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

Running title: *Hyposoter didymator* and *Metarhizium brunneum* for control of *Spodoptera littoralis*

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ABSTRACT

BACKGROUND

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

RESULTS

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

CONCLUSIONS

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

KEYWORDS

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

1 INTRODUCTION

Phytophagous insects are a major constraint to crop production and often cause huge yield losses. One of the most destructive and ubiquitous insect pests in the Mediterranean basin is the cotton leafworm, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae). This species is widespread in many northern and southern European countries, particularly Spain, Greece and Italy, and has been detected in Africa and Asia Minor.[1,2] The polyphagous habit of *S. littoralis* makes it a noxious pest on numerous economically-important crops in both greenhouses and open fields: tomato, pepper, eggplant, lettuce, artichoke, strawberry, asparagus, spinach, ornamentals and herbs.[2,3] Chemical control has been the traditional control method for *S. littoralis*.[4] However, chemical control is not a sustainable approach for the future. The environmental impact of chemical compounds threatens food and water security[5,6] and their use has led to the development of resistance in *S. littoralis* to several active ingredients, mainly among orgaphosphorus,[7] IGRs[8] and pyrethroids.[9] Moreover, chemical insecticides may be harmful for the natural enemies of insect pests.[10] As a result, research has increasingly focussed on non-chemical measures for control of *S. littoralis* with a particular emphasis on biological control.[11] Biological control agents are important alternatives to chemical pesticides and one of the principal components of any Integrated Pest Management (IPM) programme.[12] The European Directive on the sustainable use of pesticides (2009/128/EC) promotes the use of biological control as an environmentally friendly, sustainable and financially viable tool for pest control.

The endoparasitoid, *Hyposoter didymator* (Thunberg) (Hymenoptera: Ichneumonidae), is also a promising biological control agent for consideration in any IPM programme for control of noctuid pest species including *S. littoralis*.[13] This solitary koinobiont wasp is indigenous in many European countries, including Spain, and it actively searches for and parasitizes larval stages of the genera *Spodoptera*, *Heliothis* and *Helicoverpa.* As an ichneumonid wasp, *H. didymator* has been described as a polydnavirus secretor, which is injected onto the host larva during oviposition.[14] The polydnaviruses produced by hymenopteran parasitoids cause a suppression of the host immune response, affecting the presence of haemocytes in the host haemolymph.[15] As defence units that modulate the cellular immune responses,

haemocytes are a very important component of the insect immune system,[16] and the affection of the immune system of the host may increase its susceptibility to other biocontrol agents.[17] The well-known efficacy of *H. didymator* controlling several insect pests, including *S. littoralis*, have led different authors to emphasize on the interest of developing biocontrol strategies using this parasitoid.[13] Although several authors have reported difficulties rearing this parasitoid, and generally considered time-consuming and easily biassed towards males,[18] the recent develop of new rearing methods is leading to a more efficient production which could be used if a commercial *H. didymator* production is aimed.[13]

On the other hand, entomopathogenic fungi have great potential as biological control agents against many insect pests.[19,20] Their contact mode of action and ability to secrete insecticidal compounds put them at the vanguard of the global development of alternative control strategies.[19] Among them, it is worth mentioning the genus *Metarhizium*, which comprehends several species of a great efficacy as biocontrol agents, such as *Metarhizium anisopliae* (Metsch) (Hypocreales: Clavicipitaceae) and *Metarhizium brunneum* Petch (Hypocreales: Clavicipitaceae).[21] As generalist entomopathogenic fungi, *Metarhizium* spp. have a broad host range, although their virulence, and thus, their efficacy as biocontrol agents depends largely on the strain more than the species.[22] Indeed, our previous studies have reported the efficacy of several isolates of entomopathogenic fungi for control of *S. littoralis*, both by direct inoculation of larvae with the fungus[11] and by feeding larvae with leaves from endophytically-colonised plants.[23] The *M. brunneum* isolate EAMa 01/58-Su, in particular, has showed to be virulent against *S. littoralis*[11] and other economically important insect pests.[20,24]

