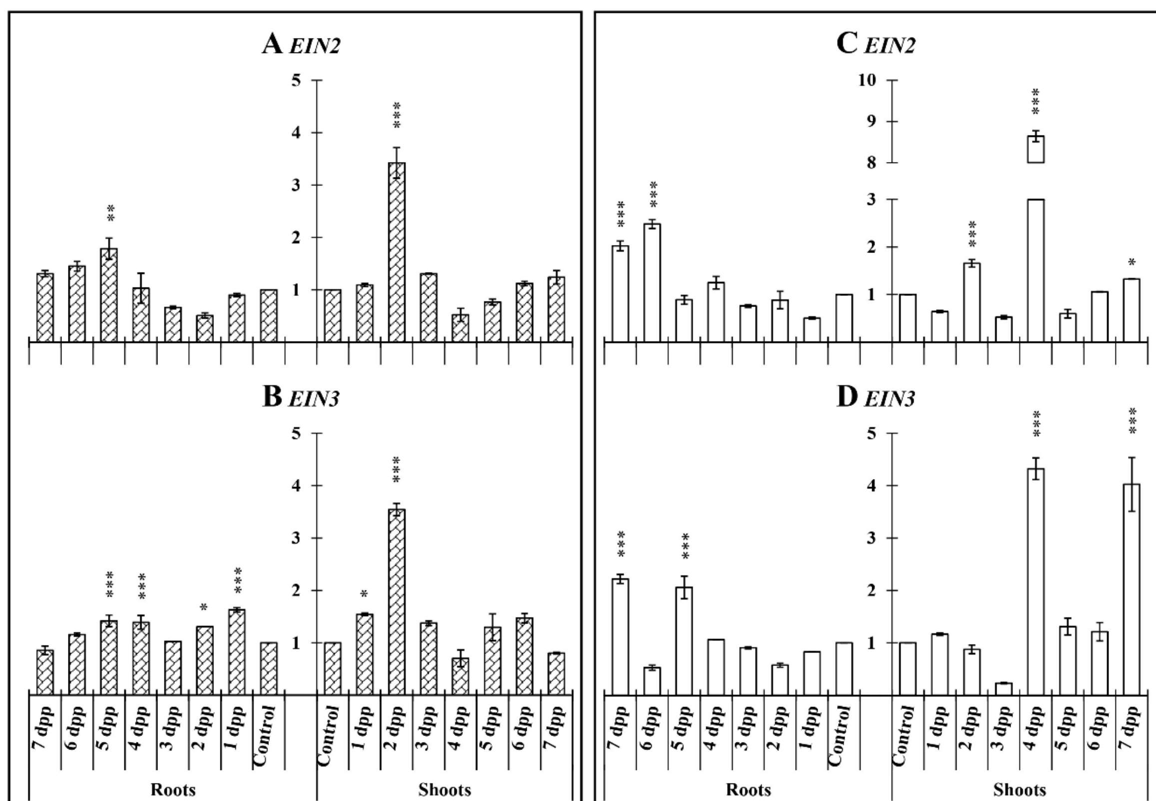

Figure III.3. Time-course evolution of the relative expression of genes involved in ET Transduction pathway on roots and shoots of *C. sativus*. Plants were primed by root immersion during 30 minutes in EAMa 01/58-Su solution with 1x10^{7} conidia/ml; plants were maintained in a hydroponic system. Samples were collected during 7 d post-priming for qRT-PCR gene expression study. Patterned bars (left) and white bars (right) represent gene expression of plants grown under Fe sufficient and Fe deficient conditions, respectively. Data of *EIN2* and *EIN3* expression represent the mean of three independent biological replicates ± S.E. The relative expression is based on the expression ratio of a target gene versus a reference gene. Bars with *, ** or *** indicate significant differences ($p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively) in relation to their respective control (+Fe40 or -Fe) according to the Dunnett's test.

In melon roots of primed plants, *EIN2* and *EIN3* showed similar relative expression levels in both Fe sufficient and deficient conditions (Figure III.4A-B and D-E). *MELO3C019787* was not detected in roots.

In melon shoots of primed plants both genes significantly enhanced their expression in comparison with their respective control at 2 and 7 dpp only under Fe deficient conditions (Figure III.4D and E). Finally, *MELO3C019787* only enhanced its relative expression level in shoots at 5 and 7 dpp under Fe sufficient and deficient conditions, respectively (Figure III.4C and E).

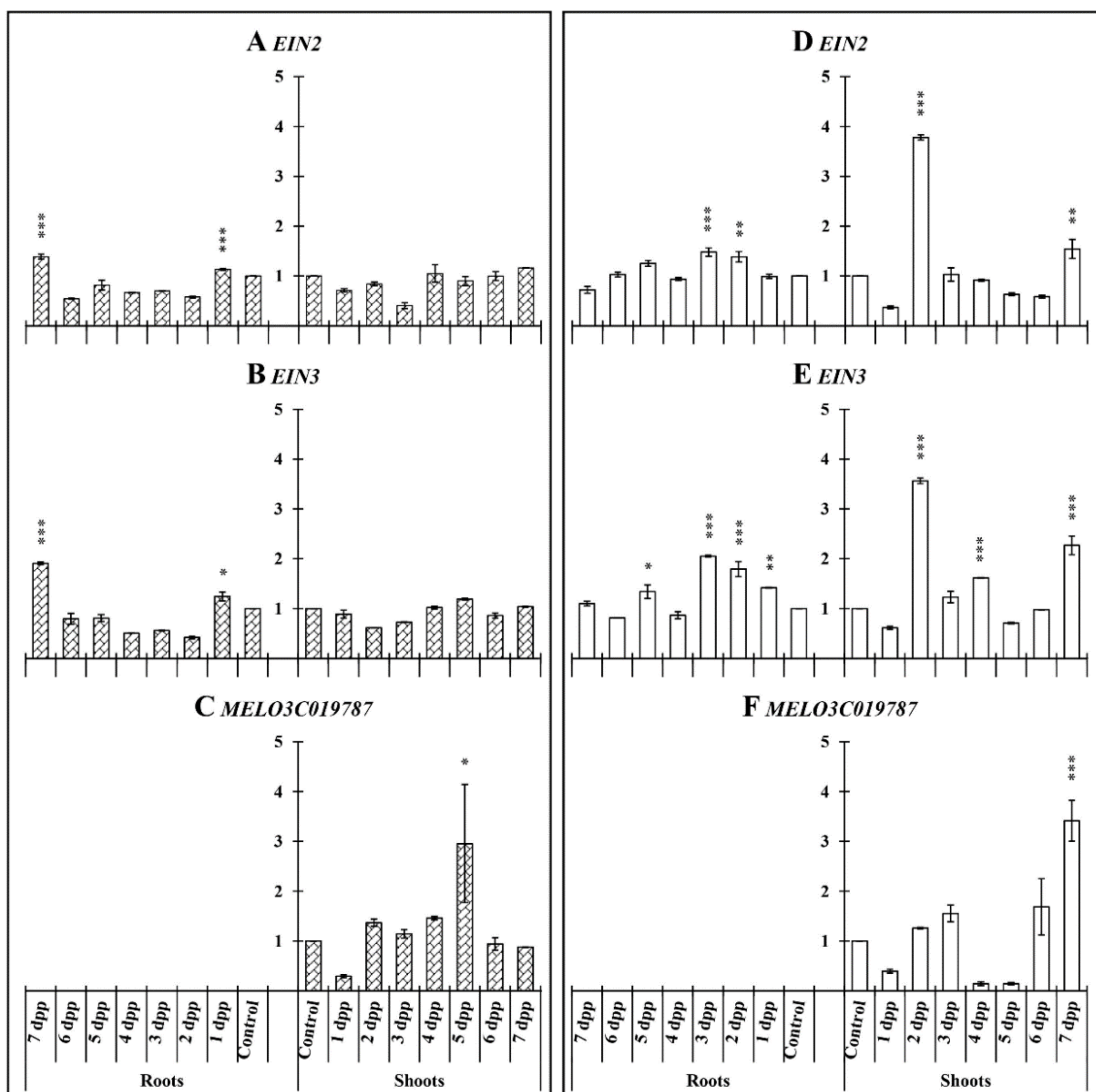

Figure III.4. Time-course evolution of the relative expression of genes involved in ET Transduction (*EIN2* and *EIN3*) and *MELO3C019787* as an ET transcription gene in roots and shoots of *C. melo*. Plants were primed by root immersion during 30 minutes in EAMa 01/58-Su solution with 1x10^7 conidia/ml; plants were maintained in a hydroponic system. Samples were collected during 7 d post-priming for qRT-PCR gene expression study. Patterned bars (left) and white bars (right) represent gene expression of plants grown under Fe sufficient and Fe deficient conditions, respectively. Data of *EIN2*, *EIN3* and *MELO3C019787* expression represent the mean of three independent biological replicates ± S.E. The relative expression is based on the expression ratio of a target gene versus a reference gene. Bars with *, ** or *** indicate significant differences ($p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively) in relation to their respective control (+Fe40 or -Fe) according to the Dunnett's test.

Genes related to JA and SA Biosynthesis. Relative expression of *LOX1, LOX2* and *PAL* was analyzed in cucumber roots and shoots. In roots *LOX1* and *PAL* showed significant increase of their relative expression in both nutritional conditions (Figure III.5A, C, D and F). *LOX2* was not detected in roots.

In cucumber shoots of primed plants significant increment of *LOX1, LOX2* and *PAL* could be observed under both nutritional conditions at different times. The relative expression increase of these genes in primed plants shoots under Fe deficient conditions reached its maximum at 4 dpp (Figure III.5D, E and F).

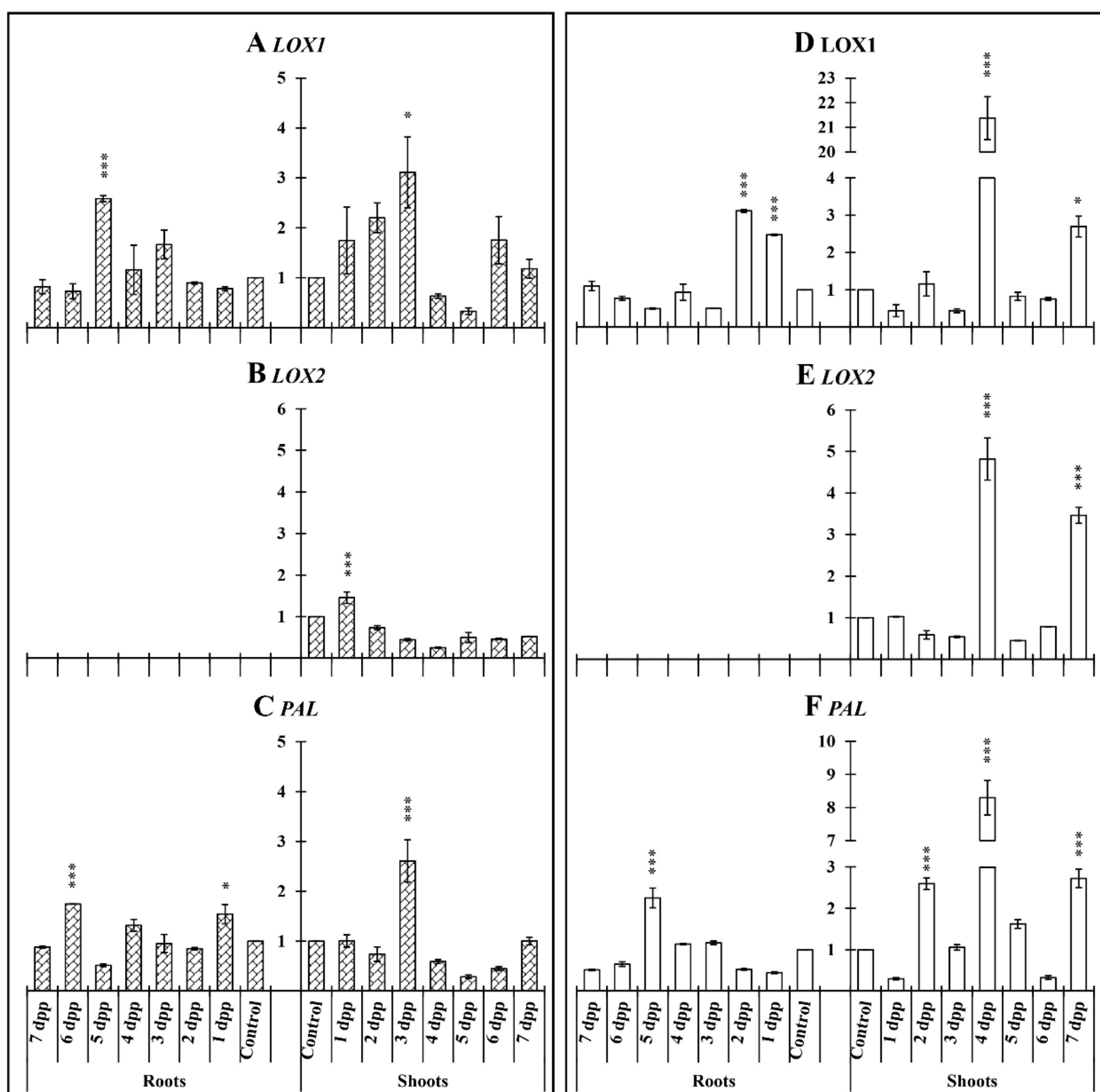

Figure III.5. Time-course evolution of the relative expression of genes involved in JA (*LOX1* and *LOX2*) and SA (PAL) biosynthesis in roots and shoots of *C. sativus*. Plants were primed by root immersion during 30 minutes in EAMa 01/58-Su solution with 1x107 conidia/ml; plants were maintained in a hydroponic system. Samples were collected during 7 d post-priming for qRT-PCR gene expression study. Patterned bars (left) and white bars (right) represent gene expression of plants grown under Fe sufficient and Fe deficient conditions, respectively. Data of LOX1, LOX2 and PAL expression represent the mean of three independent biological replicates ± S.E. The relative expression is based on the expression ratio of a target gene versus a reference gene. Bars with *, ** or *** indicate significant differences ($p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively) in relation to their respective control (+Fe40 or -Fe) according to the Dunnett's test.

In the case of melon, as in cucumber, we analyzed two genes related with JA biosynthesis, *LOX2* and *MELO3C014632* that codify a Linoleate 13S-lipoxygenase 2-1 and a gene related with the SA biosynthesis, *MELO3C014222,* a phenylalanine ammonia lyase, in roots and shoots.

In roots it was observed a significant increase of *LOX2* and *MELO3C014632* relative expression in primed plants under both nutritional conditions (Figure III.6A, B, D and E). Likewise, *MELO3COL4222* was not detected in roots.

In melon shoots of primed plants, it could be observed a significant increment of *LOX2, MELO3C014632* and *MELO3COL4222* under both nutritional conditions at different times. The relative expression increase of *LOX2* in primed plants shoots under both Fe sufficient and deficient conditions reached its maximum at 6 dpp (Figure III. 6A and D). Relative expression of *MELO3COL4222* experimented a high increase in shoots of primed plant under Fe sufficient conditions, reaching a maximum relative expression level (165-fold change) at 4 dpp (Figure III.6C).

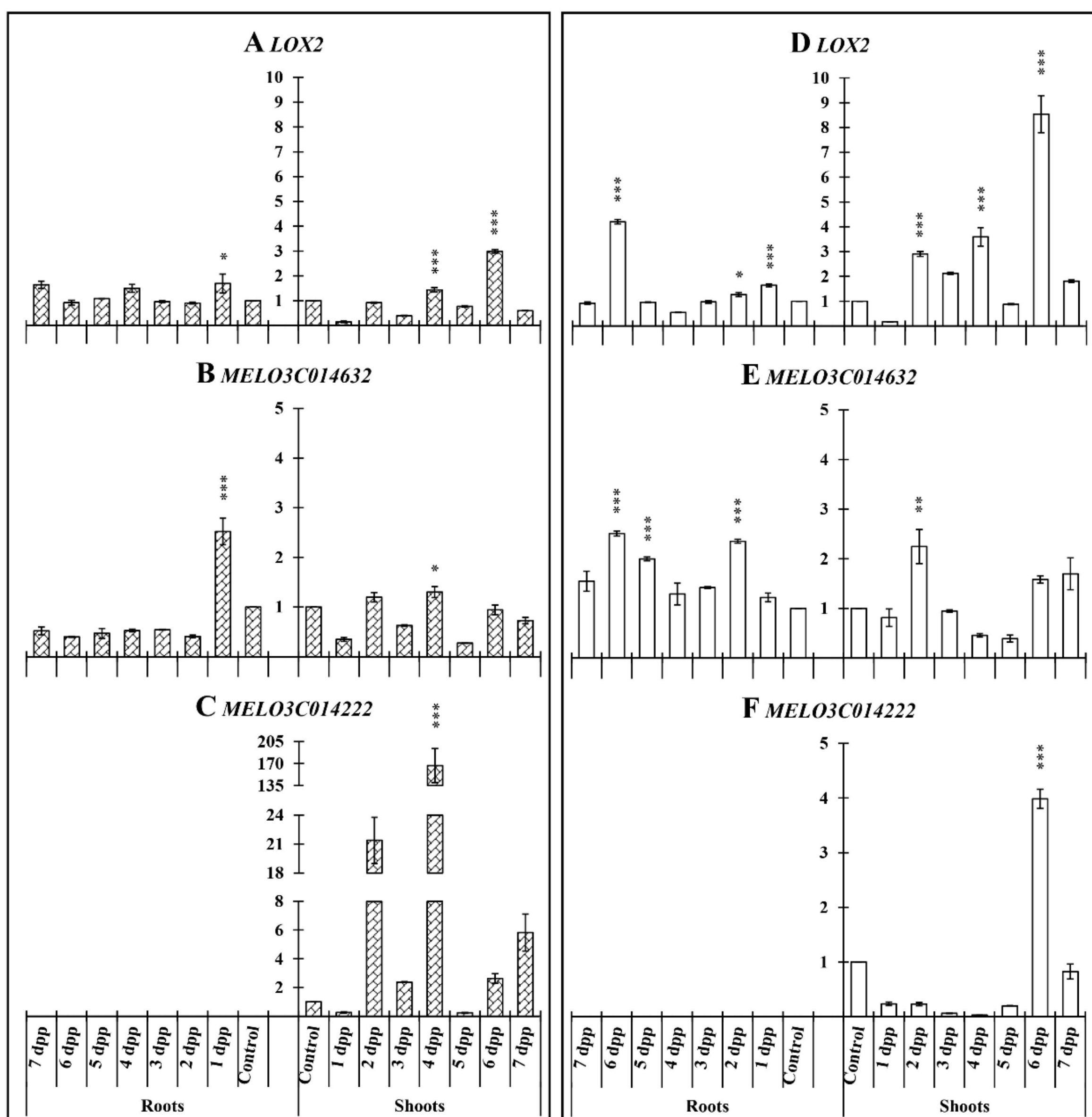

Figure III.6. Time-course evolution of the relative expression of genes involved in JA (*LOX2* and *MELO3C014632*) and SA (*MELO3C014222*) biosynthesis on roots and shoots of *C. melo*. Plants were primed by root immersion during 30 minutes in EAMa 01/58-Su solution with 1x10^{7} conidia/ml; plants were maintained in a hydroponic system. Samples were collected during 7 d post-priming for qRT-PCR gene expression study. Patterned bars (left) and white bars (right) represent gene expression of plants grown under Fe sufficient and Fe deficient conditions, respectively. Data of *LOX2*, *MELO3C014632* and *MELO3C014222* expression represent the mean of three independent biological replicates ± S.E. The relative expression is based on the expression ratio of a target gene versus a reference gene. Bars with *, ** or *** indicate significant differences ($p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively) in relation to their respective control (+Fe40 or -Fe) according to the Dunnett's test.

Pathogen related genes. Another important group of genes, *PR* proteins genes, were analyzed in this study. In cucumber we studied *PR1-1a, PR3* and *CsWRKY20.* In roots, *PR3* was the only gene detected in our experimental conditions, showing significant difference in both nutritional conditions (Figure III.7B and E).

In cucumber shoots of primed plants, we could detect and found significant differences in relative expression of all three genes studied in both nutritional conditions at different time points, except in *PR1-1a* under Fe sufficient conditions (Figure III.7A). The relative expression increase of *PR3* and *CsWRKY20* in shoots of primed plants under Fe deficient conditions reached its maximum at 4 dpp (Figure III.7E and F).

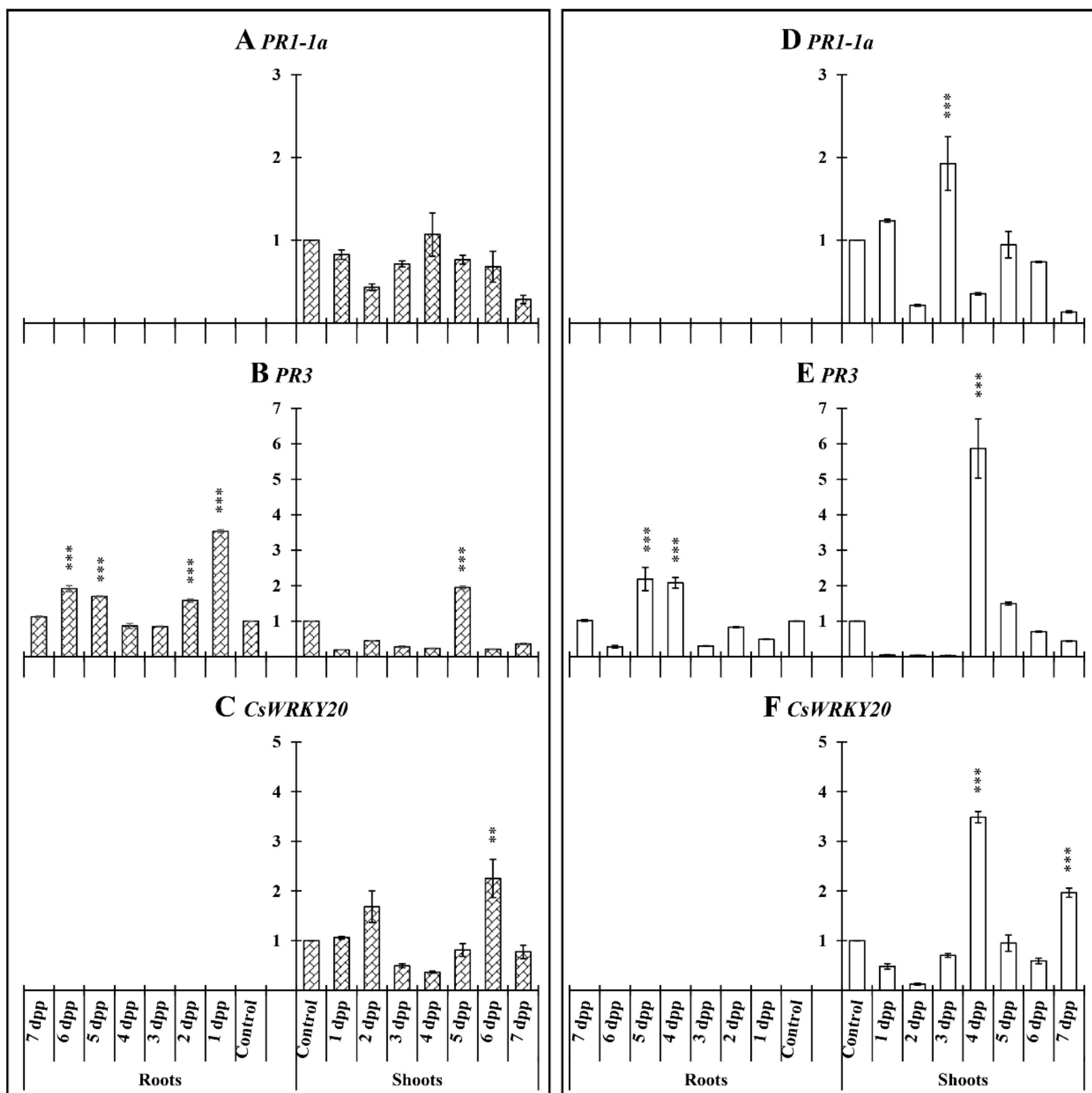

Figure III.7. Time-course evolution of the relative expression of PR proteins genes on *C. sativus* roots and shoots. Plants were primed by root immersion during 30 minutes in EAMa 01/58-Su solution with $1x10^7$ conidia/ml; plants were maintained in a hydroponic system. Samples were collected during 7 d post-priming for qRT-PCR gene expression study. Patterned bars (left) and white bars (right) represent gene expression of plants grown under Fe sufficient and Fe deficient conditions, respectively. Data of *PR1-1a, PR3* and *CsWRKY20* expression represent the mean of three independent biological replicates ± S.E. The relative expression is based on the expression ratio of a target gene versus a reference gene. Bars with *, ** or *** indicate significant differences ($p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively) in relation to their respective control (+Fe40 or -Fe) according to the Dunnett's test.

In the case of melon, the pathogen related genes studied were *PR1* and *PR9*. *PR1* was only detected in shoots while *PR9* showed significant differences in roots and shoots under both nutritional conditions (Figure III.8).

The induction of *PR9* expression in roots occurred early in primed plants under Fe deficient conditions while in Fe sufficient conditions no significant differences were observed until 4 dpp in both roots and shoots (Figure III.8B and D). In shoots, the relative expression of *PR9* increased significantly in most of the point times studied under Fe deficient conditions (Figure III.8D).

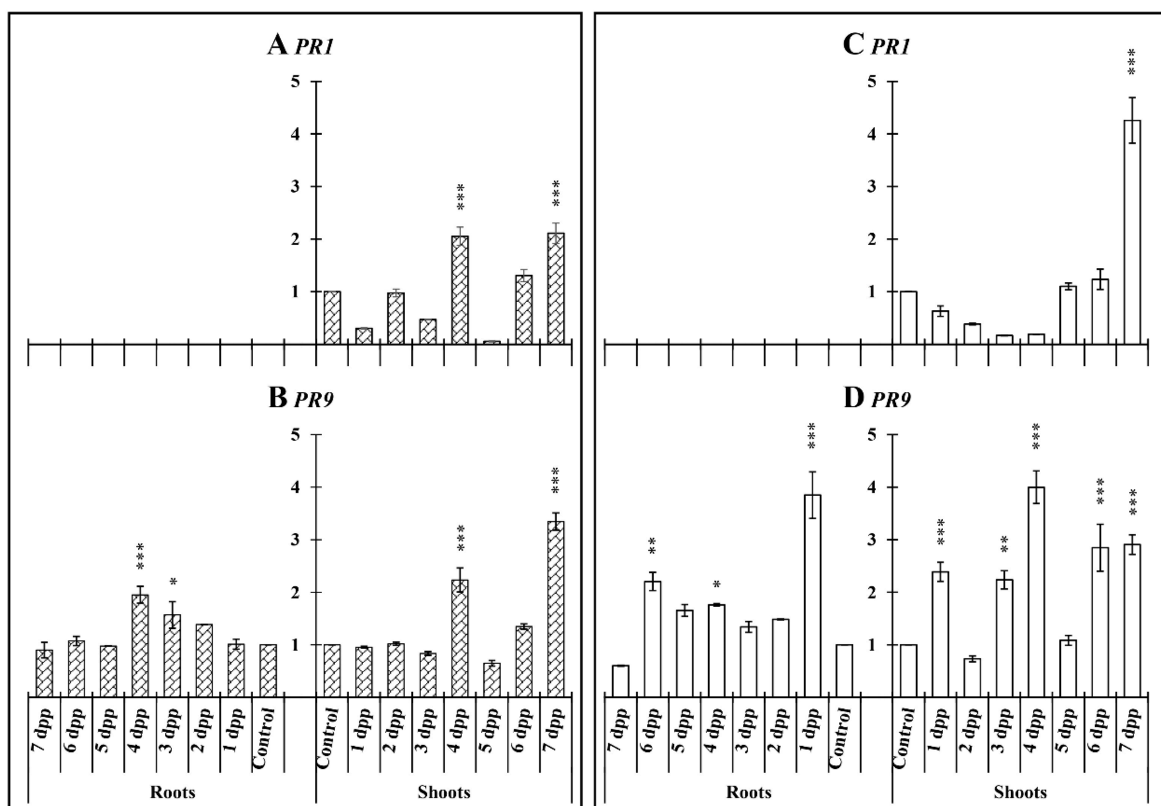

Figure III.8. Time-course evolution of the relative expression of PR proteins genes on *C. melo* in roots and shoots. Plants were primed by root immersion during 30 minutes in EAMa 01/58-Su solution with $1x10^7$ conidia/ml; plants were maintained in a hydroponic system. Samples were collected during 7 d post-priming for qRT-PCR gene expression study. Patterned bars (left) and white bars (right) represent gene expression of plants grown under Fe sufficient and Fe deficient conditions, respectively. Data of *PR1* and *PR9* expression represent the mean of three independent biological replicates ± S.E. The relative expression is based on the expression ratio of a target gene versus a reference gene. Bars with *, ** or *** indicate significant differences ($p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively) in relation to their respective control (+Fe40 or -Fe) according to the Dunnett's test.

Relative expression of genes in shoots after second priming. On the eighth day it was carried out the second priming of cucumber plants and the relative expression of genes involved in the ET biosynthesis (*ACO1*, *ACO3* and ACS7) and signaling (*EIN2* and *EIN3*) JA (*LOX1* and *LOX2*) and SA (*PAL*) biosynthesis were studied only in shoots.

ACO1, *ACO3* and *ACS7* significantly increased their relative expression in primed plants under both nutritional conditions (Figure III.9A-C), except in the case of *ACO1* in which no significant differences were observed under Fe deficient conditions (Figure III.9A). *EIN2* and *EIN3* relative expression significantly increased in primed plants under both nutritional conditions, reaching their maximum relative expression level under Fe sufficient conditions at 7dpp (Figure III.9D and E).

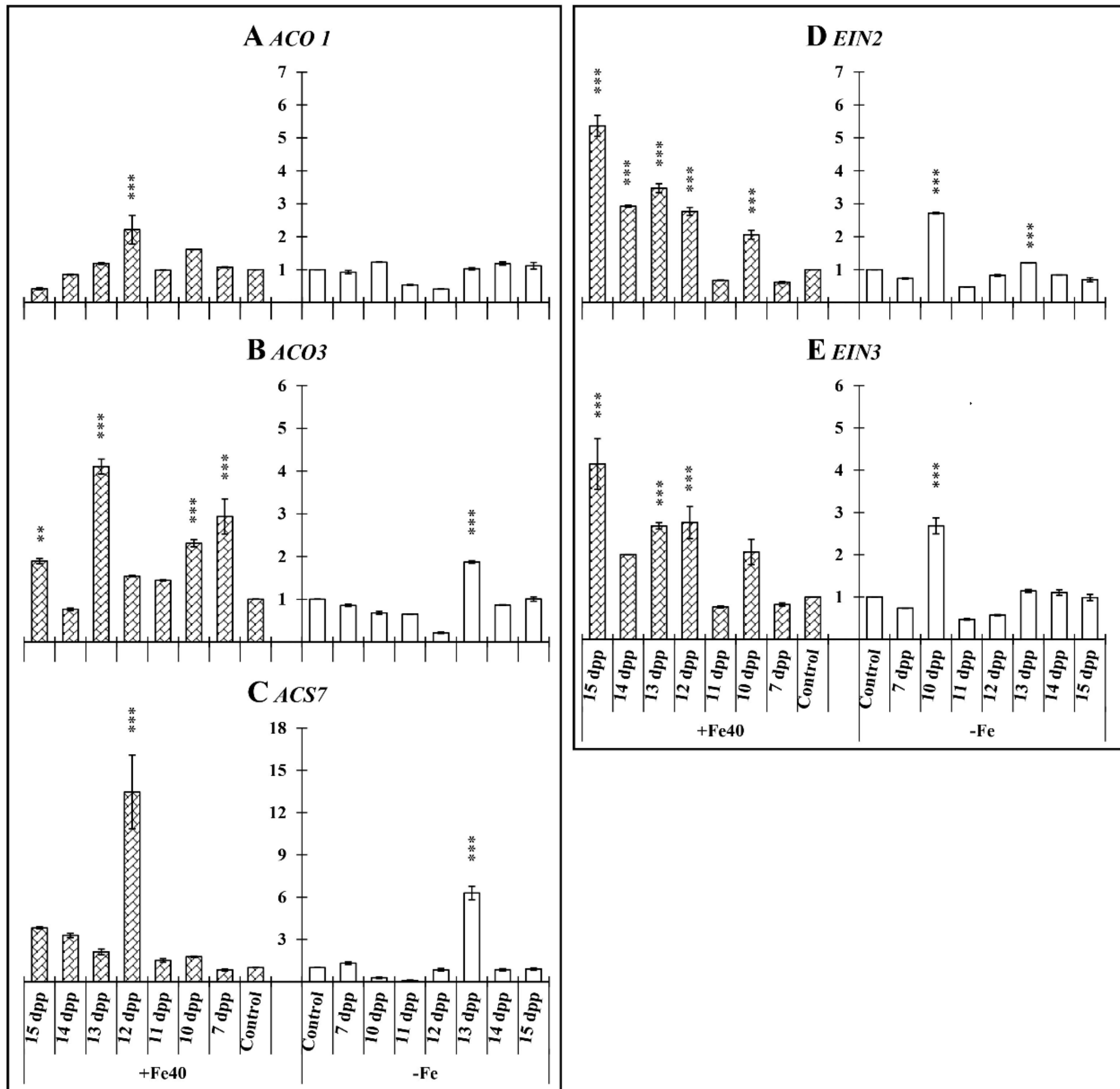

Figure III.9. Time-course evolution of the relative expression of genes involved in ET Biosynthesis (A, B and C) and ET Transduction pathway (D and E) in shoots of *C. sativus*. Plants were primed by root immersion during 30 minutes in EAMa 01/58-Su solution with $1x10^7$ conidia/ml twice, the second priming was carried out 8 d after the first one; plants were maintained in a hydroponic system. Samples for qRT-PCR gene expression study were collected at the 7th d and from 10th to 15th d after the first priming, the second priming was carried out 8 d after the first one. In the same chart, patterned bars and white bars represent gene expression of plants grown under Fe sufficient and Fe deficient conditions, respectively. Data of *ACO1*, *ACO3*, *ACS7*, *EIN2* and *EIN3* expression represent the mean of three independent biological replicates ± S.E. The relative expression is based on the expression ratio of a target gene versus a reference gene. Bars with *, ** or *** indicate significant differences ($p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively) in relation to their respective control (+Fe40 or -Fe) according to the Dunnett's test.

