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Pathogenicity of *Beauveria bassiana* and *Metarhizium anisopliae* to *Anastrepha fraterculus* (Diptera: Tephritidae) and Effects on Adult Longevity

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Abstract

Anastrepha fraterculus (Diptera: Tephritidae) is among the most important fruit pests in South America, and the use of entomopathogenic fungi is considered a promising alternative for its control. The objective of this work was to evaluate the pathogenicity of *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin on larvae and pupae of *A. fraterculus*, along with fungal effects on adult fly longevity. Fungal inoculations, fly larvae or pupae were placed in Petri dishes with 1 mL/plate, and the concentrations of 10, 15, 20 and 25 grams of commercial product/liter of water. Controls received water only. To evaluate the residual effect on adult flies, emerging adults were transferred to clean arenas and the adult longevity was monitored. *Beauveria bassiana* and *M. anisopliae* caused 93.3 and 96.7% larval mortality and 14.0 and 15.0% pupal mortality, respectively. The estimated LC50 and LC90 values were 22.56 and 40.87 g/L for *B. bassiana*, and of 23.45 and 42.02 g/L for *M. anisopliae*. Infected adult insects had shorter longevity than non-infected insects, with mean survival of 8.0 and 83.5 days for *B. bassiana* and *M. anisopliae*, respectively.

Keywords: biological control, biological insecticides, mycoinseticides, fruit fly

1. Introduction

The fruit fly *Anastrepha fraterculus* (Wiedemann 1830) (Diptera: Tephritidae) is among the most important fruit fly pests in South America, infesting more than 100 species of both native and exotic plants (Hendrichs, Vera, De Meyer, & Clarke, 2015; Zucchi, 2017). The fly damage can both direct, by the female during oviposition and larval development in the fruit, and indirect, due to penetration of secondary pathogens through oviposition injuries (Zart, Fernandes, & Botton, 2010). Among the fruit fly control techniques, the use of chemical insecticides is the most frequent, however, due to high toxicity these products are less desirable, long no-entry periods and low selectivity toward natural enemies (Garcia, Brida, Martins, Abeijon, & Lutinski, 2017).

An alternative control for these pests is the use of biological organisms which are efficient, have low environmental impacts and can be combined with other control techniques in an Integrated Pest Management Program (Lenteren, Bolckmans, Kohl, Ravensberg, & Urbaneja, 2017).

Entomopathogenic fungi stand out as a control alternative due to easy application, efficient pest control in the short time, and safety toward man and the environment (Sinha, Choudhary, & Kumari, 2016). Among the most commonly used fungi in pest control are *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin (Ascomycota: Hypocreales) which can affect the different stages of the pest development (Butt, Coates, Dubovskiy, & Ratcliffe, 2016).

When in contact with the host, the fungal spores adhere to the surface of the cuticle, germinate and produce specialized structures (appressoria) that allow penetration of the integument through a combination of enzymes and mechanical forces (Ortiz-Urquiza & Keyhani, 2013). After penetration, fungal hyphae invade and proliferate in the host hemolymph, leading to insect mortality (Mora, Castilho, & Fraga, 2016).

The action of entomopathogenic fungi on Tephritidae fruit flies has been reported for species of economic importance, and fungal efficiency was demonstrated for different phases of the insect's life cycle (Garcia et al., 2017). *Metarhizum anisopliae* caused 86.0% mortality in immature stages of *Anastrepha fraterculus* (Destéfano, Bechara, Messias, & Piedrabuena, 2005) and *B. bassiana* caused 85.0% mortality in *Anastrepha ludens* (Loew) (Sánchez-Roblero, Huerta-Palacios, Valle, Gómez, & Toledo, 2012). High pathogenicity was also observed in larvae of *Ceratitis capitata* (Wied.), where *B. bassiana* and *M. anisopliae* had 73.8% and 96.6% of mortality (Khlaywi, Khundhair, Alrubeai, Shbar, & Hadi, 2014). These fungi also caused 100% mortality of *Bactrocera zonata* (Saunders) adults at the concentration of 3×10^8 conidia/mL (Gul, Freed, Akmal, & Malik, 2015). *Beauveria bassiana* caused 80.0% mortality in *Rhagoletis indifferens* (Curran) larvae and 20.0% in pupae at a dose of 1×10^8 conidia/gram of soil (Cossentine, Thistlewood, Goettel, & Jaronski, 2010).