Combined use of multiple micro- and macro-biological control agents may enhance the effectiveness of any IPM programme. However, to ensure positive outcomes, it is important that the complex interactions between entomopathogens and arthropod natural enemies are fully understood before they are used together in IPM. Although hypocrealean fungi, including some *Metarhizium* species, have broad host ranges and may infect some non-target and/or beneficial insects, such as parasitoids, they are generally considered as organisms that have a low environmental risk.[25] Indeed, many recent studies have demonstrated both the safety of these fungi to non-target insects and the potential for their combined use

with arthropod natural enemies.[26-28] However, interactions between natural enemies in a multitrophic context are complex and should be evaluated case by case if they are to be exploited effectively for pest control.

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

2 MATERIALS & METHODS

2.1 *Spodoptera littoralis* and *Hyposoter didymator* rearings

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

A stock colony of *S. littoralis* was established and reared using the method proposed by Poitout and Bues[29] and modified by Santiago-Alvarez.[30] The detailed rearing procedure is described in one of our own previous studies.[11]

The *H. didymator* colony was established in 2016 from pupae provided by Dr Anne-Nathalie Volkoff (University of Montpellier, France), and reared following the protocol described by Schneider and Viñuela[18] with some modifications. Specifically, adult wasps (two males and one female) were placed in 12 × 5 cm methacrylate cages and provided with a solution of 10% honey. A circular hole (3 cm in diameter) covered with a net cloth allowed ventilation of the cage. For oviposition the parasitoids were routinely provided with third-instar (L3) larvae of *S. littoralis*; L3 is the best instar for obtaining high numbers of females and a low encapsulation rate.[31] Larvae of *S. littoralis* were introduced into the cages

in groups of ten with small cubes of artificial diet[30] and oviposition allowed to proceed for 24 h. After this time, the adult parasitoids were removed and the *S. littoralis* larvae transferred individually to cylindrical plastic boxes (4 cm diameter) and provided with the aforementioned diet *ad libitum* until their death due to parasitism and the subsequent emergence of L3 larvae of the parasitoid, which immediately spun cocoons and pupated. *Hyposoter didymator* pupae were incubated in the same conditions until adults emerged. Adults were fed with 10% honey. Emerging adult parasitoids were used in experiments or for rearing after they were sexed (female:male ratio = 1:2).

2.2 Inoculum preparation

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

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

To quantify lethal effects, a virulence bioassay of EAMa 01/58-Su was done against newly-emerged *H. didymator* adults. Four concentrations of conidia in suspension were prepared in a sterile solution of 0.1 % Tween 80 (10^5, 10^6, 10^7 and 10^8 conidia ml^{-1}); the control was 0.1 % Tween 80 without conidia. These concentrations were selected based on our previous studies.[11,24] Newly-emerged adult parasitoids were cold-anesthetized and sprayed, in replicate groups of ten, with conidial suspensions (or 0.1 % Tween 80 [control]) in a Potter tower (Burkard Manufacturing Co. Ltd, Rickmansworth, United Kingdom), which deposited 1.54 ± 0.06 mg cm^{-2} at 0.7 bars of pressure. The quantity of conidial suspension used for each replicate was 1 ml and there were five replicates per treatment (n = 50 adult parasitoids per treatment in total). After treatment, replicate groups of parasitoids were placed in methacrylate cages (10 × 10 × 6 cm) with covers; each cage contained a circular hole (4 cm in diameter) covered with a net cloth for ventilation. They were all provided with a liquid diet daily consisting of 10 % honey in water and incubated at 26 ± 2˚ C, 50–60 % RH in a photoperiod of 16:8 (L:D) h. Mortality was monitored daily for 5 days. Dead parasitoids were removed daily, processed as described by Quesada-Moraga et al.,[32] and inspected for fungal outgrowth as an indicator of fungal-induced mortality.

The mean lethal concentration (estimated concentration required to kill 50% of the test insects, LC_{50}) was estimated by Probit analysis,[33] after assessing fit and overdispersion with other distributions such as logit, and not getting a better fit compared to Probit analysis. The values of average survival times (ASTs) were obtained by the Kaplan-Meier method and compared using the log-rank test calculated with SPSS 15.0 software for Windows (SPSS Inc., Chicago, IL).

