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ORIGINAL PAPER

Greenhouse melon crop protection and production through the compatible use of a parasitoid with endophytic entomopathogenic ascomycetes

Fabián García-Espinoza[1,2] · Meelad Yousef-Yousef[1] · María José García del Rosal[1] · María Cuenca-Medina[1] · Enrique Quesada-Moraga[1]

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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, and for *B. bassiana*, approximately 20% of seeds from 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 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*

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

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✉ Enrique Quesada-Moraga
cr2qumoe@uco.es

[1] Departmento de Agronomía, ETSIAM, Universidad de Córdoba, Campus de Rabanales, Edificio C4 Celestino Mutis, 14071 Córdoba, Spain

[2] Departamento de Parasitología, Universidad Autónoma Agraria Antonio Narro – Unidad Laguna, Periférico Raúl López Sánchez S/N, 27054 Torreón, Coahuila, México

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. 2022; 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. 2023a, b), 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 leaf worm *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) poses a major threat to agricultural crops in the Mediterranean region (Ahmed et al. 2019; Ghoneim et al. 2020). 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. 2021). 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.

Material and methods

Biological material and growth conditions

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 1). More

Table 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	20,764
B. bassiana	EABb 04/01-Tip	Ecija (Sevilla, Spain)	Opium poppy crop	Insect (*Iraella luteipes*)	FJ972963	20,744
B. bassiana	EABb 01/33-Su	El Bosque (Cadiz, Spain)	Traditional olive orchard	Soil	FJ972969	21,149

*The Spanish type culture collection

details about these strains and their potential as biocontrol agents can be found in our previous works (Quesada-Moraga et al. 2006; Yousef et al. 2018; Garrido-Jurado et al. 2019; González-Mas et al. 2019b; Miranda-Fuentes et al. 2020, 2021; García-Espinoza et al. 2023a).

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, 2021).

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.

Inoculum, growth conditions and plant 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-min 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 1×10^8 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 min, with a 24-h 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 SF 1A-D. In the subsequent experiment, plant inoculation was exclusively carried out via leaf spraying, as depicted in SF 1E-H. For the seed coating method, seeds were submerged in a suspension of 1×10^8 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 (SF 1A), the entire plant was sprayed, and in the second experiment (SF 1E), 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. 2021). 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 and 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.

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.

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 (SF

1A). 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 (SF 1B), and larvae were confined with an organza bag for 24 h 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 (SF 1C). The parasitoids were then removed 24 h 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.

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 (SF 1E). 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 SF 1F). 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 h, the larvae were released, and four females and eight males of *H. didymator* were introduced (as illustrated in SF 1G).

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 (SF 1H).

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 (SF 1D). Assessment of colonization by conventional methods was carried out according to Garrido-Jurado et al. (2017), González-Mas et al. (2019a, b) 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 (SF 1H). 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).

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 h.

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 Fig. 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.

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 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 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 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 2).

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 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 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 (Fig. 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 (Fig. 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 ($p < 0.05$) (Fig. 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 (SF 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.

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 (Fig. 2a). Inoculation with the EABb 04/01-Tip strain by any of the three methods led to a significant increase in root fresh weight (Fig. 2b).

Table 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 ± 5.32a	0.00 ± 0.00a	8.33 ± 5.32a	11.11 ± 0.00a	33.33 ± 10.14a	20.00 ± 0.992ab	11.478 ± 0.656b	0.307 ± 0.018ab
Seed coating	5.56 ± 3.21b	0.00 ± 0.00a	5.56 ± 5.56a	0.00 ± 0.00b	11.11 ± 7.86bA	17.74 ± 0.352b	11.688 ± 0.618b	0.351 ± 0.007a
Soil drenching	11.11 ± 4.54ab	8.33 ± 2.78b	22.22 ± 4.54b	13.89 ± 6.99a	47.22 ± 11.45cB	18.65 ± 0.721ab	13.105 ± 1.017ab	0.323 ± 0.016ab
Leaves spray	47.22 ± 5.32c	8.33 ± 5.73b	19.44 ± 2.78b	11.11 ± 7.86a	77.78 ± 7.86dC	21.00 ± 1.155a	17.000 ± 1.604a	0.287 ± 0.024b
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 ± 5.32a	0.00 ± 0.00a	8.33 ± 5.32a	11.11 ± 0.00a	33.33 ± 15.30a	20.00 ± 0.992a	11.478 ± 0.656b	0.307 ± 0.018b
Seed coating	11.11 ± 4.54a	2.78 ± 2.78b	8.33 ± 5.32a	8.33 ± 2.78a	27.78 ± 9.62aA	17.75 ± 0.222a	16.115 ± 1.031a	0.364 ± 0.010a
Soil drenching	9.03 ± 5.93ab	2.78 ± 2.78b	17.71 ± 3.47b	2.78 ± 2.78b	29.51 ± 3.56aA	19.72 ± 0.751a	16.792 ± 1.231a	0.331 ± 0.014ab
Leaves spray	5.56 ± 3.21b	19.44 ± 9.49c	38.89 ± 9.62c	8.33 ± 2.78a	52.78 ± 11.45bB	18.92 ± 0.749a	16.882 ± 1.280a	0.353 ± 0.016ab
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 ± 5.32a	0.00 ± 0.00a	8.33 ± 5.32a	11.11 ± 0.00a	33.33 ± 15.30a	20.00 ± 0.992a	11.478 ± 0.656b	0.307 ± 0.018a
Seed coating	8.68 ± 5.39a	8.33 ± 5.32b	25.35 ± 8.15c	14.58 ± 5.71a	48.61 ± 13.87bB	18.33 ± 0.706a	14.556 ± 1.115ab	0.311 ± 0.019a
Soil drenching	0.00 ± 0.00b	13.89 ± 2.78c	25.00 ± 2.78c	16.67 ± 5.56b	41.67 ± 2.78abB	17.80 ± 0.424a	16.952 ± 1.204a	0.355 ± 0.012a
Leaves spray	2.78 ± 2.78b	5.56 ± 3.21b	16.67 ± 7.17b	16.67 ± 7.17b	36.11 ± 2.78aA	19.82 ± 0.841a	14.870 ± 0.964ab	0.315 ± 0.015a

