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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)

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Abstract

Four *Beauveria bassiana* and three *Metarhizium brunneum* isolates were evaluated, as soil drenches, against *Spodoptera littoralis* prepupae. Treatment efficacy was determined by assessing total mortality during development from prepupae through to pupae and adults; mortality and sub-lethal effects on reproduction were also quantified for adults emerging from surviving prepupae/pupae. All isolates were pathogenic but overall mortality varied between 31.7 and 83.3% (0% for control); average survival time was 7.5–10.5 days (14.0 days for control). From 1.7–15.0% of adults emerging from surviving prepupae/pupae were deformed (0% in control). Contact with fungal suspensions as prepupae/pupae caused a significant reduction in fecundity of emerging adult females (15–58.9%), and a significant reduction in egg viability (6.8–28.4%) compared with controls. Two isolates were selected for virulence evaluation against *S. littoralis* prepupae. The LC_{50}s were 1.7×10^7 and 1.8×10^7 conidia ml^{-1} and the median survival times were 7 and 6 days for isolates EAMa 01/58-Su and EAMb 09/01-Su, respectively. Destruxin A was present in pupae developing from prepupae treated with isolates EAMa 01/58-Su (0.010 ± 0.002 µg $pupae^{-1}$) and EAMb 09/01-Su (0.015 ± 0.003 µg $pupae^{-1}$). The use of entomopathogenic fungi as soil drenches could be a key component of *S. littoralis* IPM strategies due to direct reductions in the number of soil-dwelling life stages and, also, the significant reduction in reproductive potential of surviving adults.

Introduction

The cotton leafworm, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae), is present in approximately 80 countries across Europe, Asia and Africa (EPPO, 2018). This species is highly polyphagous and feeds on more than 100 host plants, many of them of economic importance (EPPO, 2015). Female moths lay eggs on the leaves. Larvae feed on the leaves; late instars feed mainly when it is dark, moving to the base of the plant/soil surface during the day. Last instar larvae (prepupae) burrow into the soil where they construct a clay 'cell' or cocoon in which they pupate. Adults emerge during the night and have a life span of 5–10 days (Salama and Shoukry, 1972; Pinhey, 1975; CABI and EPPO, 2011). Economic losses are associated with damage to the foliage which can lead to yield losses of approximately 50% (Russell *et al.*, 1993).

Synthetic insecticides and *Bacillus thuringiensis* Berliner are the most commonly used control strategies. However, they have become ineffective due to resistance and cross-resistance (Mosallanejad and Smagghe, 2009; Siegwart *et al.*, 2015). The new regulatory framework in Europe, defined by European regulation (CE) no. 1107/2009 and Directive 2009/128/CE of the European Parliament and of the Council, highlights the use of bio-insecticides as alternatives for the control of insect pests. They are compatible with integrated pest management (IPM) practices and considered to be environmentally friendly (Lacey, 2017). Furthermore, it has been shown that EPFs can be used successfully to control a large variety of lepidopteran pests, including *S. littoralis* (Vänninen and Hokkanen, 1997; Schulte *et al.*, 2009; Quesada-Moraga *et al.*, 2013; Resquín-Romero *et al.*, 2016*a*).

Control of *S. littoralis* by EPFs has been achieved by: applying (spraying) them directly as mycoinsecticides; applying their insecticidal secondary compounds; or a combination of both (Resquín-Romero *et al.*, 2016*a*). Most recently, EPFs have been used as endophytes (Quesada-Moraga *et al.*, 2006*a*; Sahab and Sabbour, 2011; Resquín-Romero *et al.*, 2016*a*; Sánchez-Rodríguez *et al.*, 2018). To date, studies on control of *S. littoralis* have focused on

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Table 1. Fungal isolates used this study