LOX1 relative expression experimented a significant increase at 5 dpp under both nutritional conditions, reaching its maximum expression level (506 times) at 7 dpp under Fe sufficient conditions (Figure III.10A). In the case of *LOX2* and *PAL*, similar results were obtained, significant differences in relative expression levels were observed in primed plants under both nutritional conditions (Figure III.10B and C).

Finally, PAL gene, experimented an early relative expression increase in both nutritional conditions, reaching its maximum relative expression level at 2 dpp in Fe sufficient and deficient condition (5.71 and 3.44 respectively) (Figure III.10C).

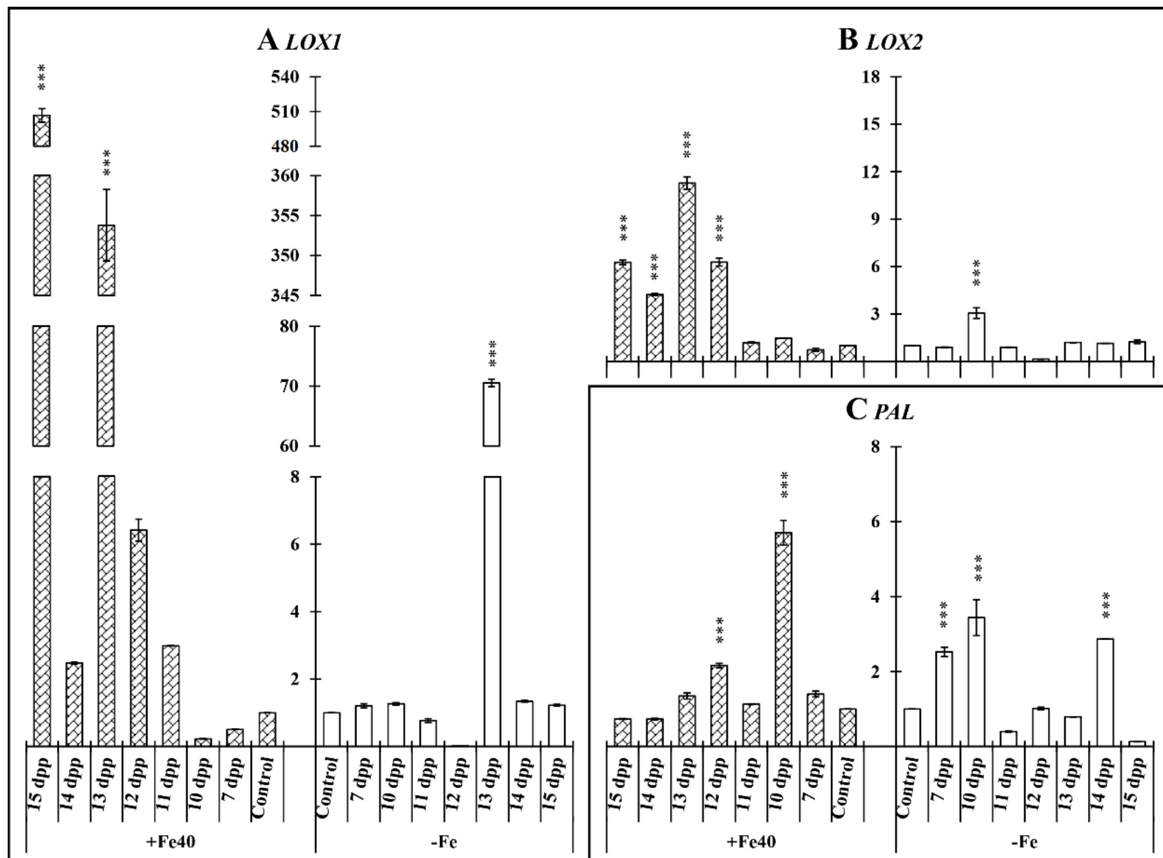

Figure III.10. Time-course evolution of the relative expression of genes involved in JA (*LOX1* and *LOX2*) and SA (*PAL*) biosynthesis in shoots of *C. sativus*. Plants were primed by root immersion during 30 minutes in EAMa 01/58-Su solution with $1x10^7$ conidia/ml; plants were maintained in a hydroponic system. Samples for qRT-PCR gene expression study were collected at the 7th d and from 10th to 15th d after the first priming, the second priming was carried out 8 d after the first one. In the same chart, patterned bars and white bars represent gene expression of plants grown under Fe sufficient and Fe deficient conditions, respectively. Data of *LOX1*, *LOX2* and *PAL* expression represent the mean of three independent biological replicates ± S.E. The relative expression is based on the expression ratio of a target gene versus a reference gene. Bars with *, ** or *** indicate significant differences ($p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively) in relation to their respective control (+Fe40 or -Fe) according to the Dunnett's test.

Lethal and sublethal effects on *S. littoralis*. A significant difference was observed in mortality. There was recorded a 4% of mortality in larvae fed with primed plants under Fe sufficient conditions ($\chi^2_{(1)}$= 2.73, *p*=0.0983), while the mortality of larvae fed with primed plants grown under Fe deficient conditions reached 8% ($\chi^2_{(1)}$= 5.39, *p*=0.0202). In the control treatments (plants without priming) of both nutrimental conditions, mortality was 0% (Figure III.11A). Abnormality of pupae presented significant differences only in the case of pupae from larvae fed with primed plants grown under Fe sufficient conditions reached a 24.88% of abnormality ($\chi^2_{(1)}$= 5.53, *p*=0.0186), while pupae from larvae fed with primed plants grown under Fe deficient conditions reached a 16.94% of abnormality ($\chi^2_{(1)}$= 2.51, *p*=0.1127) *vs.* 4 and 6% in their control, respectively (Figure III.11B).

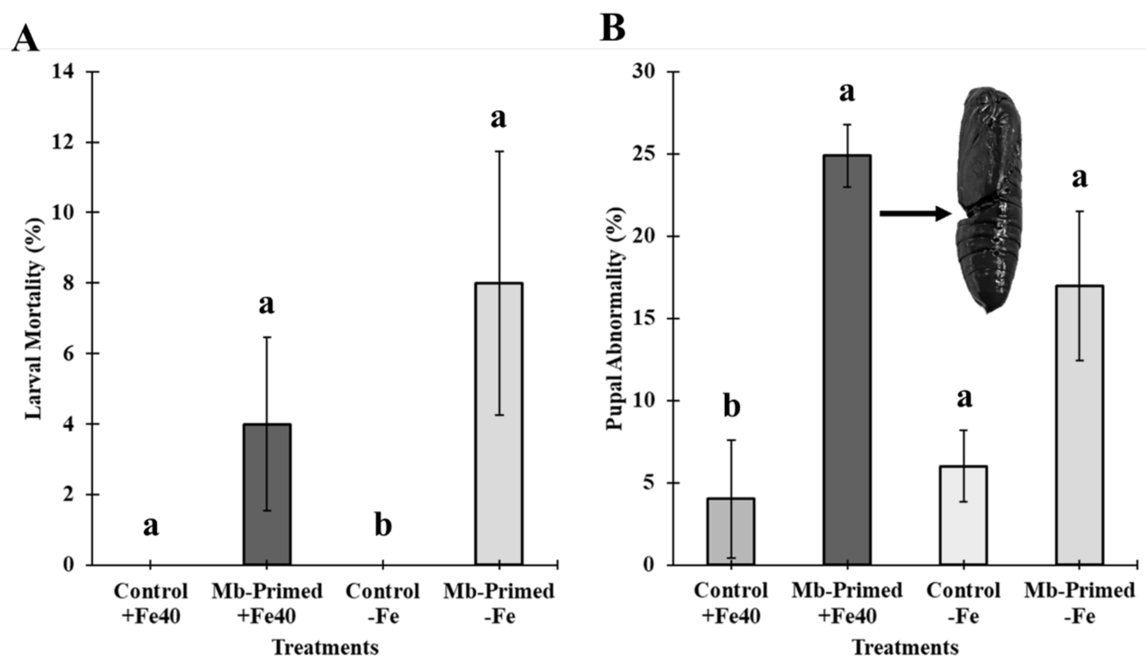

Figure III.11. Larval mortality (A) and pupal abnormality (B) of *S. littoralis* that were fed during 15 days with fragments of leaves from twice primed cucumber plants grown under Fe sufficient and deficient conditions; being the second priming applied at 8 d after the first one. Four treatments were used, namely, i) Control + Fe40 µM, ii) Mb-Primed + Fe40 µM, iii) Control -Fe and iv) Mb-Primed -Fe. Plants were primed by root immersion during 30 minutes in EAMa 01/58-Su solution with 1x10^7 conidia/ml and maintained in a hydroponic system. Letter over the bars denotes a significant difference between each treatment and its respective control, significance of the treatment was analyzed with F-test and Tukey's multiple comparisons (α < 0.05).

The time course evolution of the larval weight showed that there were not significant differences at 8 dpp, while at 16 dpp significant differences among treatments under Fe sufficient ($F_{1,97} = 115.31$, $p < 0.001$) and Fe deficient ($F_{1,96} = 29.76$, $p < 0.001$) were recorded, being larvae fed with primed plants from both nutritional conditions those that gained less body weight (Figure III.12A). On the other hand, the duration of the larval stage was prolonged by one day in those specimens that were fed with primed plants under both Fe sufficient ($F_{1,96} = 39.10$, $p < 0.001$) and Fe deficient ($F_{1,94} = 36.68$, $p < 0.001$) (Figure III.12B). Also, pupal weight showed a significant decrease of around 7-8% in respect to their control in the case of Fe deficient treatment ($F_{1,94} = 11.63$, $p < 0.001$) (Figure III.12C).

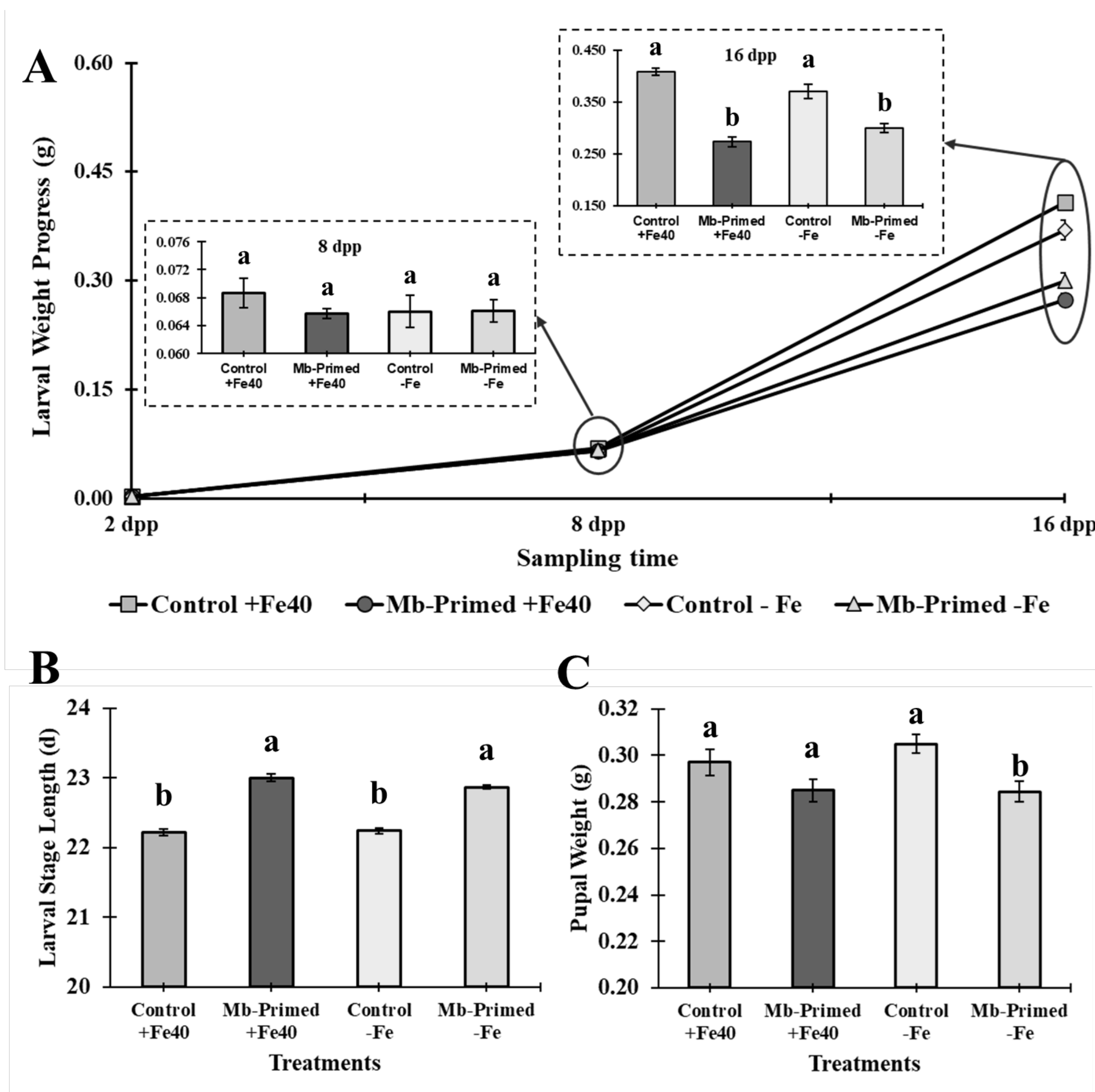

Figure III.12. Progress of larval weight (A), larval stage length (B) and weight of pupae (C) of *S. littoralis* larvae that were fed during 15 d with fragments of leaves from twice primed cucumber plants grown under Fe deficient and sufficient conditions; being the second priming applied at 8 d after the first one. Larval stage length represents the time that elapsed from the first day we fed them until the pupal stage was reached. Four treatments were used, namely, i) Control + Fe40 µM, ii) Mb-Primed + Fe40 µM, iii) Control -Fe and iv) Mb-Primed -Fe. Plants were primed by root immersion during 30 minutes in EAMa 01/58-Su solution with 1x10^7 conidia/ml and maintained in a hydroponic system. Letter over the bars denotes a significant difference between Mb-primed plants and their respective control analyzed by completely randomized ANOVA followed by a Tukey test ($p < 0.05$).

Detection and quantification of *M. brunneum* in shoots. Despite the effects observed in larvae fed with foliage of primed plants by root immersion, as well as the effects observed in pupae, the presence of *M. brunneum* was not detectable in most of the observation period, although at 6 and 7 dpp, traces of EAMa 01/58-Su DNA could be seen in the collected samples, quantifying minimum concentrations in the range of 0.03 to 0.3 pg from a total of $4x10^4$ pg of DNA per PCR reaction. Standard curves generated and other data from qPCR are shown in supplementary material.

III.3. Discussion

The phenomenon of priming is important for the development of new control methods because priming provides resistance against a broad spectrum of harmful agents significantly affecting growth and fruit or seed production (Van Hulten et al. 2006; Rasmann et al. 2012; Tiwari and Singh 2021; Tiwari et al. 2022). Since it has been showed that priming usually involves epigenetic changes, transgenerational priming phenomenon can occur (Luna and Ton 2012; Aranega-Bou et al. 2014; Tiwari and Singh 2021), as it has been showed in several works with natural and chemical compounds (Aranega-Bou et al. 2014), microorganisms such as *Pseudomonas syringae* (Luna et al. 2012; Pieterse 2012; Slaughter et al. 2012), or by herbivore attack (Pieterse 2012; Rasmann et al. 2012). Recent works have demonstrated that evolutionary relatives of *Metarhizium*, such as *Trichoderma atroviride*, can transmit the priming and the plant growth promotion effect to the next generation (De Medeiros et al. 2017; Woo et al. 2023), however inherited priming phenomenon does not always take place, since experimental conditions could be decisive (Yun et al. 2022). Furthermore, the priming and subsequent induction of resistance and Fe acquisition-related genes are usually interconnected by common regulators such as ET, JA, and NO and even, it has been recently shown that the inoculation of *Arabidopsis thaliana* plants with *Botrytis cinerea* activates Fe deficiency and resistance responses to *B. cinerea* through the induction of ET biosynthesis genes *SAM1* and *SAM2* (Romera et al. 2019; Lu and Liang 2022).

In the case of EPF, priming could be a very interesting strategy as it is an added value to the fungal efficacy when used for pest control. Usually, microorganism inducers of ISR also promote plant growth and development (Pieterse et al. 2014) and favor Fe acquisition (Pii et al. 2015, 2016; Zamioudis et al. 2015; Zhou et al. 2016; Marastoni et

al. 2019; Aparicio et al. 2023; García-Espinoza et al. 2023b). In the present work, it is showed the ability of the EPF *M. brunneum* to trigger both SA- and JA/ET-dependent priming in cucumber and melon seedlings with lethal and sublethal effects on *S. littoralis* fed on primed plants. In other works it is demonstrated that at short times, *Trichoderma* induces SA-dependent defenses and then later activates JA/ET-dependent defenses (Salas-Marina et al. 2011; Malmierca et al. 2012), similar that occurs in our study with *M. brunneum*. In the case of *Trichoderma* it is accepted that the plant can modulates *Trichoderma*-activated priming depending on the pathogen cycle, as is the case with RKN nematodes (De Medeiros et al. 2017; Martínez-Medina et al. 2017), and four timing stages can be identified (Morán-Diez et al. 2021). In addition, the undulating defense response is also effective against abiotic stresses and is more evident when the stress is present (Rubio et al. 2014). Other beneficial fungi, such as EPFs, can be expected to exert similar positive effects to a greater or lesser extent.

In general, gene expression levels obtained in cucumber and melon were similar, with a clear induction of all SA, JA, ET, and PR proteins genes in shoots of both plant species studied at different times after root priming. The results obtained evidence the crosstalk among ISR and the nutritional status of plants since in general it could be observed higher relative expression levels in shoots of primed plants under Fe deficient conditions over the 7 d after the first priming. However, after the second priming, the tendency changed and higher relative expression levels of JA, SA and ET-related genes were observed in shoots of primed plants under Fe sufficient conditions. Our results demonstrated induction of the expression of ET biosynthesis genes (*ACO1, ACO3* and *ACS7* from cucumber and *ACO1, ACO3, ACO5* and *ACS7* in melon) in shoots and roots of primed plants under both nutritional conditions at different times over the 15 monitored days Besides ET biosynthesis, relative expressions of ET signaling pathway genes, *EIN2* and *EIN3*, two key proteins in the ET signaling pathway and *MELO3CO19787,* that codify an ERF transcription factor, were significantly induced in shoots and roots under both nutritional conditions. These results would indicate that *M. brunneum* priming not only affects ET biosynthesis but ET signaling, therefore making plants more sensitive to this hormone. These results are in concordance with Aparicio et al. (2023), that have studied several genes related to ET biosynthesis (*ACO1* and *ACO3*) and signaling (*EIN2* and *EIN3*) in cucumber roots in Fe sufficient and deficient plants inoculated with the nonpathogenic strain of *Fusarium oxysporum* FO12

over 4 d and found a significant induction of the genes studied in inoculated plants at different times independently of the nutritional status.

From other hand, we studied several JA and SA- biosynthesis-related genes in cucumber (*LOX1, LOX2,* and *PAL)* and melon plants (*LOX2* and *MELO3CO14632*) with an important and significant increase in shoots of primed plants. However, the relative expression of JA and SA in roots was also enhanced by *M. brunneum* in comparison with their respective controls. For some genes like *MELO3CO14222* which codifies a phenylalanine ammonia-lyase, an enzyme involved in SA biosynthesis, the expression was only detected in shoots with high relative expression level reaching 164-fold change at the fourth dpp under Fe sufficient conditions. Similar results were obtained with PR proteins codifying genes *PR3, PR1-1a* and *CsWRKY20* in cucumber and *PR1* and *PR9* in melon plants, their relative expression levels enhanced in roots and shoots of primed plants under both nutritional conditions. It is worth to be mentioned that the second priming has led to an additional enhancement of expression level, namely the case of *LOX1* whose relative expression values reached more than a 500-fold change at 15 dpp. These results suggest that the optimization of application times would play a very important role in the resistance induction. In general, little is known about the role of EPF as ISR inducers. Some works have revealed that endophytism by *B. bassiana, M. brunneum* and *M. robertsii* lead to an increase in the relative expression of ET (*ERF-1, ACS1, WRKY51*), JA (*LOX1, LOXF, AOS, AOC, OPR7, MPI, JAZ1-5A*) and SA (*PR1,PR1-1-like, PR2, PR4 PR5, BGL, PAL, PBS1*) pathway-associated genes in grapevine, faba beans, maize, tomato and wheat (Rondot and Reineke 2019; Ahmad et al. 2020; Jensen et al. 2020; González-Guzmán et al. 2022), whereas the possible impact of such induction on insect survival and fitness remained unknown.

Our study shows lethal and sublethal effects on *S. littoralis* fed with shoots of primed cucumber plants. Even if mortality rates were not too high (up to 8%), significant sublethal effects were recorded with decreased larval and pupal weight, increased larval development time, and abnormality of pupae. The efficacy of the strain EAMa 01/58-Su of *M. brunneum* has been demonstrated against noctuid larvae with high mortality values when directly applied to the insect larvae (up to 80%) (Resquín-Romero et al. 2016a), when larvae fed with treated plants (up to 50%) (Sánchez-Rodríguez et al. 2018) and when larvae fed with or endophytically colonized plants (up to 20%) (Miranda-Fuentes et al. 2021b). However, similar to our study, no fungal

outgrowth was recorded in any of the dead larvae. In this sense, larval mortality could be explained by the capacity of this strain to produce destruxin-toxins (Ríos-Moreno et al. 2016, 2017) or by the ISR-SAR induction as shown in the present work in which larvae were fed with leaves of non-colonized primed plants that showed high relative expression levels of several genes related to ET biosynthesis (*ACO1, ACO3* and *ACS7*) and signaling (*EIN2* and *EIN3*), JA and SA biosynthesis (*LOX1, LOX2* and *PAL*). In this regard our work shows that the lethal and sublethal effects recorded were a direct consequence of *M. brunneum* priming. Related studies indicated that the effects on insect pests are outputs of endophytic colonization and the subsequent enhancement of ISR induction (Ahmad et al. 2020; Kuzhuppillymyal et al. 2021; Sari et al. 2022; Vinha et al. 2023). Likewise, other studies reported up-regulation of ET, JA, SA and PR related genes as endogenous responses of resistant genotypes against phytopathogens like *Phytophthora capsici* and *P. melonis* (Wang et al. 2020b; Hashemi et al. 2020) or as a result of the inoculation/interaction with other microorganisms like bacteria (García-Gutiérrez et al. 2013) or mycorrhizal fungi (Fiorilli et al. 2018). Recently, Di Lelio et al. (2023) showed very similar lethal and sublethal effects on *S. littoralis* larvae fed on tomato plants treated by seed coating with *Trichoderma afroharzianum.* However, these effects were attributed to gut dysbiosis as a result of plant colonization which led to resistance enhancement. In our study, we showed that the ISR-SAR induction is not necessarily related to endophytic colonization and may cause important effects on insect pest fitness.

Conclusions. Our results evidence the role of *M. brunneum* EAMa 01/58-Su strain as an ISR-SAR inducer, by triggering both SA- and JA/ET-dependent priming, and the benefits of this resistance activation for *S. littoralis* management. Also, the crosstalk between the ISR-SAR induction, insect pest control, and the Fe nutritional status of the plant is highlighted. This study contributes to the knowledge of EPF new functions that could be integrated as innovative IPM strategies.

III.4. Material and methods

Biological material. Two species of Cucurbitaceae (*Cucumis melo* L. var. Futuro and *Cucumis sativus* L. var Ashley, Semillas Fitó, S.A., Barcelona, Spain) and *S. littoralis* (Lepidoptera: Noctuidae) were used to study the effects of priming with an entomopathogenic fungus on the responses and expression of both induced and acquired systemic resistance.

Growth conditions. Plants were grown under controlled conditions as described by García et al. (2022). Briefly, seeds of both species were sterilized with 1% sodium hypochlorite for 5 minutes, stirring constantly, then washed twice with sterilized water and placed on absorbent paper moistened with 5 mM $CaCl_2$, covered with the same paper and placed at 25 ºC in the dark over 3 days (d) for germination. Then, when the plants sufficiently elongated their stems, they were transferred to a hydroponic system culture that consisted of a thin polyurethane raft with holes on which plants inserted in plastic lids were held floating on the aerated nutrient solution. Plants grew in a growth chamber at 22 ºC day/20 ºC night temperatures, with relative humidity between 50 and 70%, and a 14-h photoperiod at a photosynthetic irradiance of 300 µmol m^{-2} s^{-1} provided by white fluorescent light (10.000 lux).

The nutrient solution used was R&M (Römheld and Marschner 1981) whose composition is the following: macronutrients: 2 mM $Ca(NO_3)_2$, 0.75 mM K_2SO_4, 0.65 mM $MgSO_4$, 0.5 mM KH_2PO_4, and micronutrients: 50 µM KCl, 10 µM H_3BO_3, 1 µM $MnSO_4$, 0.5 µM $CuSO_4$, 0.5 µM $ZnSO_4$, 0.05 µM $(NH_4)6Mo_7O_{24}$, and 10 µM Fe-EDDHA.

After 10 d and 13 d of growth, for cucumber and melon, respectively, plants were separated into four groups that posteriorly constituted the 4 treatments, as described below.

The specimens of *S. littoralis* used in this work came from a colony established at the insectarium of the Agricultural and Forestry Entomology Laboratory of the University of Córdoba; the growth chamber is maintained under the following conditions: 26±2 ºC, 70±5% RH and a photoperiod of 16:8 (L:D) h (Miranda-Fuentes et al. 2020, 2021b).

Fungal strain and inoculum preparation. *Metarhizium brunneum* (EAMa 01/58-Su) strain from the culture collection of the Agronomy Department, University of Cordoba (Spain) was used in all experiments (Spanish Type Culture Collection

Accession Number 20764). Detailed information about the fungal strain can be found in García-Espinoza et al. (García-Espinoza et al. 2023b). Transient and temporary endophytic colonization of melon plants by this strain has been previously demonstrated in foliar application (Resquín-Romero et al. 2016a; Garrido-Jurado et al. 2017), and the positive effects on growth promotion and response to Fe deficiency of *M. brunneum* have been consigned previously (Raya-Díaz et al. 2017a, b; Raya D. 2017) in several cultivated species. Recently, we unraveled the direct and indirect mechanisms used by this strain for Fe acquisition by cucurbits (García-Espinoza et al. 2023b).

To provide inoculum for experiments, strain was subcultured from stored slant cultures on Potato Dextrose Agar (PDA) in Petri dishes and grown for 15 d at 25°C in darkness. Then, inoculum preparation was carried out by scraping the conidia from the Petri plates into a sterile solution of 0.1% Tween 80, followed by sonication during 5 min to homogenize the inoculum and filtration through several layers of cheesecloth to remove any mycelia. A hemocytometer (Malassez chamber; Blau Brand, Wertheim, Germany) was used to estimate conidia concentration which finally adjusted to $1x10^7$ conidia/ml by adding sterile solution of distilled water with 0.1% Tween 80.

Roots priming. Melon and cucumber plants with two true leaves were selected and placed in trays with 2.5 l of fungal inoculum suspension, previously adjusted to $1x10^7$ conidia/ml. Control plants (non-primed) were placed in trays with 2.5 l of 0.1% Tween 80. All plants were maintained in continuous agitation for 30 minutes. After that, EAMa 01/58-Su primed plants, hereinafter as Mb-Primed, and non-primed plants were transferred to two different nutritional conditions, Fe sufficient (+Fe40µM) and deficient (-Fe) so that finally four treatments were used: Control +Fe40µM (non-primed), Mb-Primed +Fe40µM, Control -Fe (non-primed) and Mb-Primed -Fe.

Relative expression of defense mechanisms related genes and effects on *S. littoralis* fitness. In a first series of experiments, the relative expression of 18 ISR and SAR related genes were studied over the 7 days post-priming (dpp) without insect pest presence. For that, samples of roots and shoots, separately, were collected daily from 1 to 7 dpp, frozen immediately with liquid nitrogen and posteriorly stored at -80 ºC. A total of 42 plants were used for each treatment and plant species (6 plants per day and treatment for each plant species). The whole assay with both species of *Cucumis* was repeated twice (Figure III.13A).

Based on the gene expression results obtained, *C. sativus* was chosen for this part of the study. A group of cucumber plants were used to study the impact of root priming by the fungus on *S. littoralis* fitness. For that, plant roots were primed as previously described and at 2 dpp, 50 larvae of *S. littoralis* (L2) were introduced and confined in methacrylate boxes to observe larval development. A second priming was applied to the roots 8 d after the first one. Larvae were fed daily with fragments of leaves of plants from their respective treatment over 15 d. After these 15 d, the larvae were fed with artificial diet until they reached the pupal stage. Larval mortality and development were monitored daily. The larval stage length, pupal abnormality and pupal weight were recorded; the larvae were weighed at the beginning of the study and at 8 and 16 dpp. Assay was set up into four treatments, as previously explained, with 5 replicates (10 larvae per replicate). The relative expression of genes was studied only in shoots after the second priming (Figure III.13B).

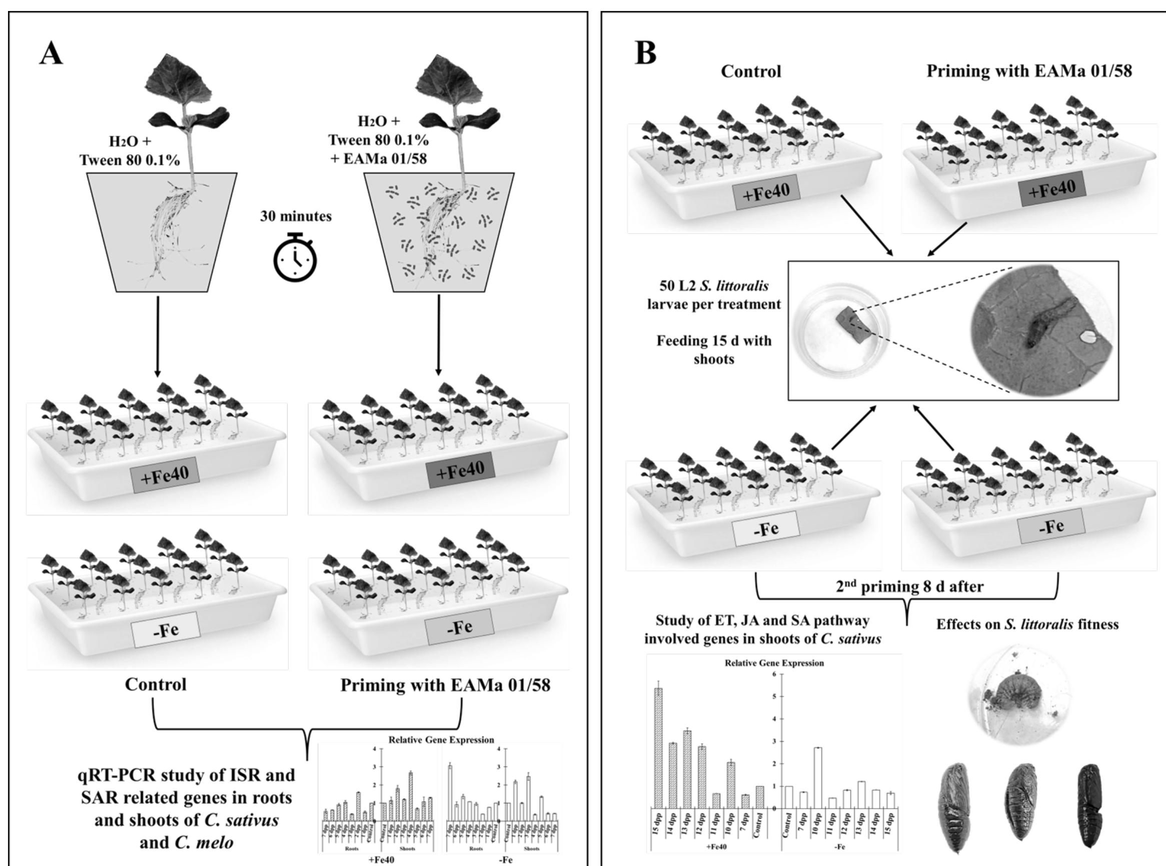

Figure III.13. Scheme of treatments and priming carried out in assays. In the first group of assays, including *C. sativus* and *C. melo*, plants were primed by root immersion during 30 minutes in a $1x10^7$ conidia/ml EAMa 01/58-Su suspension; plants were maintained in a hydroponic system. Four treatments were used, namely, i) Control + Fe40 µM (non-primed), ii) Mb-Primed + Fe40 µM, iii) Control -Fe (non-primed) and iv) Mb-Primed -Fe. Samples were collected during 7 d post-priming for qRT-PCR study of ISR and SAR genes related (A). In order to study the effects of priming in fitness of *S. littoralis*, 50 L2 larvae per treatment were fed with shoots of control and primed *C. sativus* plants during 15 d; priming was carried out twice, the second one was carried out at 8 d after the first one, for that, the roots were immersed for 30 minutes in a EAMa 01/58-Su $1x10^7$ conidia/ml suspension (B).

RNA isolation, cDNA synthesis and qRT-PCR analysis. Real-time PCR analysis was carried out as previously described García et al. (2022). Briefly, roots and leaves were ground to a fine powder with a mortar and pestle in liquid nitrogen. Total RNA was extracted using the Tri Reagent solution (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. cDNA synthesis was performed by using iScript™ cDNA Synthesis Kit (Bio-Rad laboratories, Inc, Hercules, CA, USA) from 3 µg of DNase-treated RNA as the template. As internal control 18S cDNA was amplified using the QuantumRNA Universal 18S Standards primer set (Ambion, Austin, TX, USA); the thermalcycler program was one initial cycle of 94 ºC for 5 min;

followed by cycles of 94 ºC for 45 s; 55 ºC for 45 s; 72 ºC for 1 min, with 27–30 cycles, all followed by a final 72 ºC elongation cycle of 7 min (Lucena et al. 2006; García et al. 2010, 2011, 2013).

The study of gene expression by qRT-PCR was performed in a qRT-PCR Bio-Rad CFX connect thermal cycler and the following amplification profile: initial denaturation and polymerase activation (95 ºC for 3 min), amplification and quantification repeated 40 times (94 ºC for 10 s, 57 ºC for 15 s and 72 ºC for 30 s), and a final melting curve stage of 65 ºC to 95 ºC with increment of 0.5 ºC for 5 s to ensure the absence of primer dimer or non-specific amplification products (García et al. 2022). PCR reactions were set up with 2 μL of cDNA in 23 μL of SYBR Green Bio-Rad PCR Master Mix, following the manufacturer's instructions. Standard dilution curves were performed for each primer pair to confirm appropriate efficiency of amplification (E=100±10%). Relative expression, of ET, JA and SA related genes as well as genes that codify PR proteins were studied in roots and shoots of both species, *C. sativus* and *C. melo*. Constitutively expressed *ACTIN* and *CYCLO* genes, were used as reference genes to normalize qRT-PCR results. The relative expression levels were calculated from the threshold cycles (Ct) values and the primer efficiencies by the Pfaffl method (Pfaffl 2001). Each PCR analysis was conducted on three biological replicates and each PCR reaction repeated twice.