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 h after and larvae were exposed to parasitoids (2 females and 4 males per treatment) for 24 h

[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 notsignificantly different from each other according to ANOVA followed by a Tukey test ($p<0.05$)

Roots from plants inoculated with the EAMa 01/58-Su strain by leaf spraying were also significantly higher than those from the controls (Fig. 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 (Fig. 2c).

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 (Fig. 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 (Fig. 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 (Fig. 3c).

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 (Fig. 4a, b), the plants inoculated with EABb 01/33-Su by the three inoculation methods had significantly increased lengths when compared to the control (Fig. 4c). Only plants inoculated with the EABb 04/01-Tip strain by soil drenching showed a significant increase in length at 22 DPI (Fig. 4b).

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 ($\chi^2_{(1)}=18.64$, $p=0.0001$)

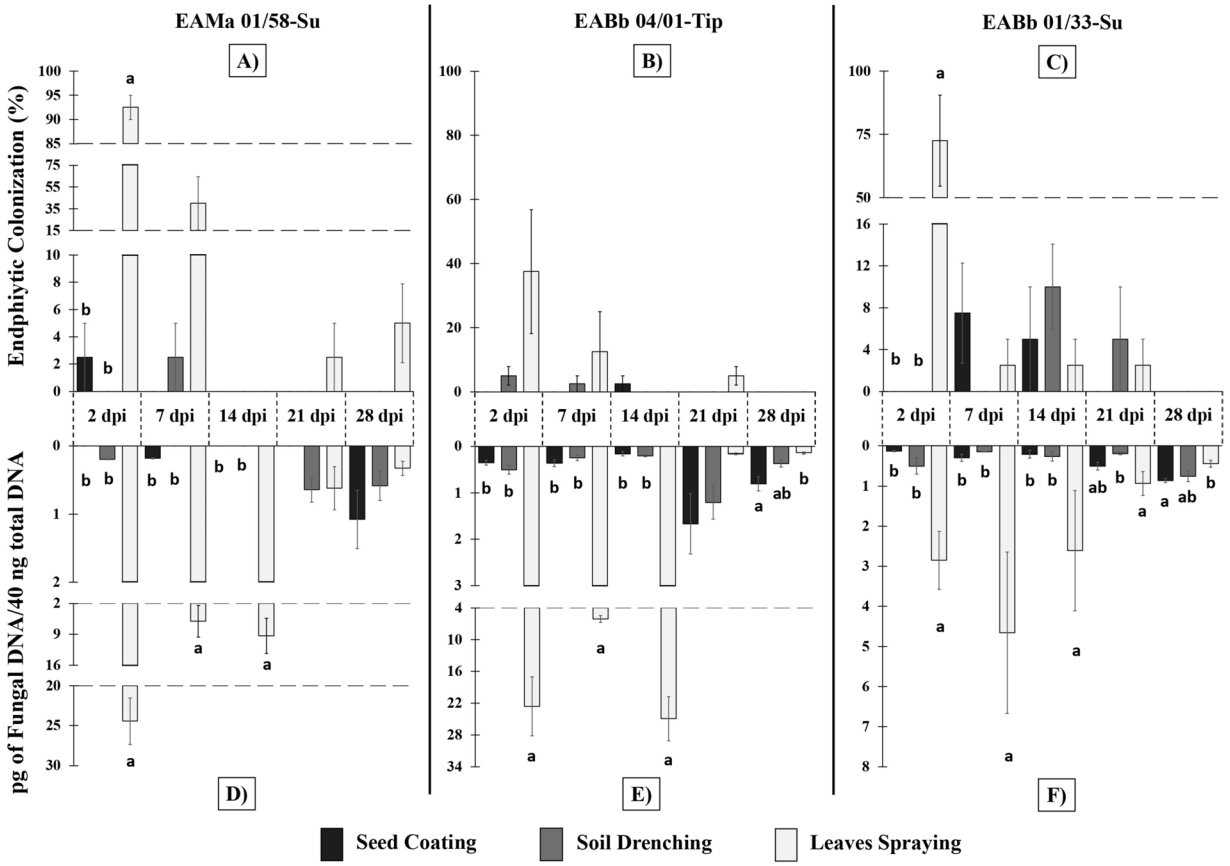

Fig. 1 Detection of endophytic presence of EAMa 01/58-Su *M. brunneum* strain (**a**, **d**) and EABb 04/01-Tip (**b**, **e**) and EABb 01/33-Su (**c**, **d**) *B. bassiana* strains by microbiological technique (up) and by qPCR (down) in melon plants inoculated by seed coating, soil drenching, and leaves 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 ($p < 0.05$)

for EABb 01/33-Su strain] (Table 3). In this scenario, relatively low significant pupal mortalities were recorded (Table 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 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 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 3).