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

in total). The number of F1 generation pupal parasitoids that developed on *S. littoralis* larvae was used as an indication of reproductive potential of fungus-treated and untreated female parasitoids in the three days following inoculation.

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

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

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

2.4.1 Fungal infection of host larvae before parasitism

In the first bioassay, we evaluated the outcomes of dual infection/ parasitism when infection occurred before parasitism. Specifically, replicate groups of early L2 *S. littoralis* were inoculated by immersion for 60 seconds in a 10^8 conidia ml^{-1} fungal suspension (10 ml). Replicate groups of control larvae were immersed in the same volume of sterile 0.1% Tween 80. Twenty-four, 48 and 72 h after immersion individual treated and control *S. littoralis* larvae were offered to individual newly-emerged mated female parasitoids (females were kept with two males the same day they emerged from cocoon and were monitored for 24 h to ensure mating occurrence; only 48-h mated females were used in all the bioassays) and oviposition allowed to proceed for 24 h. A small cube of artificial diet was introduced into each oviposition cage. The assay included the following treatments: i) three treatments in which *S. littoralis*

larvae were inoculated with the fungus and exposed to the parasitoid at different times (24, 48 and 72 h after inoculation); ii) three control treatments in which *S. littoralis* larvae were immersed in 0.1% Tween 80 and exposed to the parasitoid at 24, 48 and 72 h; iii) a control treatment in which *S. littoralis* larvae were inoculated with the fungus; iv) an absolute control treatment in which larvae were immersed in 0.1% Tween 80. There were ten replications of each treatment and control, each replication including a group of ten *S. littoralis* larvae that were inoculated and/or offered to a *H. didymator* female depending on the treatment as described before. After oviposition, the *S. littoralis* larvae were individually transferred to cylindrical plastic boxes (as described previously) and provided with artificial diet *ad libitum* until the emergence of *H. didymator* larvae and pupae. Fungus-induced mortality of *S. littoralis* larvae, the parasitoid reproductive potential and total mortality were all recorded. To determine whether mortality was due to the fungus, dead *S. littoralis* larvae were removed daily and were immediately surface-sterilised with 1 % sodium hypochlorite followed by three rinses in sterile distilled water for 1 min each. They were then placed on sterile wet filter paper in sterile Petri plates, sealed with laboratory film, incubated at 25 ˚C and inspected for fungal outgrowth.[32] The parasitoid reproductive potential, referred as *H. didymator* complete parasitism, was represented by the number of emerging parasitoid pupae.[13] Finally, total mortality was expressed as the sum of the two former variables (larvae with fungal outgrowth and larvae showing a complete parasitism) and the rest of *S. littoralis* larvae which died not evidencing fungal outgrowth nor complete parasitism, i.e. died by unknown reasons. The experiment was repeated twice with fresh fungal suspensions and a new parasitoid generation.

Total mortality (%), Parasitized larvae (%) and Larvae with fungal outgrowth (%) were analyzed using the linear mixed model: $Y = \mu + \text{treatment} + \text{experiment}$, where treatment was modeled as a fixed effect and experiment was modeled as a random effect. In order to improve the normality and homogeneity of variance of the datasets values were transformed using the arcsine transformation. The estimation method was Restricted Maximum Likelihood (REML) with Kenward-Roger's for degrees of freedom. Significance of the fixed effect (Treatment) was evaluated using the F-approximate test (α =

0.05) and means from the different treatments were compared with Tukey's test ($\alpha = 0.05$).[34] Data analyses were performed using JMP 14.2 (SAS Institute Inc., Cary, NC).

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

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

2.4.2. Parasitism of host larvae before fungal infection

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

at different times (24, 48 and 72 h after parasitization); ii) three control treatments in which *S. littoralis* larvae were inoculated with the fungus at 24, 48 and 72 h; iii) a control treatment in which *S. littoralis* larvae were exposed to the parasitoid; iv) an absolute control treatment in which larvae were immersed in 0.1% Tween 80. There were five replications of each treatment and control, each replication including a group of ten *S. littoralis* larvae that were inoculated and/or offered to a *H. didymator* female depending on the treatment as described before.