Isolate reference	Fungal species	Origin	Ecosystem	Habitat
EABb 01/33–Su	*Beauveria bassiana*	El Bosque (Cádiz, Spain)	Traditional olive orchard	Soil
EABb 01/88–Su	*Beauveria bassiana*	Vila Velha de Ficalho (Portugal)	Sunflower crop	Soil
EABb 01/103–Su	*Beauveria bassiana*	Constantina (Sevilla, Spain)	Holm oak forest	Soil
EABb 09/07-Fil	*Beauveria bassiana*	Castilblanco de los Arroyos (Sevilla, Spain)	Holm oak forest	Plant
EAMb 09/01–Su[a] (CECT 20784)	*Metarhizium brunneum*	Castilblanco de los Arroyos (Sevilla, Spain)	Holm oak forest	Soil
EAMa 01/58–Su[a] (CECT 20764)	*Metarhizium brunneum*	Hinojosa del Duque (Córdoba, Spain)	Wheat crop	Soil
EAMb 09/03–Su	*Metarhizium brunneum*	Castilblanco de los Arroyos (Sevilla, Spain)	Holm oak forest	Soil

[a]Isolates deposited in the Spanish collection of culture types (CECT) with accession number included in parenthesis.

targeting the larvae on the plant, and ignored the soil-dwelling prepupae and pupae. However, EPSs are increasingly seen as an important crop protection tool for soil-dwelling insect pests (Jaronski, 2010). EPFs can survive in the soil by cycling and multiplying in insects or roots (Leger, 2008). As such they represent an opportunity for control of *S. littoralis* prepupae and pupae, as seen for other soil-dwelling insects (Jackson *et al.*, 2000; Quesada-Moraga *et al.*, 2006*b*). The aim of the current study was to evaluate three *Metarhizium brunneum* Petch. and four *Beauveria bassiana* Bals. (Vuill.) isolates applied as soil drenches against the soil-dwelling life stages (prepupae and pupae) of *S. littoralis*. We also evaluated the sub-lethal effects of these treatments on adults that emerged from surviving pupae. The selected isolates had shown high virulence against *S. littoralis* and other pests in previous studies (Quesada-Moraga *et al.*, 2006*a*; Ortiz-Urquiza *et al.*, 2009, 2010; Lozano-Tovar *et al.*, 2013).

Material and methods

Insects

Spodopera littoralis larvae were obtained from a stock colony held at the Department of Agricultural and Forestry Sciences of the University of Cordoba (Spain), and maintained at 26 ± 2°C, 70 ± 5% RH, in a photoperiod of 16:8 (L:D) h. Larvae were fed on artificial diet consisting of 85 g of alfalfa meal, 34 g of brewer's yeast, 32 g of wheat germ, 18 g of agar-agar (Industrias ROKO, S.A., Spain), 14 g of casein (Merck KGaA, Germany); 4.5 g of ascorbic acid (Scharlab, Spain), 1.3 g of benzoic acid (Scharlab, Spain), 1.1 g of nipagin (Guinama S.L.U., Spain), 5 ml 10% formalin (formaldehyde 37–38% w/w stabilized with methanol) (Panreac, Spain) and 800 ml of distilled water (Santiago-Álvarez, 1977).

Fungal isolates

Four isolates of *B. bassiana* and three of *M. brunneum* were used in this study (table 1). These isolates are held in the culture collection of the Department of Agricultural and Forestry Sciences (AFS) of the University of Cordoba. For experiments they were grown for 15 days on malt agar (MA) in 90 cm diameter Petri dishes at 25°C in darkness. Conidial suspensions were prepared by scraping conidia from the agar into a sterile aqueous solution of 0.1% Tween 80 and filtering through a piece of cheesecloth and vortex mixing to encourage conidia into suspension. Germination tests using the methods of Yousef *et al.* (2014) were done on MA supplemented with 500 mg l^{-1} streptomycin sulfate salt (Sigma-Aldrich Chemie, China) and used to determine conidial viability; germination always exceeded 90%. The concentration of conidia in suspension was determined using a Malassez chamber and adjusted to 1.0×10^8 conidia ml^{-1} by the addition of 0.1% Tween 80.

Pathogenicity and virulence of entomopathogenic fungi as soil drenches targeted at Spodoptera littoralis prepupae