In view of the damage that *A. fraterculus* causes to many economically important fruit species and the potential efficiency of entomopathogenic fungi as part of an Integrated Pest Manegement (IPM) approach, the present study aimed to evaluate the pathogenicity of *B. bassiana* and *M. anisopliae* on larvae and pupae of *A. fraterculus* and lasting effect on adult longevity.

2. Method

Insect populations: The experiments were carried out at the Laboratório de Ecologia de Insetos of the Federal University of Pelotas-UFPel, in the district of Capão do Leão, Rio Grande do Sul, Brazil. The larvae and pupae of *A. fraterculus* were obtained from the creation of insects from the Laboratory of Insect Biology-UFPel, kept at 25±1 ºC, and 70±10% relative humidity with 12 h photophase. The insects were transferred to Petri dishes (10 cm in diameter x 1.5 cm in height) containing moist cotton and artificial diet based on refined sugar, wheat germ and beer yeast (3:1:1) (Bionis® BIONIS ® YE MF and NS), according to the methodology Salles (1992) modified by Nunes et al. (2013).

Fungal isolates: The commercial *B. bassiana* (BOVERIL® WP PL63) and *M. anisopliae* (METARRIL® WP E9) wettable powder products were obtained from Koopert Biological Systems. Conidial viability tests were carried out at the Laboratório de Micologia of the Federal University of Pelotas-UFPel, using the microculture technique, adapted from França, Marques, Torres, and Oliveira (2006). The fungal products were diluted to the concentration of 1×10^4 conidia/mL of distilled water in concentration and 100 µL of the suspensions were inoculated on potato agar dextrose medium (PDA) in 9-cm Petri dishes. The Petri dishes were incubated at 25±1 °C, with 70±10% RH, in the dark until colony forming units (CFUs) were sufficiently grown to be counted. This procedure was repeated daily for four days for each isolate used.

Pathogenicity and lethal concentration (LC_{50} and LC_{90}): Experiments were conducted in a complete randomized design with eight treatments and a control, all applied in 10 replicates, using fungal isolates following methodology by F. Q. Oliveira, Batista, Malaquias, Almeida, and R. Oliveira (2010). The commercial products were weighed in aliquots representing the concentrations recommended by the manufacturer for field application (10, 15, 20 and 25 grams of product/liter of water or 5.0×10^6, 7.5×10^6, 10.0×10^6 and 12.5×10^6 conidia/mL respectively). Products were transferred to flasks with 1 L of sterile water and agitated in vortex agitators until complete dilution. From the initial solution, aliquots of 17 µL were removed (recommended by the manufacturer) and were diluted in 1 mL of water (distilled and sterile) and inoculated into Petri dishes (9 cm) coated with two sterilized sheets of filter paper disks (autoclave at 1 atm and 121 °C for 30 minutes). The control consisted of 1 mL sterile distilled water per container applied in similar manner as the fungal treatments. After inoculation, 10 *A. fraterculus* larvae and/or pupae were inserted separately in each Petri dishes. Larvae (13-day old), corresponding to the stage in which the larva leaves the fruit towards the soil, and pupae with fully formed integument (1 day after pupation) were used in the experiments. The Petri dishes were sealed with PVC film and stored in incubator (25±1 °C, 70±10% RH, and complete darkness). Third instar larva and pupa mortality were evaluated daily until the complete emergence of the control adults (12 to 15 days).