The primers used in this study are listed in Supplementary Table III.1. Oligonucleotides used to amplify *ACO5*, *ACS2*, *ACS7*, *LOX2* (for cucumber) and *PAL* were designed by using Primer-Design software of NCBI site (NCBI 2023).

Table III.1. Gene, gene names, accession numbers and forward and reverse primers sequence studied on *C. melo* and *C. sativus* roots and shoots samples.

Hormone	Gen	Gen name / function	Accession No.	Reference	Sequence	Specie
Ethylene	*ACO1*	1-Aminocyclopropane-1-carboxylic acid oxidase 1	FN544066	(Aparicio et al. 2023)	F: TTTGGTGGCGGAGGAGAAAA R: ATGGCTTCAAACCTCGGCTC	*C. melo / C. sativus*
	ACO3	1-Aminocyclopropane-1-carboxylic acid oxidase 2	AF033583	(Aparicio et al. 2023)	F: ACTCAAAACAGTGGAACTGGA R: GGGGTACACTTCCTTCTTCTCC	*C. melo / C. sativus*
	ACO5	1-Aminocyclopropane-1-carboxylic acid oxidase 5	XM_008445975.2		F: AGCAAACCAGGAAGTGGAAGA R: GCTCCTCACATTGCTCTGAC	*C. melo*
	ACS7	1-aminocyclopropane-1-carboxylic acid synthases 7	NM_001328455.1		F: CTCGCCGGATGTCTAGCTTT R: AGCCTGTCCCGGTTCATTTT	*C. melo / C. sativus*
	EIN2	Ethylene-insensitive protein 2	KF245636	(Aparicio et al. 2023)	F: TGCCGACAAGGTTAAATGGG R: TGCTGCTGCACAATAGAAGA	*C. melo / C. sativus*
	EIN3	Ethylene-insensitive protein 3	KF245636	(Aparicio et al. 2023)	F: GCTTTCTGGGGTTGCGATTT R: CCGAACAGTCTCCCAAAGCA	*C. melo / C. sativus*
	MELO3C019787	AP2-like ethylene-responsive transcription factor		(Wang et al. 2020b)	F: CTTCGTTTTCCTATCTTCCAATCC R: CATCAACAAAGTCAAGTAGCCCTC	*C. melo*
Jasmonic acid	*LOX1*	Lipoxygenase 1	XM_004139124.1	(Hashemi et al. 2020)	F: TCTTTGCTTCAGGGTATCAC R: GCAAATTCTTCATCACTACTCC	*C. sativus*
	LOX2 (Cs)	Lipoxygenase 2	NM_001305766.1		F: GCACTTTGAGCATGTGGTTG R: AAGCTACTCTAAAGCACTCTTTTCT	*C. sativus*
	LOX2 (Cm)	Lipoxygenase 2	GQ386815	(García-Gutiérrez et al. 2013)	F: GCGTAAGGAATGGGATAGAATATATGA R: CGACGAGGATAAGGGAATTGG	*C. melo*
	MELO3C014632	Linoleate 13S-lipoxygenase 2-1		(Wang et al. 2020b)	F: AACGCCTTTCGCTGCTT R: TGTAGGACTCTGGTGGTGGA	*C. melo*

Table III.1. *Continued.*

Hormone	Gen	Gen name / function	Accession No.	Reference	Sequence	Specie
Salicylic acid	*PAL*	Phenylalanine ammonia lyase	NM_001308910.1		F: TCACTCCGCAACACGAGCA R: GGAGTGACGTTGTGGTTCAAG	*C. sativus*
	MELO3C014222	Phenylalanine ammonia lyase		(Wang et al. 2020b)	F: ATTTTGTCGGGCATCTTTG R: GCGATCTTGTTTTGGCTTCT	*C. melo*
PR proteins	*PR3*	Pathogenesis-related protein 3	NM_001308904.1	(Hashemi et al. 2020)	F: CACTGCAACCCTGACAACAACG R: AAGTGGCCTGGAATCCGACTG	*C. sativus*
	PR1-1a	Pathogenesis-related protein 1-1a	AF475286.1	(Hashemi et al. 2020)	F: CTCAAGACTTCGTCGGTGTCCA R: CGCCAGAGTTCACTAGCCTAC	*C. sativus*
	CsWRKY20	WRKY transcription factor of PR protein	XM_011653112.1	(Hashemi et al. 2020)	F: GAAATAACGTACAGAGGGAAGC R: CAGGTGCTGTTTGTTGGTTATG	*C. sativus*
	PR1	Pathogenesis-related protein 1	EU556704	(García-Gutiérrez et al. 2013)	F: GAGTGGGACAGAATAGTAGCAGGTT R: GTGCACTAGCCTACAGTCGTTGA	*C. melo*
	PR9	Pathogenesis-related protein 9	AY373372	(García-Gutiérrez et al. 2013)	F: GCATCTCGATCGTCCAAATGT R: TTGGGCTCAATACCGTGGAT	*C. melo*
Constitutive genes	*Actin*	Actin	XM_004136807	(Aparicio et al. 2023)	F: AACCCAAAGGCAAACAGGGA R: TCCGACCACTGGCATAGAGA	*C. melo / C. sativus*
	Cyclo	Cyclophilin	NM_001280769	(Aparicio et al. 2023)	F: ATTTCCTATTTGCGTGTGTTGTT R: GTAGCATAAACCATGACCCATAATA	*C. melo / C. sativus*

F, forward; R, reverse.

Detection and quantification of *M. brunneum* by quantitative PCR. DNA isolation. For each treatment, namely, Control +Fe40µM (non-primed), Mb-Primed +Fe40µM, Control -Fe (non-primed) and Mb-Primed -Fe, samples were collected from remains after feeding and stored at -20 ºC, since 2 to 7 dpp. After each sampling, vegetal material was surface-sterilized with 1% sodium hypochlorite for 2 min, rinsed twice in sterile deionized water for 2 min each, and dried on sterile filter paper (González-Mas et al. 2019c; Miranda-Fuentes et al. 2021).

Plant material was ground to a fine powder with a mortar and pestle in liquid nitrogen. Total DNA was isolated using HigherPurity™ Plant DNA Purification Kit (Canvax Biotech S.L., Córdoba, Spain) according to the manufacturer's instructions and resuspended in 100 µl of elution buffer. The concentration and quality of DNA were assessed by determination of absorbance at 260 nm and 280 nm in a NanoDrop™ 2000 (Thermo Fisher Scientific Inc.). Final concentration was homogenized to 30 ng/µl.

Quantitative PCR. Specific primer of *nrr* gene (F: TCA GGC GAT CTC GTG GTA AG, R: GGG GTG TAC TTG AGG AAT GGG) for qPCR was used (Barelli et al. 2018). Real-time PCR were performed in a qRT-PCR Bio-Rad CFX connect thermal cycler; the appliance was set to the following amplification profile: initial denaturation and polymerase activation (95 ºC for 3 min), amplification and quantification repeated 40 times (94 ºC for 10 s, 65 ºC for 15 s and 72 ºC for 30 s), and a final melting curve stage of 65 ºC to 95 ºC with increment of 0.5 ºC for 5 s to ensure the absence of primer dimer or non-specific amplification products. PCR reactions were set up with 1.3 µL of template (40 ng total) in 18.7 µL of SYBR Green Bio-Rad PCR Master Mix, following the manufacturer's instructions.

Absolute quantification was carried out according to Bell et al. (2009) and Barelli et al. (2018). A gradient of 1:4 from 40 ng to 0.61 pg of fungal and plant genomic DNA was used to set up standard curves; absolute quantification was determined by comparing threshold cycle numbers against the standard curve previously generated (Bell et al. 2009; Barelli et al. 2018).

Statistical analysis. All assays were carried out twice, representative results of both species studied are presented. The values of qRT-PCR represent the mean ± SE of three independent technical replicates. Results of relative expressions were analyzed using one-way analysis of variance (ANOVA) followed by a Dunnett's test, *

($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$) over the bars indicate significant differences in relation to the control treatment (GraphPad Prism 9.4.0, GraphPad Software, LLC, 2365 Northside Dr., Suite 560, San Diego, CA 92108 USA).

Mortality and abnormality of pupae data, expressed as percentages, were analyzed using a generalized linear mixed model with binomial distribution and logit link function. Significance of the treatment was analyzed with F-test and Tukey's multiple comparisons ($\alpha < 0.05$) (JMP 8.0, SAS Institute Inc.). Data of weight of pupae and larval stage duration were analyzed using analysis of variance (ANOVA) followed by a Tukey multiple range test, different letters over the bars indicate significant differences ($p < 0.05$) among treatments (Statistix 9.0®, Analytical Software, Tallahassee, FL, USA).

Data availability. The data of the present study are in the possession of the authors and are available for consultation under the respective request, for any additional information, please contact the corresponding author.

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Conceptualization, EQM, MYY, MJG and FGE.; methodology, FGE, MJG and MYY; formal analysis, FGE, MJG and MYY; writing, review and editing, FGE, MJG, MYY and EQM. All authors have read and agreed to the published version of the manuscript.

We declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplemental material

This is an adapted version of Supplemental material that is available online.

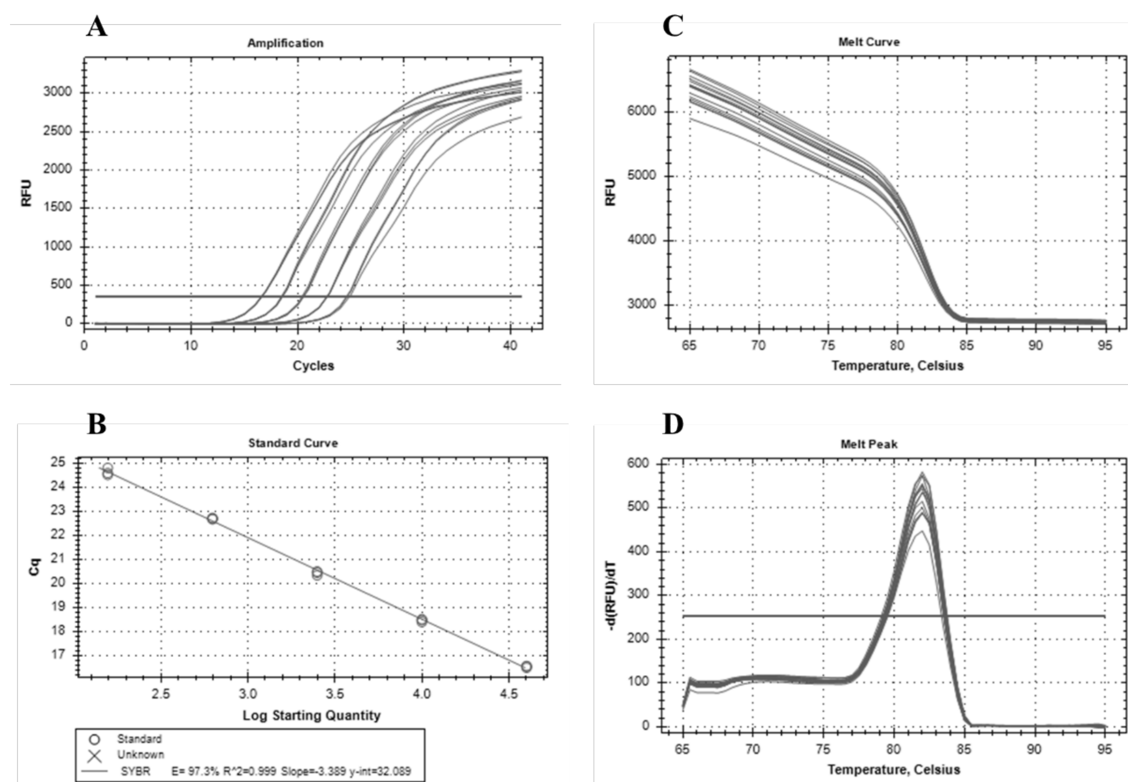

Figure III.S1. Output data from qPCR for molecular detection and quantification of *Metarhizium brunneum*. Quantification at gradual increment of number of cycles along the gradient of serial dilution (1:4 fold) (A). Figure B shows standard curve generated from serial dilutions of fungal DNA+DNA of *C. sativus* var. Ashley. Efficiency of primer was 97.3% while R^2 obtained was equal to 0.999. In Figures C and D show melting curve and melting peak, both shows the high specificity of *nrr* gene primer to identify *M. brunneum* EAMa 01/58-Su strain.

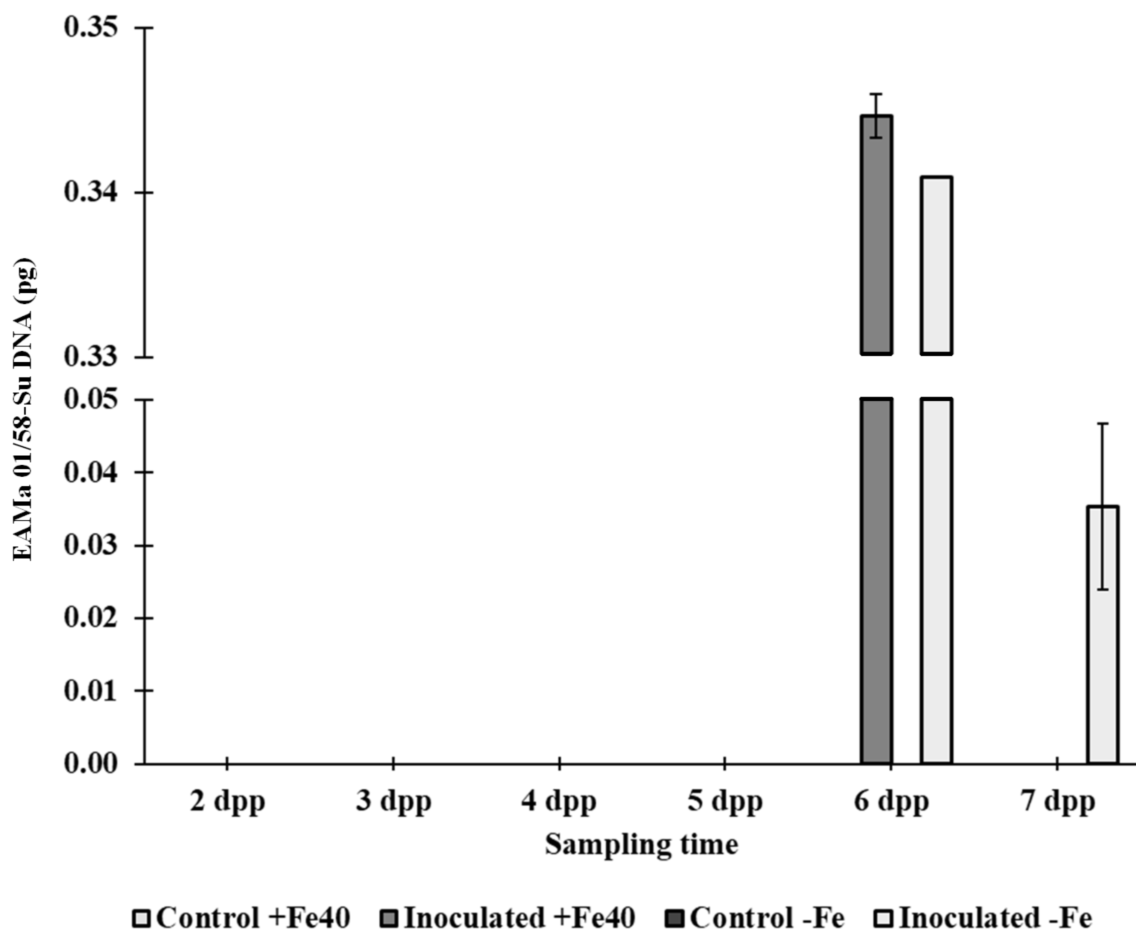

Figure III.S2. Traces of *Metarhizium brunneum* were detectable at 6 and 7 dpp on shoots of primed cucumber plants. Final concentrations were estimated by using standard curves. At 7 dpp, EAMa 01/58-Su *M. brunneum* strain was detected only in plants grown in Fe deficient conditions.

CHAPTER IV. ENTOMOPATHOGENIC FUNGI-MEDIATED SOLUBILIZATION AND INDUCTION OF Fe RELATED GENES IN MELON AND CUCUMBER PLANTS

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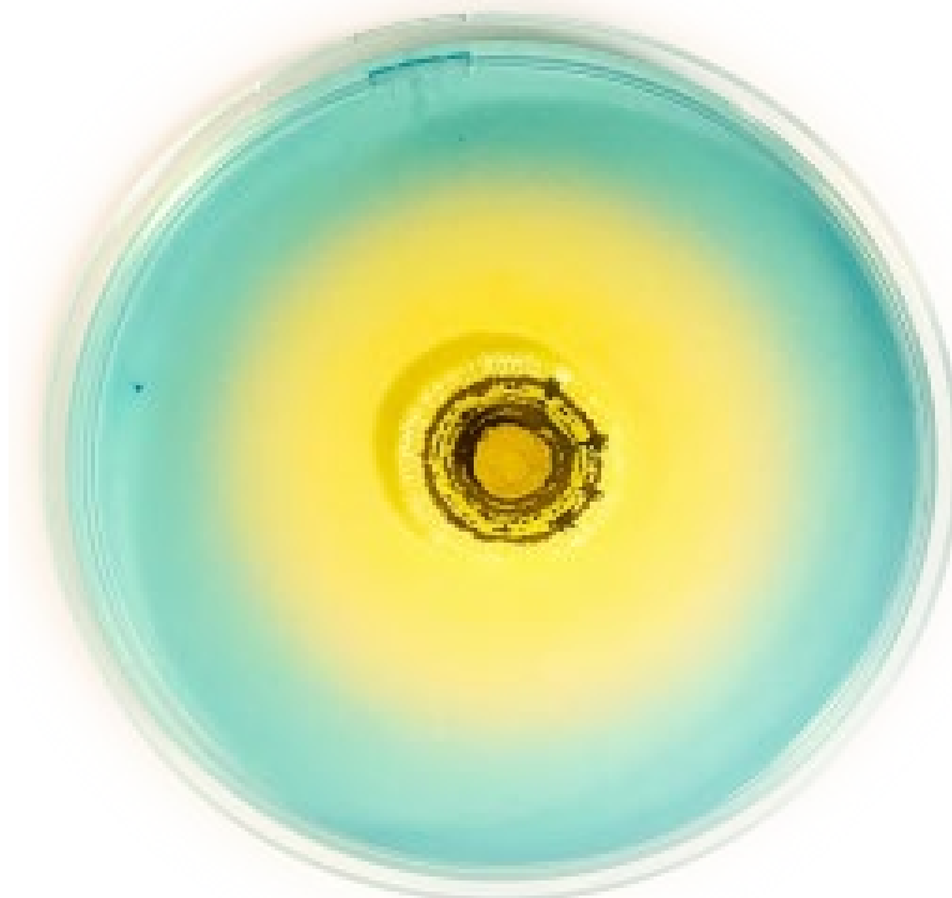

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Abstract

Endophytic insect pathogenic fungi have a multifunctional lifestyle; in addition to its well-known function as biocontrol agents, it may also help plants respond to other biotic and abiotic stresses, such as iron (Fe) deficiency. This study explores *M. brunneum* EAMa 01/58-Su strain attributes for Fe acquisition. Firstly, direct attributes include siderophore exudation (*in vitro* assay) and Fe content in shoots and in the substrate (*in vivo* assay) were evaluated for three strains of *Beauveria bassiana* and *Metarhizium bruneum*. The *M. brunneum* EAMa 01/58-Su strain showed a great ability to exudate iron siderophores (58.4% surface siderophores exudation) and provided higher Fe content in both dry matter and substrate compared to the control and was therefore selected for further research to unravel the possible induction of Fe deficiency responses, Ferric Reductase Activity (FRA), and relative expression of Fe acquisition genes by qRT-PCR in melon and cucumber plants.. In addition, root priming by *M. brunneum* EAMa 01/58-Su strain elicited Fe deficiency responses at transcriptional level. Our results show an early up-regulation (24, 48 or 72 h post inoculation) of the Fe acquisition genes *FRO1*, *FRO2*, *IRT1*, *HA1*, and *FIT* as well as the FRA. These results highlight the mechanisms involved in the Fe acquisition as mediated by IPF *M. brunneum* EAMa 01/58-Su strain.

Keywords: Entomopathogenic fungi; growth promoters; nutrient solubilization; bioavailability; iron acquisition genes; ferric reductase activity

IV.1. Introduction

Insect pathogenic fungi (IPF), which are among the most important biological control agents to be commercially developed for the management of a wide range of chewing and piercing/sucking insect pests, have multifunctional lifestyles and can interact with crops as endophytes establishing mutualistic interactions that benefits the host plant e.g., enhanced plant growth, development, immunity and resistance to biotic and abiotic stresses (Hu and Bidochka 2019; Quesada Moraga 2020; Nosheen et al. 2021). IPF can dwell internally in plant tissues including competence in the rhizosphere eliciting no disease symptoms in the plant while targeting insect pests even providing systemic protection of the plant against insect pests and contributing

to increased plant growth (Vega 2018; Branine et al. 2019; Quesada Moraga 2020). The genera *Beauveria* and *Metarhizium* are among the most studied IPF (Hu and Bidochka 2019; Sharma et al. 2021) and are considered excellent examples of fungi with multifunctional lifestyles (Barelli et al. 2016).

In recent works, IPF have been shown to be involved in plant acquisition of nutrients (Raya-Díaz et al. 2017b; Sánchez-Rodríguez et al. 2018) and plants grown in the presence of fungal partners exhibit increased growth and productivity (Barelli et al. 2016) e.g., plant inoculation with *M. brunneum*, *B. bassiana* and *Isaria farinosa*, has significant effects on growth and development of some important crops such as sorghum, wheat, sunflower and tomato (Sánchez-Rodríguez et al. 2016; Raya-Díaz et al. 2017b; Barra-Bucarei et al. 2020). Besides this, *M. brunneum* increased Fe availability on calcareous soil and alleviate Fe chlorosis in sorghum wheat and sunflower plants (Sánchez-Rodríguez et al. 2016; Raya-Díaz et al. 2017a) as well as crop protection against microbial pathogens (Barelli et al. 2016).

A lack of iron (Fe) is considered one of the major crop productivity constraints worldwide (Romera et al. 2019). Fe is a micronutrient that is essential for a range of important enzymatic processes in most organisms and in most environments Fe deficiency is not triggered by low total Fe concentrations but by low Fe bioavailability (Kraemer 2004); to over-come these limitations, bacteria, fungi, and gramineous plants (grasses) are known to sequester Fe using siderophores (Krasnoff et al. 2014). A siderophore is a low-molecular-weight Fe (III) ligand and they function as biogenic chelators with high affinity and specificity for Fe complexes (Schwyn and Neilands 1987).

According to Winkelmann (2007), both fungi and plants, unlike bacteria, are immobile organisms, therefore, to grow, both groups depend on local conditions and concentrations of nutrients, this also applies to ferric nutrition that can be improved by the secretion of siderophores and organic acids for the demineralization of other nutrients; foraging generally occurs at the tips of the growing hyphae, that is, through the propagation of the mycelium they are able to explore and exploit the resources of their environment.

Under Fe deficiency conditions, plants develop morphological and physiological responses, mainly in their roots, aimed to facilitate its acquisition (García et al. 2015, 2021a; Gattullo et al. 2018; Romera et al. 2019). The main

physiological responses are: enhanced ferric reductase activity; enhanced Fe^{2+} transport; rhizosphere acidification; and increased synthesis and/or release of organic acids, phenolic compounds, such as coumarins, and flavins, which can act as chelating and reducing Fe agents, improving its solubility for plants (Rodríguez-Celma and Schmidt 2013; Schmid et al. 2014; Fourcroy et al. 2014; Sisó-Terraza et al. 2016; Sisó-Terraza et al. 2016; Sisó-Terraza 2017; Tsai and Schmidt 2017). The main morphological responses are aimed to increase the contact surface of roots with soil and include development of subapical root hairs; of cluster roots (also named proteoid roots); and of transfer cells (Landsberg 1986; Römheld and Marschner 1986; Lucena et al. 2015; Venuti et al. 2019).

In the regulation of the Fe deficiency responses hormones and regulating substances such us ethylene and nitric oxide (NO) have been involved, which act as positive regulators (García et al. 2010, 2011). Ethylene and NO exert their function through *FIT*, a bHLH transcription factor (TF) which interacts with other TFs such as bHLH38, bHLH39, bHLH100 and bHLH101 (Kobayashi and Nishizawa 2012; Gao et al. 2019; Schwarz and Bauer 2020). All of them increase their expression under Fe deficiency conditions (Brumbarova et al. 2015). Besides bHLHs, *FIT* also interacts with MYB72 and MYB10, two other TFs essential for plant growth on low Fe conditions (Palmer et al. 2013; Zamioudis et al. 2014, 2015).

The IPF *Beauveria caledonica* has shown efficacy not only in solubilizing and transforming toxic minerals, but also in tolerating and thriving on them (Fomina et al. 2005) and the IPF *Metarhizium robertsii* has been shown to produce a complex of extracellular siderophores, including N^{α}-dimethylcoprogen (NADC) and dimerumic acid (DA) when it is cultivated under iron-depleted conditions (Krasnoff et al. 2014). Some reports indicate that *B. bassiana* is a good producer of siderophores (Barra-Bucarei et al. 2020) while others suggest that some species of *Metarhizium* are not (Ghosh et al. 2017). Compounds secreted by microorganisms may in turn help to improve the solubility of Fe in soils and plant Fe nutrition via elevated microbial activity (Jin et al. 2014). A remarkable fact is that fungi, unlike bacteria, can avoid competition for nutrients with plants (Dijkstra et al. 2013), however, there are no studies on the mechanisms used by IPF for Fe acquisition by plants. Hereby, direct and indirect mechanisms of IPF alleviation of Fe chlorosis in cucumber and melon plants have been investigated.

IV.2. Materials and methods

IV.2.1. Fungal isolates and inoculum preparation

Two isolates of *B. bassiana* (EABb 04/01-Tip and EABb 01/33-Su) and one isolate of *M. brunneum* (EAMa 01/58-Su) from the culture collection of the Agronomy Department, University of Cordoba (Spain) were used in the experiments (Table IV.1). Transient and temporary endophytic colonization of melon plants has been previously demonstrated by foliar application of these isolates (Resquín-Romero et al. 2016a; Garrido-Jurado et al. 2017).

Table IV.1. Fungal isolates used in experiments.

Isolate	Fungal Species	Origin	Agroecosystem	Habitat	GenBank Accession Number	Spanish Type Culture Collection 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 (Cadiz, Spain)	Traditional olive orchard	Soil	FJ972969	21149
EAMa 01/58–Su	*M. brunneum*	Hinojosa del Duque (Córdoba, Spain)	Wheat crop	Soil	JN900390	20764

To provide inoculum for experiments, all isolates were subcultured from stored slant cultures on Potato Dextrose Agar (PDA) in Petri dishes and grown for 15 d at 25 °C in darkness.

IV.2.2. *In vitro* study of Fe biodisponibility by production of siderophores

The in vitro study was done to investigate the abilities of fungal isolates to demineralize Fe. Prior to the test, isolates were grown in Potato Dextrose Agar (PDA) medium to obtain four-day old mycelium. This assay was repeated twice with four biological replicates per isolate.

We followed a simplified method (Srimathi and Suji 2018) of the universal chemical assay for siderophores detection (Schwyn and Neilands 1987), with $FeCl_3$ is used as FeIII source. Discs (6 mm diameter) of mycelium from each isolate (6 mm/myc) were cut from actively growing colonies (4 d) and placed at the center of Petri plates (9 cm) containing Chrome Azurol Sulfonate (CAS) agar medium. Plates

were incubated at 26 (±2) °C in darkness for 10 d (Barra-Bucarei et al. 2020). Daily from 3–10 days post inoculation (DPI) both the diameters of colonies and areas of yellow/orange halo surrounding them were measured from photographs taken using the software ImageJ (National Institute of Health, Bethesda, MD, US); the size of the orange-coloured area was indicative of the quantity of siderophores produced (Andrews et al. 2016).

IV.2.3. *In planta* and soil studies of Fe biodisponibility

To evaluate Fe acquisition in melon plants, a completely randomized design with 3 treatments (3 strains applied by soil drenching), and their respective control, with 6 replicates (plants) per treatment were used.

The substrate (Floragard, Germany) was sterilized twice in an autoclave (121 °C for 30 min), with an interval of 24 h [49]. The pots with a capacity of 500 mL, previously washed and sterilized, were filled with the sterilized substrate. Certified endophyte-free melon (*Cucumis melo* L. cv. Galia) was used as crop in all experiments, as in our previous studies (Resquín-Romero et al. 2016a; Quesada-Moraga et al. 2020). Seeds were surface sterilized according to Garrido-Jurado et al. (2017).

Inoculum preparation was carried out by scraping the conidia from the Petri plates into a sterile solution of 0.1% Tween 80, followed by sonication for 5 min to homogenize the inoculum and filtration through several layers of cheesecloth to remove any mycelia.

A hemocytometer (Malassez chamber; Blau Brand, Wertheim, Germany) was used to estimate conidia concentration which was finally adjusted to 1×10^8 conidia/mL by adding a sterile solution of distilled water with 0.1% Tween 80.

Soil drenching was carried out when the melon plants reached four true leaves stage, 30 d after seedling; 5 mL of the suspension was poured with a pipette onto the surface of the pot. Control plants were treated similarly with a sterile solution of 0.1% Tween 80. Then, at 50 DPI, elemental analysis in dry matter and substrate was carried out. For that, the substrate and vegetal material, including aerial parts and roots were dried in an oven at 60 °C for 96 h and weighed.

The content of Fe in dry matter and substrate was evaluated using the modified "Olsen Phosphorus" technique (Olsen et al. 1954). For that, both dry matter and substrate was grinded to obtain a homogeneous mixture, then, 0.2 g of sample per

replicate per treatment was added to a 100 mL precipitate glass; in a vapor extraction hood, 3 mL of nitric acid (65%) were added and covered with a watch glass, 16 h after, 1 mL of perchloric acid (70%) was added to each glass (Zasoski and Burau 1977; Bianchini and Eyherabide 1998). Fe was determined with an atomic absorption spectrophotometer (Perkin–Elmer Analyst 200).

IV.2.4. Ferric Reductase Activity and Fe acquisition gene expression

IV.2.4.1. Growth conditions and vegetal material

To study the activity of the ferric reductase and the relative expression of the Fe acquisition genes we used two species of cucurbits (*Cucumis melo* L. var. Futuro and *Cucumis sativus* L. var Ashley, Semillas Fitó, S.A., Barcelona, Spain).

Plants were grown under controlled conditions as previously described (Lucena et al. 2006). Briefly, seeds of both species were sterilized with 5% HCl for 5 min, stirring constantly, then washed twice with sterilized water and placed on absorbent paper moistened with 5 mM CaCl2, covered with the same paper and placed at 25 °C in the dark over 3 days for germination. Then, when the plants sufficiently elongated their stems, they were transferred to a hydroponic system culture that consisted of a thin polyurethane raft with holes on which plants inserted in plastic lids were held floating on the aerated nutrient solution. Plants grew in a growth chamber at 22 °C day/20 °C night temperatures, with relative humidity between 50 and 70%, and a 14-h photoperiod at a photosynthetic irradiance of 300 µmol m^{-2} s^{-1} provided by white fluorescent light (10.000 lux).

The nutrient solution used was R&M (Römheld and Marschner 1981) whose composition is the following: macronutrients: 2mM $Ca(NO_3)_2$, 0.75mM K_2SO_4, 0.65mM $MgSO_4$, 0.5mM KH_2PO_4, and micronutrients: 50µM KCl, 10µM H_3BO_3, 1µM $MnSO_4$, 0.5µM $CuSO_4$, 0.5µM $ZnSO_4$, 0.05µM $(NH_4)6Mo_7O_{24}$, and 10 µM Fe-EDDHA.

After 10 days (in the case of cucumber) and 13 days (in the case of melon) of growth, plants were separated into four groups that posteriorly constituted the 4 treatments, as described below.

IV.2.4.2. Inoculum preparation and roots priming

Metarhizium brunneum (EAMa 01/58-Su strain) was chosen to be used in this part of the study due to the properties previously shown to solubilize Fe. Inoculum

was prepared as previously described and adjusted to 1 × 10^7 conidia/mL by adding sterile solution of distilled water with 0.1% Tween 80.

Plants with two true were selected and placed in trays with 2.5 l of inoculum solution. Control plants (un-inoculated) were placed in trays with 2.5 l of 0.1% Tween 80. All plants were maintained in continuous agitation for 30 min. After that, inoculated and un-inoculated plants were transferred to two different nutritional conditions, Fe sufficient (+ Fe40µM) and deficient (-Fe) so that finally four treatments with 42 plants were used: Control + Fe40µM (un-inoculated), Inoculated + Fe40µM, Control -Fe (un-inoculated), Inoculated -Fe. Each assay with both species of *Cucumis* was repeated twice.

IV.2.4.3. Measure of Ferric Reductase Activity (FRA)

The FRA was determined as described by García et al. (2022). Previously to determine FRA, plants were subjected to a pre-treatment for 30min in plastic vessels with 50mL of a nutrient solution without micronutrients, pH 5.5. Then they were transferred into 50mL of a Fe (III) reduction assay solution for 1h. This assay solution consisted of nutrient solution without micronutrients, 100µM Fe(III)-EDTA and 300 µM Ferrozine, pH was adjusted to 5.0 with KOH. The environmental conditions during the measurement of Fe (III) reduction were the same as the growth conditions described above. FRA was determined spectrophotometrically by measuring the absorbance (562 nm) of the Fe(II)-Ferrozine complex and by using an extinction coefficient of 29.800 M^{-1} cm^{-1}. After that, roots were excised and weighed, and the results were expressed on a root fresh weight basis. Also, SPAD values (as a proxy of the chlorophyll concentration in leaf) were measured daily with a portable chlorophyllmeter (SPAD 502 Minolta Camera Co., Osaka, Japan).