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 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 4). The average survival time (AST) of *H. didymator* F1 adults

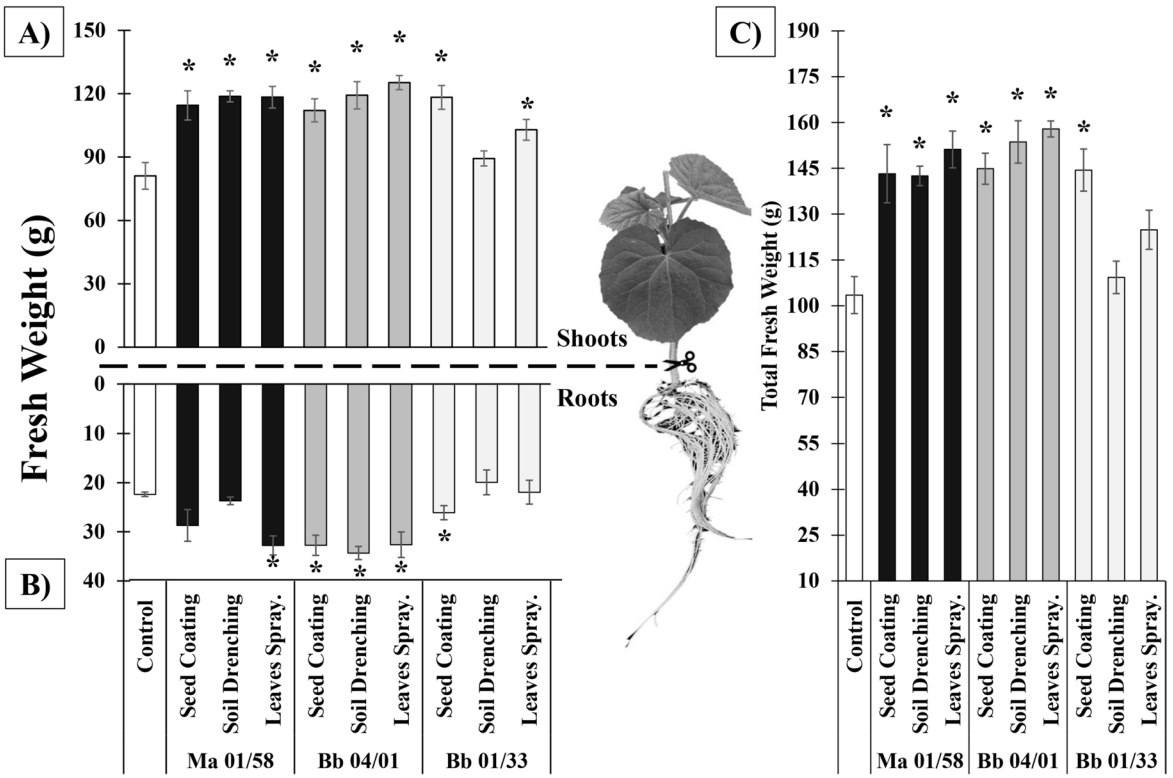

Fig. 2 Effects of endophytic entomopathogenic fungi on shoots (**a**) and roots (**b**) fresh weight and total fresh weight (**c**) of melon plants at 77 DPI (115 DAS) under greenhouse conditions. Plants were inoculated with EAMa 01/58-Su *M. brunneum* strain and EABb 04/01-Tip and EABb 01/33-Su *B. 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 ($p < 0.05$) ($n = 4$)

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 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, 2021), 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