In order to confirm mortality due to fungal infection, dead insects and non-viable pupae were removed from the inoculation Petri dish and were sterilized with sodium hypochlorite (1.0%), then alcohol (70.0%) followed by wash in sterile distilled water (Quesada-Moraga, Martin-Carballo, Garrido-Jurado, & Santiago-Alvarez, 2008). After asepsis, the insects were incubated in Petri dishes (9 cm) lined with two sheets of moist sterile filter paper (1 ml sterile distilled water). The Petri dishes with the insects were capped and sealed with plastic film and stored in incubator set at 25±1 °C, RH of 70±10%, and lights off to allow fungal development on the insect cadaver.

Sublethal effect on adult insects: The experiment was carried out in a completely randomized design with three treatments and 10 replicates. The *B. bassiana* and *M. anisopliae* strains used previously were prepared in suspensions with 12.5 × 10^6 conidia/mL, diluted in sterile distilled water and agitated in a vortex agitator to homogenize the solution. Aliquots of 17 µL were withdrawn from the initial solution, diluted again in one mL sterile distilled water and applied to Petri dishes (9 cm) containing two sheets of sterile filter paper. For the control, 1 mL of sterile distilled water (without the fungal isolate) was used per replicate (Oliveira et al., 2010).

After the inoculation, the larvae were transferred to Petri dishes, which were sealed with plastic film and stored in an incubator at 25±1 ºC and RH 70±10%, in the dark until adult emergence. The adults were transferred into transparent plastic cages (500 mL), that was covered with netting to allow ventilation and gas exchange. The insects were separated according to the treatment, repetition and date of emergence, and kept in an incubator at 25±1 ºC and RH 70±10% and 12h photophase. The insects were fed an artificial diet containing wheat germ, yeast and sugar (Salles, 1992) adapted by Nunes et al. (2013). Water was provided in moistened hydrophilic cotton placed in a Petri dish (2 mL/dish).

Evaluations were performed daily until all adult insects were dead. To confirm death by fungal infection, the dead adults were removed from the cages, sterilized in sodium hypochlorite (1.0%), alcohol (70.0%) and sterile distilled water, and incubated in Petri dishes with two sheets of sterile filter paper and 1 mL of sterile distilled water, which were capped and sealed with plastic film, then stored in a dark incubator at a temperature of 25±1 °C and RH 70±10%. Petri dishes with the insects remained in the incubator for 20 days or less if they showed symptoms of infection, following methods of Destéfano et al. (2005).

Statistical analyses: Mean mortalities were compared separately for larvae, pupae and adults in each treatment using ANOVA analysis, mean separation with Tukey's test, and a 5% confidence interval, using the program Sisvar 5.6 (Ferreira, 2011). The LC_{50} and LC_{90} values and the respective confidence intervals (95% CI) were obtained by Probit analysis using statistical software Statistica 13.0 (Statsoft 2013). To evaluate the effect of treatments on longevity of adult insects, the Kaplan-Meier analysis was performed through SPSS Statistics 22.0 (IBM SPSS Inc 2013) with a 5% confidence interval.

3. Results and Discussion

Both fungal isolates had high conidial viability, with a mean of 86.25% for *B. bassiana* and 89.75% for *M. anisopliae*, and germination between the third and the sixth day after inoculation (Figure 1). The *B. bassiana* (BOVERIL® WP PL63) and *M. anisopliae* (METARRIL® WP E9) isolates showed high conidial viability and significant mortality of *A. fraterculus* pupae and adults. These results were similar to previous work with insects of the genus *Anastrepha*, when *M. anisopliae* caused 98.7% mortality in *A. ludens* (Loew) larvae (concentration 4.8 × 10^5 conidia/mL) (Lezama-Gutiérrez et al., 2000), 86.0% in *A. fraterculus* immatures (2.52 × 10^{10} conidia/gram of soil) (Destéfano et al., 2005), and 66.0% in *Anastrepha obliqua* (Macquart) adults (1 × 10^7 conidia/mL) (Osorio-Fajardo & Canal, 2011).

