Development of Eulophidae (Hymenoptera) parasitoids in *Diatraea saccharalis* (Lepidoptera: Crambidae) pupae exposed to entomopathogenic fungi

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Abstract—*Palmistichus elaeisis* Delvare and LaSalle, *Tetrastichus howardi* (Olliff), and *Trichospilus diatraeae* Cherian and Margabandhu (Hymenoptera: Eulophidae) are promising candidates for the control of sugarcane borer, *Diatraea saccharalis* (Fabricius) (Lepidoptera: Crambidae). The fungi *Beauveria bassiana* (Balsamo) Vuillemin (Cordycipitaceae) and *Metarhizium anisopliae* (Metchnikoff) Sorokin (Clavicipitaceae) also can be used to control sugarcane borers plus leafhoppers (Hemiptera: Cercopidae) in sugarcane. This observation motivated us to investigate whether entomopathogenic fungi can affect biological aspects of these parasitoids. *Diatraea saccharalis* pupae (24 hours in age) were exposed to parasitism by three females of each eulophid species for 72 hours and then placed in small tubes. A contact surface treated with 1 mL of fungal suspension was placed inside each tube with the parasitised pupae at concentrations of 1×10^9, 5×10^9, or 10×10^9 conidia mL^{-1} of *M. anisopliae* and *B. bassiana*. Exposure to fungi reduced emergence of adult *P. elaeisis*, but not of *T. howardi* and *T. diatraeae*. Life cycle duration, progeny, and sex ratio of *P. elaeisis* were not affected by exposure. Exposure was associated with decreased longevity for both sexes of *T. howardi* and in males of *P. elaeisis*, but not at levels expected to affect their performance as biocontrol agents. In general, the exposure of eulophid species developing in pupae of *D. saccharalis* exposed to entomopathogenic fungi, did not compromise the biological aspects of these parasitoids.

Introduction

Brazil is the world's largest producer of sugarcane (Kassab *et al*. 2015). The main pests affecting this crop are the sugarcane borer, *Diatraea saccharalis* (Fabricius) (Lepidoptera: Crambidae), and the leafhopper *Mahanarva fimbriolata* (Stål) (Hemiptera: Cercopidae) (Kassab *et al*. 2014; Rossoni *et al*. 2014a, 2014b). The direct damage caused by *D. saccharalis* can lead to loss of biomass and death of apical buds in sugarcane plants (Dinardo-Miranda *et al*. 2011, 2012). Indirect effects include the reduced production of sugar and alcohol, due to the presence of microorganisms in the affected stems (Simões *et al*. 2012; Rossato *et al*. 2013).

Chemical insecticides are inefficient in controlling *D. saccharalis*, because the first instars feed on the cartridge leaves and subsequently migrate to the sugarcane stalk where they are protected from topical applied insecticides (Antigo *et al*. 2013; Oliveira *et al*. 2013). This has led to research on biological methods to control of this pest (Rodrigues *et al*. 2013).

Diatraea saccharalis is attacked by a large number of micro-Hymenoptera and several species of fungi, of particular importance are the parasitoids *Palmistichus elaeisis* Delvare and LaSalle, *Tetrastichus howardi* (Olliff), and *Trichospilus diatraeae* Cherian and Margabandhu, (Hymenoptera: Eulophidae) (Cruz *et al*. 2011; Chicera *et al*. 2012; Vargas *et al*. 2014; Pereira *et al*. 2015). The entomopathogenic fungi *Beauveria bassiana* (Balsamo) Vuillemin (Cordycipitaceae) and *Metarhizium anisopliae* (Metchnikoff) Sorokin

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(Clavicipitaceae) also can provide significant levels of control. For example, *M. anisopliae* is produced in rice and applied to sugar cane fields in Brazil to reduce populations of *M. fimbriolata* (Loureiro *et al.* 2005).