Pathogenicity assays were done in transparent containers (80 mm × 80 mm × 55 mm) containing 30 g of sterilized substrate. The substrate was compost enriched with coconut fibre and sterilized three times in an autoclave at intervals of 24 h. Then, the substrate was sieved (2 mm mesh) and dried at 60°C for 72 h. The substrate was inoculated by adding 1.7 ml of a 1.8×10^7 conidia ml^{-1} suspension and the soil was homogenized by hand mixing, providing a concentration of 1.0×10^6 conidia g^{-1} of substrate, and a water potential of −0.47 MPa (9.0% [wt.: wt.]) (Garrido-Jurado *et al.*, 2011). Control containers were treated in the same way but inoculated with 0.1% Tween 80 without conidia. Prepupae of *S. littoralis* were released into the substrate and incubated at 26 ± 2°C, 70 ± 5% RH, in a photoperiod of 16:8 h (L:D) until adult emergence. Mortality during development from prepupae to pupae and through to adult was recorded daily for up to 14 days. Pupae that failed to emerge were surface sterilized in 1% sodium hypochlorite for 5 min, rinsed twice with sterile water, and placed under humid conditions to promote fungal outgrowth and identify cause of mortality. There were three replicates, each with ten prepupae for each isolate and the control; the entire experiment was done on two occasions.

In a second series of assays, isolates that had caused more than 50% mortality in the pathogenicity assays were selected and their virulence determined using the same application method. Four concentrations of conidia were evaluated using the same method as described previously: 1.0×10^5, 1.0×10^6, 1.0×10^7 and 1.0×10^8 conidia ml^{-1} and sterile 0.1% aqueous Tween 80 for the control. The bioassay was done at 26 ± 2°C and 70 ± 5% RH. Three replicates of ten prepupae were used for each treatment and control; the entire experiment was done on two occasions. Mortality was recorded daily for up to 10 days. Pupae that failed to emerge were surface sterilized and incubated as described previously to identify cause of mortality.

Sub-lethal effects of entomopathogenic fungi in treatments targeted at Spodoptera littoralis prepupae as soil drenches

Development of pupae that had survived until day 14 of the two pathogenicity assays was followed until they emerged as adults.

Table 2. Pathogenicity of entomopathogenic fungi as soil drenches (1.0×10^8 conidia ml^{-1}) in treatments targeted at *Spodoptera littoralis* prepupae

	Mortality (mean ± SE) (%)[a]		Kaplan–Meier survival analysis	
	Total mortality	Fungal outgrowth	AST[b] (mean ± SE)	CI (95%)
Control	0 ± 0 a	0 ± 0 a	14.0 ± 0.0 c	14.0–14.0
EABb 09/07-Su	31.7 ± 3.1 b	30.0 ± 3.7 b	8.7 ± 0.4 b	7.9–9.5
EABb 01/33-Su	51.7 ± 3.1 c	41.6 ± 4.0 bc	8.5 ± 0.3 ab	7.9–9.1
EAMa 09/03-Su	55.0 ± 4.2 c	45.0 ± 4.3 bc	10.7 ± 0.3 b	10.2–11.3
EABb 01/88-Su	55.5 ± 3.7 c	36.6 ± 4.9 b	10.4 ± 0.3 ab	9.9–10.9
EABb 01/103-Su	58.3 ± 5.4 c	46.7 ± 3.3 bc	7.5 ± 0.3 a	7.0–8.0
EAMa 01/58-Su	80.0 ± 6.8 d	58.3 ± 4.0 c	8.4 ± 0.3 ab	7. 9–8.8
EAMb 09/01-Su	83.3 ± 2.1 d	55.0 ± 2.2 c	7.5 ± 0.3 a	7.0–8.0

[a]Within the same column means with the same letter are not significantly different to each other (χ^2 test, $P \leq 0.05$) according to the generalized linear model.
[b]AST: Average survival time was limited to 14 days. Within the same column means with the same letter are not significantly different to each other ($P \leq 0.05$) according to the log-rank test.

Specifically, just prior to the onset of the scotophase on the 10th day, male and female pupae from each treatment replicate and control were individually weighed and placed, in pairs (one male and one female), into new containers. As adults emerged they were placed, in their pairs, into oviposition chambers consisting of a cylindrical filter paper (150 mm × 120mm × 10 mm), closed at both ends by a layer of filter paper. Cotton wool moistened with a 10% honey solution was placed inside as food. The chambers were observed daily and egg clusters collected, surface-sterilized by immersion in 10% formalin for 10 min, and then rinsed three times with sterile water to remove any formalin residues. The disinfected egg clusters were then placed on pieces of filter paper to remove the water. Filter papers bearing egg clusters from the same replicate were placed in plastic containers (300 mm × 200 mm × 120 mm) with perforated covers and observed daily until they hatched. The total number of eggs laid per female and the proportion that hatched was recorded for each replicate.