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

Data from the dual infection/ parasitism bioassays (2.4.1 and 2.4.2) were analyzed to determine whether there were synergistic, additive or antagonistic interactions between *M. brunneum* EAMa 01/58-Su and the parasitoid *H. didymator*. A χ^2 test was done as described by Hernandez et al.[35] In this test, the expected mortality due to the effect of both treatments (M_E) was calculated from the observed mortality with the formula used by Colby:[36] $M_E = M_P + M_F - (M_P \times M_F/100)$, where M_P and M_F represent, respectively, the mortality caused by the parasitoid and the fungus corrected according to Abbott.[37] The χ^2 was calculated using the formula $\chi^2 = (M_O - M_E)^2/M_E$, where M_O is the corrected observed mortality. The obtained values were compared with the χ^2 table values for 1 degree of freedom and $P > 0.05$. If the calculated values were lower than the values of the table, the interaction between treatments was considered additive; otherwise, the interaction could be synergistic or antagonistic depending on the relationship of M_O and M_P with M_E.[11]

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

We assessed the development time of the preimaginal stages of *H. didymator* when the parasitoid developed at the expense of *S. littoralis* larvae inoculated with the EAMa 01/58-Su strain, in order to

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

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

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

In this bioassay, which aim was to ascertain whether a depletion in *S. littoralis* could be caused by *H. didymator*, individual L3 *S. littoralis* larvae were offered to individual mated female parasitoids and oviposition allowed to proceed for 24 h. The parasitoids were then removed and the *S. littoralis* larvae fed with diet and incubated, as described previously. Control larvae were treated in the same way but were not parasitized. There were three replicates, each of five larvae, for each treatment (parasitized or non-parasitized control) and each sampling day (from day one to five after parasitization). Haemolymph was

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

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

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

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

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

total). The parasitoids were removed and the larvae incubated individually and routinely fed with diet for 10 days. The parasitoid reproductive potential (represented by the number of emerged adults) was evaluated.

Choice test (oviposition preference for uninoculated vs. inoculated larvae of *S. littoralis*) was analyzed using a likelihood-ratio Chi-square test ($p \leq 0.05$) to determine whether the observed frequencies were significantly different to the expected ones under the hypothesis of no treatment effect (50% : 50%).[38] Data analysis was performed using JMP 14.2.

3 RESULTS

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

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

The reproductive potential of female parasitoids over three days was not significantly influenced by the fungal treatment based on the percentages of *S. littoralis* larvae parasitized between one and three days after fungal infection either the first day ($F_{2, 21} = 0.46$; $P = 0.6383$), the second ($F_{2, 21} = 1.51$; $P = 0.2446$) or the third ($F_{2, 21} = 0.81$; $P = 0.4593$) (Table 1).

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

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

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

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

3.2.1 Fungal infection of host larvae before parasitism

In the first bioassay, where *S. littoralis* larvae were inoculated with *M. brunneum* prior to being exposed to the parasitoid, treatments had a significant effect on the total mortality of *S. littoralis* larvae ($F_{6, 110}$ = 13.74; $P < 0.001$), which ranged from 32.8% (when parasitism occurred 24 h after fungal inoculation) to 77.0% (when parasitism occurred 48 h after fungal inoculation) (Fig. 1). Parasitism of *S. littoralis* by *H. didymator* females was significantly affected by treatment ($F_{5, 101}$ = 17.63; $P < 0.001$) with mean values of 21.4% (when parasitism occurred 24 h after fungal inoculation) to 64.9% (control: when parasitism occurred 48 h after experiment initiation). Fungal outgrowth from *S. littoralis* cadavers was not significantly affected by treatment ($F_{3, 30}$ = 1.79; P = 0.1695), which ranged from 9.0% (when parasitism occurred 24 h after fungal inoculation) to 23.0% (control: fungal inoculation only). Mortality in the absolute negative control (no fungus + no parasitoid) was of 0% and was excluded from data analysis.