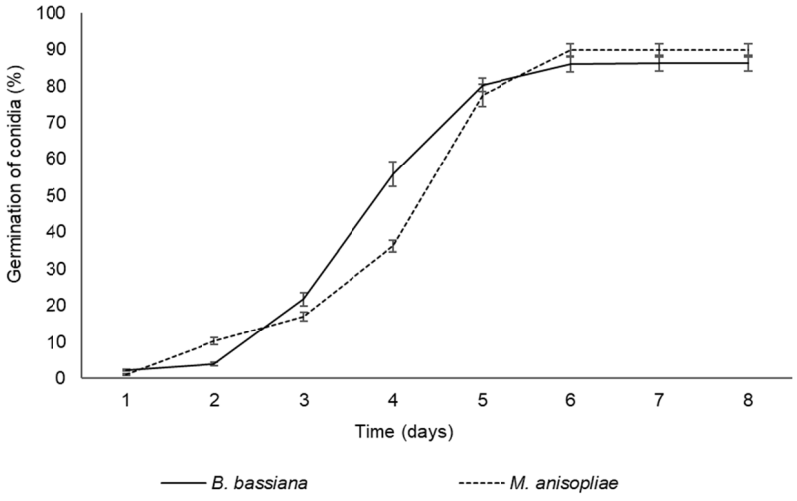

Figure 1. *Beauveria bassiana* (Balsamo) and *Metarhizium anisopliae* (Metschnikoff) conidial germination (%) of over time (days)

Pathogenicity test: All conidial concentrations of both isolates showed pathogenicity to *A. fraterculus*, with some differences seen as a function of the insect stage of development. In the highest concentration for inoculations to the larval stage, *B. bassiana* and *M. anisopliae* caused total mortality of up to 93.3% (±14.05) and 96.7% (±14.05) of insects, respectively (Table 1). Inoculations at the larval stage resulted in low larval mortality, with 10.0% (±16.09) for *B. bassiana* and 6.7% (±10.50) for *M. anisopliae*, which did not differ from the control (P = 0.600) (Table 1). Despite being infected, the insects advanced to later stages, in which the most mortality occurred. In pupae, *B. bassiana* caused 56.7% (±27.40) and *M. anisopliae* caused 53.3% (±23.30) mortality, and both treatments differed from the control (Table 1). In adults, the mortality was 36.7% (±18.90) with *B. bassiana* and 43.0% (±22.50) with *M. anisopliae*, both significantly different from the control (Table 1).

In inoculations carried out at the pupae stage, the higher mortality was 14.0% (±6.99) with *B. bassiana,* and 15.0% (±6.99) for *M. anisopliae*. Pupal inoculations did not cause infections to the insects at later stages of development (adult) (Table 2).

Our research tested *B. bassiana* against third instar larvae of *A. fraterculus*, and this isolate caused 93.3% mortality with application of 12.5×10^6 conidia/mL, which issimilar to the 96.7% mortality caused by *M. anisopliae* at the same dose. The high efficiency of these entomopathogenic fungi, aided by specific proteases produced during the infection process, which target the proteins in the insect cuticle and facilitate the fungal penetration into the insect body (Sinha et al., 2016). For tephritids, the action of these fungal metabolites was observed in *C. capitata* (Boudjelida & Soltani, 2011), with reduction and destruction of the cuticular proteins by *M. anisopliae* resulting in insect death.

Table 1. Confirmed mortality [mean ± standard deviation (SD)] in different developmental stages of *Anastrepha fraterculus* after treatment with fungal isolates *Beauveria bassiana* (Balsamo) and *Metarhizium anisopliae* (Metschnikoff) in the larval stage