Concurrent use of these fungi and parasitoids might work in a synergistic or antagonistic manner (Roy and Cottrell 2008) to enhance control *D. saccharalis*, but further research is required to clarify the nature of their interactions when co-occurring in the same host (Santos *et al.* 2006; Rossoni *et al.* 2014a, 2014b; Ibrahim 2015). This fact motivated us to investigate whether *B. bassiana* and *M. anisopliae* compromise the development of *P. elaeisis*, *T. howardi*, and *T. diatraeae* in pupae of *D. saccharalis*. Specifically, we assessed the effect of exposure to these fungi on the percentage of hosts from which parasitoids emerged, the number of parasitoids emerging per pupa, egg-to-adult development time, sex ratio, and adult longevity.

Materials and methods

Experimental work was conducted in the Biological Control of Insects (LECOBIOL) and Microbiology Laboratories of the Universidade Federal da Grande Dourados (UFGD) in Dourados, Mato Grosso do Sul, Brazil.

Source of fungi and insects

The formulations of fungi used in the experiment were the commercial products Metiê WP® (*M. anisopliae* – IBCB 425) and Ballvéria WP® (*B. bassiana* – IBCB 66) provided by the company Ballagro Agro Tecnologia (Atibaia, São Paulo, Brazil). Both formulations had more than 95% spore viability.

Diatraea saccharalis eggs were obtained from laboratory colonies maintained by LECOBIOL (UFGD). Eggs were packed into glass vials (8.5 cm diameter × 13 cm height) containing an artificial diet based on wheat germ (150 g), soybean (540 g), and the phagostimulant, sugarcane yeast (450 g) (*Saccharomyces cerevisiae* Meyen ex Hansen; Saccharomycetales: Saccaromycetaceae) to provide nutrition for the newly hatched larvae that remained *in situ* until the final instar was reached (Parra 2007). Larvae were then transferred to disposable Petri dishes (6.5 cm diameter × 2.5 cm height) with a feedback diet based on soya bean and sugarcane yeast until the formation of pupae was observed. Pupae were collected and placed in clear plastic pots (rectangular, 500 mL, 14.2 × 9.8 × 4.7 cm) where they remained until adult emergence. The adults were sexed and 50 adults (30 males and 20 females) were placed in polyvinyl-chloride-tube cages (10 cm diameter × 22 cm height), internally coated with bond paper for harvesting eggs. The cages were closed with bond paper and elastic. Eggs were collected daily, washed with a solution of copper sulfate (1%), and stored in a climatic chamber at 25 ± 2 °C, 70 ± 10% relative humidity, and 14:10 hours light:dark cycle, a methodology adapted from Parra (2007).

Populations of *P. elaeisis*, *T. howardi*, and *T. diatraeae* were maintained separately in glass tubes (2.5 cm diameter × 8.5 cm height) sealed with cotton, and fed with droplets of pure honey. To propagate these species, pupae of *D. saccharalis* (24–48 hours old) were exposed to female parasitoids. The parasitised pupae were individually placed in glass tubes and maintained at 25 ± 2 °C, 70 ± 10% relative humidity, and 14:10 light:dark hours until the emergence of adults (Vargas *et al.* 2011; Chichera *et al.* 2012).

Experimental development

For each parasitoid species, pupae of *D. saccharalis* (24 hours old) were exposed to parasitism by three females over 72 hours. Females were 24 hours in age for *T. howardi* (Pereira *et al.* 2015) and *T. diatraeae* (Pastori *et al.* 2013), and 72 hours in age for *P. elaeisis* (Andrade *et al.* 2012). These ages were selected to optimise the abundance of oocytes. After exposure, the pupae were placed in individual glass tubes (1.5 cm diameter × 10 cm height) and capped with hydrophilic cotton.

A piece of filter paper (1 × 9 cm) either untreated (control) or treated with 1 mL of fungal suspension was placed inside with the parasitised pupae each tube for 96 hours (Rossoni *et al.* 2014a, 2014b) at concentrations of 1×10^9, 5×10^9, and 10×10^9 con mL^{-1} of Metiê® (*M. anisopliae*) and Ballvéria® (*B. bassiana*). The tubes were incubated in a growth chamber (BOD) at 25 ± 2 °C, 14:10 hour light:dark cycle, and 70 ± 10% relative humidity until the emergence of parasitoids. The filter papers remained in the tubes for a period of 96 hours before removal. Thus, for each parasitoid species, data was collected for six treatments and a control, replicated 10 times (five parasitised pupae), totaling 50 pupae per treatment.