IV.2.4.4. RNA Isolation, cDNA synthesis and qRT-PCR analysis

Real-time PCR analysis was carried out as described by García et al. (2021b). Briefly, roots and true leaves were ground to a fine powder with a mortar and pestle in liquid nitrogen. Total RNA was extracted using the Tri Reagent solution (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. cDNA synthesis was performed by using iScript™ cDNA Synthesis Kit (Bio-Rad laboratories, Inc, Hercules, CA, USA) from 3 µg of DNase-treated RNA as the

template and random hexamers as the primers. As internal control 18S cDNA was amplified using the QuantumRNA Universal 18S Standards primer set (Ambion, Austin, TX, USA); the thermalcycler program was one initial cycle of 94 °C for 5 min; followed by cycles of 94 °C for 45 s; 55 °C for 45 s; 72 °C for 1 min, with 27–30 cycles, all followed by a final 72 °C elongation cycle of 7 min (Lucena et al. 2006; García et al. 2010, 2011, 2013).

The study of gene expression by qRT-PCR was performed in a qRT-PCR Bio-Rad CFX connect thermal cycler and the following amplification profile: initial denaturation and polymerase activation (95 °C for 3 min), amplification and quantification repeated 40 times (94 °C for 10 s, 57 °C for 15 s and 72 °C for 30 s), and a final melting curve stage of 65 °C to 95 °C with increment of 0.5 °C for 5 s to ensure the absence of primer dimer or non-specific amplification products. PCR reactions were set up with 2 µL of cDNA in 23 µL of SYBR Green Bio-Rad PCR Master Mix, following the manufacturer's instructions (Angulo et al. 2021; García et al. 2021a).Standard dilution curves were performed for each primer pair to confirm appropriate efficiency of amplification (E = 100 ± 10%). Relative expression of *FRO1, IRT1* and *HA1* were studied in *C. sativus* while *FRO1*, *FRO2*, *FRO3*, *FRO4*, *IRT1* and *FIT* were studied in *C. melo*. Constitutively expressed *ACTIN* (Hashemi et al. 2020) and *CYCLO* genes, were used as reference genes to normalize qRT-PCR results. Table IV.2 contents the list of primers that were used in this study. The relative expression levels were calculated from the threshold cycles (Ct) values and the primer efficiencies by the Pfaffl method (Pfaffl 2001). Each PCR analysis was conducted on three biological replicates and each PCR reaction repeated twice.

Table IV.2. Primers used in qRT-PCR analysis.

Gene	Gene function/name	Accession No.	Reference	Sequence	Species	Tissue
FRO1	Ferric reductase oxidase	AY590765	(Aparicio et al. 2023)	F: ATACGGCCCTGTTTCCACTT R: GGGTTTTGTTGTGGTGGGAA	*C. sativus*	Roots
FRO1	Ferric reductase oxidase		(Waters et al. 2014)	F: TCACAGCGATTTAGAACCAGA R: GCCTTCGAGGGAAACTTGAA	*C. melo*	Roots
FRO2	Ferric reductase oxidase		(Waters et al. 2014)	F: TCTATCTAATCCATGTGGGAGTAGC R: AACAGCGCCAGAAGGAAGAT	*C. melo*	Roots
FRO3	Ferric reductase oxidase		(Waters et al. 2014)	F: CGAAGGCTGAAGTATAAACCAAC R: ACCTTGTCCATGACTCATCACA	*C. melo*	Roots /Shoots
FRO4	Ferric reductase oxidase		(Waters et al. 2014)	F: CACCGTCGAATTGGTCCT R: TGGACTCGACGACACACTGAA	*C. melo*	Roots /Shoots
IRT1	Iron-Regulated Transporter1	AY590764	(Aparicio et al. 2023)	F: GCAGGTATCATTCTCGCCAC R: ATCATAGCAACGAAGCCCGA	*C. sativus*	Roots
IRT1	Iron-Regulated Transporter1		(Waters et al. 2014)	F: ATCCCAATGTTGCACCCGGATAGA R: AAACCGGTGGCGAGAATGATACCT	*C. melo*	Roots
HA1	ATPase	AJ703810	(Aparicio et al. 2023)	F: GGGATGGGCTGGTGTAGTTTG R: TTCTTGGTCGTAAAGGCGGT	*C. sativus*	Roots
FIT	Induced Transcription Factor		(Waters et al. 2014)	F: GACATCAACGATCAATTTGAG R: CGATCCTCGATCAAGCAA	*C. melo/C. sativus*	Roots
*Actin **	Actin	XM_004136807	(Aparicio et al. 2023)	F: AACCCAAAGGCAAACAGGGA R: TCCGACCACTGGCATAGAGA	*C. melo/C. sativus*	Roots /Shoots
*Cyclo **	Cyclophilin	NM_001280769	(Aparicio et al. 2023)	F: ATTTCCTATTTGCGTGTGTTGTT R: GTAGCATAAACCATGACCCATAATA	*C. melo/C. sativus*	Roots /Shoots

**Reference genes. F, forward; R, reverse.*

IV.2.5. Data analysis

Iron siderophore production data, total and relative content of Fe in dry matter and substrate and data of FRA were analyzed using analysis of variance (ANOVA) followed by a Tukey multiple range test, different letters over the bars indicate significant differences ($p < 0.05$) among treatments (Statistix 9.0®, Analytical Software, Tallahassee, FL, USA).

Results of relative gene expressions were analyzed using one-way analysis of variance (ANOVA) followed by a Dunnett's test, * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$) over the bars indicate significant differences in relation to the control treatment (GraphPad Prism 9.4.0, GraphPad Software, LLC, 2365 Northside Dr., Suite 560, San Diego, CA 92108 USA). Data of gene expression represent the mean of three independent technical replicates.

IV.3. Results

IV.3.1. Iron siderophores exudation

There were significant differences amongst isolates in siderophore production 10 DPI ($F_{2,21}$=117.73, p=0.000); *M. brunneum* isolate EAMa 01/58-Su was the most capable of changing the largest area of CAS agar from blue to orange (58.4%), while *B. bassiana* isolates EABb 04/01-Tip and EABb changed the color of only 24.35% and 17.88%, respectively (Figure IV.1). The timeline for Fe siderophores exudation shown in Figure IV.1B reveals the difference between the *M. brunneum* isolate and the others from 3 DPI onwards.

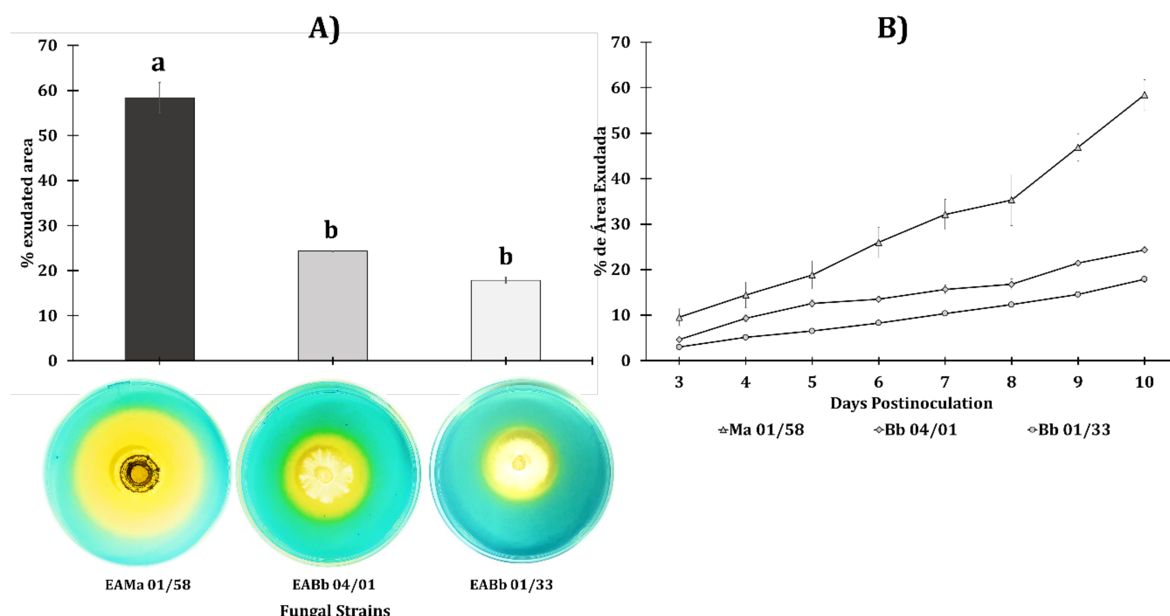

Figure IV.1. Siderophore exudation by three isolates of IPF on CAS agar medium with $FeCl_3$ as FeIII source. At the bottom, the front of plates is shown. (A) Comparison at 10 days post inoculation (DPI). Bars with different letters are significantly different to each other according to Tukey test (p < 0.05). (B) Progress (%) of colour change due to siderophore production by three isolates of IPF on CAS agar medium.

IV.3.2. Total dry matter and Fe content in dry matter and substrate

Significant differences were observed on dry matter when we compared EAMa 01/58-Su ($F_{1,8}$=10.63, p=0.0115), EABb 04/01-Tip ($F_{1,8}$=5.88, p=0.0416) and EABb 01/33-Su ($F_{1,8}$= 6.78, p=0.0314) treatments vs. control, however we can see that plants inoculated with EAMa 01/58-Su produced the highest dry matter content (Figure IV.2A). On another hand, no significant differences were observed on Fe content in dry matter when we compared each treatment vs. control [($F_{1,8}$=2.68, p=0.1400), ($F_{1,8}$=2.08, p=0.1870), ($F_{1,8}$=3.0, p=0.1213), for EAMa 01/58-Su, EABb 04/01-Tip and EABb 01/33-Su, respectively] (Figure IV.2B). In the case of relative Fe content in the substrate, only EAMa 01/58-Su treatment vs. control presented significant difference ($F_{1,6}$=7.77, p=0.0317) (Figure IV.2C); there weren't significant differences between EABb 04/01-Tip ($F_{1,6}$=3.41, p=0.1143) and EABb 01/33-Su ($F_{1,6}$=0.37, p=0.5629) treatments when were compared vs. control (Figure IV.2C).

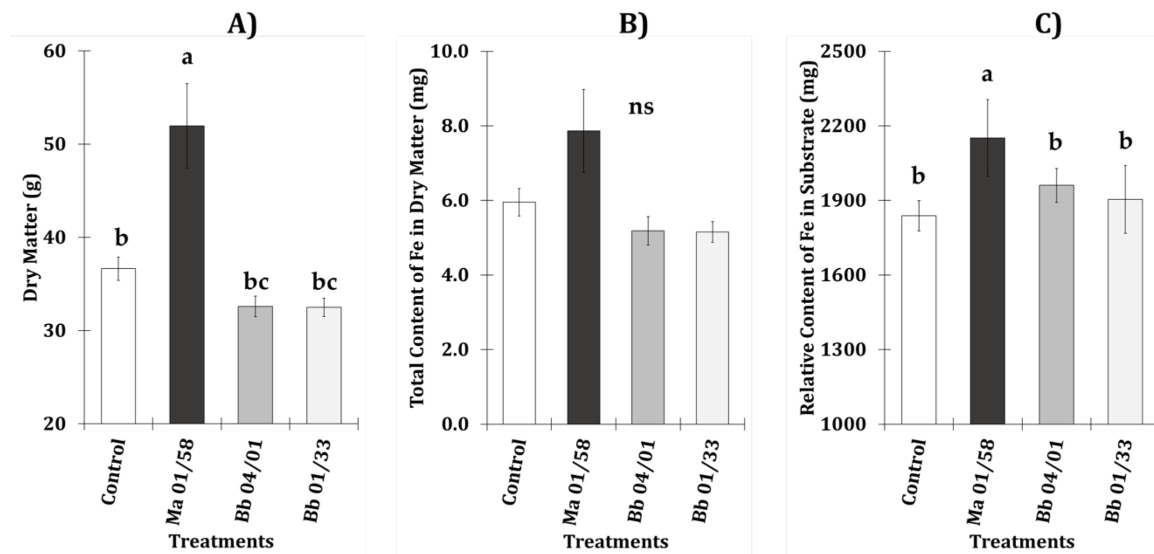

Figure IV.2. Mean (±SE) of stem and leaves dry matter weight (A), total content of Fe in dry matter (B), relative content of soluble Fe in substrate (C) measured at 50 DPI in melon plants inoculated by soil drenching. Letters over the bars denote significant difference between inoculated and control plants analyzed by completely randomized ANOVA followed by a Tukey test ($p < 0.05$).

IV.3.3. Ferric reductase activity and genes responsible of the reduction and transport of iron

In general, FRA presented higher values in cucumber and melon plants grown under Fe deficient conditions. In cucumber plants, reductase activity was higher in Fe deficient plants inoculated with *M. brunneum* (EAMa 01/58-Su strain) in comparison with their respective controls over the seven days of the study (Figure IV.3A). However, significant differences were detected between Fe deficient cucumber inoculated and un-inoculated at 4, 5 and 7 DPI [($F_{3,22}$ = 13.68, p = 0.0001), ($F_{3,20}$=35.3, p=0.0000) and ($F_{3,19}$=74.68, p=0.0000), respectively] (Figure IV.3A). In the case of melon, significant differences were found between Fe deficient plants inoculated at 3 DPI relative to the un-inoculated Fe deficient plants ($F_{3,20}$=61.23, p=0.0000) (Figure IV.4A).

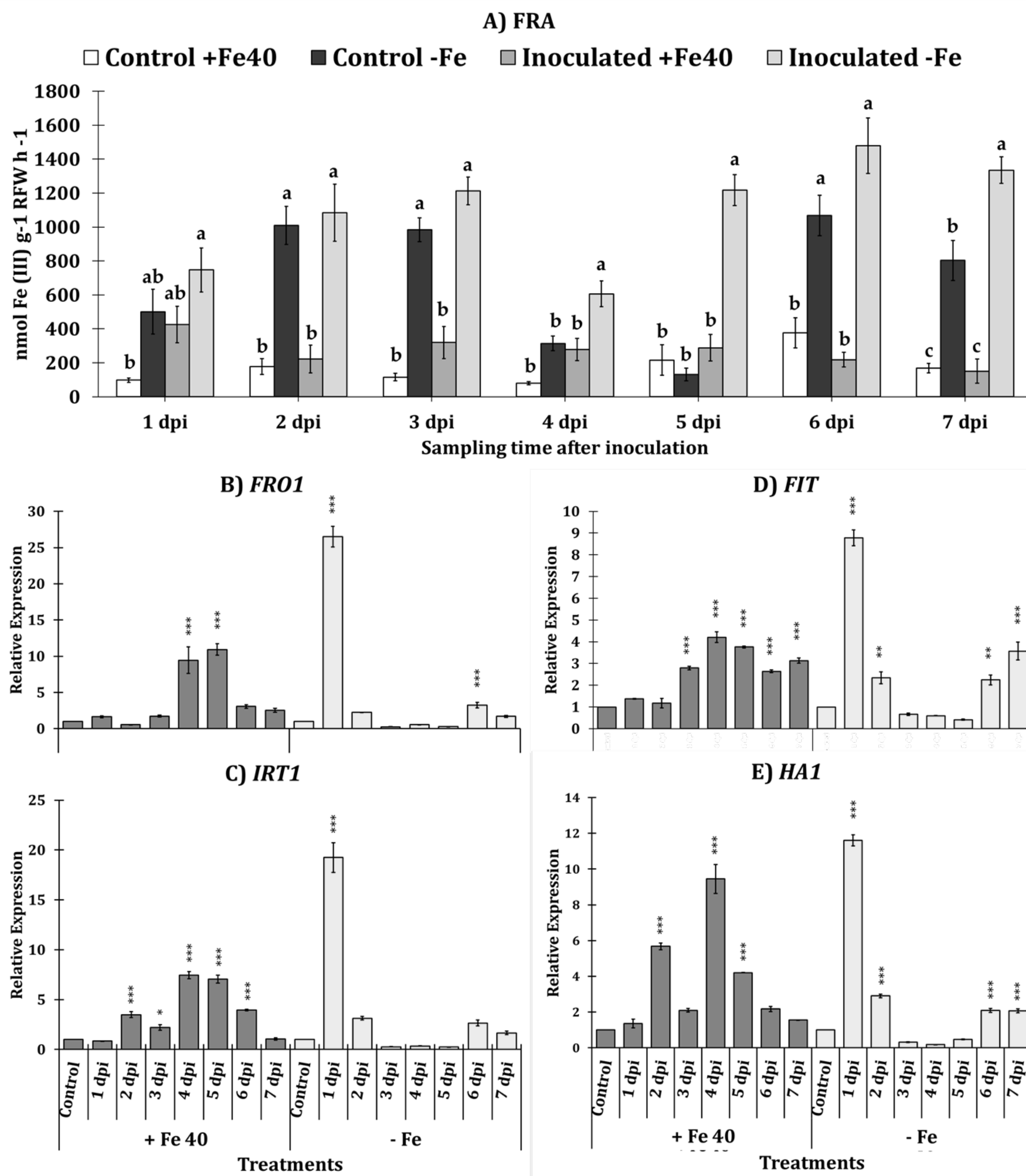

Figure IV.3. Evolution of FRA along seven days of observation and relative expression of *FRO1*, *IRT1*, *FIT* and *HA1* in *C. sativus* roots. Four treatments were used, namely, (i) Control + Fe40μM (un-inoculated), (ii) Inoculated +Fe40μM, (iii) Control –Fe (un-inoculated) and (iv) Inoculated –Fe. The expression of control treatment for each nutritional condition is presented once, at the beginning of the graph, with the relative expression comparison method used, the control is always equal to 1. Data of gene expression represent the mean of three independent technical replicates, according to the Dunnett's test, * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$) over the bars indicate significant differences in relation to the control treatment. In the case of FRA, letters over the bars denote significant difference between plants inoculated and control plants analyzed by completely randomized ANOVA followed by a Tukey test ($p < 0.05$).

Relative expression levels of Fe acquisition genes, *FRO1*, *IRT1*, *FIT* and *HA1* in cucumber are represented in Figure IV.3B–E. Fe acquisition genes experimented an increase of their expression levels after the inoculation with *M. brunneum* EAMa 01/58-Su strain in both conditions, Fe sufficient and deficient, in comparison with their respective un-inoculated controls at different times (Figure IV.3B–E). However, the relative expression levels of *FRO1*, *IRT1*, *FIT* and *HA1* reached at the first day post inoculation were much higher in Fe deficient conditions than Fe sufficient, being this increment of 26, 19, 8.8 and 11 times to *FRO1, IRT1, FIT* and *HA1* respectively (Figure IV.3B–E). In Fe sufficient conditions we observed an increase of the relative expression genes studied at different times post inoculation but in any cases the values reached were like that observed in Fe deficient conditions.

Generally, the results obtained in melon were like the ones obtained in cucumber. In this case we had the possibility to study three different genes that codify ferric reductase enzymes *FRO1, FRO2* and *FRO3* besides *IRT1* and *FIT*. As occur in cucumber roots, the relative expression of all genes studied was higher in Fe deficient conditions except in the case of *IRT1,* in which no significant differences were found in the relative expression values between Fe sufficient and deficient conditions (Figure IV.4E). *FRO1*, *FRO3* and *FIT* reached its maximum relative expression value at the second day post inoculation (Figure IV.4B,D–F) while *FRO2* did it on the third day and *IRT1* on the sixth (Figure IV.4C,E). Although *IRT1* reached its maximum relative expression level later, it also experimented a significant increase at the second day after inoculation as the rest of genes (Figure IV.4E).

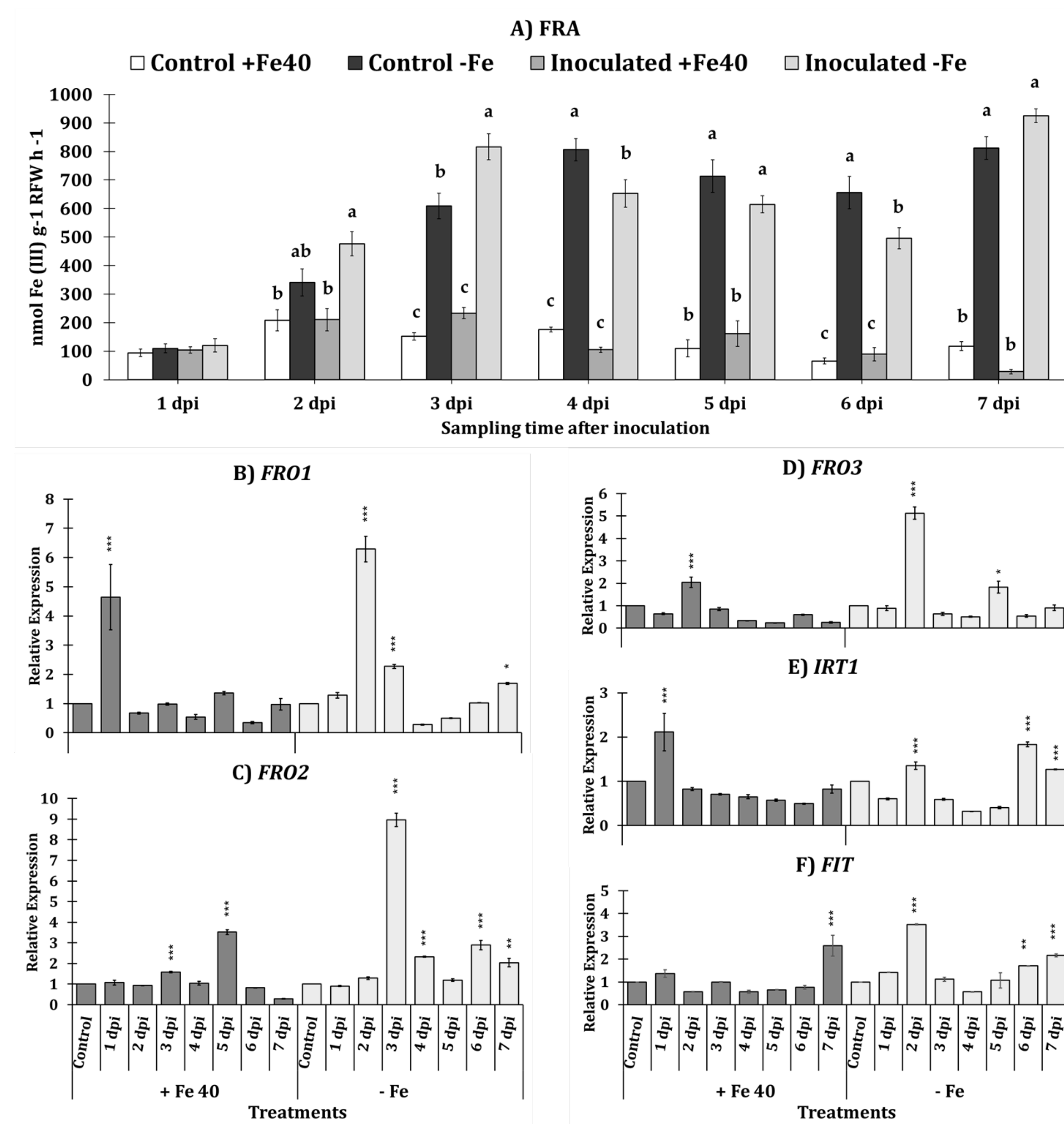

Figure IV.4. Evolution of FRA along seven days of observation and relative expression of *FRO1*, *FRO2*, *FRO3*, *IRT1* and *FIT*, in *C. melo* roots. Four treatments were used, namely, (i) Control +Fe40µM (un-inoculated), (ii) Inoculated +Fe40µM, (iii) Control –Fe (un-inoculated) and (iv) Inoculated –Fe. The expression of control treatment for each nutritional condition is presented once, at the beginning of the graph, with the relative expression comparison method used, the control is always equal to 1. Data of gene expression represent the mean of three independent technical replicates, according to the Dunnett's test, * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$) over the bars indicate significant differences in relation to the control treatment. In the case of FRA, letters over the bars denote significant difference between plants inoculated and control plants analyzed by completely randomized ANOVA followed by a Tukey test ($p < 0.05$).

Relative expression of two ferric reductase genes, *FRO3* and *FRO4,* involved in Fe^{3+} reduction in leaves were also studied in melon plants. *FRO3* and *FRO4* relative

expression significantly increased at the first day post inoculation in Fe deficient conditions. However, in the case of *FRO4* the maximum relative expression level reached occur at the second day post inoculation in Fe deficient conditions. As occur with the genes studied in roots, in Fe sufficient conditions no significant differences were observed after inoculation except at the first day post inoculation in *FRO4* where a significant increase was observed (Figure IV.5).

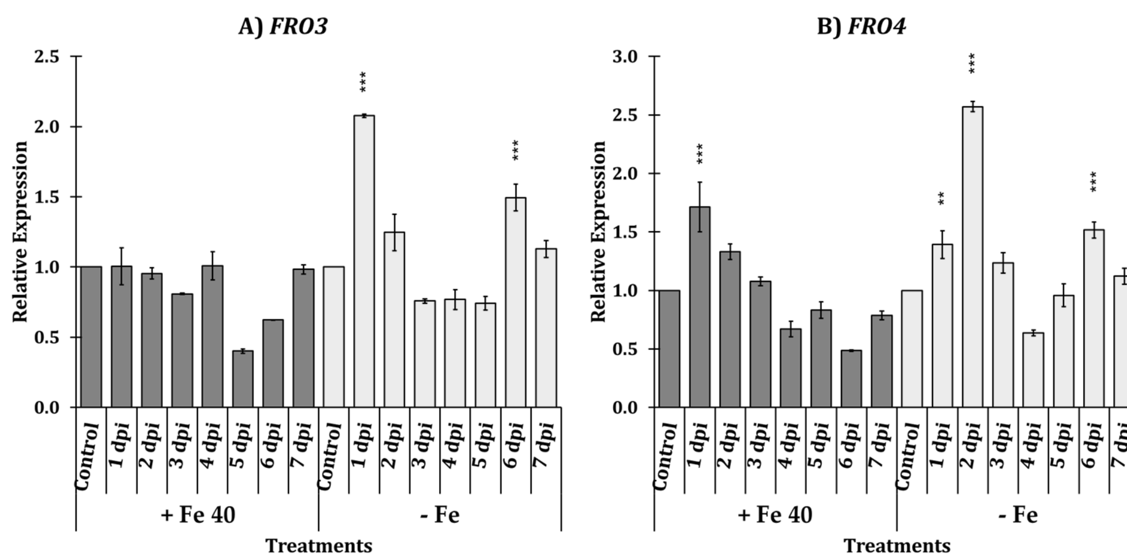

Figure IV.5. Relative expression of FRO3 (A) and FRO4 (B) on shoots of *C. melo*. Four treatments were used, namely, (i) Control + Fe40μM (un-inoculated), (ii) Inoculated + Fe40μM, (iii) Control - Fe (un-inoculated) and (iv) Inoculated - Fe. The expression of control treatment for each nutritional condition is presented once, at the beginning of the graph, with the relative expression comparison method used, the control is always equal to 1. Data of gene ex-pression represent the mean of three independent technical replicates, according to the Dunnett's test, * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$) over the bars indicate significant differences in relation to the control treatment.

In Figures IV.6 and IV.7 it is represented a panorama of FRA and general appearance of aerial parts and roots at 5 DPI. Both, cucumber (Figure IV.6) and melon (Figure IV.7) plants, began to show deficiency symptoms at 4 DPI, being more visible in the cucumber plants, where leaves with a higher degree of chlorosis were observed. In both species, the roots of the plants that grew with sufficient Fe had a more elongated appearance and less abundant secondary roots as it can be seen in the picture. Also, in cucumber plants, SPAD values from 4 to 7 DPI, have shown to be significantly different between treatments ($F_{3,95}$=42.11, p=0.0000), especially in those grown under Fe deficient conditions (Figure IV.6B), nonetheless, plants grown in Fe

sufficient conditions show higher chlorophyll content; in the case of melon, inoculated plants grown under Fe deficient conditions, were those that presented higher chlorophyll content with significant difference respect to other treatments ($F_{3,85}$=14.89, p=0.0000) (Figure IV.7B).

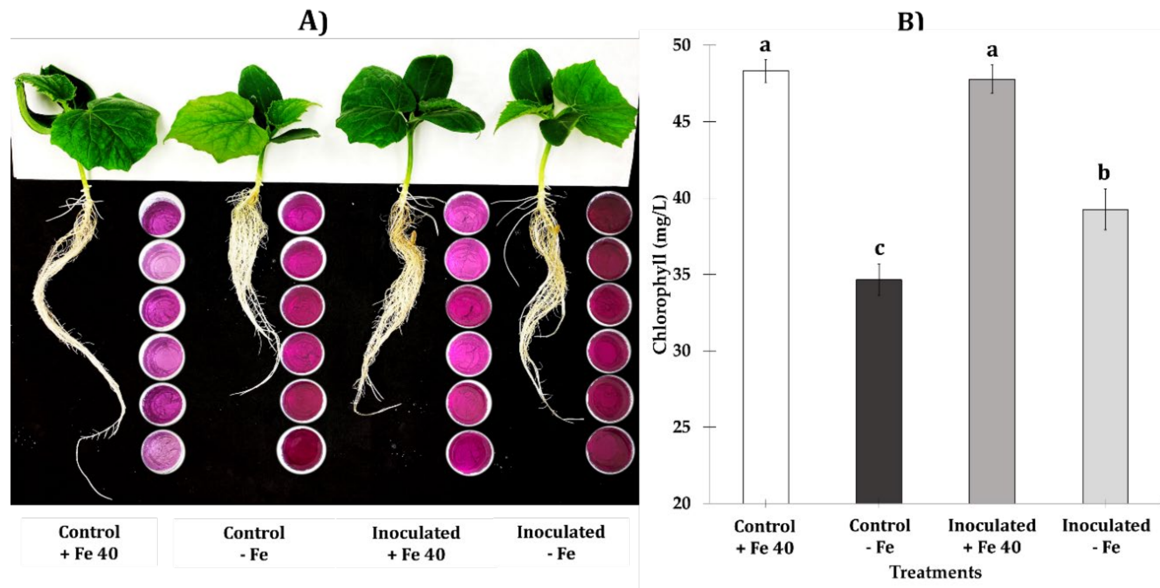

Figure IV.6. General panorama of FRA on roots of *C. sativus*. On the right side of the roots, the indicator solution containers can be seen at 5 DPI, the FRA is generally highly induced, however, as can be seen in the intense purple color, inoculated plants exceed their respective control; shoots of inoculated plants did not show sever symptoms of chlorosis like occurred in plants without inoculation. (B) Mean of SPAD values from at 7 DPI showed significant difference between control and inoculated plants grown in Fe deficient conditions, exceeding inoculated plants their respective control. Letter over the bars denote significant difference between plants inoculated and control plants analyzed by completely randomized ANOVA followed by a Tukey test (p < 0.05).

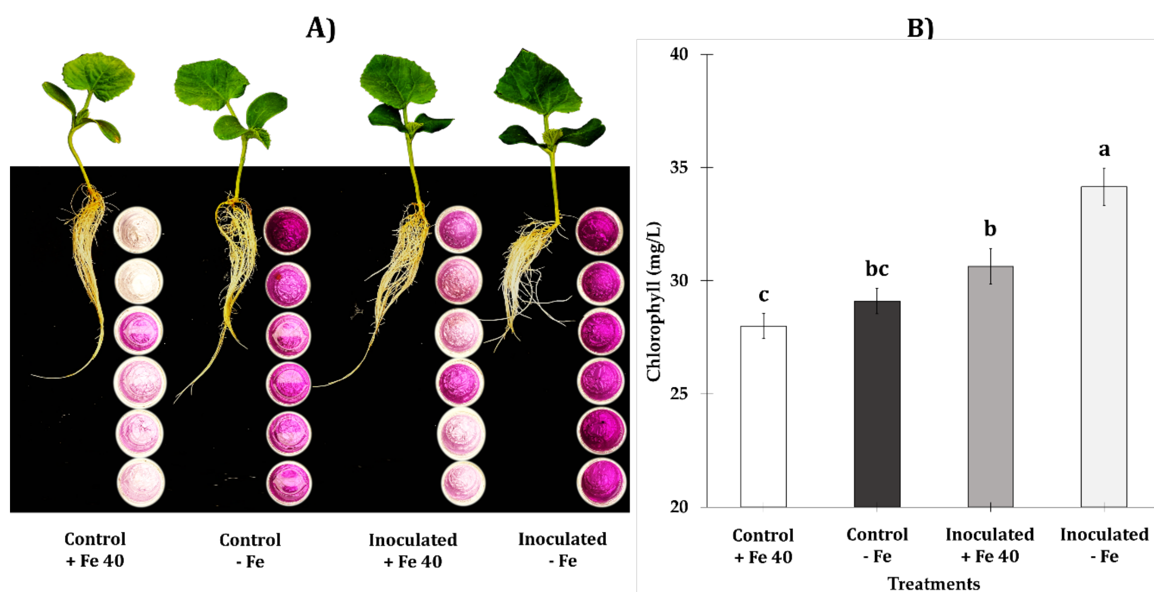

Figure IV.7. General panorama of FRA on roots of *C. melo* at 5 DPI. On the right side of the roots, the indicator solution containers can be seen at 5 DPI with clearly significant difference between controls and inoculated plants. Also, abundant secondary roots growth can be observed; in shoots of inoculated plants did not show chlorosis symptoms. (B) Mean of SPAD values at 7 DPI revealed significant difference with inoculated plants exceed their respective controls, being the plants grown in Fe deficient conditions those that reached higher values in chlorophyl content. Letter over the bars denote significant difference between plants inoculated and control plants analyzed by completely randomized ANOVA followed by a Tukey test ($p < 0.05$).

IV.4. Discussion

The discovery of new functions for IPF as plant endophytes and growth promoters, and their competence in the rhizosphere have enabled the expansion of their use, thus providing added value to their main use as biological control agents against a wide variety of insects and mites harmful to cultivated plants (Quesada-Moraga et al. 2020; Quesada Moraga 2020). In this sense, many studies have shown that IPF represent an excellent alternative to control agricultural pests (Moonjely et al. 2016; Sánchez-Rodríguez et al. 2018; Yousef et al. 2018; Brunner-Mendoza et al. 2019; Stone and Bidochka 2020; Barra-Bucarei et al. 2020). Indeed, several studies have shown the efficacy of species from the genera *Metarhizium* and *Beauveria* to control herbivores in crops like olive, corn, wheat, tomato, sunflower, melon and soybean amongst others (Yousef et al. 2018; González-Mas et al. 2019a; Barra-Bucarei et al. 2020; Agbessenou et al. 2020). Besides, they play other roles beyond pest control with direct and indirect benefits for plant growth through nutrient mobilization

and/or mediation of trophic relationships (Raya-Díaz et al. 2017b; Sánchez-Rodríguez et al. 2018; Moonjely and Bidochka 2019; Miranda-Fuentes et al. 2021b; Quesada-Moraga et al. 2022). Increasing the bioavailability of nutrients through phytohormones production and improvement of water transport are ways that IPF promote plant growth directly; they also benefit plants through indirect mechanisms involving induction of systemic resistance to harmful organisms (Barra-Bucarei et al. 2020).