Treatment Grams/L (Conidia/mL)	Total (Mort.±SD)	Larvae (Mort.±SD)	Pupae (Mort.±SD)	Adults (Mort.±SD)
Beauveria bassiana (Balsamo)				
10 (5.00×10^6)	50.0±17.6b	3.3±10.5a	30.0±10.53b	16.7±17.56b
15 (7.50×10^6)	63.3±24.59bc	6.7±14.05a	33.3±22.2b	23.3±27.43b
20 (10.00×10^6)	76.3±16.10cd	3.0±10.5a	36.6±10.5bc	36.7±18.91b
25 (12.50×10^6)	93.3±14.05d	10.0±16.09a	56.7±27.4c	26.6±21.07b
Control	0.0±0.00a	0.0±0.00a	0.0±0.00a	0.0±0.00a
F (df)	10.27 (3)	0.60 (3)	3.91 (3)	1.49 (3)
Value P	0.0001	0.619	0.0163	0.2336
Metarhizium anisopliae (Metschnikoff)				
10 (5.00×10^6)	50.0±17.56b	0.0±0.00a	20.0±17.2b	30.0±10.53b
15 (7.50×10^6)	86.4±17.21c	0.0±0.00a	43.4±23.56bc	43.0±22.49b
20 (10.00×10^6)	56.6±14.05b	3.2±14.05a	33.4±22.2bc	20.0±17.2b
25 (12.50×10^6)	96.7±14.05c	6.7±10.5a	53.3±23.3c	36.7±24.5b
Control	0.00±0.00a	0.0±0.00a	0.0±0.00a	0.0±0.00a
F (df)	17.29 (3)	1.320 (3)	4.409 (3)	2.610 (3)
Value P	0.0001	0.2829	0.0097	0.0664

Note. The means for the same fungus followed by different letters within the same column are significantly different (P < 0.05). Mort., Mortality; SD, Standard Deviation.

Table 2. Confirmed mortality [mean ± standard deviation (SD)] in different developmental stages of *Anastrepha fraterculus* exposed to treatments with fungal isolates *Beauveria bassiana* (Balsamo) and *Metarhizium anisopliae* (Metschnikoff) in the pupal stage

Treatment Grams/L (Conídia/mL)	*B. bassiana*		*M. anisopliae*	
	Pupae (Mort.±SD)	Adults (Mort.±SD)	Pupae (Mort.±SD)	Adults (Mort.±SD)
10 (5.00×10^6)	5.0±5.27ab	0.0±0.00a	7.0±4.83ab	0.0±0.00a
15 (7.50×10^6)	14.0±6.99c	0.0±0.00a	13.0±6.74b	0.±0.00a
20 (10.00×10^6)	10.0±4.71bc	0.0±0.00a	15.0±6.99b	0.0±0.00a
25 (12.50×10^6)	11.0±5.67bc	0.0±0.00a	11.0±7.37b	0.0±0.00a
Control	0.0±0.00a	0.0±0.00a	0.0±0.00a	0.0±0.00a
F (df)	4.271 (3)	0.00 (0)	2.692 (3)	0.00 (0)
Value P	0.111	0.00	0.606	0.00

Note. The means for the same fungus followed by different letters of the same column are significantly different ($P < 0.05$). Mort., Mortality; SD, Standard Deviation.

Lethal concentrations (LC_{50} and LC_{90}): The LC_{50} and LC_{90} values for *B. bassiana* and *M. anisiopliae* did not differ significantly between the isolates, but there were significant differences between the developmental stages of the insect. After larval inoculations, the insects progressed to the pupal stage, and this stage was affected, with lower LC_{50} and LC_{90} (22.56 g/L and 40.87 g/L for *B. bassiana*, and 23.45 gr/L and 42.02 gr/L for *M. anisopliae*, respectively (Table 3). For inoculations at the pupal stage, higher product concentrations were required, with LC_{50}'s at 95.73 g/L and 83.13 g/L for *B. bassiana* and LC_{90}'s at 175.15 g/L and 150.90 g/L for *M. anisopliae* (Table 4).

