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Effect of entomopathogenic fungi on the control of the coffee berry borer in the laboratory

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ABSTRACT
Among the management and control tactics of the coffee berry borer *Hypothenemus hampei* (Ferrari), there is the use of entomopathogenic fungi. Due to the importance of prospecting isolates of entomopathogenic fungi for the control of the coffee berry borer, the objective of this study was to evaluate the efficiency of 26 isolates of entomopathogenic fungi in the control of this insect pest in the laboratory. The coffee berry borers were immersed in a solution adjusted to the concentration of 1 to 3 × 10^8 conidia/mL of each isolate and the control treatment (sterilized water). After seven days total mortality and confirmed mortality were evaluated. The isolates that caused the highest mortality and two commercial isolates were selected for evaluation of lethal concentration (LC_{50} and LC_{90}) and lethal time (LT_{50} and LT_{90}). Coffee berry borers were treated at different conidia concentrations for lethal concentration to assess total and confirmed mortality. For a lethal time, the coffee berry borers were treated at the concentration of 108 conidia/mL of the selected isolates, and, after two days and every 24 hours until the eighth day, the number of dead individuals was verified. Among the 26 isolates evaluated, 24 presented mortality higher than the control treatment, and three presented mortality higher than 85%. In the LC_{50} and LC_{90} assays, the IBCB 353 and IBCB 364 isolates were more lethal to *H. hampei*. In the LT_{50} and LT_{90} assays, the IBCB 66 and IBCB 353 isolates caused lethality in a shorter time.

Keywords: bioassay; biological control; Hypocreales; neotropical fungi.

INTRODUCTION

The coffee culture, *Coffea arabica* L. and *Coffea canephora* Pierre, is of great importance worldwide. In addition, this beverage is the most consumed in the world. Among the difficulties faced by coffee growers, is the sustainable control of pests and diseases to provide a quality product to consumers. Among the main pests in this crop, is the coffee berry borer *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae, Scolytinae). This insect pest requires the adoption of monitoring and control tactics since it causes quantitative and qualitative damage to coffee (JOHNSON et al. 2020; SOUZA, 2019).

Adult females of *H. hampei* initiate injury to the coffee fruit when they pierce the crown and build a gallery in the endosperm, in which eggs are deposited. After hatching, the larvae feed on the seed, reducing productivity (VEGA et al., 2011). Therefore, the intensity of infestations caused by this insect causes damage to coffee growers. These damages can be quantitative, due to premature fruit drop and weight loss, due to the insect's feeding, or qualitative, due to the change in the type of grain according to the number of defects and the quality of the drink, which can be altered by the presence of microorganisms (MATIELLO, 2008).

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MATIELLO (2008) evaluated quantitative and qualitative damage caused by *H. hampei* in three naturally infested conilon coffee plantations. The quantitative losses of coffee mass in coconut ranged from 0.64 to 11%, and processed coffee mass ranged from 0.99 to 22.73%. The qualitative damages were related to the number of defects and types of coffee that increased as the number of fruits infested by the insect increased. Both losses and damages had a linear increase, according to the increase in the level of infestation, due to the consumption of the fruit by the larvae and adults of the coffee berry borer. Thus, the management of the coffee berry borer is important to avoid losses caused by the insect infestation in the field.

All the factors that constitute the coffee ecosystem and its multiple interactions must be considered to effectively control the coffee berry borer. It is important to know the phenology of the crop, such as flowering times and fruit age; the factors favorable to the occurrence and feeding of the borer; as well as biology and breeding habits, in order to adopt control tools, maintaining the insect population level in a way that does not cause damage and does not affect productivity (BUSTILLO et al., 1998; CAMILO et al., 2003).

Among the control tools of *H. hampei*, is the use of entomopathogenic fungi that can be prospected to reduce the population density of this insect. The biological control of the coffee berry borer is mainly done by means of products based on *Beauveria bassiana* Bals. Vuill. (Hypocreales: Cordycipitaceae), a fungus commonly found in the holes opened by the coffee berry borer, as in the crown of the coffee fruit in rainy times (PARRA; REIS, 2013), and *Metarhizium anisopliae* (Metsch.) Sorokin (Hypocreales: Cordycipitaceae, Clavicipitaceae). These two species are the most studied entomopathogens used in microbial pest control due to specificity, selectivity, compatibility with other methods, and environmental safety (ALMEIDA et al., 2007).

Currently, for the biological control of *H. hampei* in Brazil, there are 15 microbiological insecticides registered, for six isolates of entomopathogenic fungi of the species *B. bassiana* and *M. anisopliae*, five isolates of *B. bassiana* (BV 13, CG 716, GHA, IBCB 66 e PL 63) and an isolate of *M. anisopliae* (IBCB 425) associated with the isolate of IBCB 66 (AGROFIT, 2022). However, there is a great diversity of genera that still need to be explored, such as *Cordyceps*, *Fusarium*, and *Paecilomyces*.