Determination of destruxin A in Spodoptera littoralis *pupae*

Destruxin A production in *S. littoralis* was determined for *M. brunneum* isolates EAMb 09/01-Su and EAMa 01/58-Su only. Prepupae were treated by soil drenching, as described previously for the pathogenicity assay, with the exception that they were treated individually ($n = 10$ per isolate) and not in groups. Mortality was recorded up until adult emergence. Destruxin A was extracted from all pupae following the protocol of Ríos-Moreno *et al.* (2017). Briefly, individual pupae were lyophilized and powdered in a porcelain mortar. The powder from each pupa was then mixed with 5 ml of dichloromethane: ethyl acetate (1:1 v/v), shaken for 2.5 h at 100 rpm and sonicated for 30 min before evaporation in a flow chamber. The pellet was re-suspended in 1 ml of methanol: acetonitrile (1:1 v/v) and filtered through a 0.2 µm filter. Dextruxin A content was analyzed by a research support service (SCAI) from the University of Cordoba (Spain), using an Agilent Technologies 1200-HPLC tandem mass spectrometry Q Trap AB Sciex 5500 (AB SCIEX, Darmstadt, Germany). Briefly, a Phenomenex C18 (150 mm Kinetex × 2.10 mm, 2.7 µm) column set at 35°C was used for separation, and 10 µl samples from each replicate were injected. The mobile phase consisted of 0.01% aqueous formic acid solution (solvent A) and MeOH (solvent B) at a flow of 0.25 ml min^{-1}. The eluent gradient profile was as follows: 0 min, 5% B; 15 min, 65% B; and 15.50 min, 90% B. The eluent was returned to 5% B after 0.5 min and maintained for 2 min to allow column equilibration.

Statistical analysis

Mortality data and female fertility data were analyzed using a generalized linear model with binomial distribution and logit link function. Female fecundity data were modeled using a generalized linear model with a Poisson distribution and log link function using JMP 8 software. Treatment comparisons were made using a χ^2 test ($P < 0.05$). Average survival times (ASTs) were obtained using Kaplan–Meier survivorship analysis and compared using the log-rank test calculated with IBM SPSS 25.0 software. Median lethal concentrations (LC_{50}) were estimated by probit analysis (Finney, 1971), and the median survival time (MST) were calculated using IBM SPSS 25.0 software. Destruxin A data were analyzed by ANOVA and the means were compared using the Tukey's honest significant difference (HSD) test.

Results

Pathogenicity of entomopathogenic fungi as soil drenches targeted at Spodoptera littoralis *prepupae*

Significant differences in total mortality were found between the isolates [$\chi^2(7) = 140.23$, $P < 0.001$]. Total mortality ranged between 31.7 and 83.3% for EABb 09/07-Su and EAMb 09/01-Su, respectively (table 2). Average survival times ranged between 10.7 days for EAMa 09/03-Su and 7.5 days for EABb 01/103-Su and EAMb 09/01-Su, respectively (table 2). No mortality was observed in the controls. With respect to mortality that could be directly attributed to the fungus (i.e. cadavers with fungal outgrowth), significant differences were also found between the isolates [$\chi^2(7) = 79.48$, $P < 0.001$]. Confirmed fungus-induced mortality ranged between 30 and 58.3% for EABb 09/07-Su and EAMa 01/58-Su, respectively (table 2). When we consider the life-stage at which mortality occurred we can see that 30 and 58.3% mortality due to isolates EAMb 09/07-Su and EAMa 01/58-Su occurred between the prepupal and pupal stage; mortality between the pupal and adult stage was related to deformity of