To measure the effect of fungi exposure on parasitoid fitness, data were recorded for percentage of exposed pupae with adult parasitoid emergence, the number of parasitoids emerging per pupa, and development time (the number of days from date of pupa exposure until emergence of adult parasitoids). Data also were recorded for the frequency of female progeny and the longevity of adult parasitoids. This latter assessment was measured for, 20 females and 20 males of each species of parasitoid, randomly selected for each treatment.

Statistical analyses

To test for an effect of fungi exposure, separate Scott-Knott tests (critical $P = 0.05$) were performed using percentage emergence, number of progeny, developmental time, female frequency, and adult longevity as dependent variables, with treatment as the independent variable. These tests were performed for each combination of fungi and parasitoids. Comparisons were not made across species, because differences in natural history could potentially confound the interpretation of the data.

Results

Fitness parameters for the three parasitoid species developing in pupae exposed to the different concentrations of *B. bassiana* and *M. anisopliae* are reported in Table 1.

The emergence of *T. howardi* and *T. diatraeae* from pupae was not affected ($P > 0.05$) by fungal exposure. The emergence of the parasitoid *P. elaeisis* was affected ($P = 0.01$), with values ranging from 67% to 98%. Development time, progeny, and the female frequency of *P. elaeisis* were not affected by fungal exposure ($P < 0.05$). Adult longevity values for *P. elaeisis* females did not differ among treatments, but the values for males ranged from 9.95 ± 1.01 to 14.80 ± 0.29 days.

The development time of *T. howardi* ranged from 17.54 ± 0.19 to 18.28 ± 0.18 days, which may be related to the natural history of this parasitoid. The number of progeny of *T. howardi* emerging from pupae ranged 123.80 ± 2.44 to 146.82 ± 2.84 days, the highest value being that of the control. The female frequency of *T. howardi* ranged from 0.82 ± 0.05 to 0.92 ± 0.09 for all treatments. The longevity of males and females of *T. howardi* was affected by treatment, with values ranging from 8.80 ± 0.53 to 19.95 ± 0.78 days.

The development time of *T. diatraeae* was affected by treatments ($P = 0.01$), and ranged from 17.80 ± 1.74 to 19.30 ± 0.06 days. The number of progeny of *T. diatraeae* emerging from pupae of exposed to fungal treatments ranged from 283.10 ± 2.8 to 307.66 ± 2.89. The frequency of females for *T. diatraeae* ranged from 0.90 ± 0.08 to 0.93 ± 0.19 across different fungal treatments. The longevity of *T. diatraeae* males and females did not differ among treatments.

Discussion

The negligible variations in emergence of *T. howardi* and *T. diatraeae* from hosts in contact with *B. bassiana* and *M. anisopliae* fungi suggest that the exposure of parasitised pupae to the biopesticides did not affect parasitoid fitness. This observation has been demonstrated in the parasitoids *Trichogramma galloi* Zucchi (Hymenoptera: Trichogrammatidae) in *D. saccharalis* eggs treated with isolated IPA 159E (Broglio-Micheletti *et al*. 2006) and in *Cotesia flavipes* (Cameron) (Hymenoptera: Braconidae) exposed to the microbial insecticides Biometha WP Plus® (*M. anisopliae*), Biovéria G® (*B. bassiana*), Metarril WP® (*M. anisopliae*), Boverril WP® (*B. bassiana*), and Metiê WP® (*M. anisopliae*) (Rossoni *et al*. 2014a, 2014b). The parasitoids *Psyttalia concolor* (Szépligeti) and *Psyttalia cosyrae* (Wilkinson) (Hymenoptera: Braconidae) successfully emerged from *Ceratitis capitata* (Weidemann) and *Ceratitis cosyra* (Walker) (Diptera: Tephritidae) exposed to *M. anisopliae* in treated soil at 0 and 183 days after treatment application, indicating that the fungus did not exert an adverse effect on their development (Ekesi *et al.* 2005). Similar results were observed for the parasitoid *Phradis morionellus* (Holmgren) (Hymenoptera: Ichneumonidae) developing in *Meligethes aeneus* (Fabricius) (Coleoptera: Nitidulidae); *i.e.*, *M. anisopliae* was shown to be effective in controlling the pest beetle,