Table 3. Lethal concentrations (LC_{50} and LC_{90}) (grams/liter) (confidence intervals) of commercial fungal isolates of *Beauveria bassiana* and *Metarhizium anisopliae* to larvae, pupae and adults of *Anastrepha fraterculus* after treatments in the larval stage of the insect

		LC_{50} gr/L (95%FL)	F (df)/P
Larvae	*B. bassiana*	135.8 (105.42-166.29)b	85.6 (1.8)/0.000015
	M. anisopliae	122.2 (48.04-196.33)b	11.436 (1.8)/0.009769
Pupae	*B. bassiana*	22.6 (20.80-24.33)a	248.1 (1.8)/<0.001
	M. anisopliae	23.5 (21.41-25.48)a	206.3 (1.8)/<0.001
Adults	*B. bassiana*	35.0(31.21-38.70)ab	193.7 (1.8)/<0.001
	M. anisopliae	32.5 (30.37-34.56)ab	500.8 (1.8)/<0.001
		LC_{90} gr/L (95%FL)	F (df)/P
Larvae	*B. bassiana*	243.2 (186.04-300.40)b	85.6 (1.8)/0.000015
	M. anisopliae	212.5 (76.59-348.11)b	11.4 (1.8)/0.009769
Pupae	*B. bassiana*	40.9 (36.75-45.00)a	248.1 (1.8)/<0.001
	M. anisopliae	42.0 (37.32– 46.72)a	206.3 (1.8)/<0.001
Adults	*B. bassiana*	63.5 (55.25-71.92)ab	193.7 (1.8)/<0.001
	M. anisopliae	63.1 (57.99-68.27)ab	500.8 (1.8)/<0.001

Note. The means followed by different letters within the same column are significantly different ($P < 0.05$).

Table 4. Lethal concentrations (LC_{50} and LC_{90}) (grams/liter) (confidence intervals) of commercial fungal isolates of *Beauveria bassiana* and *Metarhizium anisopliae* to larvae, pupae and adults of *Anastrepha fraterculus* after treatments in the pupal stage of the insect

		LC_{50} gr/L(95%FL)	F (df)/P
Pupae	*B. bassiana*	95.7 (80.67-110.79)a	158.9 (1.8)/0.000001
	M. anisopliae	83.1 (70.56-95.69)a	163.3 (1.8)/0.000001
		LC_{90} gr/L(95%FL)	F (df)/P
Pupae	*B. bassiana*	175.1 (144.29-202.83)a	158.3 (1.8)/0.000001
	M. anisopliae	150.9 (126.15-175.65)a	163.3 (1.8)/0.000001

Note. The means followed by different letters within the same column are significantly different ($P < 0.05$).

Sublethal effect on adult insects: When inoculated in the larval phase of the insect, *B. bassiana* and *M. anisopliae* isolates had effects on adults, reducing their longevity (Figure 2). The mean longevity of adults infected with *B. bassiana* was 8.1 (±4.50) days and *M. anisopliae* was 7.80 (±4.61) days, differing significantly from the control (F: 131.68; df: 2; P: < 0.001). Untreated adults had a longevity of 71.1 (±17.79) and 75.7 (±31.81) days, which was not significantly different from the control insects, with 83.5 (±19.79) days (F: 0.68; df: 2; P: 0.51) (Figure 2).

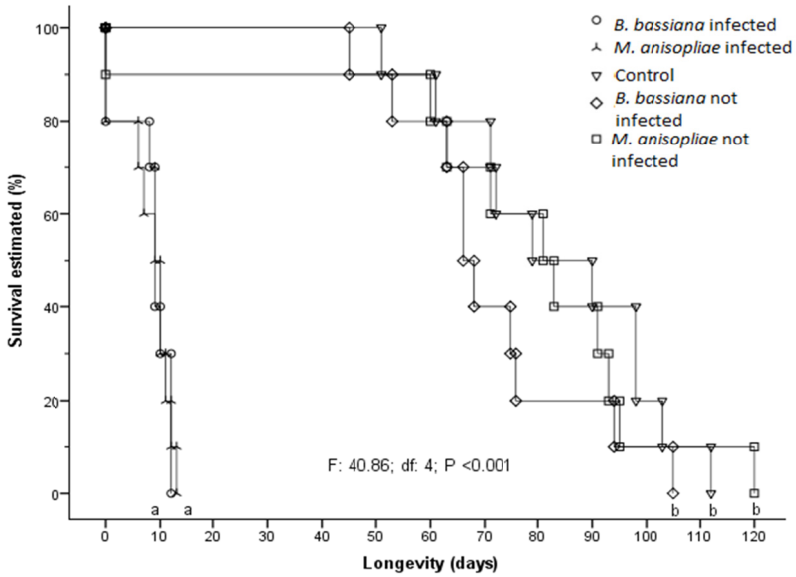

Figure 2. Longevity of adult *Anastrepha fraterculus* (days) inoculated or not with *Beauveria bassiana* (Balsamo) or *Metarhizium anisopliae* (Metschnikoff) during the larval stage. Curves identified by different letters, differ significantly from each other ($P < 0.05$)