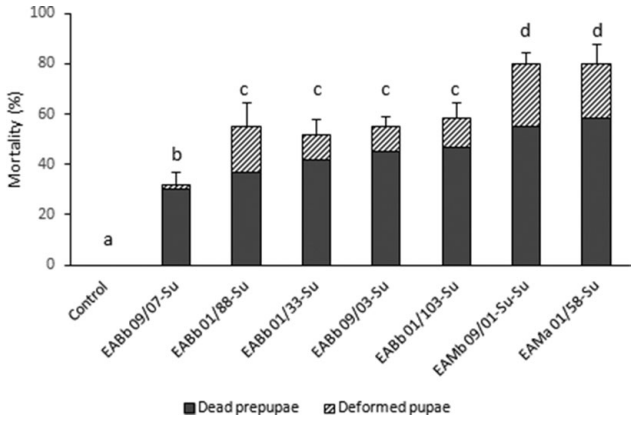

Figure 1. Relative percent mortality during development from prepupae to pupae (solid bars) and proportion (%) of deformed adults emerging (lined bars) of *Spodoptera littoralis* treated with soil drenches of fungi (1.0×10^8 conidia ml^{-1}). Within the same column means with the same letter are not significantly different to each other (χ^2 test, $P \leq 0.05$) according to the generalized linear model. Mortality limited to 14 days.

pupae and ranged between 1.7% for EAMb 09/07-Su and 25% for EAMb 09/01-Su, respectively (fig. 1).

Virulence of entomopathogenic fungi as soil drenches targeted at Spodoptera littoralis *prepupae*

Virulence (multiple dose) assays were done with isolates that attained 50% mortality in the pathogenicity assays (EABb 01/33-Su, EAMa 09/03-Su, EABb 01/88-Su, EABb 01/103-Su, EAMa 01/58-Su and EAMb 09/01-Su). However, only *M. brunneum* isolates EAMb 09/01-Su and EAMa 01/58-Su achieved 50% mortality in the virulence assay. Maximum mortalities achieved by the other isolates were: 36.7% for EABb 01/88-Su; 41.7% for EABb 01/33-Su; 45% for EAMa 09/03-Su; and 46.7% for EABb 01/103-Su. Therefore, probit analysis was only possible for isolates EAMb 09/01-Su and EAMa 01/58-Su (table 3). No χ^2 values were significant ($\alpha = 0.05$), indicating a good fit of the regression lines. Isolate EAMa 01/58-Su was the most virulent with an LC_{50} of 1.7×10^7 conidia ml^{-1} ($= 9.6 \times 10^5$ conidia $g^{-1}$of substrate), but there was no statistically significant difference between the two isolates. The shortest MST was observed for isolate EAMa 01/58-Su at 6.0 days, but it was not significantly shorter than the MST for isolate EAMb 09/01-Su, which was 7.0 days (table 3). Both regression lines had low slopes (0.3 and 0.2 for EAMb 09/01-Su and EAMa 01/58-Su, respectively) indicating only small changes in activity per unit change in conidial concentration. The relative potency and the 95% fiducial limits of isolate EAMa 01/58-Su compared with isolate EAMb 09/01-Su was 1.08 (0.1–12.4) (table 3).

Sub-lethal effects of entomopathogenic fungi on Spodoptera littoralis *prepupae when applied as soil drenches*

Fungal treatments had no significant effect on the weight of pupae [$\chi^2(7) = 0.6423$, $P = 0.9987$], but significantly affected the number of deformed adults emerging from them [$\chi^2(7) = 67.03$, $P < 0.001$] and their fecundity [$\chi^2(7) = 189.98$, $P < 0.001$] and fertility [$\chi^2(7) = 599.21$, $P < 0.001$] (table 4). Mean pupal weight ranged between 185 mg for EABb 01/33-Su and 285.3 mg for EAMa 09/03-Su, respectively. The mean weight of the control pupae was 253.1 mg. There was a significant increase in the proportion of deformed adults emerging from surviving prepupae/pupae treated with isolates EABb 01/88-Su, EABb 01/103-Su, EAMa 01/58-Su and EAMb 09/01-Su. No deformed adults emerged from control prepupae/pupae or from prepupae/pupae treated with isolates EAMa 09/03-Su, EABb 09/07-Su and EABb 01/33-Su.

Highest mean values for fecundity were 65 and 62.1 eggs per female and was achieved by adults emerging from surviving prepupae/pupae treated with isolate EABb 01/88-Su and the control, respectively; there was no statistically significant difference between these values. The lowest fecundity (25 eggs per female) was achieved by adults emerging from prepupae/pupae treated with isolate EAMb 09/01-Su and it was significantly lower than all the values achieved in the other treatments. The lowest mean egg fertility (as measured by hatch rate) was 69.4% for adults emerging from surviving prepupae/pupae treated with isolates EABb 01/88-Su and EAMb 09/01-Su. Egg fertility of adults emerging from the fungus treatments was significantly lower than for control adults (96.9%).