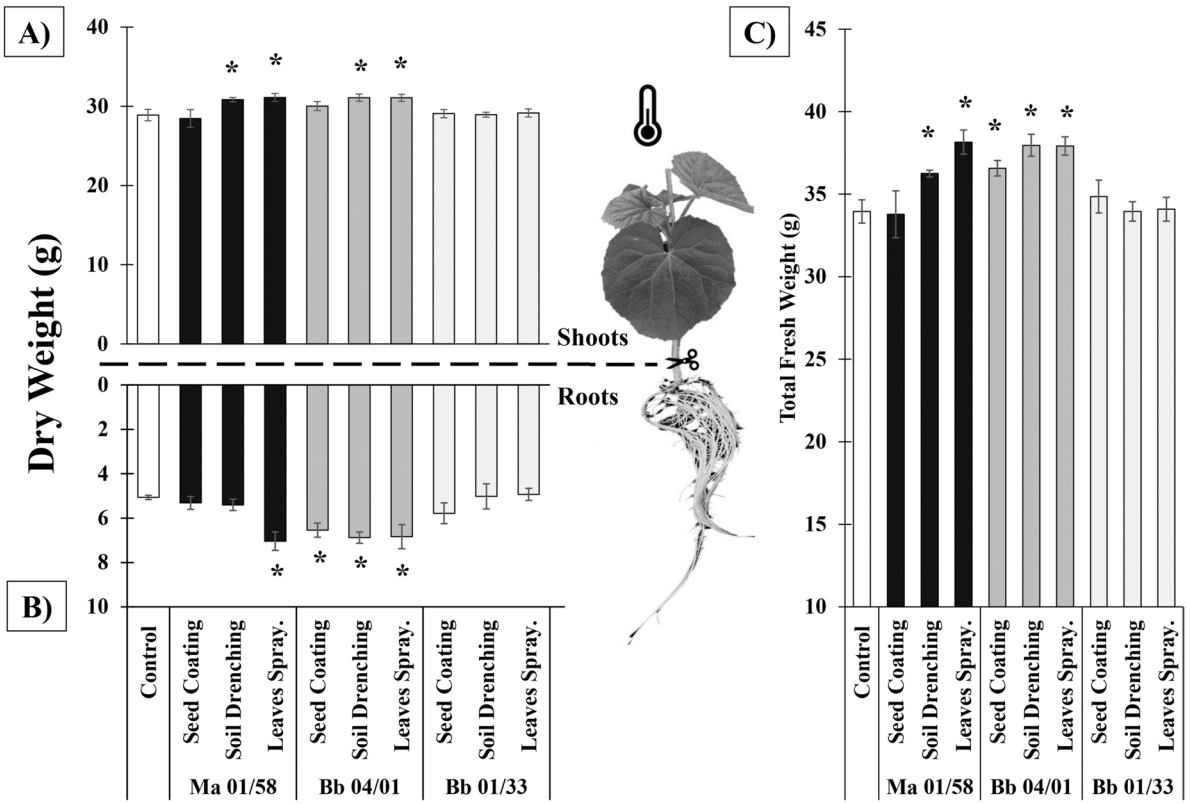

Fig. 3 Effects of endophytic entomopathogenic fungi on shoots (**a**) and roots (**b**) dry weight and total dry weight (**c**) of melon plants at 77 DPI (115 DAS) under greenhouse conditions. Plants were inoculated with EAMa 01/58-Su *M. brunneum* strain and EABb 04/01-Tip and EABb 01/33-Su *B. bassiana* 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 60 °C for 96 h. 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 ($p < 0.05$) ($n = 4$)

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. 2021).

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. 2022; 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. 2017; 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. 2022; Torkaman et al. 2023), *M. brunneum* (Jaber and Enkerli 2016; Gupta et al. 2022; Posada-Vergara et al. 2023), *Metarhizium robertsii* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae) (Liao et al. 2017; Ahmad et al. 2020, 2022) and *Lecanicillium*

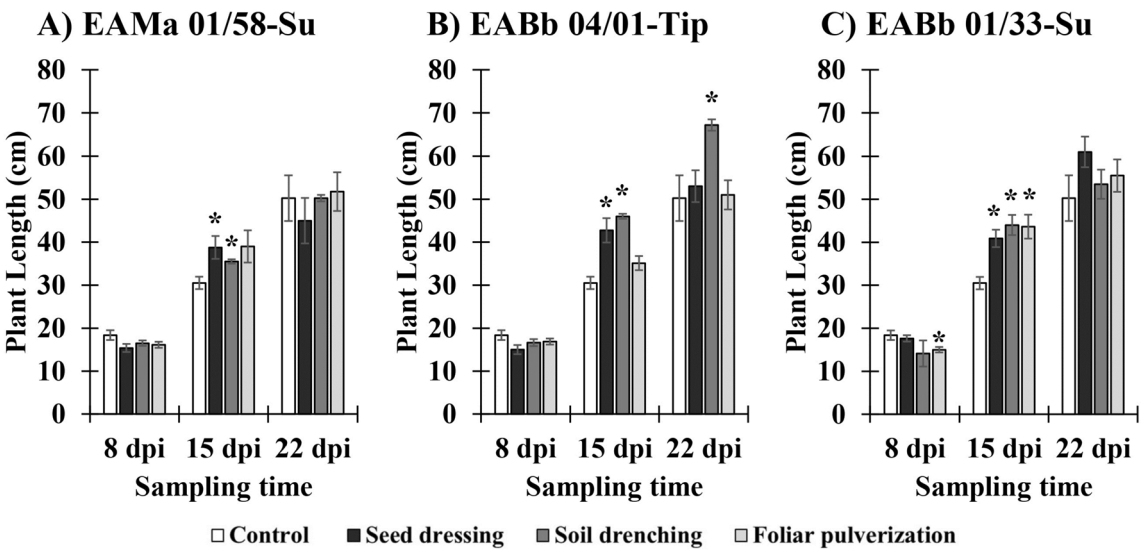

Fig. 4 Effects of endophytic entomopathogenic fungi on plant length at 8, 15 and 22 DPI under greenhouse conditions. Plants were inoculated with EAMa 01/58-Su *M. brunneum* strain and EABb 04/01-Tip and EABb 01/33-Su *B. bassiana* strains by seed coating, soil drenching and leaves spraying; from 2 to 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 ($p < 0.05$) ($n = 4$)