Table 1. Biological aspects of Eulophidae parasitoids that emerged from pupae of *Diatraea saccharalis* (Lepidoptera: Crambidae) following exposure to various concentrations of *Beauveria bassiana* (Cordycipitaceae) and *Metarhizium anisopliae* (Clavicipitaceae).*

Treatments	Emergence	Duration of the life cycle	Progeny	Sex ratio	Longevity: Males	Longevity: Females
Palmistichus elaeisis						
Control	97.50 ± 0.79 a	21.43 ± 0.06 a	98.96 ± 0.09 a	0.92 ± 2.43 a	11.90 ± 1.21 b	12.40 ± 0.97 a
1×10^9 con mL^{-1} *Beauveria bassiana*	86.50 ± 0.60 a	22.16 ± 0.44 a	93.98 ± 0.03 a	0.87 ± 0.92 a	10.55 ± 1.19 b	12.00 ± 1.05 a
5×10^9 con mL^{-1} *Beauveria bassiana*	59.66 ± 0.26 c	21.88 ± 0.28 a	104.50 ± 0.09 a	0.92 ± 0.18 a	14.45 ± 1.12 a	13.40 ± 0.57 a
10×10^9 con mL^{-1} *Beauveria bassiana*	67.49 ± 0.57 c	21.52 ± 0.35 a	94.82 ± 0.20 a	0.89 ± 2.63 a	13.25 ± 0.16 a	13.15 ± 0.44 a
1×10^9 con mL^{-1} *Metarhizium anisopliae*	78.83 ± 0.28 b	22.70 ± 0.53 a	88.86 ± 0.05 a	0.94 ± 2.01 a	9.95 ± 1.01 b	11.20 ± 1.46 a
5×10^9 con mL^{-1} *Metarhizium anisopliae*	91.50 ± 0.59 a	21.98 ± 0.30 a	98.50 ± 0.10 a	0.94 ± 2.47 a	11.60 ± 1.57 b	13.40 ± 0.96 a
10×10^9 con mL^{-1} *Metarhizium anisopliae*	98.00 ± 0.47 a	21.98 ± 0.32 a	101.82 ± 0.15a	0.91 ± 2.31 a	14.80 ± 0.29 a	12.75 ± 0.10 a
P	0.01	ns	ns	ns	0.05	ns
CV	19.36	–	–	–	21.53	–
Tetrastichus howardi						
Control	100.00 ± 0.00 a	17.50 ± 0.04 c	146.82 ± 2.84 a	0.86 ± 0.01 b	39.00 ± 0.97 a	19.95 ± 0.78 a
1×10^9 con mL^{-1} *Beauveria bassiana*	96.00 ± 2.66 a	17.96 ± 0.12 b	137.71 ± 271 a	0.87 ± 0.02 b	23.65 ± 0.50 b	14.90 ± 0.62 b
5×10^9 con mL^{-1} *Beauveria bassiana*	100.00 ± 0.00 a	18.12 ± 0.03 b	144.30 ± 2.84 a	0.92 ± 0.09 a	31.15 ± 0.95 a	18.75 ± 0.53 a
10×10^9 con mL^{-1} *Beauveria bassiana*	100.00 ± 0.00 a	18.28 ± 0.18 b	141.46 ± 2.87 a	0.82 ± 0.05 b	13.85 ± 1.99 c	8.80 ± 0.53 b
1×10^9 con mL^{-1} *Metarhizium anisopliae*	100.00 ± 0.00 a	18.08 ± 0.20 b	127.77 ± 2.34 b	0.85 ± 0.04 b	12.95 ± 0.60 c	11.40 ± 0.46 b
5×10^9 con mL^{-1} *Metarhizium anisopliae*	100.00 ± 0.00 a	17.54 ± 0.19 c	123.80 ± 2.44 b	0.92 ± 0.07 a	11.90 ± 0.90 c	12.10 ± 0.79 b
10×10^9 con mL^{-1} *Metarhizium anisopliae*	98.00 ± 2.00 a	18.87 ± 0.18 a	139.59 ± 2.18 a	0.83 ± 0.05 b	25.00 ± 1.62 b	18.80 ± 0.39 a
P	ns	0.01	0.01	0.05	0.01	0.01
CV	–	2.71	14.82	9.24	23.28	21.52
Trichospilus diatraeae						
Control	100.00 ± 0.00 a	19.30 ± 0.06 a	299.08 ± 2.04 a	0.93 ± 0.04 a	9.60 ± 0.74 a	17.50 ± 1.90 a
1×10^9 con mL^{-1} *Beauveria bassiana*	100.00 ± 0.00 a	18.76 ± 0.02 b	283.10 ± 2.88 b	0.92 ± 0.02 a	9.90 ± 0.23 a	13.40 ± 0.95 a
5×10^9 con mL^{-1} *Beauveria bassiana*	100.00 ± 0.00 a	17.80 ± 1.74 f	284.16 ± 2.62 b	0.90 ± 0.08 b	10.50 ± 0.52 a	14.95 ± 1.90 a
10×10^9 con mL^{-1} *Beauveria bassiana*	98.00 ± 2.00 a	18.02 ± 0.05 e	301.53 ± 2.48 a	0.91 ± 0.11 b	9.05 ± 0.52 a	12.30 ± 1.50 a
1×10^9 con mL^{-1} *Metarhizium anisopliae*	100.00 ± 0.00 a	18.44 ± 0.08 c	302.54 ± 1.60 a	0.92 ± 0.06 a	8.00 ± 0.28 a	15.95 ± 0.90 a
5×10^9 con mL^{-1} *Metarhizium anisopliae*	100.00 ± 0.00 a	18.16 ± 0.97 d	307.66 ± 2.89 a	0.92 ± 0.05 a	8.45 ± 0.59 a	14.65 ± 1.97 a
10×10^9 con mL^{-1} *Metarhizium anisopliae*	100.00 ± 0.00 a	18.30 ± 0.06 d	303.18 ± 2.45 a	0.93 ± 0.19 a	9.75 ± 0.54 a	17.50 ± 1.96 a
P	ns	0.01	0.01	0.05	ns	ns
CV	–	1.2	12.21	2.01	–	–