However, little is known about direct and indirect mechanisms used by IPF for Fe acquisition in plants, although many studies indicated that IPF alleviate Fe chlorosis symptoms as in previous studies (Raya-Díaz et al. 2017a, b), *M. brunneum* EAMa 01/58-Su was also the best growing in culture medium with low Fe availability. In the same way, Raya-Díaz et al. (2017) showed that *M. brunneum* EAMa 01/58-Su applied to the soil at high doses (5×10^8 conidia ml^{-1}) alleviated Fe chlorosis symptoms in sorghum plants grown in calcareous soil, and increased plant height and inflorescence production of sunflowers grown in calcareous and non-calcareous soils.

Our in vitro study demonstrated the ability of *M. brunneum* isolate EAMa 01/58-Su to demineralize Fe being the most effective in producing Fe siderophores, with 58.4% of surface siderophores exudation 10 DPI, while *B. bassiana* isolates EABb 04/01-Tip and EABb 01/33-Su only achieved 24.3% and 17.8% of surface siderophores exudation, respectively. The increase of Fe availability resulting from application of a specific isolate could either be due to secretion of organic acids, thus reducing the pH of the medium, or through release of siderophores that chelate not only Fe but also other nutrients such as Zn, Mn and Cu (Krasnoff et al. 2014; Raya-Díaz et al. 2017b). There are few reports about IPF activity as solubilizers of nutrients. Some studies showed similar data using the well-known genus *Trichoderma* (Sánchez-Montesinos et al. 2020) and others using the saprophyte *Aspergillus niger* showing abilities as phosphorus solubilizers (Pal and Ghosh 2018; Baron et al. 2018, 2020; Naeem et al. 2022). Recent studies by Barra-Bucarei et al. (2020) showed differences between five isolates of *B. bassiana*. Although four of them were able to produce siderophores, isolates RGM-731 and RGM-644 highlighted by their high siderophores exudation capacity, 73% and 81%, respectively. Our results show the capacity of IPF to solubilize nutrients at the isolate-specific level, which contributes to our knowledge of these fungi and their function as plant growth promoters.

In higher plants two different strategies have been described; Strategy I which includes all plants except grasses and Strategy II that it is confined to grasses; dicots or Strategy I, is characterized by the necessity to reduce Fe^{3+}, to Fe^{2+}, prior to its absorption, this reduction is mediated by a ferric reductase located in the plasma membrane of the epidermal root cells codified by *FRO2* gene in *Arabidopsis thaliana*. Once Fe^{3+} has been reduced, it is transported into the cells by a Fe^{2+} transporter codified by *IRT1* in *A. thaliana* (Marschner et al. 1986; Brown and Jolley 1988; Marschner and Römheld 1994; Ivanov et al. 2012; Kobayashi and Nishizawa 2012; Naranjo-Arcos and Bauer 2016; Romera et al. 2019; García et al. 2021a, b). Some plants species also induce H+ -ATPases responsible for rhizosphere acidification (Lucena et al. 2006). This work shows for the first time a role of an IPF as elicitor of the Fe deficiency responses in Strategy I plants. However, in the bibliography it can be found some examples of microorganisms e.g., bacteria and fungi, that induce Fe deficiency responses, ferric reductase activity and relative expression of the Fe acquisition genes. Some genera of saprophytic, phytopathogenic fungi, including mycorrhizae, such as *Paelomyces*, *Aspergillus*, *Penicillium*, *Gliocladium*, *Trichoderma*, *Gongronella*, *Fusarium*, among others, have been recorded as capable of solubilizing nutrients such as P and K (Vera et al. 2002; Baron et al. 2018; Zhao et al. 2020). Among them one of the most studied species is *Azospirillum brasilense*, cucumber plants inoculated with *A. brasilense* showed higher ferric reductase activity and relative expression of the Fe acquisition genes, *FRO1, IRT1, FIT, HA1* and *FRO3* (Pii et al. 2016; Zhou et al. 2016). Similar results were obtained in *A. thaliana* plants inoculated with *Bacillus subtilis* and *Pseudomonas simiae* (Zhang et al. 2009; Zamioudis et al. 2015). Relative to the fungus species we found arbuscular mycorrhizal (Rahman et al. 2020; Kabir et al. 2020), *Trichoderma asperellum* and *Trichoderma harzianum* (Zhao et al. 2014; Martínez-Medina et al. 2017b). Recently, Lucena et al. (2021) found that two yeast strain, *Debaromyces hansenii* and *Hansenula polymorpha* were able to induce Fe deficiency responses in cucumber plants. However, any works relative to IPF as Fe deficiency responses inductor can be found in the literature.

In this work the ability of *M. brunneum* 01/58-Su strain to induce Fe deficiency responses have been studied in two Cucurbitaceae species, *C. sativus* and *C. melo*. The results obtained show that *M. brunneum* 01/58-Su strain clearly induced the Ferric reductase activity and the relative expression of the Fe acquisition genes, *FRO, IRT1,*

HA1 and *FIT* in both species. These new skills of *M. brunneum* 01/58-Su strain confer him an added value to its use as an excellent biological control agent and highlight the direct and indirect mechanisms involved in the Fe acquisition as mediated by an IPF.

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CHAPTER V. GREENHOUSE MELON CROP PROTECTION AND PRODUCTION THROUGH THE COMPATIBLE USE OF A PARASITOID WITH ENDOPHYTIC ENTOMOPATHOGENIC ASCOMYCETES

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Abstract

This study delves into the compatible use of a parasitoid with multifunctional endophytic Entomopathogenic Ascomycetes (EA) in IPM under greenhouse conditions. The parasitoid *Hyposoter didymator* was evaluated against *Spodoptera littoralis* in a multitrophic system with melon plants that were endophytically colonized by one of three EA strains (*Metarhizium brunneum* [one] or *Beauveria bassiana* [two]). In the first scenario, plants were inoculated by three different methods, and after infestation with noctuid larvae, the parasitoid was released at a 1:20 ratio. Microbiological and molecular techniques allowed the identification of progressive colonization throughout the whole plant life cycle. Indeed, *B. bassiana* was detected in approximately 20% of seeds from new fruits. The parasitoid was shown to be compatible with all strains and application methods, with total mortality rates ranging from 11.1% to 77.8%. Significant lethal and sublethal effects, a decrease in pupal weight and mortality of pupae showing abnormalities and an extension of the immature developmental times were observed for different strain–application method combinations. Additionally, the fungal treatments improved crop growth, as revealed by the significant gains in plant weight. In a second scenario (by inoculating plants with the fungi only by leaf spraying), and after infestation with noctuid larvae, the parasitoid was released at a 1:10 ratio, which revealed the remote fungal effect from the inoculation point and confirmed the compatibility of the parasitoid-EA-based strategy. These findings underscore the compatible use of a parasitoid with endophytic EA for *S. littoralis* control that can additionally exploit their multifunctionality for sustainable crop production.

Keywords: Entomopathogenic fungi, integrated pest management, multitrophic relationships, *Hyposoter didymator*, greenhouse conditions, *Spodoptera littoralis*

V.1. Introduction

The global population is currently undergoing exponential growth, and it is projected that by 2050, the world's population will approach nearly 10 billion individuals (Reid and Greene 2012; Julot and Hiller 2021; United Nations 2023). This fact presents a formidable challenge for agricultural food production for a growing global population in accordance with the principles of agricultural sustainability (Abrol and Shankar 2014; Tiwari and Singh 2021; Patel et al. 2022; Singh and Rale 2022). In this context, according to the FAO, between 20 and 40% of global crop production is lost annually to pests (FAO, 2019). Invasive insects cost the world economy approximately US$70 billion annually, while plant diseases cost it approximately $220 billion (FAO 2019; Julot and Hiller 2021). Consequently, pest control emerges as a primary concern in crop production, and the use of chemical pesticides has experienced a significant and alarming surge in recent decades, becoming a central element of the prevailing crop production system. To provide some perspective, 370 million kilograms of pesticides were sold within the European Union in 2018 (Jacquet et al. 2022). Despite their effectiveness, many of these chemical pesticides are linked to a plethora of adverse consequences for both human health and the environment (Reid and Greene 2012; Julot and Hiller 2021; Adeleke et al. 2022). Aligned with the European Commission's "Green Deal," numerous countries have integrated the reduction of pesticide usage as a primary objective within their public policies, with the aim of promoting sustainable agriculture (Julot and Hiller 2021; Jacquet et al. 2022).

Within this framework, the exploration of potential environmentally friendly entomopathogenic endophytic microorganisms such as entomopathogenic ascomycetes (EA) has the potential for establishing a stable and pest-free ecosystem, ultimately fostering higher and more sustainable crop productivity (Solter et al. 2017; Parewa et al. 2018; Quesada-Moraga et al. 2020; Quesada Moraga 2020). These fungi are recognized as excellent biocontrol tools to be used in IPM programs since they can infect a wide range of arthropod pests with a unique mode of action by contact through the integument, playing a key role in crop pest control (Quesada-Moraga et al. 2020). Among EA, the genera *Metarhizium* and *Beauveria* are also considered excellent examples of fungi with multifunctional lifestyles (Barelli et al. 2016) that

positively impact plant growth and immunity against generalist herbivores (Gange et al. 2019) and other biotic (Gupta et al. 2022b; Posada-Vergara et al. 2022, 2023; García-Espinoza et al. 2023a) and abiotic stresses (Khan et al. 2012, 2015; García-Espinoza et al. 2023b; Chaudhary et al. 2023).

The effectiveness of several endophytic EA against some of the most destructive piercing–sucking melon pests, such as *Aphis gossypii* Glover (Hemiptera: Aphididae), has been well documented (Resquín-Romero et al. 2016b; Garrido-Jurado et al. 2017; González-Mas et al. 2019a). In addition, the response of melon plants to EA colonization in terms of defense induction (González-Guzmán et al. 2022; García-Espinoza et al. 2023b, a), which can ultimately influence multitrophic interactions involving melon, *A. gossypii* and their natural enemies, predators and parasitoids, such as *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) and *Aphidius colemani* (Dalman) (Hymenoptera: Braconidae), respectively (González-Mas et al. 2019a; Quesada-Moraga et al. 2022), has also been documented.

Regarding chewing pests, the cotton leafworm *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) poses a major threat to agricultural crops in the Mediterranean region (Ahmed et al. 2019). This pest shares its habitat with the koinobiont solitary parasitoid *Hyposoter didymator* (Thunberg) (Hymenoptera: Ichneumonidae), which plays a crucial ecological role as a native parasitoid in southern Spain. In this context, Miranda-Fuentes et al. (2020) observed in laboratory settings that *H. didymator* and *Metarhizium brunneum* Petch (Hypocreales: Clavicipitaceae) EAMa 01/58-Su strains were compatible for controlling these noctuid pests and even enhancing fungal performance due to parasitization. Similarly, under the same laboratory conditions, *S. littoralis* larvae feeding on *M. brunneum*-colonized plants did not affect the reproductive potential of the parasitoid, and both the fungus and parasitoid larvae were found to coexist within the same larval host (Miranda-Fuentes et al. 2021b). We hereby provide a step forward: the evaluation of the biocontrol potential of the parasitoid *H. didymator* for controlling *S. littoralis* on melon plants inoculated with endophytic EA under greenhouse conditions. Additionally, we aimed to assess the possible added beneficial impact of fungal application on melon plant growth in this multitrophic system.

V.2. Material and methods

V.2.1. Biological material and growth conditions

V.2.1.1. Fungal strains

One strain of *M. brunneum* (EAMa 01/58-Su) and two strains (EABb 04/01-Tip and EABb 01/33-Su) of *Beauveria bassiana* (Bals.) Vuill (Ascomycota: Hypocreales) from the culture collection of the Agronomy Department, University of Cordoba (Spain) were used in this work (Table V.1). More details about these strains and their potential as biocontrol agents can be found in our previous works (Quesada-Moraga et al. 2006a; Yousef et al. 2018; Garrido-Jurado et al. 2019; González-Mas et al. 2019c; Miranda-Fuentes et al. 2020, 2021b; García-Espinoza et al. 2023a).

Table V.1. *Metarhizium brunneum* and *Beauveria bassiana* strains used in this study.

Fungal Species	Strain	Origin	Agroecosystem	Habitat	GenBank Accession Number	CECT* Accession Number
M. brunneum	EAMa 01/58-Su	Hinojosa del Duque (Córdoba, Spain)	Wheat crop	Soil	JN900390	20764
B. bassiana	EABb 04/01-Tip	Ecija (Sevilla, Spain)	Opium poppy crop	Insect (*Iraella luteipes*)	FJ972963	20744
B. bassiana	EABb 01/33-Su	El Bosque (Cadiz, Spain)	Traditional olive orchard	Soil	FJ972969	21149

* The Spanish Type Culture Collection

V.2.1.2. Insects

The *S. littoralis* and *H. didymator* specimens used in this work came from a colony established at the insectarium of the Agricultural and Forestry Entomology Laboratory of the University of Cordoba. The growth chamber was maintained under the following conditions: 26±2 °C, 70±5% RH and a photoperiod of 16:8 (L:D) h (Miranda-Fuentes et al. 2020, 2021b).

The pollinator *Bombus terrestris* L. (Hymenoptera: Apidae), used in the second experiment to obtain fruits and seeds, was acquired from a commercial stock of Koppert S.A.

V.2.2. Inoculum, growth conditions and plants inoculation methods

To acquire the inoculum, the procedure was carried out following the methods described in García-Espinoza et al. (2023a, b). In brief, the three strains were subcultured from their stored slant cultures on potato dextrose agar (PDA) in Petri

dishes and allowed to grow for 15 days at a temperature of 25 °C in complete darkness. Subsequently, the inoculum preparation process involved scraping conidia from the Petri plates into a sterile solution of 0.1% Tween 80. To ensure homogenization of the inoculum, it underwent a 5-minute sonication step. The final step involved filtration through multiple layers of cheesecloth to eliminate any residual mycelia. A hemocytometer (Malassez chamber; Blau Brand, Wertheim, Germany) was used to estimate the conidia concentration, which was finally adjusted to $1x10^8$ conidia/ml by adding a sterile solution of distilled water with 0.1% Tween 80.

In all experiments, certified endophyte-free melon seeds (*Cucumis melo* L. cv. Galia) were employed. These seeds, which had undergone prior surface sterilization following the method outlined by Garrido-Jurado et al. (2017), were germinated. Germination was carried out in 500 ml sterilized pots filled with universal black peat (Floragard, Germany), which was subjected to double sterilization in an autoclave at 121 °C for 30 minutes, with a 24-hour interval, as described by González-Mas et al. (2019a). Three distinct application methods—namely, seed coating, soil drenching, and leaf spraying—were employed for each fungal strain. In the initial experiment, a completely randomized design was implemented, consisting of a total of nine treatments (comprising 3 strains and 3 application methods) and an untreated control group. There were four replicates (plants) for each treatment, as shown in Figure V.S1A-D. In the subsequent experiment, plant inoculation was exclusively carried out via leaf spraying, as depicted in Figure V.S1E-H. For the seed coating method, seeds were submerged in a suspension of $1x10^8$ conidia/ml solution in Falcon tubes on a rotary shaker at 12 rpm for 4 h. Then, seeds were sown in pots previously prepared at 1 cm of profundity. On the other hand, soil drenching and leaf spraying were carried out when the melon plants reached the 4 true leaf stage, 30 d after seed coating treatment. For soil drenching, 5 ml of the conidial suspension was poured with a pipette onto the surface of the pot.

In the case of leaf spraying, for the first experiment (Figure V.S1A), the entire plant was sprayed, and in the second experiment (Figure V.S1E), only the 2 true basal leaves were sprayed (the adaxial and abaxial leaf surfaces) with 2 ml of conidia suspension using an aerograph (piston compressor of 23 l/min, 15-50 PSI and 0.3 mm nozzle diameter, Artesania Latina S.A., Madrid, Spain) (Miranda-Fuentes et al. 2021b). To avoid contamination by run-off, soil and uninoculated parts of the plants were

protected with aluminum foil and plastic bags, respectively. Control plants were sprayed with a sterile solution of 0.1% Tween 80. After soil drenching and leaf spraying, treated and control plants were covered with plastic bags for 48 h and maintained in the growth chamber.

The pots were kept in a greenhouse environment from October to December. During this period, the plants were watered three times a week, and their nutritional requirements were met using Nutrichem 60 fertilizer (N: 20, P: 20, K: 20) (Miller Chemical & Fertilizer Corporation, Hanover, Pennsylvania, USA). Fertilizer was added to the irrigation water at a rate of 1 g/l, and this procedure was carried out twice a month.

V.2.3. Compatibility between EA strains and *H. didymator* for *S. littoralis* control under greenhouse conditions

The compatibility between EA strains and the parasitoid *H. didymator* for controlling *S. littoralis* was investigated in two greenhouse experiments.

V.2.3.1. Experiment 1

The aim of the first experiment, as the first scenario, was to explore the impact of EA strains and inoculation methods on the fitness of *S. littoralis* within a multitrophic system that also involved the presence of the parasitoid. Melon plants with four well-developed true leaves were inoculated as described in "Inoculum, growth conditions and plant inoculation methods" section using the strains EAMa 01/58-Su of *M. brunneum* and EABb 04/01-Tip and EABb 01/33-Su of *B. bassiana* by three inoculation methods: seed coating, soil drenching and leaf spraying. For the leaf spraying method, the entire aerial part of the plant was sprayed (Figure V.S1A). The treated plants were organized in groups of four per treatment within the greenhouse and enclosed in anti-aphid mesh cages. At 2 days postinoculation (DPI), 10 L3 (third-instar larvae) *S. littoralis* larvae were released in the second pair of true leaves (Figure V.S1B), and larvae were confined with an organza bag for 24 hours to ensure contact with the plant and the inoculum. Following this period, the textile bag was removed, and two females and four males of the parasitoid *H. didymator* were introduced per cage for each treatment (Figure V.S1C). The parasitoids were then removed 24 hours later, leaving only the *S. littoralis* larvae on the plants.

Larvae fed on the plants for 5 days. Then, the larvae were carefully removed from the plants and individually placed in methacrylate boxes. Here, they were provided with melon leaves from their respective treatments for an additional three days. Subsequently, they were fed an artificial diet until they reached the pupal stage. Throughout the entire process, starting from the introduction of L3 larvae until they reached the pupal stage, daily monitoring was conducted to record any instances of mortality.

V.2.3.2. Experiment 2

The aim of the second experiment, considered the second scenario, was to delve into the effect of endophytic colonization on *S. littoralis* fitness and *H. didymator* reproductive potential. For this assay, plants were inoculated with the three EA strains only by leaf spraying.

Plants with four well-developed true leaves were inoculated with the EAMa 01/58-Su *M. brunneum* strain and the EABb 04/01-Tip and EABb 01/33-Su *B. bassiana* strains. In this scenario, only the two basal leaves of each plant were subjected to spraying as detailed in "Inoculum, growth conditions and plant inoculation methods" section (Figure V.S1E). The effects of the fungi were assessed on both *S. littoralis* larvae and the *H. didymator* parasitoid by introducing new L3 larvae onto the sprayed and unsprayed leaves.

At 2 DPI, 10 L3 *S. littoralis* larvae were released on sprayed or unsprayed leaves. For the first case, the larvae were enclosed within organza bags, following the procedures outlined in “Experiment 1” section (shown in Figure V.S1F). In the case of larvae that fed on unsprayed leaves, the sprayed leaves were carefully isolated to prevent any contact between the larvae and the inoculum. After 24 hours, the larvae were released, and four females and eight males of *H. didymator* were introduced (as illustrated in Figure V.S1G).

With the aim of reaching fruit production, *B. terrestris* bumblebees were introduced during the flowering stage for a week to achieve pollination, and 4 specimens were introduced (4 times a week) per treatment. Once the fruits had matured, which occurred 115 days after sowing (DAS), they were removed to study the endophytic colonization both in the mesocarp and in the seeds (Figure V.S1H).

II.2.4. Endophytic colonization by microbiological technique and by qPCR

The assessment and follow-up of the endophytic colonization of the three strains evaluated were carried out both by conventional microbiological techniques and by molecular detection and quantification by qPCR. Endophytic colonization monitoring was carried out from leaves collected at 2, 7, 14, 21 and 28 d after leaf spraying and soil drenching (Figure V.S1D). Assessment of colonization by conventional methods was carried out according to Garrido-Jurado et al. (2017), González-Mas et al. (2019) and Miranda-Fuentes et al. (2021). Following the same methodology, endophytic colonization was also evaluated on fruits (including mesocarp and seeds) collected at 115 DAS in the second experiment (Figure V.S1H). Endophytic colonization was expressed and represented as a percentage, according to the number of fragments that presented growth of EA.

Molecular identification by qPCR was carried out as described by García-Espinoza et al. (2023a). Briefly, plant material was ground to a fine powder with a mortar and pestle in liquid nitrogen. Total DNA was isolated using a HigherPurity™ Plant DNA Purification Kit (Canvax Biotech S.L., Córdoba, Spain) according to the manufacturer's instructions and resuspended in 100 µl of elution buffer. The concentration and quality of DNA were assessed by measuring absorbance at 260 nm and 280 nm in a NanoDrop™ 2000 (Thermo Fisher Scientific Inc.). The final concentration was homogenized until it reached 30 ng/µl. To identify and quantify the EAMa 01/58-Su *M. brunneum* strain, the primer pair of the *nrr* gene (F: TCA GGC GAT CTC GTG GTA AG, R: GGG GTG TAC TTG AGG AAT GGG) was used (Barelli et al. 2018), while in the case of the two strains of *B. bassiana* (EABb 04/01-Tip and EABb 01/33-Su), the ITSII rRNA gene (F: GCC GGC CCT GAA ATG G, R: GAT TCG AGG TCA ACG TTC AGA AG) pair primer was used (Bell et al. 2009). Real-time PCRs were performed in a qRT–PCR Bio-Rad CFX Connect thermal cycler. Absolute quantification was carried out according to Bell et al. (2009) and Barelli et al. (2018). To set up the standard curves, a gradient of 1:4 from 40 ng to 0.16 pg of fungal and plant genomic DNA was used; absolute quantification was determined by comparing threshold cycle numbers against the standard curve previously generated (Bell et al. 2009; Barelli et al. 2018).

V.2.5. Assessment of melon growth promotion

At 8, 15, 22 and 28 DPI, measurements of plant length were recorded to assess plant growth. Subsequently, at 77 DPI (115 days after sowing), the fresh weight of both the aerial parts and roots was measured. To determine the weight of dry matter, which includes both aerial parts and roots, plant material was placed in paper envelopes and dried in an oven at 60 °C for 96 hours.

V.2.6. Statistical analysis

Mortality data, expressed as percentages, were analyzed using a generalized linear mixed model with binomial distribution and logit link function. The significance of the treatment was analyzed with the F test and Tukey's multiple comparisons ($p < 0.05$) (JMP 8.0, SAS Institute Inc.). Data on pupae weight, larval stage duration and plant growth were analyzed using analysis of variance (ANOVA) followed by a Tukey multiple range test among treatments (Statistix 9.0®, Analytical Software, Tallahassee, FL, USA). Means were compared by the HSD All-Pairwise Comparisons method. Different letters within columns or over the bars, as specified in the Figure or Table legends, indicate significant differences ($p < 0.05$). The *H. didymator* mortality data were subjected to Kaplan–Meier survival analysis to calculate average survival time (AST) values in days and compared by the log-rank test calculated with IBM SPSS 25.0 software (SPSS Inc., Chicago, IL, USA).

The qPCR values represent the mean ± SE of four independent replicates. Fungal DNA quantification data were analyzed using one-way analysis of variance (ANOVA) followed by a Tukey test.

V.3. Results

In the first scenario, the melon plants were inoculated with the fungal strains by soil drenching, seed coating or plant spraying and then infested with noctuid larvae and parasitoids released at a 1:20 ratio. Fungal inoculation had a significant impact on larval mortality ($\chi^2_{(1)}$=75.99, p=0.0001), with higher total mortality rates observed for the EAMa 01/58-Su *M. brunneun* strain applied by spraying ($\chi^2_{(1)}$=47.72, p=0.0001), whereas the percentage of dead *S. littoralis* pupae, including those that showed abnormalities, was significant for all strains and application methods [EAMa 01/58-Su ($\chi^2_{(3)}$=58.06, p=0.0001), EABb 04/01-Tip ($\chi^2_{(3)}$=103.92, p=0.0001) and EABb 01/33-Su ($\chi^2_{(3)}$=39.47, p=0.0001)] (Table V.2). There were significant pupal mortality rates ranging between 16.67 and 38.89%, mainly for the soil drenching inoculation and leaf spray treatments (Table V.2). Indeed, the EAMa 01/58-Su and EABb 04/01-Tip strain application methods significantly affected the total mortality of *S. littoralis* [EAMa 01/58-Su ($\chi^2_{(2)}$= 163.35, p=0.0001), EABb 04/01-Tip ($\chi^2_{(2)}$= 29.19, p=0.0001)] (Table V.2). In this context, for the EAMa 01/58-Su strain, there were significant differences in mortality rates between seed coating and soil drenching ($\chi^2_{(1)}$= 75.99, p=0.0001), between seed coating and leaf spraying ($\chi^2_{(1)}$=163.05, p=0.0001) and between soil drenching and leaf spraying ($\chi^2_{(1)}$=18.73, p=0.0001); in the case of EABb 04/01-Tip, a significant difference was recorded between seed coating ($\chi^2_{(1)}$=22.73, p=0.0001) and soil drenching ($\chi^2_{(1)}$=19.05, p=0.0001) compared to leaf spray (Table V.2).

Table V.2. Lethal and sublethal effects of endophytic entomopathogenic fungi on *S. littoralis* larvae that were fed on colonized melon leaves *in planta* for 5 days and for 3 additional days on colonized leaf fragments.

Treatment	Larval mortality (%)[1]	Pupal mortality (%)		Parasitization (%)[1]	Total mortality (%)[1,2]	Larval development time[3] (d)	Pupal development time[3] (d)	Pupal weight[3] (g)
		With abnormalities[1]	Total[1]					
EAMa 01/58-Su	($\chi^2_{(3)}$= 166.41, p=0.0001)	($\chi^2_{(3)}$= 89.74, p=0.0001)	($\chi^2_{(3)}$= 58.06, p=0.0001)	($\chi^2_{(3)}$= 80.45, p=0.0001)	($\chi^2_{(3)}$= 171.07, p=0.0001)	($F_{3,94}$ = 3.27, p = 0.0294)	($F_{3,81}$ = 5.09, p = 0.0029)	($F_{3,94}$ = 3.13, p = 0.0295)
Control	13.89 ± 5.32 a	0.00 ± 0.00 a	8.33 ± 5.32 a	11.11 ± 0.00 a	33.33 ± 10.14 a	20.00 ± 0.992 ab	11.478 ± 0.656 b	0.307 ± 0.018 ab
Seed coating	5.56 ± 3.21 b	0.00 ± 0.00 a	5.56 ± 5.56 a	0.00 ± 0.00 b	11.11 ± 7.86 bA	17.74 ± 0.352 b	11.688 ± 0.618 b	0.351 ± 0.007 a
Soil drenching	11.11 ± 4.54 ab	8.33 ± 2.78 b	22.22 ± 4.54 b	13.89 ± 6.99 a	47.22 ± 11.45 cB	18.65 ± 0.721 ab	13.105 ± 1.017 ab	0.323 ± 0.016 ab
Leaves spray.	47.22 ± 5.32 c	8.33 ± 5.73 b	19.44 ± 2.78 b	11.11 ± 7.86 a	77.78 ± 7.86 dC	21.00 ± 1.155 a	17.000 ± 1.604 a	0.287 ± 0.024 b
EABb 04/01-Tip	($\chi^2_{(3)}$= 8.97, p=0.0297)	($\chi^2_{(3)}$= 131.78, p=0.0001)	($\chi^2_{(3)}$= 103.92, p=0.0001)	($\chi^2_{(3)}$= 21.04, p=0.0001)	($\chi^2_{(3)}$= 29.90, p=0.0001)	($F_{3,106}$ = 2.0, p = 0.1186)	($F_{3,89}$ = 5.88, p = 0.0011)	($f_{3,106}$ = 3.06, p = 0.0316)
Control	13.89 ± 5.32 a	0.00 ± 0.00 a	8.33 ± 5.32 a	11.11 ± 0.00 a	33.33 ± 15.30 a	20.00 ± 0.992 a	11.478 ± 0.656 b	0.307 ± 0.018 b
Seed coating	11.11 ± 4.54 a	2.78 ± 2.78 b	8.33 ± 5.32 a	8.33 ± 2.78 a	27.78 ± 9.62 aA	17.75 ± 0.222 a	16.115 ± 1.031 a	0.364 ± 0.010 a
Soil drenching	9.03 ± 5.93 ab	2.78 ± 2.78 b	17.71 ± 3.47 b	2.78 ± 2.78 b	29.51 ± 3.56 aA	19.72 ± 0.751 a	16.792 ± 1.231 a	0.331 ± 0.014 ab
Leaves spray.	5.56 ± 3.21 b	19.44 ± 9.49 c	38.89 ± 9.62 c	8.33 ± 2.78 a	52.78 ± 11.45 bB	18.92 ± 0.749 a	16.882 ± 1.280 a	0.353 ± 0.016 ab
EABb 01/33-Su	($\chi^2_{(3)}$= 71.41, p=0.0001)	($\chi^2_{(3)}$= 55.54, p=0.0001)	($\chi^2_{(3)}$= 39.47, p=0.0001)	($\chi^2_{(3)}$= 5.12, p=0.1632)	($\chi^2_{(3)}$= 9.64, p=0.0219)	($F_{3,101}$ = 1.95, p = 0.1271)	($F_{3,84}$ = 5.45, p = 0.0018)	($f_{3,101}$ = 1.94, p = 0.1276)
Control	13.89 ± 5.32 a	0.00 ± 0.00 a	8.33 ± 5.32 a	11.11 ± 0.00 a	33.33 ± 15.30 a	20.00 ± 0.992 a	11.478 ± 0.656 b	0.307 ± 0.018 a
Seed coating	8.68 ± 5.39 a	8.33 ± 5.32 b	25.35 ± 8.15 c	14.58 ± 5.71 a	48.61 ± 13.87 bB	18.33 ± 0.706 a	14.556 ± 1.115 ab	0.311 ± 0.019 a
Soil drenching	0.00 ± 0.00 b	13.89 ± 2.78 c	25.00 ± 2.78 c	16.67 ± 5.56 b	41.67 ± 2.78 abB	17.80 ± 0.424 a	16.952 ± 1.204 a	0.355 ± 0.012 a
Leaves spray.	2.78 ± 2.78 b	5.56 ± 3.21 b	16.67 ± 7.17 b	16.67 ± 7.17 b	36.11 ± 2.78 aA	19.82 ± 0.841 a	14.870 ± 0.964 ab	0.315 ± 0.015 a

Melon plants were inoculated with the strains EAMa 01/58-Su of *M. brunneum*, EABb 04/01-Tip and EABb 01/33-Su of *B. bassiana* by seed coating, soil drenching and leaves spraying. At 2 DPI, newly L3 *S. littoralis* larvae were released on plants, *H. didymator* adults were released 24 hours after and larvae were exposed to parasitoids (2 females and 4 males per treatment) for 24 hours.

[1]Means ± SE within columns, for each strain and control, with the same lowercase letter are not significantly different from each other according to the Tukey HSD test (p<0.05). [2]Means ± SE of total mortality, for each strain, with the same uppercase letter are not significantly different from each other according to the Tukey test (p<0.05). [3]Means ± SE within columns, for each strain, with the same letter are not significantly different from each other according to ANOVA following by a Tukey test (p<0.05).

The parasitoid was shown to be compatible with the fungal strains for *S. littoralis* control ($\chi^2_{(1)}$, $p \geq 0.05$) for all strains and application methods, with mortality rates ranging from 11.11% to 77.78% (Table V.2). Indeed, the three fungal strains led to a significant extension of the noctuid pupal development time ($F_{3,81}$ =5.09, p=0.0029), whereas *M. brunneum* also caused an increase in the larval development time ($F_{3,94}$ =3.27, p=0.0294) and a decrease in the noctuid pupal weight ($F_{3,94}$ =3.13, p=0.0295). The lowest pupal weight was recorded for those specimens fed on plants inoculated by soil drenching and leaf spraying (Table V.2).

In addition, the endophytic colonization of plants was assessed over time, with the three strains being able to colonize melon plants, whereas the intensity of colonization over time was strain and application method dependent, as shown by both microbiological and qPCR techniques (Figure V.1). In plants inoculated by leaf spraying, the presence of the EAMa 01/58-Su, EABb 04/01-Tip, and EABb 01/33-Su strains as endophytes was detected using both microbiological techniques and qPCR at all observation time points. Specifically, through microbiological techniques, EAMa 01/58-Su was detected at 2, 7, 21, and 28 DPI, EABb 04/01-Tip was detected at 2, 7, and 21 DPI, and EABb 01/33-Su was detected at 2, 7, 14, and 21 DPI (Figure V.1A-C). In contrast, qPCR analysis showed a similar prevalence of all three strains at 2, 7, and 14 DPI, which was significantly different from the levels observed at 21 and 28 DPI (p< 0.05) (Figure V.1D-E). No fungal presence was microbiologically detected in the mesocarp of fruits from inoculated plants with any of the strains used, except in the seeds from inoculated plants with the EABb 04/01-Tip strain, in which 20% of seeds presented fungal growth (Figure V.S1 2). This result was confirmed by qPCR. In 33.3% of fruits from plants sprayed with EABb 04/01-Tip, a reading of 1.68±0.19 pg/40 ng of total DNA per qPCR was recorded, while EABb 01/33-Su was detected in 50% of fruits from plants sprayed with this strain, showing 0.73±0.05 pg/40 ng of total DNA per qPCR. However, the presence of the EAMa 01/58-Su *M. brunneum* strain in seeds was not detectable.