Table 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 ± 3.06a	0.00 ± 0.00a	0.00 ± 0.00a	89.44 ± 4.10a	94.72 ± 3.06a
Sprayed leaves	35.28 ± 10.19b	0.00 ± 0.00a	2.50 ± 2.50b	38.89 ± 7.29c	76.67 ± 5.27aA
Unsprayed leaves	33.33 ± 6.42b	3.70 ± 3.70b	3.70 ± 3.70b	51.85 ± 7.41b	88.89 ± 6.42aA
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 ± 3.06a	0.00 ± 0.00a	0.00 ± 0.00a	89.44 ± 4.10a	94.72 ± 3.06a
Sprayed leaves	28.33 ± 5.45c	0.00 ± 0.00a	2.50 ± 2.50b	48.89 ± 10.51b	79.72 ± 6.94aB
Unsprayed leaves	15.74 ± 7.91b	0.00 ± 0.00a	0.00 ± 0.00a	84.26 ± 7.91a	100.00 ± 0.00aA
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 ± 3.06a	0.00 ± 0.00a	0.00 ± 0.00a	89.44 ± 4.10a	94.72 ± 3.06a
Sprayed leaves	7.78 ± 4.84a	0.00 ± 0.00a	0.00 ± 0.00a	87.22 ± 6.26a	95.00 ± 2.89aA
Unsprayed leaves	16.20 ± 11.12b	8.33 ± 8.30b	8.33 ± 8.30b	60.19 ± 7.58b	84.72 ± 9.72aA

Melon plants were previously inoculated by leaves spraying with 1×10^8 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 h later and larvae were exposed to parasitoids (4 females and 8 males per treatment) for 24 h. 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 ($p < 0.05$)

Table 4 Sublethal effects on F1 *H. didymator* that developed on *S. littoralis* larvae after being fed on sprayed and unsprayed leaves of melon plants

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 ± 0.23a	18.36 ± 0.20a	25.14 ± 0.46b	24.24	26.04
Ma 01/58 spray	12.71 ± 0.30b	19.46 ± 0.30b	28.92 ± 1.79c	25.40	32.43
Ma 01/58 unsprayed	13.44 ± 0.38b	20.38 ± 0.44b	27.75 ± 0.68b	23.00	32.50
Bb 04/01 spray	12.56 ± 0.29b	19.08 ± 0.31a	25.67 ± 1.03b	23.66	27.68
Bb 04/01 unsprayed	11.59 ± 0.23a	18.26 ± 0.24a	23.90 ± 0.31a	23.30	24.49
Bb 01/33 spray	12.00 ± 0.29a	18.68 ± 0.35a	24.82 ± 0.59b	23.66	25.98
Bb 01/33 unsprayed	11.83 ± 0.11a	18.55 ± 0.29a	24.64 ± 0.61b	23.45	25.83

Plants were inoculated with EAMa 01/58-Su, EABb 04/01-Tip and EABb 01/33-Su by leaves spraying

[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

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. 2022; 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 1×10^8 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. 2021). 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.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10340-023-01735-0.

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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.

References

Abrol DP, Shankar U (2014) Pesticides, food safety and integrated pest management. In: Pimentel D, Peshin R (eds) Integrated pest management. Springer, Dordrecht, pp 167–199. https://doi.org/10.1007/978-94-007-7796-5_7

Adedayo AA, Babalola OO (2023) Fungi that promote plant growth in the rhizosphere boost crop growth. J Fungi 9:239. https://doi.org/10.3390/jof9020239

Adeleke BS, Ayilara MS, Akinola SA, Babalola OO (2022) Biocontrol mechanisms of endophytic fungi. Egypt J Biol Pest Control 32:46. https://doi.org/10.1186/s41938-022-00547-1

Agbessenou A, Akutse KS, Yusuf AA, Ekesi S, Subramanian S, Khamis FM (2020) Endophytic fungi protect tomato and nightshade plants against *Tuta absoluta* (Lepidoptera: Gelechiidae) through a hidden friendship and cryptic battle. Sci Rep 10:10. https://doi.org/10.1038/s41598-020-78898-8

Ahmad I, del Jiménez-Gasco M, Luthe DS, Shakeel SN, Barbercheck ME (2020) Endophytic *Metarhizium robertsii* promotes maize growth, suppresses insect growth, and alters plant defense gene expression. Biol Control 144:104167. https://doi.org/10.1016/j.biocontrol.2019.104167

Ahmad I, del Jiménez-Gasco M, Luthe DS, Barbercheck ME (2022) Endophytic *Metarhizium robertsii* suppresses the phytopathogen, *Cochliobolus heterostrophus* and modulates maize defenses. PLoS ONE 17:e0272944. https://doi.org/10.1371/journal.pone.0272944

Ahmed KS, Mikhail WZA, Sobhy HM, Radwan EMM, Salaheldin TA (2019) Impact of nanosilver-profenofos on cotton leafworm, *Spodoptera littoralis* (Boisd.) larvae. Bull Natl Res Center 43:46. https://doi.org/10.1186/s42269-019-0076-z

Barelli L, Moonjely S, Behie SW, Bidochka MJ (2016) Fungi with multifunctional lifestyles: endophytic insect pathogenic fungi. Plant Mol Biol 90:657–664. https://doi.org/10.1007/s11103-015-0413-z

Barelli L, Moreira CC, Bidochka MJ (2018) Initial stages of endophytic colonization by *Metarhizium* involves rhizoplane colonization. Microbiology 164:1531–1540. https://doi.org/10.1099/mic.0.000729

Batool R, Umer MJ, Shabbir MZ, Wang Y, Ahmed MA, Guo J, He K, Zhang T, Bai S, Chen J, Wang Z (2022) Seed Myco-priming improves crop yield and herbivory induced defenses in maize by coordinating antioxidants and Jasmonic acid pathway. BMC Plant Biol. https://doi.org/10.1186/s12870-022-03949-3