Finally, there was a significant effect of fungal treatment on the reproductive potential of the F1 generation of female parasitoids ($F_{1, 10}$ = 9.33; P = 0.01); 48.8% of *S. littoralis* larvae were parasitized by F1 female parasitoids that originated from fungus-treated hosts compared with 65.9% by F1 female parasitoids originating from control hosts that had not been treated with fungus.

3.2.2 Parasitism of host larvae before fungal infection

In the second bioassay, where *S. littoralis* larvae were offered to the parasitoid prior to fungal inoculation, the total mortality of *S. littoralis* larvae was significantly influenced by treatment ($F_{6, 28}$ = 20.46; P < 0.001) with values ranging from 10% (fungus inoculation alone at 72 h) to 60.3% (fungus inoculation 48 h after parasitism) (Fig. 1). Treatment also had a significant effect on the proportion of cadavers with fungal outgrowth ($F_{5, 24}$ = 4.12; P = 0.0076), ranging from 8.0% (fungus inoculation alone at 72 h) to 30.0% (fungus inoculation 48 h after parasitism). However, there were no significant differences in reproductive potential ($F_{3, 16}$ = 1.07; P = 0.3886), with parasitism values that ranged from 30.0% (fungus inoculation 24 h after parasitism) to 40.0% (control parasitized larvae). When averaging the results, larvae exposed to parasitoids had an average mortality caused by the fungus of 22%, while for larvae not exposed to parasitoids, the value was 15.7%. Mortality in the absolute negative control (no fungus + no parasitoid) was of 0% and was excluded from data analysis.

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

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

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

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

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

The development time of the preimaginal stages of *H. didymator* individuals developed at the expense of both fungus-treated and non-treated *S. littoralis* larvae were scored. The fungal treatment applied to *S. littoralis* larvae had no significant effect on the development time of parasitoid larvae ($F_{1,28}$ = 1.51; P = 0.223), which was of 10.1 days (*H. didymator* larvae emerging from fungus-treated *S. littoralis* larvae) and 10.7 days (*H. didymator* larvae emerging from non-treated *S. littoralis* larvae). However, the fungal treatment had a significant effect on the pupal development time of the parasitoid, causing a slight yet significant reduction ($F_{1,28}$ = 3.9; P = 0.01) in the pupal development time of *H. didymator* when *S. littoralis* larvae were inoculated with the fungus (6 day) versus the non-inoculated control (6.73 days).

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

The total haemocyte count (THC) varied significantly both with time after treatment ($F_{4,20}$ = 7.07; P = 0.0010) and by treatment ($F_{1,20}$ = 86.25; P < 0.0001) and there was a significant interaction between the two (time × treatment) ($F_{4,20}$ = 6.66; P = 0.0014). The haemolymph extracted from parasitized *S. littoralis* larvae had significantly fewer haemocytes than control larvae at 72 h ($F_{1,20}$ = 39.67; P < 0.0001), 96 ($F_{1,20}$ = 15.21; P = 0.0009) and 120 h ($F_{1,20}$ = 52.18; P < 0.0001) (Fig. 2).

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

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

4 DISCUSSION

Interactions between entomopathogenic fungi and parasitoids have been reported in many papers with mixed results. Some indicate high compatibility between these two biological control agents with no negative effects of the fungus on the parasitoid,[39] or even describe the potential to use parasitoids as vectors of entomopathogenic fungi.[40] However, other studies indicated antagonistic interactions between the two agents.[41] Despite this, the majority of investigations have shown that combined use of entomopathogenic fungi and parasitoids within IPM programmes is always effective with a suitable adaptation of release times, i.e. which agent is applied first, and the correct timing of applications.[42]