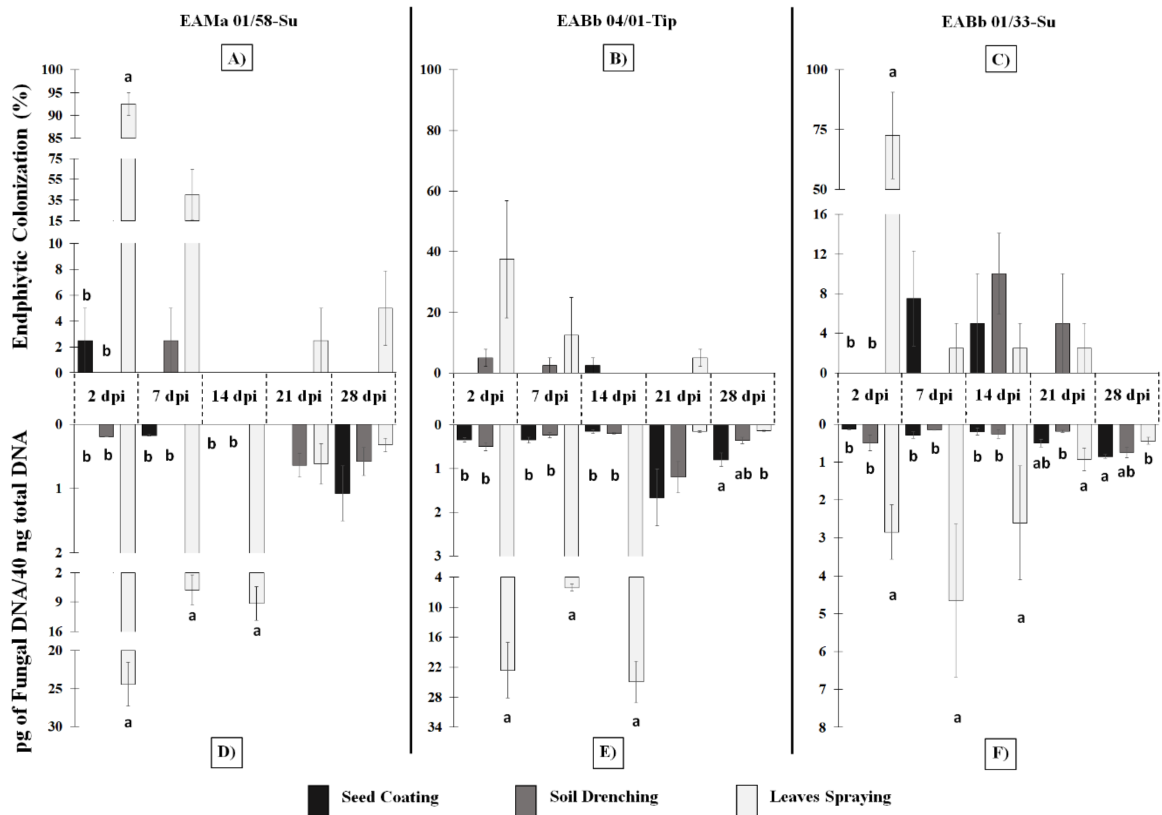

Figure V.1. Detection of endophytic presence of EAMa 01/58-Su *M. brunneum* strain (A and D) and EABb 04/01-Tip (B and E) and EABb 01/33-Su (C and D) *B. bassiana* strains by microbiological technique (up) and by qPCR (down) in melon plants inoculated by seed coating, soil drenching, and leaves spraying. Samples were collected at 2, 7, 14, 21 and 28 days post-inoculation from untreated leaves. Endophytic colonization is expressed as a percentage of melon leaf fragments in which fungal growth was observed; molecular detection and quantification is expressed in fungal DNA picograms (pg) relative to 40 nanograms (ng) of total DNA per reaction. For qPCR quantification, bars represent the mean values of two technical replicates from each of four independent biological replicates. Letter over the bars denotes a significant difference between plants treated with each strain by seed coating, soil drenching or leaves spraying analyzed by sampling time by completely randomized ANOVA followed by a Tukey test ($p < 0.05$).

In general, higher growth rates at 77 DPI (115 DAS) were observed in plants inoculated with EA, regardless of the specific strain or method of application used, with significant differences ($p < 0.05$) found in foliar fresh weight from all treatments except in those plants inoculated by soil drenching with the EABb 01/33-Su strain (Figure V.2A). Inoculation with the EABb 04/01-Tip strain by any of the three methods led to a significant increase in root fresh weight (Figure V.2B). Roots from plants inoculated with the EAMa 01/58-Su strain by leaf spraying were also significantly higher than those from the controls (Figure V.2B). There was a significant increase in the plant fresh weight in all inoculated plants except for the EABb 01/33-Su strain by soil drenching and leaf spraying (Figure V.2C).

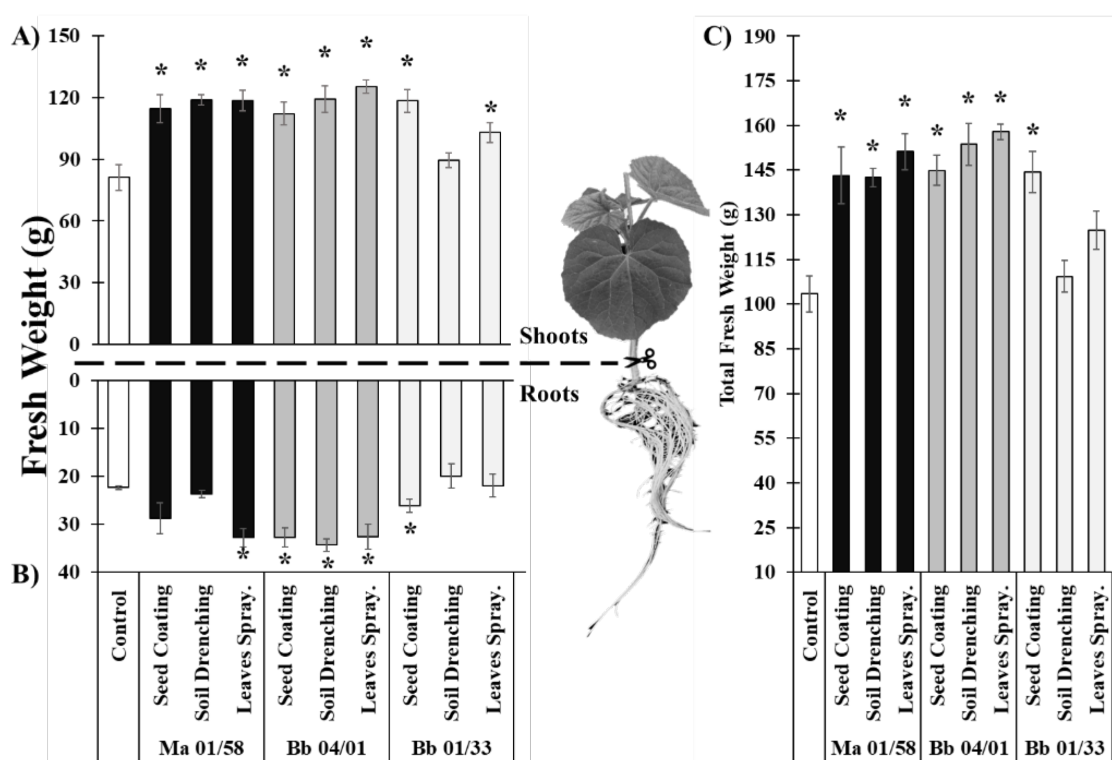

Figure V.2. Effects of endophytic entomopathogenic fungi on shoots (A) and roots (B) fresh weight and total fresh weight (C) of melon plants at 77 DPI (115 DAS) under greenhouse conditions. Plants were inoculated with EAMa 01/58-Su *M. brunneum* strain and EABb 04/01-Tip and EABb 01/33-Su *B. bassiana* strains by seed coating, soil drenching and leaves spraying; from 2 to 6 DPI plants were infested with 10 *S. littoralis* larvae which posteriorly were recollected and grown under laboratory conditions. Asterisks over the bars (mean ± SE) denote significant difference between treatments and control. Data were analyzed by completely randomized ANOVA followed by a Tukey test ($p < 0.05$) (n=4).

Significant differences ($p < 0.05$) were also found in foliar dry weight from plants inoculated with EAMa 01/58-Su or EABb 04/01-Tip strains by soil drenching and leaf spraying (Figure V.3A). The EAMa 01/58-Su strain only increased the dry root weight of plants inoculated by leaf spraying, while the EABb 04/01-Tip strain significantly increased the root dry weight of plants inoculated by all three inoculation methods used (Figure V.3B). Total dry weight was significantly different in plants inoculated with EAMa 01/58-Su (by soil drenching and leaf spraying) and EABb 04/01-Tip (by all inoculation methods used) strains when compared to controls (Figure V.3C).

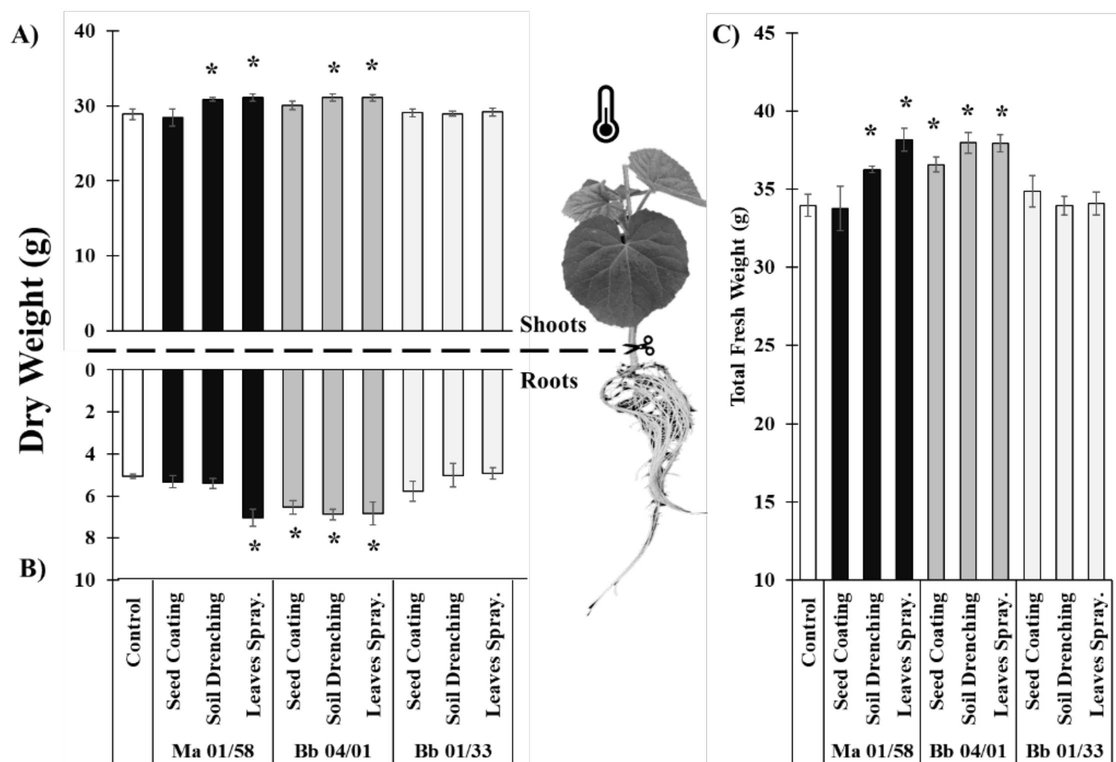

Figure V.3. Effects of endophytic entomopathogenic fungi on shoots (A) and roots (B) dry weight and total dry weight (C) of melon plants at 77 DPI (115 DAS) under greenhouse conditions. Plants were inoculated with EAMa 01/58-Su *M. brunneum* strain and EABb 04/01-Tip and EABb 01/33-Su *B. bassiana* strains by seed coating, soil drenching and leaves spraying; from 2 to 6 DPI plants were infested with 10 *S. littoralis* larvae which posteriorly were recollected and grown under laboratory conditions. To obtain shoots and roots dry matter weight, the vegetal material was placed in paper envelopes and dried in a stove at 60º C for 96 hours. Asterisks over the bars (mean ± SE) denotes significant difference between treatments and control. Data were analyzed by completely randomized ANOVA followed by a Tukey test ($p < 0.05$) (n=4).

Significant differences ($p < 0.05$) were also found in the length of plants inoculated with EAMa 01/58-Su and EABb 04/01-Tip strains by seed coating and soil drenching. At 15 DPI (Figure V.4A and B), the plants inoculated with EABb 01/33-Su by the three inoculation methods had significantly increased lengths when compared to the control (Figure V.4C). Only plants inoculated with the EABb 04/01-Tip strain by soil drenching showed a significant increase in length at 22 DPI (Figure V.4B).

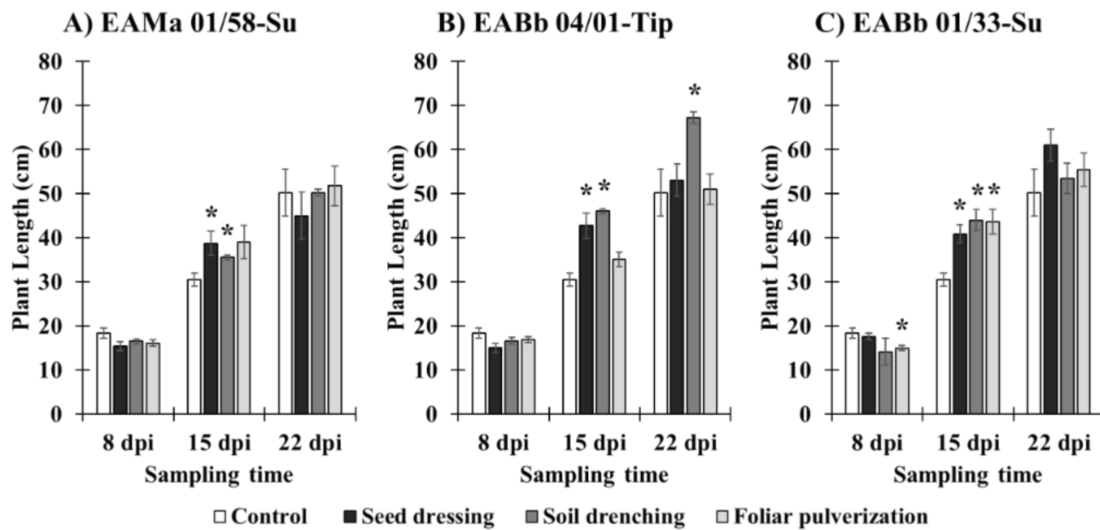

Figure V.4. Effects of endophytic entomopathogenic fungi on plant length at 8, 15 and 22 DPI under greenhouse conditions. Plants were inoculated with EAMa 01/58-Su *M. brunneum* strain and EABb 04/01-Tip and EABb 01/33-Su *B. bassiana* strains by seed coating, soil drenching and leaves spraying; from 2 to 6 DPI plants were infested with 10 *S. littoralis* larvae which posteriorly were recollected and grown under laboratory conditions. Asterisks over the bars (mean ± SE) denotes significant difference between treatments and control. Data were analyzed by completely randomized ANOVA followed by a Tukey test ($p < 0.05$) ($n=4$).

In a second scenario, significant larval *S. littoralis* mortality rates of 33-35% (for EAMa 01/58-Su strain), 15-28% (for EABb 04/01-Tip strain) and 16% (for EABb 01/33-Su strain) were observed after feeding larvae on sprayed or unsprayed melon leaves [sprayed ($\chi^2_{(1)}$=84.44, p=0.0001) and unsprayed leaves ($\chi^2_{(1)}$=69.61, p=0.0001) for EAMa 01/58-Su strain, sprayed leaves ($\chi^2_{(1)}$=60.35, p=0.0001) and unsprayed leaves ($\chi^2_{(1)}$=17.45, p=0.0001) for EABb 04/01-Tip and unsprayed leaves ($\chi^2_{(1)}$=18.64, p=0.0001) for EABb 01/33-Su strain] (Table V.3). In this scenario, relatively low significant pupal mortalities were recorded (Table V.3).

The reproductive potential of the parasitoid *H. didymator,* as indicated by its parasitization rates, varied from 38.89% to 89.44%, being significantly influenced by the fungal strain and application method when larvae fed on both sprayed and unsprayed leaves [($\chi^2_{(2)}$= 54.14, p=0.0001) for EAMa 01/58-Su, ($\chi^2_{(2)}$= 32.53, p=0.0001) for EABb 04/01-Tip and ($\chi^2_{(2)}$= 13.02, p=0.0015) for EABb 01/33-Tip strain] (Table V.3). It must be highlighted that the EAMa 01/58-Su strain, which caused higher larval mortality ratios, also led to the lower reproductive potential of the parasitoid (Table V. 3). Nonetheless, no significant differences were detected in the total mortality rates of the control larvae and the larvae exposed to melon leaves challenged by EAMa 01/58-Su ($\chi^2_{(2)}$= 4.25, p=0.1196), EABb 04/01-Tip ($\chi^2_{(2)}$= 4.76, p=0.0925) or EABb 01/33-Tip ($\chi^2_{(2)}$= 1.30, p=0.5217) strains, regardless of whether the *S. littoralis* larvae fed on sprayed or unsprayed melon leaves (Table V.3).

Table V.3. Lethal and sublethal effects of endophytic entomopathogenic fungi on *S. littoralis* larvae that were fed on sprayed or unsprayed melon leaves *in planta* for 5 days and for 3 additional days fed on colonized leaf fragments.

Treatment	Larval Mortality (%)[1]		Pupal Mortality (%)				Parasitization (%)[1]		Total Mortality (%)[1,2]	
			With abnormalities[1]		Total[1]					
EAMa 01/58-Su	($\chi^2_{(2)}$= 96.04, p=0.0001)		($\chi^2_{(2)}$= 28.58, p=0.0001)		($\chi^2_{(2)}$= 19.64, p=0.0001)		($\chi^2_{(2)}$= 54.14, p=0.0001)		($\chi^2_{(2)}$= 4.25, p=0.1196)	
Control Tween 0.1%	5.28 ± 3.06	a	0.00 ± 0.00	a	0.00 ± 0.00	a	89.44 ± 4.10	a	94.72 ± 3.06	a
Sprayed leaves	35.28 ± 10.19	b	0.00 ± 0.00	a	2.50 ± 2.50	b	38.89 ± 7.29	c	76.67 ± 5.27	aA
Unsprayed leaves	33.33 ± 6.42	b	3.70 ± 3.70	b	3.70 ± 3.70	b	51.85 ± 7.41	b	88.89 ± 6.42	aA
EABb 04/01-Tip	($\chi^2_{(2)}$= 60.48, p=0.0001)		($\chi^2_{(2)}$= 0.00, p=1.00)		($\chi^2_{(2)}$= 20.07, p=0.0001)		($\chi^2_{(2)}$= 32.53, p=0.0001)		($\chi^2_{(2)}$= 4.76, p=0.0925)	
Control Tween 0.1%	5.28 ± 3.06	a	0.00 ± 0.00	a	0.00 ± 0.00	a	89.44 ± 4.10	a	94.72 ± 3.06	a
Sprayed leaves	28.33 ± 5.45	c	0.00 ± 0.00	a	2.50 ± 2.50	b	48.89 ± 10.51	b	79.72 ± 6.94	aB
Unsprayed leaves	15.74 ± 7.91	b	0.00 ± 0.00	a	0.00 ± 0.00	a	84.26 ± 7.91	a	100.00 ± 0.00	aA
EABb 01/33-Su	($\chi^2_{(2)}$= 19.89, p=0.0001)		($\chi^2_{(2)}$= 63.50, p=0.0001)		($\chi^2_{(2)}$= 63.50, p=0.0001)		($\chi^2_{(2)}$= 13.02, p=0.0015)		($\chi^2_{(2)}$= 1.30, p=0.5217)	
Control Tween 0.1%	5.28 ± 3.06	a	0.00 ± 0.00	a	0.00 ± 0.00	a	89.44 ± 4.10	a	94.72 ± 3.06	a
Sprayed leaves	7.78 ± 4.84	a	0.00 ± 0.00	a	0.00 ± 0.00	a	87.22 ± 6.26	a	95.00 ± 2.89	aA
Unsprayed leaves	16.20 ± 11.12	b	8.33 ± 8.30	b	8.33 ± 8.30	b	60.19 ± 7.58	b	84.72 ± 9.72	aA

Melon plants were previously inoculated by leaves spraying with1x10^8 conidia/ml of EAMa 01/58-Su, EABb 04/01-Tip and EABb 01/33-Su strains. At 2 DPI, newly L3 *S. littoralis* larvae were confined into a textile bag to ensure they consumed either sprayed or unsprayed leaves. *H. didymator* adults were released 24 hours later and larvae were exposed to parasitoids (4 females and 8 males per treatment) for 24 hours. The plants were maintained under greenhouse conditions.

[1]Means ± SE within columns, for each strain and control, with the same lowercase letter are not significantly different from each other according to the Tukey's HSD test (p<0.05). [2]Means ± SE of total mortality, for each strain, with the same uppercase letter are not significantly different from each other according to the Tukey test (p<0.05).

Finally, there were sublethal effects observed in some life parameters of the F1 parasitoid generation. Specifically, pupal development time showed a significant elongation in parasitoids that developed on *S. littoralis* larvae fed melon plants inoculated with the EAMa 01/58-Su strain. The development time was 12.71 days in the sprayed leaf treatment ($F_{1,44}$=6.42, p=0.0150) and 13.44 days in the unsprayed leaf treatment ($F_{1,39}$=13.61, p=0.0007) compared to the control group, which showed 11.71 days for this parameter (Table V.4). Likewise, in these treatments, a significant elongation of the preimaginal development time was also observed, showing 18.36 d for the control and 19.46 d and 20.38 d for those parasitoids developed on *S. littoralis* larvae fed sprayed ($F_{1,40}$=9.25, p=0.0042) and unsprayed leaves ($F_{1,35}$=16.47, p=0.0003), respectively (Table V.4). The average survival time (AST) of *H. didymator* F1 adults that developed on *S. littoralis* larvae fed on plants inoculated with EAMa 01/58-Su was 28.91 d (sprayed leaves) and 27.75 d (unsprayed leaves), which was significantly different from the control in specimens that developed on *S. littoralis* larvae that fed on sprayed leaves ($F_{1,40}$=9.39, p=0.0039) (Table V.4).

Table V.4. Sublethal effects on F1 *H. didymator* that developed on *S. littoralis* larvae after being fed on sprayed and unsprayed leaves of melon plants. Plants were inoculated with EAMa 01/58-Su, EABb 04/01-Tip and EABb 01/33-Su by leaves spraying.

Treatment	Pupal development time ± SE (d)[1]	Preimaginal stage ± SE (d)[1,2]	AST ± SE (d)[3]	Confidence Interval (95%)	
				Lower limit	Upper limit
Control	11.71 ± 0.23 a	18.36 ± 0.20 a	25.14 ± 0.46 b	24.24	26.04
Ma 01/58 Spray	12.71 ± 0.30 b	19.46 ± 0.30 b	28.92 ± 1.79 c	25.40	32.43
Ma 01/58 Unsprayed	13.44 ± 0.38 b	20.38 ± 0.44 b	27.75 ± 0.68 b	23.00	32.50
Bb 04/01 Spray	12.56 ± 0.29 b	19.08 ± 0.31 a	25.67 ± 1.03 b	23.66	27.68
Bb 04/01 Unsprayed	11.59 ± 0.23 a	18.26 ± 0.24 a	23.90 ± 0.31 a	23.30	24.49
Bb 01/33 Spray	12.00 ± 0.29 a	18.68 ± 0.35 a	24.82 ± 0.59 b	23.66	25.98
Bb 01/33 Unsprayed	11.83 ± 0.11 a	18.55 ± 0.29 a	24.64 ± 0.61 b	23.45	25.83

[1]Means ± SE within columns with the same letter are not significantly different from each other according to ANOVA followed by a Tukey's HSD test (p<0.05). [2]Preimaginal stage duration is counted from day of parasitization until the emergence of adults. [3]AST: Average Survival Time of F1 *H. didymator* adults, means ± SE within columns with the same letter are not significantly different from each other according to the log rank test (p<0.05). AST is limited at 42 days.

V.4. Discussion

The multifunctionality of endophytic entomopathogenic ascomycetes extends their possible use beyond pest control and paves the way for new tools and applications in IPM and crop production in protected crops (Quesada Moraga, 2020). Among them, an IPM strategy based upon the combined use of a natural enemy with endophytic entomopathogenic fungi either applied directly to target the pest or indirectly targeting the crop (Quesada-Moraga et al. 2022). While our previous work sheds light on the compatibility of the *H. didymator* – entomopathogenic fungus system when the fungal biocontrol agent targets *S. littoralis* larvae (Miranda-Fuentes et al. 2020, 2021b), the possible multitrophic impact of the fungus as an endophyte on parasitoids and even on crop growth under real pest control greenhouse scenarios remained unknown.

The strains EAMa 01/58-Su, EABb 04/01-Tip, and EABb 01/33-Su successfully colonized the melon plants, and their endophytic presence was identified through both microbiological techniques and qPCR. The highest amount of fungal DNA was recorded in plants that were inoculated via leaf spraying. Interestingly, the *B. bassiana* EABb 04/01-Tip strain was reisolated from 20% of F1 seeds, which supports previous work demonstrating the vertical transmission of this fungal strain (Quesada-Moraga et al. 2014). The first scenario designed in the present study reveals the compatibility of the parasitoid with the three fungal strains when they target pest larvae by colonizing the plant. Likewise, the effect of the endophytic EA strains, even if lower than when they were directly sprayed onto the pest larvae (Miranda-Fuentes et al. 2020), led to significant larval mortality and anomalous pupation that strengthened the combined effect of the parasitoid and the fungus. Plant factors related to the endophytic EA-induced systemic defense responses in melon upon priming through the leaves, seeds or roots could be the possible cause of the observed fungal-related mortality rates and sublethal developmental effects (García-Espinoza et al. 2023a), which is further supported by the lack of fungal outgrowth from the cadavers of *S. littoralis* larvae feeding on EA-colonized melon leaves (Miranda-Fuentes et al. 2021b).

The present research provides strong evidence of the multifunctionality of the selected EA strains, as indicated by their compatibility with the parasitoid for *S. littoralis* control while benefiting plant growth (Quesada-Moraga et al. 2020; Gupta et

al. 2022b; Posada-Vergara et al. 2022; García-Espinoza et al. 2023a). Likewise, significant differences in both the total and shoot and root fresh and dry weight of melon plants were observed for most of the fungal strain-combination method combinations. These findings agree with previous recent work showing the importance of EA as a promoter of plant growth (Raya-Díaz et al. 2017b; Sánchez-Rodríguez et al. 2018; Tall and Meyling 2018; Gonzalez-Guzman et al. 2021; Mantzoukas et al. 2022; Batool et al. 2022; Adedayo and Babalola 2023; García-Espinoza et al. 2023b) and highlight the expanding role of EA beyond its traditional function in insect pest control (Quesada Moraga 2020; Quesada-Moraga et al. 2022).

The second scenario explored in our work, with a higher ratio of parasitoids released and inoculation of the basal leaves of melon plants, was conducive to better testing the translocation of the EA strains in the plant and their possible effect on the reproductive potential of the parasitoid. The fact that larval mortality rates were similar when the larvae fed on sprayed and unsprayed leaves again suggests the existence of direct and indirect effects on the pest larvae related to the fungal colonization of the melon plant. Several studies have reported that the effects of endophytic EA on target pests can be attributed to the presence of fungal inoculum in plant tissues, as shown for *B. bassiana* (Jaber and Enkerli 2016; Jensen et al. 2020; Agbessenou et al. 2020; Silva et al. 2020; Gupta et al. 2022b; Torkaman et al. 2023), *M. brunneum* (Jaber and Enkerli 2016; Gupta et al. 2022b; Posada-Vergara et al. 2023), *Metarhizium robertsii* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae) (Liao et al. 2017; Ahmad et al. 2020, 2022) and *Lecanicillium lecanii* (Zimm.) Zare & W. Gams (Hypocreales: Clavicipitaceae) (Mejía and Espinel 2022), whereas as stated before, some EA can act by inducing systemic defense responses in plants even by priming them (Rondot and Reineke 2019; Ahmad et al. 2020; Gupta et al. 2022b; Posada-Vergara et al. 2022; Van Hee et al. 2023; García-Espinoza et al. 2023a). Interestingly, the second scenario also revealed the sublethal effects caused by the three EA strains on the noctuid larvae and pupae as previously reported by Resquín-Romero et al. (2016a), who found a weight reduction in *S. littoralis* larvae treated with *B. bassiana* EABb 01/33-Su and *M. brunneum* EAMb 09/01-Su strains at a concentration of $1x10^8$ conidia/ml. Likewise, Kalvnadi et al. (2018) reported a significant reduction in pupal weight in F1 *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) descendants treated with sublethal doses of *B. bassiana*. Even if there are several previous works

reporting the compatibility of parasitoids with insect pests directly exposed to EA as detailed in a revision conducted by Quesada-Moraga et al. (2022), information on parasitoid behavior when parasitized insect hosts feeding on EA-colonized plants is very scarce. Indeed, Jensen et al. (2020) and Oreste et al. (2016) suggested that the inoculation of plants with endophytic EA may indeed affect beneficial insects, making a key issue in evaluating this interaction before establishing any multitrophic system for IPM. In our work, the reproductive potential of the parasitoid *H. didymator* was not affected by any of the EA strain and application method combinations, demonstrating in all cases a parasitization capacity similar to that previously reported in laboratory settings (Miranda-Fuentes et al. 2021b). Moreover, although a slight extension in larval development time and pupation time was observed in parasitoids emerging from *S. littoralis* larvae that had fed on inoculated plants (through direct or indirect contact), the average survival time was not reduced.

The present research represents a significant step forward in the pursuit of sustainability in food production by fully integrating macrobials such as the parasitoid *H. didymator* with endophytic *M. brunneum* and *B. bassiana* within real greenhouse agriculture settings. Our research underscores the compatible use of the endophytic EAMa 01/58-Su *M. brunneum* strain and EABb 04/01-Tip and EABb 01/33-Su *B. bassiana* strains with the parasitoid *H. didymator* for a sustainable IPM strategy for controlling *S. littoralis* in greenhouse conditions that can additionally exploit their multifunctionality for melon crop production.

Author contributions

EQM, MYY and FGE conceptualized the experiments; FGE MCM, MJG and MYY performed assays and assessments; FGE, MYY and MJG analyzed the data; FGE and MYY prepared the original draft; writing, review and editing, FGE, EQM, MYY, MJG and MCM. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

The data of this study are owned by the authors and can be viewed upon request. For further information, please contact the corresponding author. Supplementary material is available in additional files in the web version of this work.

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Declarations

Conflict of interest. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary information

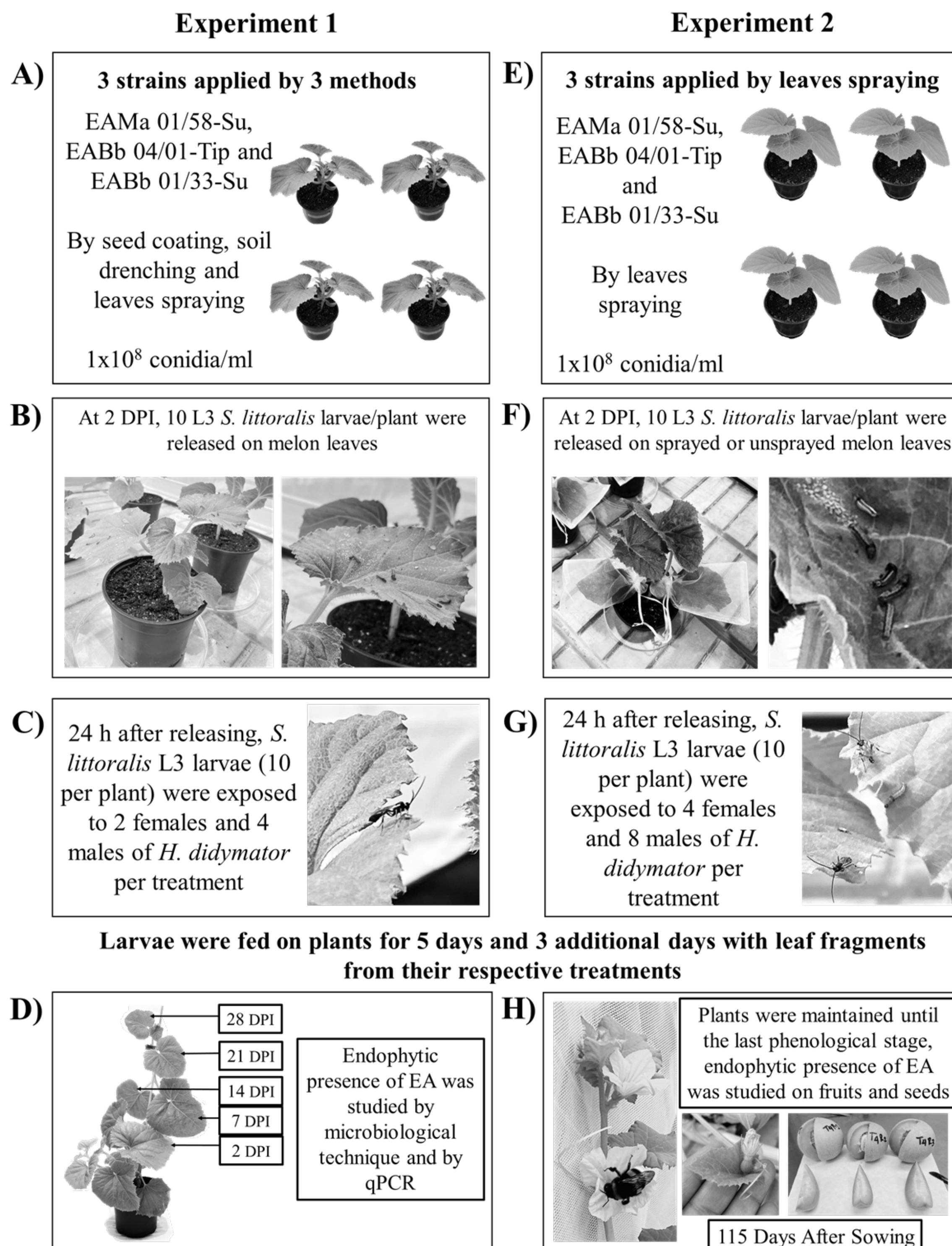

Figure V.S1. Schematic illustration of the experimental design.