Bell AS, Blanford S, Jenkins N, Thomas MB, Read AF (2009) Real-time quantitative PCR for analysis of candidate fungal biopesticides against malaria: Technique validation and first applications. J Invertebr Pathol 100:160–168. https://doi.org/10.1016/j.jip.2009.01.006

Chaudhary PJ, Raghunandan BL, Patel HK, Mehta PV, Patel NB, Sonth B, Dave A, Bagul SY, Divya M, Jain D, Alsahli AA, Kaushik P (2023) Plant growth-promoting potential of entomopathogenic fungus *Metarhizium pinghaense* AAUBC-M26 under elevated salt stress in tomato. Agronomy 13:1577. https://doi.org/10.3390/agronomy13061577

FAO (2019) Food and Agriculture Organization of the United Nations. New standards to curb the global spread of plant pests and diseases. Available from: https://www.fao.org/news/story/en/item/1187738/icode/ (21 Nov 2023).

Gange AC, Koricheva J, Currie AF, Jaber LR, Vidal S (2019) Meta-analysis of the role of entomopathogenic and unspecialized fungal endophytes as plant bodyguards. New Phytol 223:2002–2010. https://doi.org/10.1111/nph.15859

García-Espinoza F, Quesada-Moraga E, García del Rosal MJ, Yousef-Yousef M (2023a) Entomopathogenic fungi-mediated solubilization and induction of Fe related genes in melon and cucumber plants. J Fungi 9:258. https://doi.org/10.3390/jof9020258

García-Espinoza F, García MJ, Quesada-Moraga E, Yousef-Yousef M (2023b) Entomopathogenic fungus-related priming defense mechanisms in cucurbits impact *Spodoptera littoralis* (Boisduval) fitness. In: Druzhinina IS (Ed) Applied and environmental microbiology 89. https://doi.org/10.1128/aem.00940-23

Garrido-Jurado I, Resquín-Romero G, Amarilla SP, Ríos-Moreno A, Carrasco L, Quesada-Moraga E (2017) Transient endophytic colonization of melon plants by entomopathogenic fungi after foliar application for the control of *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae). J Pest Sci 90:319–330. https://doi.org/10.1007/s10340-016-0767-2

Garrido-Jurado I, Resquín-Romero G, Yousef-Naef M, Ríos-Moreno A, Quesada-Moraga E (2019) Soil drenching with entomopathogenic fungi for control of the soil-dwelling life stages and adults of the same generation of *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae). Bull Entomol Res 110:242–248. https://doi.org/10.1017/S000748531900052X

Ghoneim K, Hamadah K, Waheeb H (2020) Bioefficacy of Farnesol, a common Sesquiterpene, on the survival, growth, development, and morphogenesis of *Spodoptera littoralis* (Lepidoptera: Noctuidae). Egyptian Academic Journal of Biological Sciences 12: 71–99. Available from: http://eajbsf.journals.ekb.eg/.

Gonzalez-Guzman A, Raya-Diaz S, Sacristán D, Yousef M, Sánchez-Rodríguez AR, Barrón V, del Campillo MC, Torrent J (2021) Effects of entomopathogenic fungi on durum wheat nutrition and growth in the field. Eur J Agron. https://doi.org/10.1016/j.eja.2021.126282

González-Guzmán A, Rey M-D, Froussart E, Quesada-Moraga E (2022) Elucidating the effect of endophytic entomopathogenic fungi on bread wheat growth through signaling of immune response-related hormones. Appl Environ Microbiol 88:e00882–e00922. https://doi.org/10.1128/aem.00882-22

González-Mas N, Cuenca-Medina M, Gutiérrez-Sánchez F, Quesada-Moraga E (2019a) Bottom-up effects of endophytic *Beauveria bassiana* on multitrophic interactions between the cotton aphid, *Aphis gossypii*, and its natural enemies in melon. J Pest Sci 92:1271–1281. https://doi.org/10.1007/s10340-019-01098-5

González-Mas N, Sánchez-Ortiz A, Valverde-García P, Quesada-Moraga E (2019b) Effects of endophytic entomopathogenic ascomycetes on the life-history traits of *Aphis gossypii* Glover and its interactions with melon plants. InSects 10:165. https://doi.org/10.3390/insects10060165

Gupta R, Keppanan R, Leibman-Markus M, Rav-David D, Elad Y, Ment D, Bar M (2022) The entomopathogenic fungi *Metarhizium brunneum* and *Beauveria bassiana* romote systemic immunity and confer resistance to a broad range of pests and pathogens in tomato. Phytopathology 112:784–793. https://doi.org/10.1094/PHYTO-08-21-0343-R

Jaber LR, Enkerli J (2016) Effect of seed treatment duration on growth and colonization of *Vicia faba* by endophytic *Beauveria bassiana* and *Metarhizium brunneum*. Biol Control 103:187–195. https://doi.org/10.1016/j.biocontrol.2016.09.008

Jacquet F, Jeuffroy MH, Jouan J, Le Cadre E, Litrico I, Malausa T, Reboud X, Huyghe C (2022) Pesticide-free agriculture as a new paradigm for research. Agron Sustain Dev. https://doi.org/10.1007/s13593-021-00742-8

Jensen RE, Cabral C, Enkegaard A, Steenberg T (2020) Influence of the plant interacting entomopathogenic fungus *Beauveria bassiana* on parasitoid host choice-behavior, development, and plant defense pathways. PLoS ONE 15:e0238943. https://doi.org/10.1371/journal.pone.0238943

Julot J-F, Hiller N (2021) Exploring the benefits of biocontrol for sustainable agriculture. IEEP (Ed) Institute for European Environmental Policy, Brussels, Belgium Available from: www.ieep.eu.