In the present study, we measured direct lethal effects and pre-mortality effects of *M. brunneum* EAMa 01/58-Su on adult *H. didymator*. We also evaluated compatibility between these two agents when used together but released at different times and in different orders. The results showed that the parasitoid was susceptible to infection following direct contact with relatively high concentrations of conidia, with a 20.7% reduction in AST compared with the control. Few studies have addressed both direct lethal effects and pre-mortality effects of entomopathogenic fungi on parasitoids. However, our results confirm those obtained by Castillo et al. who found that direct application of the fungus *Beauveria bassiana* Bals. (Vuill) (Hypocreales: Clavicipitaceae) (10^8 conidia ml^{-1}) caused a 22% reduction in adult longevity of the eulophid endoparasitoid *Phymastichus coffea* LaSalle (Hymenoptera: Eulophidae).[43] Furthermore, Matias da Silva et al. showed that adult stages of the braconid endoparasitoid, *Cotesia flavipes* Cam. (Hymenoptera: Braconidae), were susceptible to *B. bassiana* and *M. anisopliae*.[44] Similarly, our previous

work with the same strain (EAMa 01/58-Su) and the same concentration (10^8 conidia ml^{-1}) that we used in the present article, showed that the fungus caused a mortality of 21% on adults of the cosmopolitan parasitoid *Psyttalia concolor* Szepliget (Hymenoptera: Braconidae).[24]

On the other hand, we calculated the LC_{50} of *H. didymator* inoculated with the EAMa 01/58-Su strain, which was of 1.85×10^6 conidia ml^{-1}. Although our results are consistent with those obtained for this strain and other parasitoids,[24] it is shown that the susceptibility of *H. didymator* to EAMa 01/58-Su is higher than those evidenced by different insect pests.[11,20] This result is of a great importance to develop a suitable strategy for biological control as it allows to compare the susceptibility of the parasitoid with other insects. Moreover, the reproductive potential of *H. didymator* females, over three days, was not affected by direct applications of the fungus on parasitoid adults, even at high conidial concentrations (10^7 and 10^8 conidia ml^{-1}), as no significant differences in *S. littoralis* parasitization were scored during this time for any treatment (Table 1). As the most productive copulation/egg-laying period for *H. didymator* females and males is 36 h after emergence,[13] our results show that fungus-treated *H. didymator* females would have plenty of time to parasitize *S. littoralis* larvae before being killed by the fungus (≈ 4 days after treatment), even if they were inoculated as soon as they emerged. It is worth stressing that direct contact between the fungus and the parasitoid represents the worst-case scenario under field conditions and could be prevented, or at least reduced, if fungus is applied after parasitization. These results are similar to those obtained by other authors, who found that the entomopathogenic fungus *B. bassiana* did not affect the reproductive potential of the parasitoid *Tamarixia triozae* (Burks) (Hymenoptera: Eulophidae) despite reducing their life expectancy.[45] Furthermore, Labbe et al. reported that the use of commercial isolates of entomopathogenic fungi had no effect on survival rates of the parasitoid, *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae), and even increased parasitism rates.[28] Other authors have reported that prior inoculation with entomopathogenic fungi could affect fitness of the parasitoid wasp *Trybliographa rapae* Westwood (Hymenoptera: Figitidae), reducing its life expectancy but increasing its oviposition rates as an adaptation in response to the presence of the fungus.[26]

Our compatibility bioassays, in which we assayed two different strategies (inoculation before and after parasitization) to control *S. littoralis*, demonstrated high compatibility between *M. brunneum* EAMa 01/58-Su and *H. didymator* since the effect of combined use of fungus and parasitoid on total mortality of *S. littoralis* larvae was additive in both scenarios. In the first scenario (fungal inoculation before exposure to the parasitoid), the time that parasitoids were released following fungal inoculation did influence the total mortality of *S. littoralis* larvae; the lowest mortality was obtained when parasitism occurred 24 h after fungal inoculation and highest mortality was obtained when parasitism occurred 48 h after fungal inoculation. Nonetheless, the mortality caused by both the parasitoid (=parasitization) and the fungus (=fungal outgrowth) was slightly higher when applied alone than when combined with the each other, yet the total mortality was higher when applied together. Of interest, the combined use of the two agents has an additive effect in all combinations with parasitoid time releasing-dependent mortality. Many studies have indicated that the time between fungal inoculation and subsequent parasitism is an important factor affecting the likelihood of both agents successfully completing their development within the same host.[46] Emami et al. showed that, increasing the release interval for the parasitoid *Aphidius colemani* Viereck (Hymenoptera: Braconidae) after *B. bassiana* application for control of green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) reduced the number of parasitoid pupae developing and the percent emerging as adults.[47] The same result was reported by Mohammed and Hatcher, who found that when *M. persicae* treated with the fungus *Lecanicillium muscarium* Zare & Gams (Hypocreales: Cordycipitaceae) were offered to the parasitoid *A. colemani* they were less likely to be parasitized if they were offered to the parasitoid 3-4 days after fungal infection than if they were offered 1-2 days after fungal infection.[27] In our study, we think that the time between fungal infection and subsequent parasitism was not of great importance since the only day that parasitism was significantly lower (24 h), it was both in the combined treatment including fungus + parasitoid (EFP24) and in the treatment including parasitoid alone (P24). That led us to think that the most crucial factor for the parasitoid, and the reason why a lower parasitization was recorded the first day both in the EFP24 and P24 treatments, was the larval instar of the host. It has been shown that *H. didymator* only parasitizes second (L2) or third (L3) instar *S. littoralis* larvae.[13,31] Earlier and later larval stages are considered as low-quality hosts because