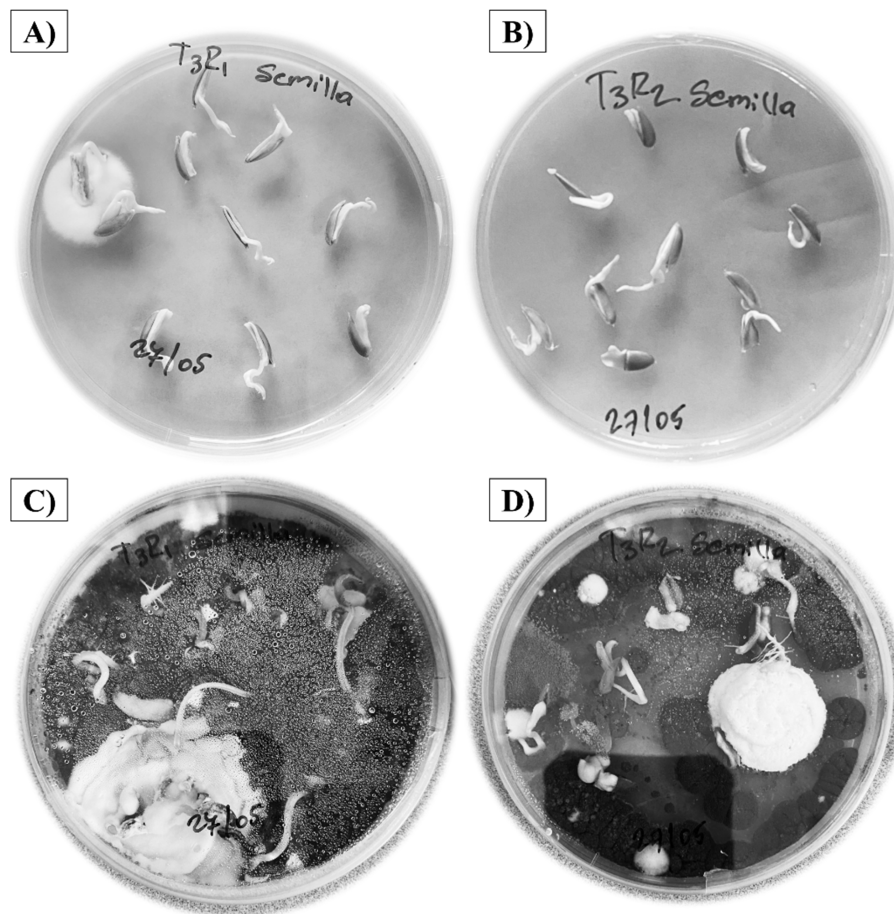

Figure V.S2. Endophytic presence of EABb 04/01-Tip *B. bassiana* isolate was detected by microbiological technique in melon seeds. A and B show fungal growth from seeds 10 days after plating, C and D show the same plates 30 days after.

CHAPTER VI. GENERAL DISCUSSION

The EA are microorganisms commonly present in ecosystems and that, over time, have gone from being saprophytes to endophytes and to occupying the role of pathogens of insect, forming part of the symbiont lifestyle of plants (Barelli et al. 2016). For this reason, EA can interact with crops and establish mutualistic relationships, that end up in multiple benefits to plants (Barelli et al. 2016; Gange et al. 2019; Quesada-Moraga et al. 2020; Quesada Moraga 2020). In addition to their well-known role as biological pest control agents, these benefits go further, for example, the promotion of growth and an improvement in the acquisition of nutrients, and the activation of the plant defense systems, the latter helping to improve tolerance to both biotic and abiotic stresses, especially in response to herbivore attack (Sánchez-Rodríguez et al. 2016, 2018; Raya-Díaz et al. 2017b; Kuzhuppillymyal-Prabhakarankutty et al. 2020; González-Guzmán et al. 2020; Kuzhuppillymyal et al. 2021; Gonzalez-Guzman et al. 2021; González-Guzmán et al. 2022). Also, it is of special interest to study EA as members of multitrophic systems, in which they are capable of mediating insect-plant relationships and where the potential compatibility they have with other pest control agents such as predators and parasitoids is evident, thus leading them to be key components in IPM programs (González-Mas et al. 2019a; Miranda-Fuentes et al. 2020, 2021; Quesada Moraga 2020; Quesada-Moraga et al. 2022).

In the chapters II and III, the molecular bases of the defense mechanisms that elicited when plants are challenged or primed by EA with different inoculation methods were explored, recording, for the first time that the three strains of EA can induce SR through both the JA and SA pathways in addition to activating genes related to PR proteins. In the same way, using qPCR, the endophytic progressive colonization of melon plants was monitored, even detecting traces of fungal DNA in F1 seeds. Chapter V of this Doctoral Thesis addresses in real greenhouse conditions the application of three strains of EA that have previously demonstrated their effectiveness to control key pests including *S. littoralis* at the laboratory level (Quesada-Moraga et al. 2006a; Yousef et al. 2018; Garrido-Jurado et al. 2019; González-Mas et al. 2019b; Miranda-Fuentes et al. 2020, 2021) to control *S. littoralis*. Also, their compatibility with the parasitoid *H. didymator* was studied.

On the other hand, the multifunctionality of EA was also confirmed, demonstrating their role as crop bodyguards capable not only of promoting plant

development but also conferring tolerance to herbivores, thus mitigating the damage that pests could cause to our crops. Likewise, EA are also capable of relieving abiotic stresses, in this case, stress due to a scarcity of nutrients, specifically Fe. In chapters III and IV of this Doctoral Thesis, the studied *M. brunneum* strain demonstrated, also by molecular techniques, that it can mediate the acquisition of Fe and inducing transcriptional responses to the deficiency of this important nutrient. Interestingly, it was also demonstrated that there is a crosstalk between the presence of biotic stress and the induction of both ISR and SAR, with phytohormones such as ET, JA and SA being involved in this process.

The Chapter II was dedicated to unveiling novel insights into the interactions between EA and melon plants, revealing an EA-mediated antibiosis and tolerance response. Melon plants inoculated with EA exhibited positive responses to short- and long-term scenarios of *S. littoralis* infestation, displaying increased fresh and dry weights while inducing sublethal effects on the *S. littoralis* larvae (larval development time and pupal weight). In this chapter, the molecular quantification revealed a high relative expression of ET and JA-related genes, suggesting a complex regulatory network. Interestingly, despite minimal traces of EA detected in plant tissues, the relative expression increase of studied genes was mainly attributed to priming. In addition to the traditional downregulation of defense genes with endophytic establishment, this study showed an undulating expression profile, demonstrating the ability of EA strains to induce defense responses. Consequently, this phenomenon produces an EA-induced tolerance, trough the expression of defense traits, particularly induced by JA and ET signaling, benefiting the plant not only a quick recovery after an attack by herbivores but also improving its growth rate. Although the potential uses of EA beyond their traditional role of controlling insect pests, have been noted previously, the mechanisms underlying the plant antibiosis and tolerance to herbivore attack and its response to priming by EA as mediated by fungal strain, application method and the presence of insect have been subjected to scarce studies. This Chapter contributes significantly to understanding the mechanisms behind plant antibiosis and tolerance to herbivore attacks as mediated by EA. It highlights the potential of EAMa 01/58-Su, EABb 04/01-Tip, and EABb 01/33-Su strains in priming plants for future attacks, showcasing their role beyond pest control in promoting a “state of readiness” in plants. This research advances our knowledge of the tritrophic

relationship, emphasizing once more time the multifaceted effects of EA on plants and their intricate responses to herbivore pressure.

The Chapter III explores the phenomenon of priming as a crucial aspect for developing effective control methods by conferring resistance against a broad spectrum of harmful agents. Priming, often associated with epigenetic changes, can lead to transgenerational effects, as demonstrated in various works with natural compounds, microorganisms like *P. syringae*, and herbivore attacks (Luna et al. 2012; Pieterse 2012; Rasmann et al. 2012). Evolutionary relatives of *Metarhizium*, such as *T. atroviride*, have shown the ability to transmit priming and plant growth promotion effects to the next generation (De Medeiros et al. 2017; Woo et al. 2023). In this way, Chapter IV, focuses on the EAMa 01/58-Su *M. brunneum* strain, demonstrating its capacity to induce both SA and JA/ET-dependent priming in cucumber and melon pants. This priming results in lethal and sublethal effects on *S. littoralis* larvae fed on primed plants. The undulating defense response against abiotic stresses is highlighted, indicating its efficacy even under stress conditions. Gene expression analysis reveals a crosstalk ISR, SAR, and the nutritional status of plants. The increase in the relative expression levels of SA, JA, ET, and pathogenesis-related proteins were influenced by both nutritional conditions and priming. Being this in concordance with some previous works, which contribute to the understanding of EA as ISR-SAR inducers (Ahmad et al. 2020, 2022; Gupta et al. 2022b; Posada-Vergara et al. 2022, 2023). Previous studies on EA, including *B. bassiana* and *M. robertsii*, have shown increased expression of genes related to ET, JA, and SA pathways in various plants (Ahmad et al. 2020, 2022; Jensen et al. 2020; Batool et al. 2022; Gupta et al. 2022b; Posada-Vergara et al. 2022, 2023; Iida et al. 2023). However, the impact of such induction on insect survival and fitness remained unknown. In this chapter. the lethal and sublethal effects on *S. littoralis* larvae are attributed directly to *M. brunneum* priming, highlighting the potential of EA in IPM strategies. The study advances our knowledge of EA functions, particularly *M. brunneum*, as an ISR-SAR inducer with implications for innovative IPM strategies, providing a comprehensive understanding of the crosstalk between plant defenses, nutritional status, and resistance induction.

Delving deeper, in the Chapter IV of this Doctoral Thesis, the mechanisms through which *M. brunneum* facilitates the solubilization and acquisition of iron by plants were studied. Previous studies from AGR-163 Agricultural Entomology

research group have recognized the potential of EAMa 01/58-Su *M. brunneum* strain as plant endophytes and growth promoters, offering additional benefits to their primary function in pest management. The Chapter V delves into the less-explored aspect of Fe acquisition mechanisms employed by EA in plants. While EAMa 01/58-Su strain is known to alleviate Fe chlorosis symptoms, little is known about the direct and indirect mechanisms involved. The in vitro study revealed the ability of *M. brunneum* EAMa 01/58-Su isolate to demineralize Fe and produce Fe siderophores, contributing to increased Fe availability. Notably, *M. brunneum* exhibits superior siderophore exudation compared to *B. bassiana* isolates. Strategy I plants (non-grass plants), involves reducing Fe^{3+} to Fe^{2+} before absorption, mediated by a ferric reductase. This work reveals, for the first time, the role of an EA as an elicitor of Fe deficiency responses in Strategy I plants, specifically inducing FRA and the expression of Fe acquisition genes. This novel finding contrasts with existing references, which have focused on other microorganisms inducing Fe deficiency responses, including bacteria, fungi, and mycorrhizae (Romera et al. 2019; Sánchez-Montesinos et al. 2020; Aparicio et al. 2023). This Chapter expands our understanding of EA, showcasing *M. brunneum* ability to induce Fe deficiency responses in cucurbit plants. This newfound skill adds value to its role as a biological control agent, emphasizing the intricate direct and indirect mechanisms involved in Fe acquisition mediated by EA.

Finally, into the research conducted for this Doctoral Thesis, Chapter V examines the compatibility of three EA strains, namely, EAMa 01/58-Su EABb 04/01-Tip, and EABb 01/33-Su), applied by seed coating, soil drenching and leaves spraying, and the use of the parasitoid *H. didymator* to control the cotton leafworm in greenhouse scenarios. Also, this part of the thesis explores the multifunctionality of EA and their potential applications in IPM and protected crop production. It was observed that strains successfully colonize melon plants, with the highest fungal DNA levels in plants inoculated via leaf spraying. Notably, EABb 04/01-Tip *B. bassiana* strain shows vertical transmission through F1 seeds, supporting previous findings (Quesada-Moraga et al. 2014). A first scenario reveals the compatibility of *H. didymator* with the three fungal strains targeting *S. littoralis* larvae, leading to a significant larval mortality and sublethal effects such as the decrease of pest fitness and elongation of the immature development time. This Chapter provides evidence of the multifunctionality of selected strains, emphasizing compatibility with the

parasitoid for pest control while promoting plant growth, recording significant differences in inoculated melon plant weight respect to control. These findings support the role of EA as plant growth promoters, expanding beyond insect pest control (Barelli et al. 2016; Dara 2019b; Quesada Moraga 2020). A second scenario, designed to test fungal translocation and its effects on parasitoid reproduction, indicates similar larval mortality rates and sublethal effects on the noctuid larvae and pupae for sprayed and unsprayed leaves, reinforcing the importance of evaluating interactions before establishing multitrophic systems for IPM. This Chapter represents a significant step towards sustainability by integrating parasitoids with EA in real greenhouse conditions. In all cases, the parasitoid *H. didymator* showed a similar parasitization capacity as previously reported in laboratory settings (Miranda-Fuentes et al. 2020; Miranda-Fuentes et al. 2021), and its reproductive potential remained unaffected by any combination of EA strain and application method. Furthermore, although a slight increase in larval development time and pupation time was observed in parasitoids emerging from *S. littoralis* larvae feeding on inoculated plants (through direct or indirect contact), average survival time was not reduced.

The results presented in this Doctoral Thesis demonstrate and reinforce the multifunctionality of EA. In addition to proving once again their excellent biocontrol potential, the indirect mechanisms through which they function as crop bodyguards are also revealed, either to confer tolerance to biotic and abiotic stresses or to induce SR and promote plant development. The results reinforce the use of EA as a sustainable alternative to reduce synthetic pesticides application. Going even further, they are in the front line to overcome the challenges faced by today agriculture. Undoubtedly, a cornerstone for sustainable food production programs.

CHAPTER VII. CONCLUSIONS

The objectives of this Doctoral Thesis were addressed through a series of experiments, resulting in four scientific articles. The first chapter, currently under review for publication, "Guardians within: Entomopathogenic ascomycete-driven antibiosis and compensatory growth combine to protect melon plants from herbivore damage" (Chapter II), produced conclusions number **1**, **2** and **3**. Conclusion number **4** derived from the article "Entomopathogenic fungus-related priming defense mechanisms in cucurbits impact *Spodoptera littoralis* (Boisduval) fitness" (Chapter III), published in the journal ***Applied and Environmental Microbiology***. The article titled "Entomopathogenic fungi-mediated solubilization and induction of Fe related genes in melon and cucumber plants" published in the ***Journal of Fungi***, which constitutes the Chapter V of this Doctoral Thesis led to the conclusion number **5**. Finally, the study conducted under greenhouse conditions, titled "Greenhouse melon crop protection and production through the compatible use of a parasitoid with endophytic entomopathogenic ascomycetes" (Chapter V), published in ***Journal of Pest Science***, led to conclusions number **1**, **2**, and **6**.

1. Endophytic colonization by the selected entomopathogenic ascomycete *M. brunneum* and *B. bassiana* strains by leaf spraying, soil drenching and seed coating is demonstrated in all experiments, and it is even systemic and progressive throughout the entire melon phenology, with *B. bassiana* detected in the seeds of F1 generation.
2. The application of the selected entomopathogenic ascomycete *M. brunneum* and *B. bassiana* strains by three inoculation methods induces antibiosis and tolerance against *S. littoralis* while promoting melon plant growth in controlled conditions, underscoring the multifunctionality of these fungi and their novel function as plant bodyguards.
3. This bodyguard function was related to the fungal-related up-regulation in the relative expression of ethylene (*ACO1*, *ACO3*, *EIN2*, *EIN3*) and jasmonic acid (*LOX2*)-related genes specially in *S. littoralis* infested plants.
4. In controlled conditions, *M. brunneum* EAMa 01/58-Su strain triggers both SA- and JA/ET-dependent priming in cucumber and melon plants, as an ISR-SAR inducer, resulting in a significant decrease in *S. littoralis* fitness. Interestingly, this response was a fungal *priming*, not necessarily linked to plant tissue colonization.

5. The *M. brunneum* 01/58-Su strain is a model of the multifunctionality of an entomopathogenic fungus as confirmed by the crosstalk between its SR induction potential and the relative expression of iron acquisition genes in cucurbit species.
6. The selected entomopathogenic ascomycete *M. brunneum* and *B. bassiana* strains are compatible with the parasitoid *H. didymator* for *S. littoralis* control under greenhouse conditions, with the parasitoid release ratio to be decided based upon economic criteria.

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ADDITIONAL PUBLICATIONS AND CONTRIBUTIONS

Conference proceedings and other contributions

García-Espinoza, F., Yousef-Yousef, M., Garrido-Jurado, I., and Quesada-Moraga, E. (2021). Multifunctionality of endophytic entomopathogenic fungi: plant growth promotion and *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) control in melon. ***2021 International Congress on Invertebrate Pathology and Microbial Control & 53rd Annual Meeting of the Society for Invertebrate Pathology***, (p. 107).

García-Espinoza, F., García, M. J., Quesada-Moraga, E., and Yousef-Yousef, M. (2022). Expresión de genes de resistencia en *Cucumis melo* L. y *Cucumis sativus* L. (Cucurbitales: Cucurbitaceae) inoculadas con *Metarhizium bruneum* Petch (Hypocreales: Clavicipitaceae). In Sociedad Española de Entomología Aplicada (Ed.), ***XII Congreso Nacional de Entomología Aplicada*** (p. 136). Sociedad Española de Entomología Aplicada.

García-Espinoza, F., Quesada-Moraga, E., and Yousef-Yousef, M. (2022). Hongos entomopatógenos endófitos para el control de *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) en un sistema multitrófico en condiciones de invernadero. In Sociedad Española de Entomología Aplicada (Ed.), ***XII Congreso Nacional de Entomología Aplicada*** (p. 123). Sociedad Española de Entomología Aplicada.

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García-Espinoza, F., García del Rosal, M. J., Quesada-Moraga, E., and Yousef-Yousef, M. (2023). Contribución directa e indirecta del hongo entomopatógeno *Metarhizium brunneum* (Pecth) en la solubilización y adquisición del hierro en plantas de melón y pepino. ***Horticultura*** (june), 50–59. Available from: https://goo.su/sA7WlY

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Este hallazgo supone un importante avance en el conocimiento de nuevas aptitudes de estos hongos y su función como promotores de crecimiento de las plantas

CONTRIBUCIÓN DIRECTA E INDIRECTA DEL HONGO ENTOMOPATÓGENO *METARHIZIUM BRUNNEUM* (PECTH) EN LA SOLUBILIZACIÓN Y ADQUISICIÓN DEL HIERRO EN PLANTAS DE MELÓN Y PEPINO

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Los hongos entomopatógenos (HE) tienen un estilo de vida multifuncional; además de su función bien conocida como agentes de biocontrol, también pueden ayudar a las plantas a responder a otros estreses bióticos y abióticos, como la deficiencia de hierro (Fe). Este estudio explora la capacidad de la cepa EAMa 01/58-Su del HE *Metarhizium brunneu*m para inducir respuestas de adquisición de *Fe in vitro* e *in vivo*. La cepa EAMa 01/58-Su mostró una gran capacidad para exudar sideróforos de hierro (58.4% de exudación en medio CAS agar) y proporcionó un mayor contenido de Fe en ma-teria seca y en sustrato en comparación con el control. Se observó, una regulación positiva (24, 48 o 72 h post-inoculación) de la actividad de la reductasa férrica así como de la expresión de los genes de adquisición de Fe FRO1, FRO2, IRT1, HA1 y FIT, revelando así el potencial de la cepa EAMa 01/58-Su del HE *M. brunneum* para inducir los mecanismos implicados en la adquisición de Fe.

INTRODUCCIÓN

Los hongos entomopatógenos (HE) se encuentran entre los agentes de control micro-biano de plagas más importantes que se han desarrollado comercialmente, aunque algunas especies de ascomicetos entomopatógenos, presentan estilos de vida multifuncionales y pueden interactuar con las plantas cultivadas como endófitos o como competentes en la rizosfera, interacciones mu-tualistas que benefician a la planta hospedante tales como la promoción de su crecimiento, e incluso mejor respuesta a otros estreses de tipo biótico y abiótico (Hu & Bidochka, 2019; Nosheen et al., 2021; Quesada-Moraga, 2020).

El hierro (Fe) es un micronutriente esencial para una serie de procesos enzimáticos importan-tes en la mayoría de los organismos y en la mayoría de los entornos y su deficiencia se considera una de las principales limitaciones de la productividad de los cultivos en todo el mundo (Romera et al., 2019). La deficiencia de Fe no se desencadena por bajas concentraciones totales de Fe, sino por una baja biodisponibilidad de este en el suelo (Kraemer, 2004). Para superar estas limitaciones, se sabe que algunas bacterias, hongos y gramíneas secuestran Fe utilizando sideróforos (Krasnoff et al., 2014).

En condiciones de deficiencia de Fe, las plantas desarrollan respuestas morfológicas y fisio-lógicas, principalmente en sus raíces, destinadas a facilitar su adquisición (García et al., 2015, 2021; Gattullo et al., 2018; Romera et al., 2019). En plantas de Estrategia I, las principales respuestas fisiológicas son: aumento de la actividad de la reductasa férrica, incremento del transporte de Fe2+, acidificación de la rizosfera y aumento de la síntesis y/o liberación de ácidos orgánicos, compuestos fenólicos, como cumarinas y flavinas, que pueden actuar como agentes quelantes y reductores de Fe, mejorando su solubilidad para las plantas (Rodríguez-Celma & Schmidt, 2013; Sisó-Terraza et al., 2016; Tsai & Schmidt, 2017). En la regulación de las respuestas de deficiencia de Fe se han implicado hormonas y sustancias reguladoras como el etileno (ET) y el óxido nítrico (NO), que actúan como reguladores positivos (García et al., 2010, 2011).

Nuestros trabajos recientes han demostrado el papel de varias especies de HE en la adquisición de nutrientes por parte de las plantas, con un mayor crecimiento y productividad de plantas de sorgo, trigo, girasol y tomate inoculadas con cepas de los HE *Metarhizium brunneum* Petch., *Beauveria bassiana* (Balsamo) Vuil. e *Isaria farinosa* (Holmsk.) Fr. (Raya-Díaz et al., 2017; Sán-chez-Rodríguez et al., 2018). En el caso de M. brunneum, se observó un aumento de la disponibilidad de Fe en suelos calcáreos y un

alivio la clorosis férrica en plantas de sorgo, trigo y girasol (Raya-Díaz et al., 2017; Sánchez-Rodríguez et al., 2016). Sin embargo, no hay estudios sobre los mecanismos por los que los HE mejoran la adquisición de Fe por las plantas. En este trabajo, se han estudiado los mecanismos directos e indirectos que utilizan los HE para aliviar la clorosis férrica en plantas de pepino y melón.

MATERIAL Y MÉTODOS

Cepas fúngicas y preparación del inóculo. En los ensayos se utilizaron dos cepas de *B. bassiana* (EABb 04/01-Tip y EABb 01/33-Su) y una cepa de *M. brunneum* (EAMa 01/58-Su) de la colección de cultivos de la Unidad de Entomología Agrícola del Departamento de Agronomía de la Universidad de Córdoba (España) con Códigos de Acceso a la Colección Española de Cultivos Tipo 20744, 21149 y 20764, respectivamente. La colonización endofítica transitoria y temporal de plantas de melón se ha demostrado previamente mediante la aplicación foliar de estas cepas (Garrido-Jurado et al., 2017; Resquín-Romero et al., 2016). Para proporcionar inóculo para los experimentos, las tres cepas se sub-cultivaron a partir de cultivos almacenados en patata dextrosa agar (PDA) en placas de Petri y se cultivaron durante 15 días a 25 °C en la oscuridad.

Estudio in vitro de la biodisponibilidad de Fe mediante la producción de sideróforos. El estudio *in vitro* se realizó para estudiar las capacidades de las distintas cepas de hongos para desmineralizar Fe. Antes del ensayo, las cepas se cultivaron en medio de agar dextrosa de patata (PDA) para obtener micelio de cuatro días de edad. Este ensayo se repitió dos veces con cuatro réplicas biológicas por cepa. Seguimos un método simplificado (Srimathi & Suji, 2018) del ensayo químico universal para la detección de sideróforos (Schwyn & Neilands, 1987). Discos 6 mm de diámetro de micelio de cada cepa se cortaron de colonias en crecimiento activo (4 d) y se colocaron en el centro de placas de Petri (9 cm) que contenían medio de agar Cromo Sulfonato de Azurol (CAS). Las placas se incubaron a 26 (±2) °C en la oscuridad durante 10 días (Barra-Bucarei et al., 2020). Diariamente, a partir de los 3 y hasta 10 días después de la inoculación (dpi), se midió tanto el diámetro de la colonia y el área de halo amarillo/naranja que la rodeaba, el tamaño del área de color naranja era indicativo de la cantidad de sideróforos producidos (Andrews et al., 2016; Barra-Bucarei et al., 2020).

Estudios de biodisponibilidad de Fe en plantas y suelo. Para evaluar la adquisición de Fe en plantas, se utilizó un diseño completamente aleatorizado con 3 tratamientos (3 cepas aplicadas por empapado del suelo), y su respectivo control, con 6 réplicas (plantas) por tratamiento. Semilla certificada de melón (*Cucumis melo* L. cv. Galia), con una desinfección superficial (Garrido-Jurado et al., 2017), fue sembrada en sustrato en macetas de 500 ml, tanto las macetas como el sustrato fueron debidamente esterilizados.

La preparación del inóculo se llevó a cabo raspando los conidios de las placas de Petri en una solución estéril de 0,1% Tween 80, seguido de sonicación durante 5 minutos para homogeneizar el inóculo y filtración a través de gasa para eliminar el micelio. Con ayuda de una cámara de Malassezse estimó la concentración

Plantas de pepino.

de conidios que finalmente se ajustó a 1x10^8 conidios/ml mediante la adición de una solución estéril de agua destilada con 0,1% Tween 80. El empapado del suelo se llevó a cabo cuando las plantas de melón alcanzaron la etapa de cuatro hojas verdaderas, 5 ml de la suspensión se vertieron con una pipeta sobre la superficie de la maceta. Las plantas control fueron tratadas de manera similar con una solución estéril de 0,1% Tween 80. Luego, a 50 dpi, se realizó un análisis elemental en materia seca y sustrato. Para ello, el sustrato y el material vegetal, incluidas las partes aéreas y las raíces, se secaron en un horno a 60 °C durante 96 h y se pesaron.

El contenido de Fe en materia seca y sustrato se evaluó utilizando la técnica modificada de "Fósforo Olsen" (Olsen et al., 1954). El contenido de Fe se determinó con un espectrofotómetro de absorción atómica (Perkin-Elmer Analyst 200).

Actividad de la reductasa férrica y expresión génica de adquisición de Fe

Condiciones de crecimiento y material vegetal. Para estudiar la actividad de la reductasa férrica y la expresión relativa de los genes de adquisición de Fe se utilizaron dos especies de cucurbitáceas (*C. melo* L. var. Futuro y *Cucumis sativus* L. var Ashley, Semillas Fitó, S.A., Barcelona, España). Las plantas se mantuvieron en un sistema hidropónico, en condiciones controladas en una cámara de crecimiento a temperaturas diurnas de 22 °C / 20 °C nocturnas, con humedad relativa entre 50 y 70%, y un fotoperíodo de 14 h a una irradiancia fotosintética de 300 µmol m^{-2} s^{-1} proporcionado por luz fluorescente blanca (10.000 lux) (Lucena et al., 2006). Para el mantenimiento del sistema hidropónico se utilizó la solución nutritiva Romheld & Marschner (R&M) (Römheld & Marschner, 1981). Después de 10 días (en el caso del pepino) y 13 días (en el caso del melón) de crecimiento, las plantas se separaron en cuatro grupos que posteriormente constituyeron los 4 tratamientos, como se describe a continuación.

Preparación del inóculo e inoculación por inmersión. La cepa EAMa 01/58-Su de *M. brunneum* fue elegida para esta parte del estudio debido a las propiedades previamente demostradas para solubilizar Fe. El inóculo se preparó como se describió anteriormente y se ajustó a 1x10^7 conidios/ml mediante la adición de solución estéril de agua destilada con 0.1% Tween 80.

Las plantas con dos hojas verdaderas fueron seleccionadas y colocadas en bandejas con 2,5 l de solución de inóculo. Las plantas control (no inoculadas) se colocaron en bandejas con 2,5 l de Tween 80% al 0.1%. Todas las plantas se mantuvieron en agitación continua durante 30 min. Después de eso, las plantas inoculadas y no inoculadas se transfirieron a dos condiciones nutricionales diferentes, Fe suficiente (+ Fe40µM) y deficiente (-Fe) para que finalmente se usaran cuatro tratamientos con 42 plantas: Control + Fe40µM (no inoculado), Inoculado + Fe40µM, Control - Fe (no inoculado), Inoculado - Fe. Cada ensayo con ambas especies se repitió dos veces.

Determinación de la actividad de la reductasa férrica (FRA). La FRA se determinó tal y como se describe en García et al., (2022) por el método colorimétrico de la Ferrozina. Tras medir la absorbancia a 562nm, las raíces se cortaron y pesaron. Los resultados se expresaron en relación con el peso fresco de raíz. Además, se tomaron los valores de SPAD diariamente (como un proxy de la concentración de clorofila en la hoja) (SPAD 502 Minolta Camera Co., Osaka, Japón).

Aislamiento de ARN, síntesis de ADNc y análisis de qRT-PCR. El análisis de PCR en tiempo real se llevó a cabo tal y como describe García et al., (2021). Las raíces y las hojas verdaderas se homogenizaron con nitrógeno líquido en un mortero hasta obtener un polvo fino. Para el aislamiento de ARN se utilizó Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH, EUA) y para la síntesis de ADNc se partió de 3µg de ARN total y se usó el kit iScriptTM cDNA Synthesis (Bio-Rad laboratories, Inc, Hercules, CA, EUA) siguiendo la metodología propuesta por García et al., (2011, 2013).

El estudio de la expresión génica mediante qRT-PCR se realizó en un termociclador Bio-Rad CFX, utilizando para ello la máster mix SYBR Green de Bio-Rad siguiendo las instrucciones del fabricante [19,58]. Cada reacción se realizó en un volumen final de 25 µL de los que 2 µL eran de ADNc. Se estudió la expresión relativa de los genes *FRO1*, *IRT1* y *HA1* (Aparicio et al., 2023) en pepino, mientras que, en melón, se estudiaron los genes *FRO1*, *FRO2*, *FRO3*, *FRO4*, *IRT1* y *FIT* (Aparicio et al., 2023; Waters et al., 2014). Los niveles de expresión relativa se calcularon a partir de los valores de los ciclos umbral (Ct) y la eficiencia de los primeros por el método Pfaffl (Pfaffl, 2001). Cada análisis de PCR se realizó en tres réplicas biológicas y cada reacción de PCR se repitió dos veces.

Análisis de datos. Los datos de producción de sideróforos de hierro, el contenido total y relativo de Fe en materia seca y sustrato y los datos de FRA se analizaron mediante análisis de varianza (ANOVA) seguido de una prueba de rango múltiple de Tukey, diferentes letras sobre las barras indican diferencias significativas ($p < 0.05$) entre los tratamientos (Statistix 9.0®, Analytical Software, Tallahassee, FL, EUA). Los resultados de las expresiones génicas relativas se analizaron mediante análisis unidireccional de varianza (ANOVA) seguido de una prueba de Dunnett, * ($p < 0.05$), ** ($p < 0.01$) o *** ($p < 0.001$) sobre las barras indican diferencias significativas en relación con el tratamiento de con-

trol (GraphPad Prism 9.4.0, GraphPad Software, LLC, 2365 Northside Dr., Suite 560, San Diego, CA 92108 USA). Los datos de expresión génica representan la media de tres réplicas técnicas independientes.

RESULTADOS

Exudación de sideróforos. Los resultados demostraron diferencias significativas entre las tres cepas en la producción de sideróforos a los 10 dpi (F2,21 = 117.73, p = 0.001); la cepa EAMa 01/58-Su de *M. brunneum* demostró mayor capacidad de cambiar de color el área del medio CAS agar de azul a naranja (58.4%), mientras que las cepas EABb 04/01-Tip y EABb 01/33-Su de *B. bassiana* cambiaron el color de solo 24.35% y 17.88%, respectivamente (Figura 1A). En la Figura 1B se observa la evolución de la exudación de sideróforos de Fe, misma que revela diferencia entre la cepa de *M. brunneum* y los otros a desde los 3 dpi.

Contenido total de materia seca y Fe en materia seca y sustrato. Se observaron diferencias significativas en la materia seca cuando se compararon los tratamientos con EAMa 01/58-Su ($F_{1,8}$ = 10.63, p = 0.0115), EABb 04/01-Tip ($F_{1,8}$ = 5.88, p = 0.0416) y EABb 01/33-Su ($F_{1,8}$ = 6.78, p = 0.0314) vs. control, sin embargo, podemos ver que las plantas inoculadas con EAMa 01/58-Su produjeron el mayor contenido de materia seca (Figura 2A). Por otro lado, no se observaron diferencias significativas en el contenido de Fe en materia seca cuando comparamos cada tratamiento vs. control [($F_{1,8}$ = 2.68, p = 0.1400), ($F_{1,8}$ = 2.08, p = 0.1870), ($F_{1,8}$ = 3.0, p = 0.1213), para EAMa 01/58-Su, EABb 04/01-Tip y EABb 01/33-Su, respectivamente] (Figura 2B). En el caso del contenido relativo de Fe en el sustrato, solo el tratamiento con EAMa 01/58-Su vs. control presentó diferencia significativa ($F_{1,6}$ = 7.77, p = 0.0317) (Figura 2C).

Actividad de la reductasa férrica y genes responsables de la reducción y el transporte de hierro. En general, FRA presentó valores más altos en plantas de pepino y melón cultivadas en condiciones deficientes de Fe. En plantas de pepino, la actividad reductasa fue mayor en plantas deficientes en Fe inoculadas con la cepa EAMa 01/58-Su de *M. brunneum* en comparación con sus respectivos controles durante los siete días del estudio (Figura 3A). Sin embargo, se detectaron diferencias significativas entre plantas de pepino deficientes en Fe inoculadas y no inoculadas a 4, 5 y 7 dpi [($F_{3,22}$ = 13.68, p = 0.001), ($F_{3,20}$ = 35.3, p = 0.001) y ($F_{3,19}$ = 74.68, p = 0.001), respectivamente] (Figura 3A). En el caso del melón, se encontraron diferencias significativas entre las plantas deficientes en Fe inoculadas a 3 dpi en relación con las plantas deficientes en Fe no inocu-ladas ($F_{3,20}$ = 61.23, p = 0.001) (Figura 4A).