The LC_{50} and LC_{90} values were more consistent when the fungi were inoculated at the *A. fraterculus* larval stage. The high efficiency of treatments applied to larvae is due to thinner and weaker integument, and the high mobility of the insects which result in greater contact with a larger number of conidia (Cossentine et al., 2010). However, the infected insect is able to pass from the larval to the pupal stage. Because the process of conidia adhesion, germination and tegument penetration lasts about 30 hours, that is sufficient time for the insect to go through the remaining of the larval stage (Mora et al., 2016; Bechara, Destéfano, Bresil, & Messias, 2011) and pupate. Third instar *A. fraterculus* larvae infected with *M. anisopliae* are able to pupate, but the insect dies when the pathogen develops sufficiently during the pupal or adult phase of the insect.

Pupae of *A. fraterculus* showed low susceptibility when exposed to fungal isolates, with only 14.0% mortality when exposed to *B. bassiana* (7.5×10^6 conidia/mL) and 15.0% for *M. anisopliae* (10.0×10^6 conidia/mL). The reduced mortality is due to sclerosis of the pupal cuticle, which hinders the penetration of the pathogen (Cossentine et al., 2010; Wilson et al., 2017).

After application to the larval stage, *B. bassiana* (12.5×10^6 conidia/mL) caused 36.7% mortality in adult insects and *M. anisopliae* (7.5×10^6 conidia/mL) caused 43.0% mortality of adults. These pathogens usually cause host death in the larval or pupal stages, about 24 to 96 hours after conidial adhesion to the insect cuticle (Sinha et al., 2016; Garcia et al., 2017). However, some insects may survive to adulthood even when infected, perhaps due to a strong immune system, which produces antimicrobial peptides and phenoloxidases, or encapsulates the hyphae, reducing or preventing fungal development and the synthesis of toxins (Dubovskiy et al., 2013; Lu & Leger, 2016). In addition, hemolymph pressure, pH and temperature in insect hemocele may contribute to delayed pathogen development, resulting in insect mortality only after adult emergence (Meyling & Eilenberg, 2007; Ortiz-Urquiza & Keyhani, 2013).

Adult *A. fraterculus* that developed from inoculated pupae did not show fungal growth; however, pupal mortality by pathogen action was observed. The absence of infected adults from pupal inoculations may be due to the rapid kill during the pupal phase due to toxic metabolites secreted by fungi soon after pupal cuticle penetration (Sinha et al., 2016). The sublethal effect of fungi on adult insects from inoculations of immature stages has been reported previously (Bechara et al., 2011; Bissoli, Correia, & Barbosa, 2014). However, with the present study, we observed 20.0% mortality on the first day after the adult emergence, reaching 50.0% mortality by the sixth day, and total mortality by the 12th day. The mortality of adult insects in this period prevents damage to fruits because it occurs before the end of the preoviposition period (7 and 14 days after adult emergence) (Zart et al., 2010).

4. Conclusions

Beauveria bassiana and *M. anisopliae* showed high efficiency in the control of *A. fraterculus*, with lower lethal concentrations (LC_{50} and LC_{90}) in third instar larvae inoculations.

The pathogenicity of *B. bassiana* to *A. fraterculus* larvae was evaluated for the first time, showing promising effects similar to those by the fungus *M. anisopliae*.

The sublethal effect of these entomopathogenic fungi on adult insects after conidial applications to the larvae reduced adult longevity. Additional field work is needed to verify the efficacy of similar treatments in the field.

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