Kalvnadi E, Mirmoayedi A, Alizadeh M, Pourian HR (2018) Sub-lethal concentrations of the entomopathogenic fungus, *Beauveria bassiana* increase fitness costs of *Helicoverpa armigera* (Lepidoptera: Noctuidae) offspring. J Invertebr Pathol 158:32–42. https://doi.org/10.1016/j.jip.2018.08.012

Khan AL, Hamayun M, Khan SA, Kang S-M, Shinwari ZK, Kamran M, ur Rehman S, Kim J-G, Lee I-J (2012) Pure culture of *Metarhizium anisopliae* LHL07 reprograms soybean to higher growth and mitigates salt stress. World J Microbiol Biotechnol 28:1483–1494. https://doi.org/10.1007/s11274-011-0950-9

Khan AL, Hussain J, Al-Harrasi A, Al-Rawahi A, Lee I-J (2015) Endophytic fungi: resource for gibberellins and crop abiotic stress resistance. Crit Rev Biotechnol 35:62–74. https://doi.org/10.3109/07388551.2013.800018

Liao X, Lovett B, Fang W, St Leger RJ (2017) *Metarhizium robertsii* produces indole-3-acetic acid, which promotes root growth in *Arabidopsis* and enhances virulence to insects. Microbiology (united Kingdom) 163:980–991. https://doi.org/10.1099/mic.0.000494

Mantzoukas S, Daskalaki E, Kitsiou F, Papantzikos V, Servis D, Bitivanos S, Patakioutas G, Eliopoulos PA (2022) Dual action of *Beauveria bassiana* (Hypocreales; Cordycipitaceae) endophytic strains as biocontrol agents against sucking pests and plant growth biostimulants on melon and strawberry field plants. Microorganisms 10:2306. https://doi.org/10.3390/microorganisms10112306

Mejía C, Espinel C (2022) In vitro versus in planta: Comparing the compatibility of *Akanthomyces lecanii* with pesticides against *Trialeurodes vaporariorum*. J Appl Entomol 146:1272–1280. https://doi.org/10.1111/jen.13064

Miranda-Fuentes P, Quesada-Moraga E, Aldebis HK, Yousef-Naef M (2020) Compatibility between the endoparasitoid *Hyposoter didymator* and the entomopathogenic fungus *Metarhizium brunneum*: a laboratory simulation for the simultaneous use to control *Spodoptera littoralis*. Pest Manag Sci 76:1060–1070. https://doi.org/10.1002/ps.5616

Miranda-Fuentes P, Yousef-Yousef M, Valverde-García P, Rodríguez-Gómez IM, Garrido-Jurado I, Quesada-Moraga E (2021) Entomopathogenic fungal endophyte-mediated tritrophic interactions between *Spodoptera littoralis* and its parasitoid *Hyposoter didymator*. J Pest Sci 94:933–945. https://doi.org/10.1007/s10340-020-01306-7

Oreste M, Bubici G, Poliseno M, Tarasco E (2016) Effect of *Beauveria bassiana* and *Metarhizium anisopliae* on the *Trialeurodes vaporariorum-Encarsia formosa* system. J Pest Sci 89:153–160. https://doi.org/10.1007/s10340-015-0660-4

Parewa HP, Meena VS, Jain LK, Choudhary A (2018) Sustainable crop production and soil health management through plant growth-promoting rhizobacteria. In: Role of rhizospheric microbes in soil: stress management and agricultural sustainability. Springer Singapore, pp 299–329. https://doi.org/10.1007/978-981-10-8402-7_12

Patel P, Kumar S, Kumar A (2022) Plant microbiota: a prospect to *Edge off* postharvest loss. In: Biocontrol Mechanisms of Endophytic Microorganisms. Elsevier, 261–284. https://doi.org/10.1016/B978-0-323-88478-5.00006-7

Posada-Vergara C, Lohaus K, Alhussein M, Vidal S, Rostás M (2022) Root colonization by fungal entomopathogen systemically primes belowground plant defense against cabbage root fly. Journal of Fungi 8:1–18. https://doi.org/10.3390/jof8090969

Posada-Vergara C, Vidal S, Rostás M (2023) Local competition and enhanced defense: How *Metarhizium brunneum* inhibits *Verticillium longisporum* in oilseed rape plants. Journal of Fungi 9:796. https://doi.org/10.3390/jof9080796

Quesada Moraga E (2020) Entomopathogenic fungi as endophytes: their broader contribution to IPM and crop production. Biocontrol Sci Tech 30:864–877. https://doi.org/10.1080/09583157.2020.1771279

Quesada-Moraga E, Carrasco-Díaz J-A, Santiago-Álvarez C (2006) Insecticidal and antifeedant activities of proteins secreted by entomopathogenic fungi against *Spodoptera littoralis* (Lep., Noctuidae). J Appl Entomol 130:442–452. https://doi.org/10.1111/j.1439-0418.2006.01079.x