Determination of destruxin A in Spodoptera littoralis *pupae*

Production of destruxin A by both isolates was confirmed in infected *S. littoralis* pupae; there was no significant difference in the titer of destruxin A between the two isolates ($P = 0.29$). Despite this, EAMb 09/01-Su-infected pupae contained a 1.5-fold higher titer of destruxin A than EAMa 01/58-Su-infected pupae. The mean titer of destruxin A per infected pupa was 0.015 ± 0.003 and 0.010 ± 0.002 µg for isolates EAMb 09/01-Su and EAMa 01/58-Su, respectively.

Discussion

This is the first study to report the successful use of soil drenching with EPFs for control of the soil-dwelling stages of *S. littoralis* (prepupae, pupae and adults emerging from surviving prepupae/pupae). There has been only one other study on pathogenicity of EPF against *S. littoralis* in the soil, but in this case the prepupae and pupae were inoculated by immersion in the EPF (*Cordyceps fumosorosea*) before being placed on the soil (Hussein *et al.*, 2013). The results of our current study indicate variation in susceptibility of *S. littoralis* prepupae to the EPFs we evaluated and that this was independent of fungal genera. Greatest mortality (exceeding 80%) was achieved when *M. brunneum* isolates EAMa 01/58-Su and EAMb 09/01-Su were used as the soil drench. This figure is similar to that obtained in the aforementioned study by Hussein *et al.* (2013) who achieved 83.3 and 64.5% mortality using *C. fumosorosea* against prepupae and pupae of *S. littoralis*, respectively. However, our results are in contrast with the results of Ahmed and El-Katatny (2007) and El-Katatny (2010) who reported low/no mortality in pupae (10%) and prepupae (0%) of *S. littoralis* larvae treated with *B. bassiana* isolate IMI 386701, although they did observe higher larval (90%) and pupal mortality (80%) when spraying *B. bassiana* isolate IMI 382302 at 10^8 conidia ml^{-1}. Also, Amer *et al.* (2008) reported mortality rates of 60% (2^{nd} instar larvae of *S. littoralis*) and 55% (4^{th} instar larvae of *S. littoralis*) after exposure to filter papers sprayed with a suspension of *M. anisopliae* conidia.

The high virulence of isolates EAMa 01/58-Su and EAMb 09/01-Su against prepupae and pupae is consistent with our previous studies on these isolates with other pest species. For example, these isolates also achieved high mortality in prepupae and

Table 3. Probit analysis of the log-dose mortality response and Median Survival Time (MST) of *Spodoptera littoralis* prepupae treated with soil drenches of two isolates of *Metarhizium brunneum*

Isolate	Regression equation	Se[a]	χ^2 (2 d.f.)	LC_{50} (conidia ml^{-1})	Confidence interval (CI 95%, conidia ml^{-1})		Relative potency	MST (days)	Confidence interval (CI 95%, days)	
					Lower	Upper			Lower	Upper
EAMb 09/01–Su	$y = 0.3x + 3.2$	0.075	0.544	1.8×10^7	3.6×10^6	4.5×10^8	1	7.0	5.5	8.5
EAMa 01/58–Su	$y = 0.2x + 3.3$	0.074	0.059	1.7×10^7	3.6×10^6	4.6×10^8	1.08	6.0	4.8	7.2

[a]Slope error.

pupae of the dipteran fruit flies, *Ceratitis capitata* Wied. and *Bactrocera oleae* Rossi (Garrido-Jurado *et al.*, 2011; Yousef *et al.*, 2013). This is interesting as EPFs often have no or low efficacy against pupae of dipterans because the dipteran puparium is a barrier to penetration and outgrowth of EPFs (Kaaya and Munyinyi, 1995; De la Rosa *et al.*, 2002; Cossentine *et al.*, 2010). Lepidoptera have obtect pupae with appendages attached closely to the body and surrounded by a cocoon, while Diptera have coarctate pupae enclosed in the cuticle of the third stage larvae (Alfaro-Moreno, 2005), which may negatively affect fungal penetration.