Notes:
* Means followed by the same letter do not differ by the Scott-Knott test, with up to 5% probability.
con mL^{-1}, conidia of *B. bassiana* and/or *M. anisopliae* per mL; CV, coefficient of variation.

but did not affect the development of the parasitoid (Husberg and Hokkanen 2001).

Emergence of female *P. elaeisis* was reduced with exposure *B. bassiana* and *M. anisopliae*, but development time, number of progeny, and female frequency were not affected. A similar result was observed for *C. flavipes* following exposure to these fungi (Rossoni *et al*. 2014a, 2014b). In certain instances, parasitoids may be susceptible to infection by entomopathogenic fungi, but can still reproduce, thus, fungi and parasites can be applied simultaneously for pest control (Nielsen *et al*. 2005).

Development times of *T. howardi* and *T. diatraeae* in hosts exposed to fungi were affected, but the variation across treatments was small, *i.e.*, 1.28 days for *T. diatraeae* and 0.78 days for *T. howardi*. This suggests that the observed variation was unrelated to treatment and maybe attributed to natural history. This, observation has been reported for these parasitoids in research conducted by Vargas *et al*. (2011), Rodrigues *et al*. (2013), Costa *et al*. (2014a, 2014b), Glaeser *et al*. (2014), and Pereira *et al*. (2015). These results differ from those found in *Aphidius colemani* (Viereck) (Hymenoptera: Braconidae), which displayed a decreased development time when its host, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), was treated with a suspension of *B. bassiana* (Emami *et al*. 2013).