Quesada-Moraga E, López-Díaz C, Landa BB (2014) The hidden habit of the entomopathogenic fungus *Beauveria bassiana*: first demonstration of vertical plant transmission. PLoS ONE. https://doi.org/10.1371/journal.pone.0089278

Quesada-Moraga E, Yousef-Naef M, Garrido-Jurado I (2020) Advances in the use of entomopathogenic fungi as biopesticides in suppressing crop insect pests. In: Birch NN, Glare T (eds) Biopesticides for sustainable agriculture. Burleigh Dodds Science Publishing, Cambridge, pp 63–98. https://doi.org/10.19103/AS.2020.0073.05

Quesada-Moraga E, Garrido-Jurado I, Yousef-Yousef M, González-Mas N (2022) Multitrophic interactions of entomopathogenic fungi in BioControl. Biocontrol 67:457–472. https://doi.org/10.1007/s10526-022-10163-5

Raya-Díaz S, Sánchez-Rodríguez AR, Segura-Fernández JM, Del Campillo MDC, Quesada-Moraga E (2017) Entomopathogenic fungi-based mechanisms for improved Fe nutrition in sorghum plants grown on calcareous substrates. PLoS ONE 12:e0185903. https://doi.org/10.1371/journal.pone.0185903

Reid A, Greene SE (2012) How microbes can help feed the world. A report from the American Academy of Microbiology. Washington, DC. Available from: https://www.ncbi.nlm.nih.gov/books/NBK559436/#. Accessed 21 Nov 2023

Resquín-Romero G, Garrido-Jurado I, Quesada-Moraga E (2016a) Combined use of entomopathogenic fungi and their extracts for the control of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae). Biol Control 92:101–110. https://doi.org/10.1016/j.biocontrol.2015.10.007

Resquín-Romero G, Garrido-Jurado I, Delso C, Ríos-Moreno A, Quesada-Moraga E (2016b) Transient endophytic colonizations of plants improve the outcome of foliar applications of mycoinsecticides against chewing insects. J Invertebr Pathol 136:23–31. https://doi.org/10.1016/j.jip.2016.03.003

Rondot Y, Reineke A (2019) Endophytic *Beauveria bassiana* activates expression of defense genes in grapevine and prevents infections by grapevine downy mildew *Plasmopara viticola*. Plant Pathol 68:1719–1731. https://doi.org/10.1111/ppa.13089

Sánchez-Rodríguez AR, Raya-Díaz S, Zamarreño ÁM, García-Mina JM, del Campillo MC, Quesada-Moraga E (2018) An endophytic *Beauveria bassiana* strain increases spike production in bread and durum wheat plants and effectively controls cotton leafworm (*Spodoptera littoralis*) larvae. Biol Control 116:90–102. https://doi.org/10.1016/j.biocontrol.2017.01.012

Silva ACL, Silva GA, Abib PHN, Carolino AT, Samuels RI (2020) Endophytic colonization of tomato plants by the entomopathogenic fungus *Beauveria bassiana* for controlling the South American tomato pinworm, *Tuta absoluta*. CABI Agricult Biosci 1:3. https://doi.org/10.1186/s43170-020-00002-x

Singh P, Rale V (2022) Applications of microbial biosurfactants in biocontrol management. In: Biocontrol mechanisms of endophytic microorganisms. Elsevier, Amsterdam, pp 217–237. https://doi.org/10.1016/B978-0-323-88478-5.00009-2

Solter LF, Hajek AE, Lacey LA (2017) Exploration for entomopathogens. In: Lacey LA (ed) Microbial control of insect and mite pests: from theory to practice. Elsevier Inc., London, pp 13–23. https://doi.org/10.1016/B978-0-12-803527-6.00002-0

Tall S, Meyling NV (2018) Probiotics for plants? Growth promotion by the entomopathogenic fungus *Beauveria bassiana* depends on nutrient availability. Microb Ecol 76:1002–1008. https://doi.org/10.1007/s00248-018-1180-6

Tiwari M, Singh P (2021) Plant defense priming: a new tool for sustainable global food security. In: Singh Shekhawa G (ed) Agricultural innovations and sustainability. Agrobios Research, Jodhpur, pp 133–153. Available from: https://www.researchgate.net/publication/355771884. Accessed 21 ov 2023

Torkaman Z, Talaei-Hassanloui R, Khorramnejad A, Pashaei MR (2023) Effects of endophytism by *Beauveria bassiana* (Cordycipitaceae) on plant growth, *Fusarium* (Nectriaceae) disease, and Sunn pest *Eurygaster integriceps* (Hemiptera: Scutelleridae) in wheat (Poaceae). Can Entomol 155:1–17. https://doi.org/10.4039/tce.2022.43

United Nations (2023) United Nations. Global Issues. Population. Available from: https://www.un.org/en/global-issues/population (November 21, 2023).

Van Hee S, Stockmans I, Alınç T, Cusumano A, Jacquemyn H, Lievens B (2023) Effects of plant-beneficial fungi on plant growth and herbivore resistance under contrasting fertilizer conditions. Plant Soil. https://doi.org/10.1007/s11104-023-06220-2

Yousef M, Alba-Ramírez C, Jurado IG, Mateu J, Díaz SR, Valverde-García P, Quesada-Moraga E (2018) *Metarhizium brunneum* (Ascomycota; Hypocreales) treatments targeting olive fly in the soil for sustainable crop production. Front Plant Sci 9:1. https://doi.org/10.3389/fpls.2018.00001