Fungal infection represents a challenge to the insect immune system which responds by producing phenoloxidase (PO) (González-Santoyo and Córdoba-Aguilar, 2012). In *Spodoptera litura*, and in dipteran and coleopteran hosts, PO activity increases with increasing larval instar and decreases in pupae (Dorrah, 2009; Shi and Sun, 2010). PO activity decreases in larvae when fungal infection occurs (Bali and Kaur, 2013). Therefore, differences in PO activity in lepidopteran larvae and pupae can modulate susceptibility to fungal infection (Kaur *et al.*, 2011).

The current work shows that the soil drenching technique with isolates EAMa 01/58-Su and EAMb 09/01-Su (at 1.8×10^7 conidia ml^{-1}) achieved high levels of mortality in prepupae and pupae of *S. littoralis* within 6–7 days. Similar results were obtained against the rice cutworm *Spodoptera litura* Fab. pupae after soil drenching with suspensions of 2.4×10^8 conidia ml^{-1} of *M. anisopliae* (85.8% mortality) or *Lecanicillium muscarium* (79.5% mortality); this was higher than that achieved following soil drenching with 1.2×10^6 conidia ml^{-1} *B. bassiana* suspensions (50% of mortality) against 6th instar *S. litura* larvae (Anand *et al.*, 2009; Agrawal and Simon, 2017). Overall, soil drenching has great potential for control of soil-dwelling stages of pests.

The fungal treatments had no effect on pupal weight, which was similar to the study of Gosselin *et al.* (2009), following applications of spinosad against *Agrotis ipsilon* Huf. In contrast, Resquín-Romero *et al.* (2016*b*) found that the weights of *S. littoralis* larval decreased, compared with the control, when they fed on plants colonized endophytically with EPFs. Leckie *et al.* (2008) also observed a decrease in the weight of *Helicoverpa zea* larvae fed on diets supplemented with *B. bassiana*, compared with the control.

Compared with the control, there was also an increase in the proportion of deformed adult *S. littoralis* emerging (1.7–15%) in treatments receiving fungal soil drenches. However, not all isolates resulted in adult deformity. This is consistent with Hussein *et al.* (2013) who reported that 6.7% of *C. fumosorosea*- treated larvae and 32.3% of *C. fumosorosea*- treated pupae emerged as malformed adults. Malformation in adults has been described previously following exposure of 2nd and 4th instar *S. littoralis* larvae to *M. anisopliae*-sprayed filter papers (Amer *et al.*, 2008). In our study, *B. bassiana* isolates EABb 01/88-Su and EABb 01/103-Su caused the highest proportions of deformed adults (15%). Both these isolates are known to produce toxic proteins that enhance their virulence; their extracts cause temporary tetanic paralysis in *Galleria mellonela* (L.) larvae (Ortiz-Urquiza *et al.*, 2010). Tetanic paralysis occurs as a result of the membrane Ca^{2+} channels opening; it influences chitin synthesis which can result in deformities (Doucet and Retnakaran, 2016).

There was also a significant decrease (15–58.9% reduction) in fecundity of *S. littoralis* adults emerging from pupae that had survived in the fungus treatments. It has been reported previously that application of the ethyl acetate fraction of volatiles from several EPFs can result in a similar reduction in the number of eggs laid by *S. litura* adults (Moorthi *et al.*, 2015). With respect to egg fertility, all fungal treatments significantly decreased the proportion of eggs that hatched compared with the control (6.8–28.4% reduction). This is consistent with the study of Malarvannan *et al.* (2010) on *S. litura* although less extreme; they found total inhibition of egg hatch after application of a 2.4×10^7 conidia ml^{-1} suspension of *B. bassiana* to adults. Similarly, Pirali-Kheirabadi *et al.* (2007) reported a 10–90% reduction in hatch rate of *Rhipicephalus* (*Boophilus*) *annulatus* (Say) eggs treated with *M. anisopliae*, *B. bassiana* and *Lecanicillium psalliotae* isolates. Gindin *et al.* (2006) and Dembilio *et al.* (2010) found that egg hatch in *Rhynchophorus ferrugineus* (Olivier) treated with *B. bassiana* was reduced by 80–82% and 32.8%, respectively. In all these experiments fungal applications were made to adults or directly on to eggs; in our experiment the inoculum was received by the prepupae, but we still observed a significant reduction in egg hatch rate.