they provide few nutrients and have strong immune responses that prevent parasitoid development.[48] In our study, we used early L2 *S. littoralis* larvae, which are suboptimal for *H. didymator*,[48] which may explain why parasitization was higher in all treatments from the second day (48 h) onwards (72 h). On the other hand, the lack of significant differences on the larval death with fungal outgrowth in any treatment (including or not exposure to parasitoid) suggests that parasitism does not interfere with the fungus. It is worth stressing that there was a certain percentage of mortality due to unknown reasons (neither complete parasitism nor larval mortality with fungal outgrowth) in most treatments (Fig. 1). However, its relative value was very low compared with total mortality except in the treatment including inoculation alone (EF control). As stated before, mortality due to unknown reasons was not scored in the absolute negative control (no fungus + no parasitoid) in any assay or repetition, what indicates that this mortality was caused by the biocontrol agents. In fact, there are two reasons to justify that mortality: in the treatments including only parasitoid, it is likely caused by incomplete parasitism, i.e. parasitization of *S. littoralis* without a complete develop of the parasitoid larva, causing the host premature death; incomplete parasitism causing *S. littoralis* larval death after exposure to *H. didymator* females has been described and may reach high values depending on the rearing method, larval age or instar and other factors.[13] On the other hand, we think that the high mortality without fungal outgrowth in the treatment including only inoculation with the fungus is likely due to the production of entomotoxic substances by the fungus. Resquin-Romero et al. inoculated *S. littoralis* larvae by an immersion in conidial suspensions (10^8 conidia ml^{-1}) of different strains of *M. brunneum* and *B. bassiana*, using the same methodology we presented here.[11] The authors reported high rates of larval death without fungal outgrowth after inoculation with some of the strains, especially with the EAMa 01/58-Su strain, which was due to the toxins produced by the fungus.[11] Similar results were obtained by Yousef et al. when using the EAMa 01/58-Su strain against *P. concolor*, with fungal outgrowth values depending on the experimental methods.[24]

In the second scenario (exposure to the parasitoid before fungal inoculation), the time between parasitism and subsequent fungal inoculation had no effect on the total overall mortality since no significant differences were scored any day in the same treatments (Fig. 1); nonetheless total mortality in