The number of progeny for *T. howardi* and *T. diatraeae* emerging from pupae was affected by treatment, but this maybe due to other factors. Previous research has indicated that the numbers of produced maybe related to the quantity of eggs and toxins deposited during egg laying by the female parasitoid, the immune response of the host, and host biomass (Andrade *et al*. 2010; Cusumano *et al*. 2010; Harvey *et al*. 2013). Inadequate doses of toxins injected by female parasitoid may reduce numbers of progeny emerging from the host (Cusumano *et al*. 2010; Harvey *et al*. 2013). Host hemocytes can encapsulate the eggs and larvae of parasitoids to reduce parasitoid emergence (Strand 2008; Andrade *et al*. 2010). Variations in host biomass also can affect parasitoid propagation, as shown for *T. diatraeae* in pupae of *D. saccharalis* (Glaeser 2011).

The frequency of female progeny for *P. elaeisis*, *T. howardi*, and *T. diatraeae* was not predictably affected by treatment, suggesting that host exposure to fungi did not affect sex ratio. Frequency exceeded 0.82; 0.50 is the minimum value required by quality control processes (Navarro 1998). This high female bias increases the efficiency of parasitism in field releases, because females are responsible for subsequent generations (Rodrigues *et al*. 2013; Costa *et al*. 2014a, 2014b; Barbosa *et al*. 2015). For example, a low frequency of females has been shown can to compromise the parasitism efficiency of parasitoids (Pereira *et al*. 2009a, 2009b, 2010, 2011). We therefore propose that the changes in sex ratio of Eulophidae within pupae observed in this experiment can be attributed to the availability of host resources, as reported for *T. diatraeae* in pupae of *D. saccharalis* and *Tenebrio molitor* Linnaeus (Coleoptera: Tenebrionidae) (Chichera *et al*. 2012; Favero *et al*. 2013, 2014), *P. elaeisis* in pupae of *Anticarsia gemmatalis* Hübner (Lepidoptera: Erebidae), *Bombyx mori* Linnaeus (Lepidoptera: Bombycidae), and *D. saccharalis* (Pereira *et al*. 2009a, 2009b, 2010, 2013; Chichera *et al*. 2012), and *T. howardi* in pupae of *D. saccharalis* (Vargas *et al*. 2011; Costa *et al*. 2014a; Pereira *et al*. 2015) and *Erinnyis ello* (Linnaeus) (Lepidoptera: Sphingidae) (Barbosa *et al*. 2015).

The longevity of females of *P. elaeisis* and *T. diatraeae* emerging from pupae was not affected by treatment. However, a decreased longevity was observed in both sexes of *T. howardi* and in males of *P. elaeisis*. It has previously been suggested that this biological characteristic correlates positively with increased parasitism in field conditions (Pratissoli *et al*. 2007). Thereby, a greater longevity can increase the search and parasitism capabilities of parasitoid females, contributing to the reduction of the pest population and an increased effectiveness in controlling outbreaks. However, we believe that the reduction of this biological feature in *T. howardi* and *P. elaeisis* was insufficient do compromise the propagation and parasitism of these parasitoids.

In general, the exposure of pupae parasitised by *P. elaeisis*, *T. howardi*, and *T. diatraeae* to entomopathogenic fungi did not affect the development or biological characteristics of these parasitoids. This observation is important, given that, entomopathogens can come into contact with pupae parasitised by these species under field conditions. Because, these biological characteristics (emergence, duration of life cycle, progeny, and sex ratio) are correlated with the biological qualities of parasitoids, we propose that use of these parasitoid species is compatible with the entomopathogenic fungi *B. bassiana* and *M. anisopliae*.

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