The destruxin A content of pupae was 0.015 ± 0.003 and 0.010 ± 0.002 μg $pupa^{-1}$ for isolates EAMb 09/01-Su and EAMa 01/58-Su, respectively. These values are low compared with the study of Ríos-Moreno *et al.* (2018), who found 0.031 μg g^{-1} in *S. littoralis* larvae. Destruxins target different components of the host, inhibiting pupal and wing development (Meng *et al.*, 2013). Interestingly, deformed pupae were only observed when isolates EABb 01/88-Su, EABb 01/103-Su, EAMa 01/58-Su and EAMb 09/01-Su were used. Isolates EABb 01/88-Su and EABb 01/103-Su are known to produce toxic proteins, as described previously, while isolates EAMa 01/58-Su and EAMb 09/01-Su are known to produce destruxin A (Ortiz-Urquiza *et al.*, 2010; Garrido-Jurado *et al.*, 2017; Ríos-Moreno *et al.*, 2017). Moorthi *et al.* (2015) also found a relationship between deformed pupae in *S. litura* (4–18%) and secretion of metabolites in *B. bassiana, Paecilomyces varioty* and *C. fumosorosea*.

In conclusion, this study reveals the potential of soil drenching with EPFs for control of *S. littoralis* prepupae in soil and the subsequent reduction in reproductive potential of the adults that

Table 4. Weight of pupae, proportion of deformed adults and sub-lethal reproductive effects on adults (fecundity and fertility) of *Spodoptera littoralis* prepupae treated with soil drenches of fungi (1.0×10^8 conidia ml^{-1})

Treatment	Weight of pupae (mean ± SE) (mg)	Effect on reproduction		Deformed adults (mean ± SE) (%)
		Fecundity (mean ± SE) (number of eggs $female^{-1}$)	Fertility (mean ± SE) (% of hatched eggs)	
Control	253.1 ± 9.6 a	62.1 ± 12.1 a	96.9 ± 1.7 a	0.0 ± 0.0 a
EABb 01/88-Su	261.2 ± 10.8 a	65.0 ± 17.9 a	69.4 ± 6.5 bd	15.0 ± 2.2 b
EAMa 09/03-Su	285.3 ± 10.1 a	52.8 ± 9.2 b	87.9 ± 3.9 ef	0.0 ± 0.0 a
EABb 01/103-Su	251.9 ± 8.6 a	45.2 ± 10.5 bc	76.6 ± 7.2 c	15.0 ± 2.2 b
EABb 09/07-Su	267.8 ± 15.0 a	38.8 ± 11.3 cd	90.3 ± 3.7 f	0.0 ± 0.0 a
EAMa 01/58-Su	218.0 ± 7.4 a	36.0 ± 8.5 cd	85.0 ± 5.5 e	1.7 ± 1.7 b
EABb 01/33-Su	185.0 ± 12.0 a	32.6 ± 5.7 d	85.1 ± 4.2 be	0.0 ± 0.0 a
EAMb 09/01-Su	204.1 ± 9.4 a	25.5 ± 1.8 e	69.4 ± 9.1 d	3.3 ± 2.1 b

Within the same column means with the same letter are not significantly different to each other (χ^2 test, $P \leq 0.05$) according to the generalized linear model. Living pupae from the abovementioned first bioassay series, three replicates each, with the entire experiment repeated, were allowed to continue their development and placed in new containers with the same number of pupae per sex. Dead adults and sub-lethal reproductive effects on live adults were measured at the end of the experiment.

emerged from surviving pupae. As the intention of this study was to evaluate the direct effects of EFP on *S. littoralis* prepupae in the soil, it was necessary to remove other biotic factors in the soil, i.e. we used an artificial sterile substrate; this may have affected the viability of the fungi. Further studies in non-sterile soil should now be done with the most promising isolates to obtain more realistic data. However, these preliminary results show that soil drenching with EPFs could contribute to the suppressive potential of the soil and has potential for use in *S. littoralis* IPM strategies that disrupt the pest life cycle. This would also contribute to reducing the number of insecticide applications made during the crop season.

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Author contributions. IGJ, GRR and EQM conceived and designed the experiments. IGJ and GRR did the experiments. IGJ analyzed the data. MY and ARM determined the destruxin A content. IGJ, GRR and EQM wrote the manuscript and all authors read and approved the manuscript.

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