Los niveles de expresión relativa de los genes de adquisición de Fe, *FRO1, IRT1, FIT* y *HA1* en pepino se representan en la figura 3B–E. Los genes de adquisición de Fe experimentaron un au-mento de sus niveles de expresión tras la inoculación con la cepa EAMa 01/58-Su de *M. brunneum* en ambas condiciones, Fe suficiente y deficiente, en comparación con sus respectivos controles no inoculados en diferentes momentos (Figura 3B–E). Aunque en condiciones de Fe suficiente se observó

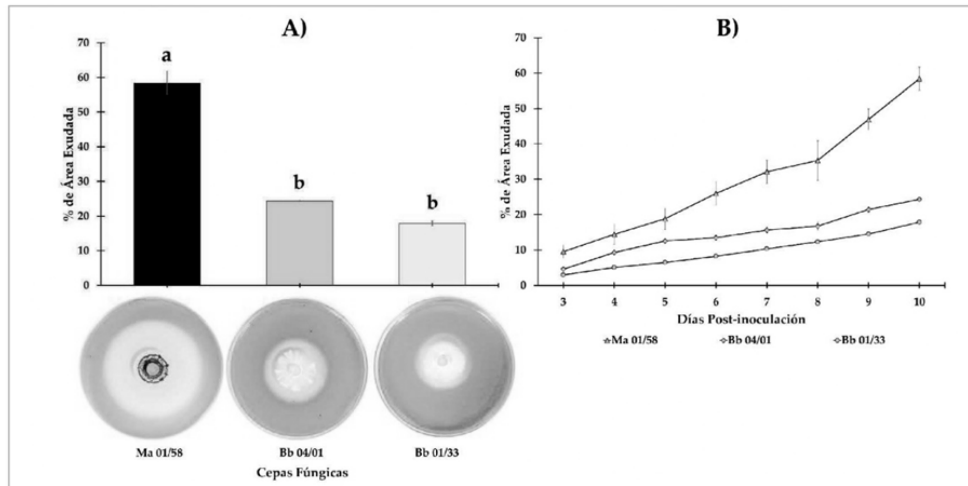

Figura 1. A) Comparación a los 10 días después de la inoculación (dpi) de la exudación de sideróforos por tres cepas de hongos entomopatógenos en medio de CAS agar con FeCl3 como fuente de FeIII. En la parte inferior, se muestra la parte frontal de las placas. B) Progreso (%) del cambio de color debido a la producción de sideróforos por tres cepas de HE en medio de agar CAS.

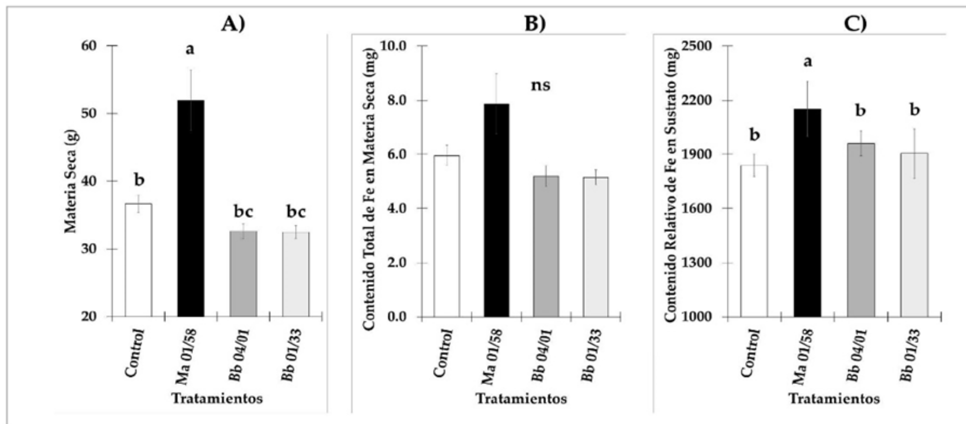

Figura 2. Media (±SE) del peso de la materia seca del tallo y las hojas (A), contenido total de Fe en materia seca (B), contenido relativo de Fe soluble en sustrato (C) medido a 50 dpi en plantas de melón inoculadas por tratamiento al suelo.

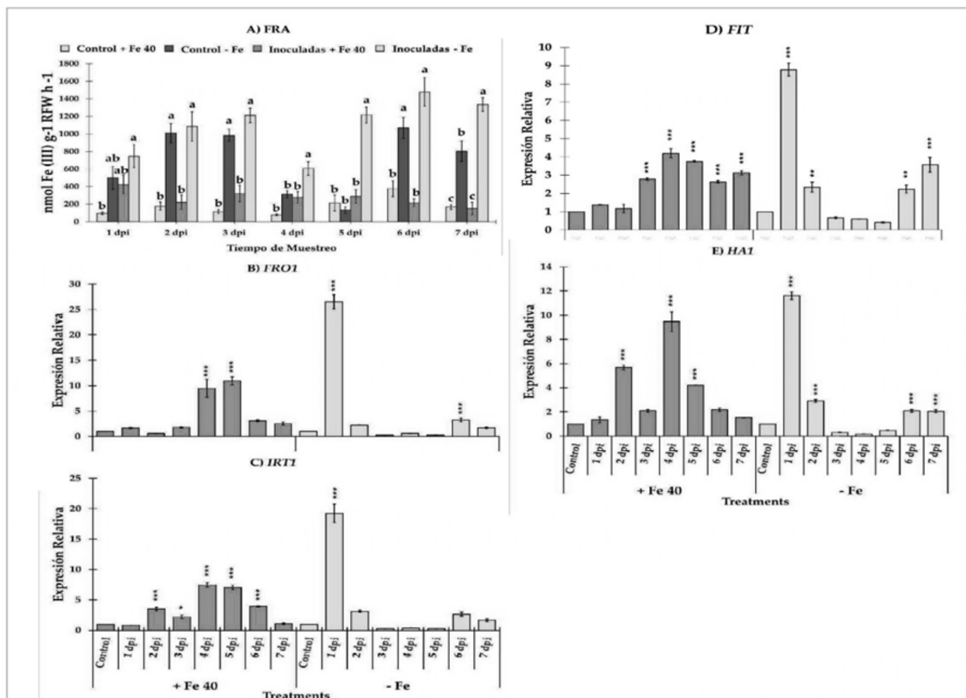

Figura 3. Evolución de FRA a lo largo de siete días de observación y expresión relativa de FRO1, IRT1, FIT y HA1 en raíces de pepino. Se utilizaron cuatro tratamientos, a saber, (i) Control + Fe40µM (no inoculado), (ii) Inoculado + Fe40µM, (iii) Control - Fe (no inoculado) y (iv) Inoculado - Fe. Los datos de expresión génica representan la media de tres réplicas técnicas independientes, según la prueba de Dunnett, * ($p < 0.05$), ** ($p < 0.01$) o *** ($p < 0.001$) sobre las barras indican diferencias significativas en relación con el tratamiento control. En el caso de FRA, la letra sobre las barras denota diferencia significativa entre las plantas inoculadas y las plantas control analizadas por ANOVA completamente aleatorizado seguido de una prueba de Tukey ($p < 0.05$).

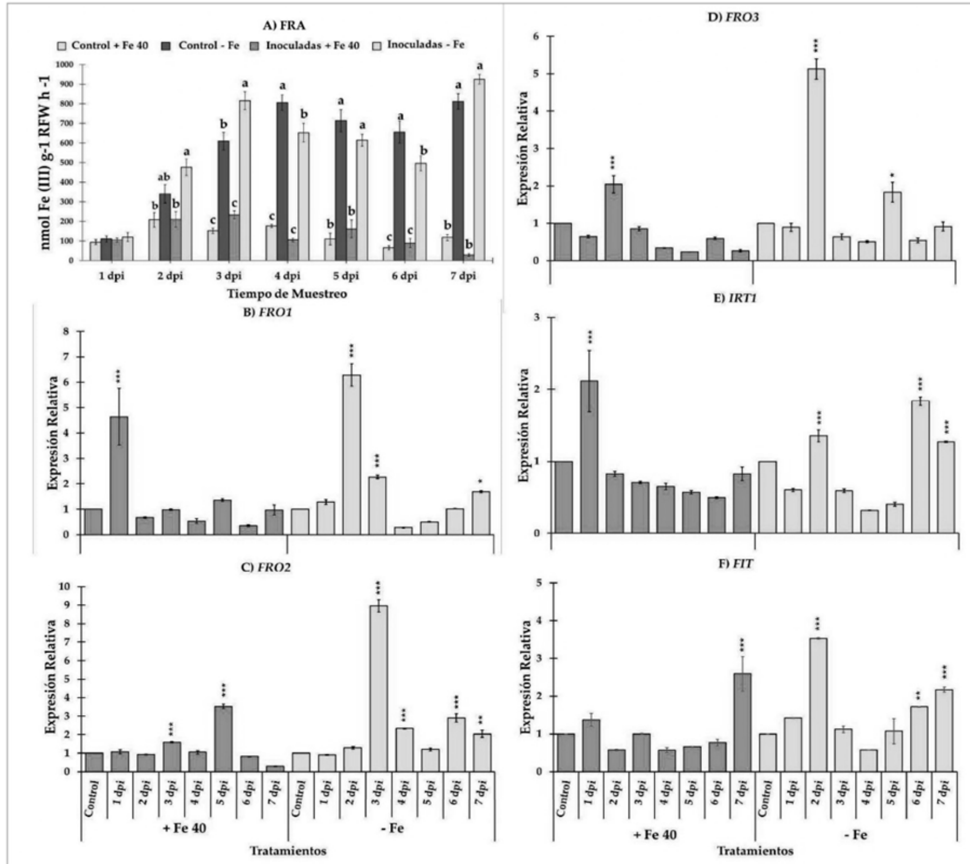

Figura 4. Evolución de FRA a lo largo de siete días de observación y expresión relativa de FRO1, FRO2, FRO3, IRT1 y FIT, en raíces de melón. Se utilizaron cuatro tratamientos, a saber, (i) Control + Fe40μM (no inoculado), (ii) Inoculado + Fe40μM, (iii) Control - Fe (no inoculado) y (iv) Inoculado - Fe. Los datos de expresión génica representan la media de tres réplicas técnicas independientes, según la prueba de Dunnett, * ($p < 0.05$), ** ($p < 0.01$) o * ($p < 0.001$) sobre las barras indican diferencias significativas en relación con el tratamiento control. En el caso de FRA, la letra sobre las barras denota diferencia significativa entre las plantas inoculadas y las plantas control analizadas por ANOVA completamente aleatorizado seguido de una prueba de Tukey ($p < 0.05$).**

un aumento de la expresión relativa de los genes estudiados, en ningún caso los valores alcanzados fueron similares a los observados en condiciones deficientes de Fe. En el caso del melón se estudiaron tres genes diferentes que codifican la enzima reductasa férrica como *FRO1*, *FRO2* y *FRO3* además de *IRT1* y *FIT*. Al igual que ocurre en las raíces de pepino, la expresión relativa de todos los genes estudiados fue mayor en condiciones de deficiencia de Fe, excepto en el caso de *IRT1*, en el que no se encontraron diferencias significativas entre plantas cultivadas en condiciones de Fe suficiente y deficiente (Figure 4E). *FRO1*, *FRO3* y *FIT* alcanzaron su valor máximo de ex-presión relativa al segundo día después de la inoculación (Figure 4B, D–F) mientras que *FRO2* lo hizo el tercer día e *IRT1* el sexto (Figura 4C y E).

En las figuras 5 y 6 se representa un panorama de FRA y apariencia general de partes aéreas y raíces a 5 dpi. Tanto las plantas de pepino (Figura 5) como las de melón (Figura 6), comenzaron a mostrar síntomas de deficiencia a los 4 dpi, siendo más visible en las plantas de pepino, donde se observaron hojas con un mayor grado de clorosis. En ambas especies, las raíces de las plantas que crecieron con suficiente Fe tenían un aspecto más alargado y raíces secundarias menos abundantes como se puede ver en la imagen. En plantas de pepino, los valores SPAD de 4 a 7 dpi, han demos-trado ser

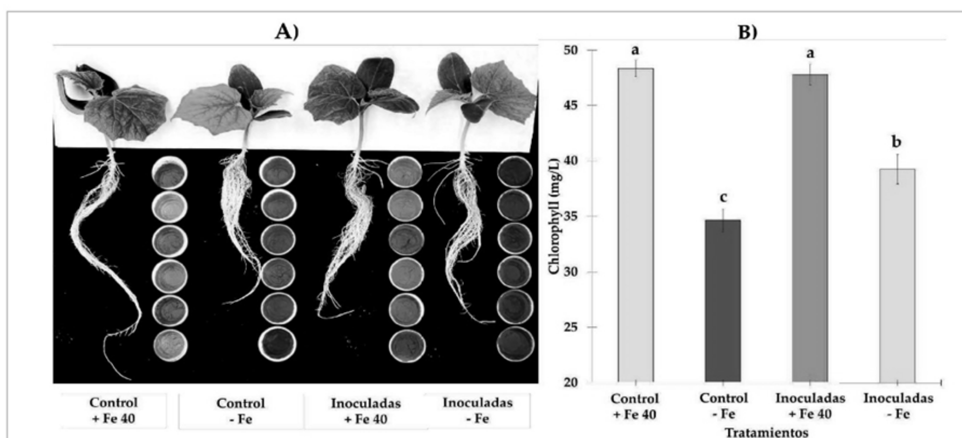

Figura 5. A) Panorama de la actividad de la reductasa férrica (FRA) en raíces de pepino. En el lado derecho de las raíces se pueden ver los contenedores de la solución indicadora a 5 dpi, FRA, en general está muy inducida, sin embargo, como se puede ver en el color púrpura intenso, las plantas inoculadas rebasan su respectivo control; los brotes de plantas inoculadas no mostraron síntomas severos de clorosis como ocurrió en las plantas sin inocula-ción. B) Las medias de los valores de SPAD de 7 dpi mostraron diferencia significativa entre las plantas control y plantas inoculadas cultivadas en condiciones deficientes de Fe. Se utilizaron cuatro tratamientos, a saber, (i) Control + Fe40μM (no inoculado), (ii) Inoculado + Fe40μM, (iii) Control - Fe (no inoculado) y (iv) Inoculado - Fe.

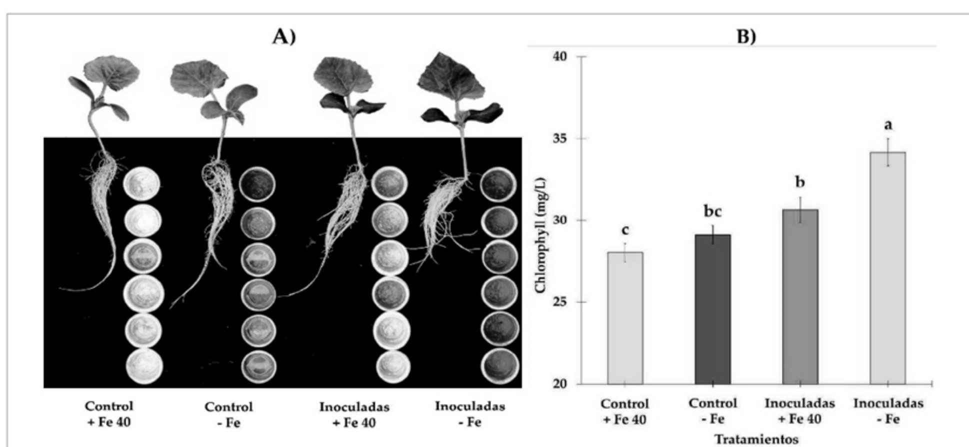

Figura 6. A) Panorama de la actividad de la reductasa férrica (FRA) en raíces de melón. En el lado derecho de las raíces se pueden ver los contenedores de solución indicadora a 5 dpi, FRA presenta claramente diferencia significativa entre los controles y las plantas inoculadas. Se puede observar también un abundante crecimiento de raíces secundarias; los brotes de plantas inoculadas no mostraron síntomas de clorosis. B) Las medias de los valores de SPAD a 7 dpi revelaron diferencia significativa en plantas inoculadas que superan sus respectivos controles, siendo las plantas cultivadas en condiciones deficientes en Fe las que alcanzaron valores más altos en contenido de clorofila. Se utilizaron cuatro tratamientos, a saber, (i) Control + Fe40μM (no inoculado), (ii) Inoculado + Fe40μM, (iii) Control - Fe (no inoculado) y (iv) Inoculado - Fe.

significativamente diferentes entre los tratamientos ($F_{3,95} = 42.11$, $p = 0.001$), especial-mente en aquellos cultivados bajo condiciones deficientes de Fe (Figura 5B), sin embargo, las plantas cultivadas en condiciones suficientes de Fe muestran un mayor contenido de clorofila; en el caso del melón, plantas inoculadas cultivadas en condiciones deficientes de Fe, fueron las que presentaron mayor contenido de clorofila con diferencias significativas respecto a otros trata-mientos ($F_{3,85} = 14.89$, $p = 0.001$) (Figura 6B).

DISCUSIÓN

Muchos estudios han demostrado que los HE representan una excelente alternativa para el control de plagas de insectos y ácaros en cultivos herbáceos y leñosos claves para la agricultura plagas (Quesada-Moraga et al., 2020). Además, el descubrimiento de nuevas funciones para los HE como endófitos y promotores del crecimiento vegetal, y su competencia en la rizosfera han permi-tido la ampliación de su uso, proporcionando así un valor añadido a su uso principal como agentes de control microbiano de plagas (Quesada-Moraga, 2020; Quesada-Moraga et al., 2020). Más allá, los HE pueden llevar asociados otros beneficios directos e indirectos para el crecimiento de las plantas a través de la movilización de nutrientes y/o la mediación de las relaciones tróficas (Mi-randa-Fuentes et al., 2021; Moonjely & Bidochka, 2019; Quesada-Moraga et al., 2022; Raya-Díaz et al., 2017; Sánchez-Rodríguez et al., 2018). El aumento de la biodisponibilidad de nutrientes a través de la producción de fitohormonas y la mejora del transporte de agua son formas en las que los HE promueven directamente el crecimiento de las plantas; también benefician a las plantas a través de mecanismos indirectos que implican la inducción de resistencia sistémica frente a estreses bióticos (Barra-Bucarei et al., 2020).

Aunque hay estudios previos en los que se muestra un alivio de los síntomas de clorosis férrica tras el tratamiento con HE (Raya-Díaz et al., 2017; Raya-Díaz et al., 2017), muy poco se conoce acerca de los mecanismos directos e indirectos utilizados por los HE para

promover la adquisición de Fe en plantas, motivo por el cual ese ha sido el principal objetivo de este trabajo. En un trabajo de Raya-Díaz et al., (2017) mostraron que *M. brunneum* EAMa 01/58-Su aplicado al suelo en dosis altas (5 x 10^8 conidios ml^{-1}) alivió los síntomas de clorosis de Fe en plantas de sorgo cultivadas en suelos calcáreos, y aumentó la altura de las plantas y la producción de inflorescencia de girasoles cultivados en suelos calcáreos y no calcáreos.

Nuestro estudio in vitro demostró la capacidad de la cepa EAMa 01/58-Su de *M. brunneum* para desmineralizar el Fe siendo la más efectiva en la producción de sideróforos de Fe, con un 58.4% de exudación de sideróforos superficiales de 10 dpi, mientras que las cepas de B. bassiana EABb 04/01-Tip y EABb 01/33-Su solo lograron el 24.3% y el 17.8% de exudación de sideróforos superficiales, respectivamente. El aumento de la disponibilidad de Fe resultante de la aplicación de una cepa específica podría deberse a la secreción de ácidos orgánicos, reduciendo así el pH del medio, o a través de la liberación de sideróforos que quelatan no solo Fe sino también otros elementos como Zn, Mn y Cu (Krasnoff et al., 2014; Raya-Díaz et al., 2017). Algunos estudios mostraron datos similares utilizando el conocido género *Trichoderma* (Sánchez-Montesinos et al., 2020) y otros utilizando el saprofito *Aspergillus* niger mostrando habilidades como solubilizadores de fósforo (Baron et al., 2018, 2020; Naeem et al., 2022; Pal & Ghosh, 2018). Estudios recientes de Ba-rra-Bucarei et al., (2020) mostraron diferencias entre cinco cepas de *B. bassiana*, aunque cuatro de ellos fueron capaces de producir sideróforos, las cepas RGM-731 y RGM-644 destacaron por su alta capacidad de exudación de sideróforos, 73% y 81%, respectivamente. Nuestros resultados muestran la capacidad de los HE para solubilizar nutrientes como el Fe a nivel específico de cepa, así como la inducción de respuestas de deficiencia de Fe. Este hallazgo supone un importante avance en el conocimiento de nuevas aptitudes de estos hongos y su función como promotores de crecimiento de las plantas.

CONCLUSIONES

En este trabajo se ha estudiado la capacidad de la cepa *M. brunneum* 01/58-Su para inducir respuestas de deficiencia de Fe en dos especies de *Cucurbitaceae*, *C. sativus* y *C. melo*. Los resul-tados obtenidos muestran que la cepa EAMa 01/58-Su de *M. brunneum* indujo claramente la acti-vidad de la reductasa férrica y la expresión relativa de los genes de adquisición de Fe, FRO, IRT1, HA1 y FIT en ambas especies. Estas nuevas habilidades de la cepa EAMa 01/58-Su de *M. brunneum* le confieren un valor añadido a su uso como excelente agente de control biológico y ponen de ma-nifiesto los mecanismos directos e indirectos implicados en la adquisición de Fe mediada por un HE.■

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REFERENCIAS

Consultar con los autores.

DECLARACIÓN

El presente trabajo es una adaptación del trabajo original titulado "Entomopathogenic FungiMediated Solubilization and Induction of Fe Related Genes in Melon and Cucumber Plants" publicado recientemente en el Journal of Fungi (https://doi.org/10.3390/jof9020258).

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Strain-specific demineralization of Fe, P and K by insect pathogenic fungi

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Summary

Some endophytic insect pathogenic fungi (IPF) have multifunctional lifestyles, particularly *Beauveria* spp. and *Metarhizium* spp.; in addition to their well-known function as biocontrol agents, these fungi may also help plants respond to other biotic and abiotic stresses, such as nutrient deficiency, which is a major global constraint to crop productivity. This study explores three IPF isolates (*M. brunneum* EAMa 01/58-Su, *B. bassiana* EABb 04/01-Tip and EABb 01/33-Su) for their *in vitro* attributes as potential growth promoters. Our study demonstrated a strong isolate-specific ability to demineralize nutrients in *M. brunneum* and *B. bassiana*, specifically Fe, K and P, normally scarce or with low availability in the soil. At 10 days post inoculation (DPI), EAMa 01/58-Su strain achieved 58.4% surface siderophores exudation) compared with isolates EABb 04/01-Tip and EABb 01/33-Su achieved only 24.3% and 17.8% surface siderophores exudation, respectively. Isolate EABb 01/33-Su attained 94.84 of RES, the highest indices of P-solubilization. Isolate EABb 04/01-Tip had an index of 2.98 on Premono Scale, a higher K-solubilization index than the other two isolates. This study provides key knowledge about IPF nutrient assimilation behavior as a function of isolate dependence.

Key words: Entomopathogenic fungi; Growth promoters; Nutrient solubilization

Resumen

Algunos hongos endófitos patógenos de insectos (IPF) tienen estilos de vida multifuncionales, particularmente *Beauveria* spp. y *Metarhizium* spp.; además de su función bien conocida como agentes de control biológico, estos hongos también pueden ayudar a las plantas a responder a otros estreses bióticos y abióticos, como la deficiencia de nutrientes, que es una limitación global importante para la productividad de los cultivos. Este estudio explora tres cepas de IPF (*M. brunneum* EAMa 01/58-Su, *B. bassiana* EABb 04/01-Tip y EABb 01/33-Su) por sus atributos *in vitro* como potenciales promotores del crecimiento. Los resultados muestran una fuerte especificidad de las cepas para desmineralizar nutrientes, específicamente Fe, K y P, normalmente escasos o con baja disponibilidad en el suelo. A 10 días post inoculación (DPI) la cepa EAMa 01/58-Su alcanzó un 58.4 % de exudación de sideróforos en comparación EABb 04/01-Tip y EABb 01/33-Su que alcanzaron sólo 24.3 % y 17.8 %, respectivamente. La cepa EABb 01/33-Su alcanzó 94.84 de Eficiencia Relativa de Solubilización, el índice más alto de solubilización de P. La cepa EABb 04/01-Tip tuvo un índice de solubilización de K de 2.98 en la escala Premono, mayor que los índices de las otras cepas. Este estudio proporciona conocimientos clave sobre el comportamiento de asimilación de nutrientes de los IPF en función de la cepa.

Palabras clave: Hongos entomopatógenos; Promotores de crecimiento; Solubilización de nutrientes

1. Introduction

The discovery of new functions for IPF as plant endophytes and growth promoters, and their competence in the rhizosphere have enabled the expansion of their use, thus providing added value to their main use as biological control agents against a wide variety of insects and mites harmful to cultivated plants (Quesada-Moraga et al. 2020). In this sense, many studies have shown that IPF represent an excellent alternative to control agricultural pests (Barra-Bucarei et al. 2020). Besides, they play other roles beyond pest control with direct and indirect benefits for plant growth through nutrient mobilization and/or mediation of trophic relationships (Raya-Díaz et al. 2017; Miranda-Fuentes et al. 2020). Increasing the bioavailability of nutrients alongside the production of phytohormones and improvement of water transport are ways that IPF promote plant growth directly; they also benefit plants

through indirect mechanisms involving the induction of systemic resistance to harmful organisms (Barra-Bucarei et al. 2020). However, little is known about nutrient solubilization by IPF at the isolate level. This study investigated the ability of three isolates of *M. brunneum* and *B. bassiana* to solubilize nutrients.

It is important to point out that the attributes of IPF are highly dependent on the fungal isolate and the plant species, and these relationships need to be studied on a case-by-case basis. For this reason, the objective of this study was to determine the capacity of three well-studied IPF isolates for the solubilization of Fe, P, and K.

2. Materials and Methods

2.1. Fungal Isolates and Inoculum Preparation

Two isolates of *B. bassiana* (EABb 04/01-Tip and EABb 01/33-Su) and one isolate of *M. brunneum* (EAMa 01/58-Su) from the culture collection of the Agronomy Department, University of Cordoba (Spain) were used in all experiments. To provide inoculum for experiments, all isolates were subcultured from stored slant cultures on Potato Dextrose Agar (PDA) in Petri dishes and grown for 15 d at 25°C in darkness. Prior to each of the following tests, isolates were grown in PDA medium to obtain four-day-old mycelium. Each of the *in vitro* assays were repeated twice with four replicates per isolate.

For the production of iron siderophores, we followed a simplified method of the universal chemical assay for the detection of siderophores, according to Barra-Bucarei et al. (2020); quantification and measurement were carried as in Andrews et al. (2016). The phosphate solubilization ability of fungal isolates was determined using a phosphate medium from the National Institute of Botanical Research (NBRIP) (Nautiyal 1999), as modified in Barra-Bucarei et al. (2020); the overall Relative Efficiency of Solubilization (RES) index was calculated using the formula:

RES = (Halo zone Diameter / Colony Diameter) * 100

Daily from 9 - 15 DPI, the diameters of colonies (front of plates) and the hyaline halo (back of plates), were measured to determine the progress of solubilization.

In the case of K, discs of 6 mm of mycelium were inoculated into Petri plates (9 cm) with modified Aleksandrov medium (Rajawat et al. 2016). Solubilization index was estimated and expressed in the Premono scale (Premono et al. 1996).

Statistical analyses of data were done using Statistix 9.0® (Analytical Software, Tallahassee, FL, USA). Analysis of variance (ANOVA) was used to compare differences in these variables. When significant differences between treatments were identified ($p < 0.05$), means were compared using the LSD All-Pairwise Comparisons method.

3. Results

There were significant differences amongst isolates in siderophore production 10 DPI ($F_{2,21} = 117.73$, $p = 0.001$); *M. brunneum* isolate EAMa 01/58-Su was the most capable of changing the largest area of CAS agar from blue to orange (58.4%), while *B. bassiana* isolates EABb 04/01-Tip and EABb changed the color by only 24.35% and 17.88%, respectively (Figure 1). The timeline for Fe siderophores exudation is shown in Figure 1B revealing the difference between the *M. brunneum* isolate and the others from 3 DPI onwards.

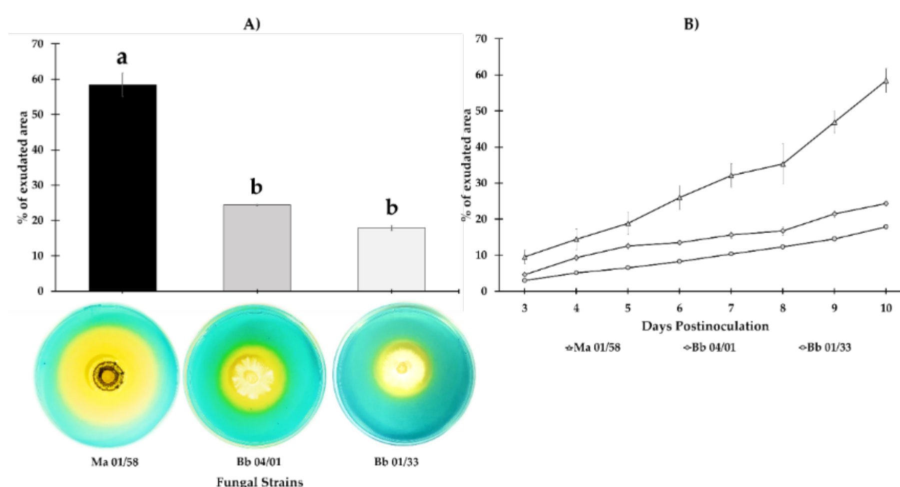

Figure 1. Siderophore exudation by three isolates of IPF on CAS agar medium with $FeCl_3$ as FeIII source. At the bottom, the front of the plates is shown. A) Comparison at 10 days post inoculation (DPI). B) Progress (%) of color change due to siderophore production by three isolates of IPF on CAS agar medium.

Significant differences were also observed amongst isolates in P solubilization ($F_{2,23} = 141.15$, $p = 0.001$) 15 DPI; the highest RES value was achieved by *B. bassiana* isolate EABb 01/33-Su (94.84%), followed by *B. bassiana* isolate EABb 04/01-Tip (52.68%), while *M. brunneum* isolate EAMa 01/58-Su achieved only 3.4% of RES (Figure 2A). The growth of the colonies did not exceed the diameter of the transparent halo of solubilization, which was observed at the back of the plates. Isolate EABb 01/33-Su had the greatest capacity for P solubilization from 9 to 15 DPI compared with the other strains as shown in Figure 2B.

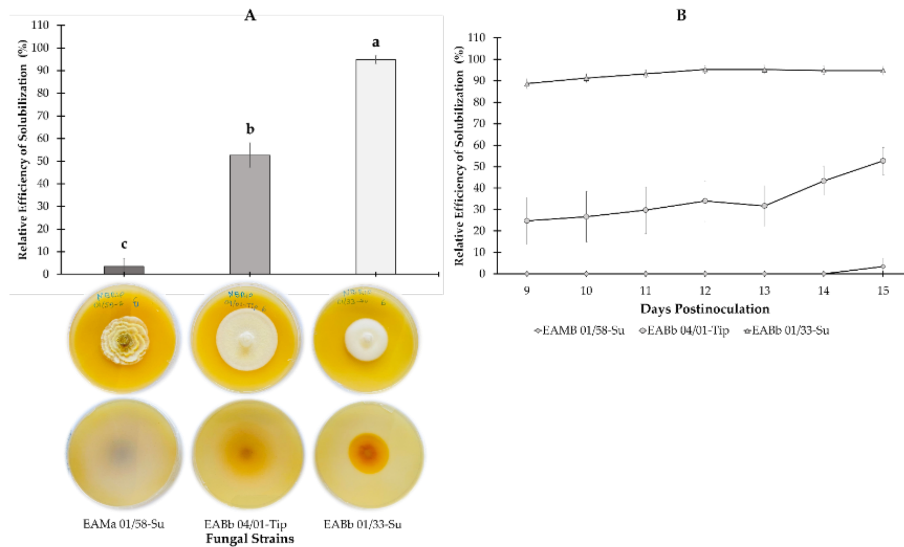

Figure 2. P solubilization by three isolates of IPF in NBRIP solid medium. At the bottom, the front and back of the plates are shown. A) Comparison at 15 DPI. B) Progress (% of RES) of P solubilization by three isolates of IPF on NBRIP medium.

Finally, significant differences ($F_{2,23}$ = 74.06, p = 0.001) were observed amongst isolates in their capacity to solubilize K-mineral at 10 DPI. Values of 2.97 and 2.53 on the Premono scale were achieved by *B. bassiana* isolates EABb 04/01-Tip and EABb 01/33-Su respectively (Figure 3A). *M. brunneum* isolate EAMa 01/58-Su achieved the lowest solubilization index (2.06 on the Premono scale). Figure 3B shows the progress of K solubilization expressed in RES throughout the evaluation period. *B. bassiana* isolate EABb 04/01-Tip was most capable from 7 DPI onwards.

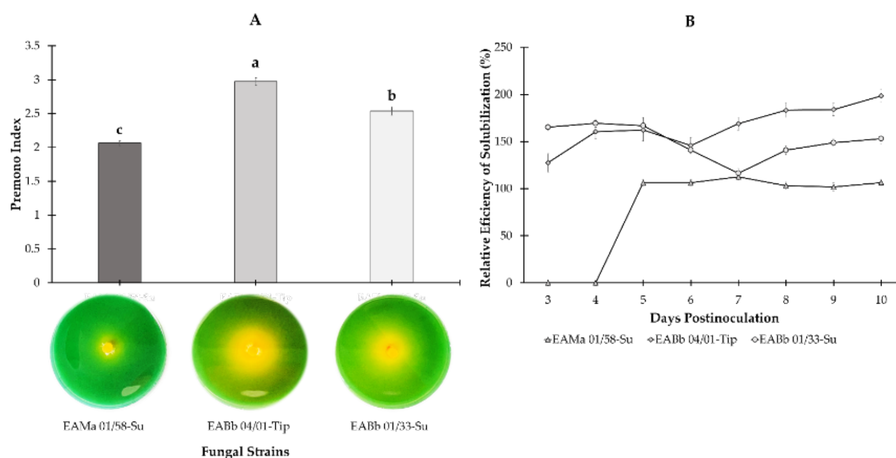

Figure 3. Solubilization of K-mineral in Aleksandrov medium by three isolates of IPF. A) Comparison at 10 DPI. At the bottom, the front of the plates is shown. B) Progress of K solubilization expressed in RES on Aleksandrov medium throughout the evaluation period.

4. Conclusions

Our *in vitro* study demonstrated the isolate-specific ability of *M. brunneum* and *B. bassiana* to demineralize nutrients such as Fe, K and P, which contributes to our knowledge of these fungi and their function as plant growth promoters. On other hand, our recent work demonstrated that the *M. brunneum* EAMa 01/58-Su strain, besides the excellent capacity to produce siderophores, mediated solubilization and induction of Fe-related genes in melon and cucumber plants (García-Espinoza et al. 2023).

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