those treatments including parasitoid was significantly higher than that scored in the treatments lacking them (EF24, EF48 and EF72). No significant differences in parasitism were scored for any day or treatment, what shows that further inoculation does not interfere with the development of *H. didymator*. However, significant differences in larvae with fungal outgrowth were scored, with its maximum value when parasitism occurred 48 hours before fungal application (PEF48). Interestingly, *S. littoralis* larval mortality due to the fungus was higher in this combined treatment than when the fungus was applied first (all treatment including fungus and parasitoid in the first scenario: EFP24, EFP48 and EFP72) or alone (EF control in the first scenario and EF24, EF48 and EF72 in the second scenario). Most studies on tritrophic interactions amongst parasitoids, their hosts, and entomopathogenic fungi, have focused on the negative effects of the fungus on parasitoid development within the same host; few studies have considered changes in the host susceptibility to the fungus after parasitoid oviposition.[46] Labbe et al. found that, in whiteflies, application of *B. bassiana* after parasitism by *E. formosa* had no effect on either the abundance of the parasitoid or parasitism rates.[28] Furthermore, Mohammed and Hatcher showed that, in *M. persicae*, application of the fungus, *L. muscarium*, 3 - 7 days after parasitism by *A. colemani* had no effect on the proportion of aphids that were parasitized.[27] It is possible that the fungus may outcompete immature parasitoids within the host, but there are no reports of the fungus invading parasitoid tissues when they are both attacking the same host;[46,49] however, neither of these studies considered the influence of the parasitoid on host susceptibility to the fungus. King and Bell have shown that the noctuid moth *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) was more susceptible to the hypocrealean fungus, *Nomuraea rileyi* (Farl.) Kepler, Rehner & Humber (Hypocreales: Clavicipitaceae), if it was already parasitized by the braconid *Microplitis croceipes* (Cresson) (Hymenoptera: Braconidae).[17] Furthermore, Powell et al. reported that *Metopolophium dirhodum* (Walker) (Hemiptera: Aphididae) aphids that had been parasitized for 2 days were more susceptible to infection by the entomophthoralean fungus *Pandora neoaphidis* Humber (Entomophthorales: Entomophthoraceae) than unparasitized ones;[49] this is similar to our results for inoculation 48h after parasitism (PEF48). We hypothesise that this may occur because parasitism reduces immunity to subsequent infection; we showed that parasitized *S. littoralis* larvae had significantly fewer haemocytes than unparasitized larvae. Since haemocytes are a very important

component of the insect immune system, a lower number of haemocytes may lead to a lower immune response.[16] Furthermore, THC in control larvae increased over the five-day observation period, particularly between days 3 and 5; this may be because larvae moult from L2 to L3 between days 3 and 5. In general, THC increases with the larval age reaching a maximum in pre-pupae[50] although THC also tends to increase prior to each moult, decrease at moulting, and then increase again.[16] Other studies have reported the same effect of parasitism on THC.[51] When comparing both scenarios, we observed that parasitism was the factor which has most contributed to *S. littoralis* total mortality. Since all *S. littoralis* larvae were offered to the parasitoid in early L2 instar in the second scenario, parasitization, and thus total mortality, was lower than in the treatments of the first scenario where larvae were offered from the second day (EFP48, EFP72, P48, P72). This finding is interesting and reinforces our idea that larval age is a crucial factor for achieving a sustainable *S. littoralis* control in the assayed conditions.

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

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

used together with *M. anisopliae* against the Mediterranean flour moth, *Anagasta kuehniella* (Zeller) (Lepidoptera: Pyralidae).[52]

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

5 CONCLUSIONS

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

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

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

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

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

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AUTHORS' CONTRIBUTIONS

EQM, HKA and MY conceived and designed the research. MY and PMF conducted the experiments. MY and PMF analyzed the data. MY, PMF and EQM wrote the manuscript. All authors read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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FIGURE CAPTIONS

Figure 1. Total percent mortality of *Spodoptera littoralis* larvae at 24- 48- and 72-h: number with *Metarhizium brunneum* fungal outgrowth + parasitized by *Hyposoter didymator* + died by an

unknown reason. Letters show statistical comparisons between treatments within each assay (A or B) (Tukey's test; α=0.05).

(A) Fungal inoculation before exposure to the parasitoid. EFP, *S. littoralis* larvae exposed to the parasitoid 24- 48- and 72 h after inoculation with the fungus. P, larvae only exposed to the parasitoid at 24- 48- 72 h after starting the experiment. EF control, larvae only inoculated with the fungus;

(B) Exposure to the parasitoid before fungal inoculation. PEF, *S. littoralis* larvae inoculated with the fungus 24- 48- 72h after exposure to the parasitoid. EF, larvae only inoculated with the fungus at 24- 48- 72h after starting the experiment. P control, larvae only exposed to the parasitoid;

(A)(B) Both assays included an absolute negative control in which *S. littoralis* larvae were treated with aqueous 0.1% Tween 80 solution. The total mortality was of 0% and was not included in the analyses.

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