Therefore, it is important to conduct studies with new isolates of entomopathogenic fungi for the manufacture of microbiological products to control the coffee berry borer. Thus, based on the described before, the objective of this work was to evaluate the efficiency of entomopathogenic fungal isolates in the control of coffee berry borer in the laboratory, to increase the potential for the use of these biological control agents.

MATERIAL AND METHODS

Origin and obtaining of isolates

Twenty-six isolates of entomopathogenic fungi (Table 1) were used, eight of which came from the Instituto Biológico de São Paulo, SP, Brazil, three from the Universidade Federal de Uberlândia, Monte Carmelo, MG, Brazil, one from a commercial product; and 14 were directly isolated from naturally colonized adults of the coffee berry borer found in coffee plantations in Patos de Minas, MG, Brazil, coordinates 18°44'13"S e 46°39'40"W. The fungi were identified at the genus level through colony morphology and optical microscopy and preserved by the technique CASTELLANI (1939).

Table 1. Isolates and origin of entomopathogenic fungi.

Isolated	Species	Origin
CMAA 1306	*Beauveria bassiana*	Commercial product
IBCB 170	*Beauveria bassiana*	Instituto Biológico de São Paulo
IBCB 66	*Beauveria bassiana*	Instituto Biológico de São Paulo
NCTB 04	*Beauveria* sp.	Nooa Ciência e Tecnologia Agrícola LTDA.
NCTB 05	*Beauveria* sp.	Nooa Ciência e Tecnologia Agrícola LTDA.
NCTB 06	*Beauveria* sp.	Nooa Ciência e Tecnologia Agrícola LTDA.
NCTB 07	*Beauveria* sp.	Nooa Ciência e Tecnologia Agrícola LTDA.
NCTB 08	*Beauveria* sp.	Nooa Ciência e Tecnologia Agrícola LTDA.
NCTB 13	*Beauveria* sp.	Nooa Ciência e Tecnologia Agrícola LTDA.

Continue...

Table 1. Continuation...

Isolated	Species	Origin
NCTB 15	*Beauveria* sp.	Nooa Ciência e Tecnologia Agrícola LTDA.
NCTB 15.1	*Beauveria* sp.	Nooa Ciência e Tecnologia Agrícola LTDA.
NCTB 16	*Beauveria* sp.	Nooa Ciência e Tecnologia Agrícola LTDA.
NCTB 18	*Beauveria* sp.	Nooa Ciência e Tecnologia Agrícola LTDA.
UFUMCB 1	*Beauveria* sp.	Universidade Federal de Uberlândia
UFUMCB 3	*Beauveria* sp.	Universidade Federal de Uberlândia
IBCB 130	*Cordyceps fumosorosea*	Instituto Biológico São Paulo
NCTI 01	*Cordyceps* sp.	Nooa Ciência e Tecnologia Agrícola LTDA.
NCTC 01	*Cordyceps* sp.	Nooa Ciência e Tecnologia Agrícola LTDA.
NCTC 02	*Clonostachys* sp.	Nooa Ciência e Tecnologia Agrícola LTDA.
UFUMCF	*Fusarium* sp.	Universidade Federal de Uberlândia
IBCB 348	*Metarhizium anisopliae*	Instituto Biológico de São Paulo
IBCB 353	*Metarhizium anisopliae*	Instituto Biológico de São Paulo
IBCB 364	*Metarhizium anisopliae*	Instituto Biológico de São Paulo
IBCB 383	*Metarhizium anisopliae*	Instituto Biológico de São Paulo
IBCB 391	*Metarhizium anisopliae*	Instituto Biológico de São Paulo
IBCB 425	*Metarhizium anisopliae*	Instituto Biológico de São Paulo
NCTP 08	*Paecilomyces* sp.	Nooa Ciência e Tecnologia Agrícola LTDA.

Source: Elaborated by the authors.

Selection of isolates

The isolates were chopped in potato dextrose agar (PDA) medium and incubated at 25°C for 10 days or until they reached sporulation, depending on each isolate (Fig. 1). After sporulation, 20 to 50 mL of sterile distilled water solution + 0.2% were added to the plate with culture grown from each isolate, and, with the aid of drigalski handle, the conidia were incorporated into the solution. Subsequently, the solution containing conidia and mycelium was stirred in a vortex agitator and sieved in a 100-mesh sieve. The concentration of the solutions was verified in the Neubauer chamber and, when necessary, adjusted to the concentration of 1 to 3×10^8 conidia/mL, adding more water solution with Tween or conidia. With the adjusted concentrations, 0.5 mL of solution was added to cryogenic tubes of 2 mL to perform the treatment in insects.

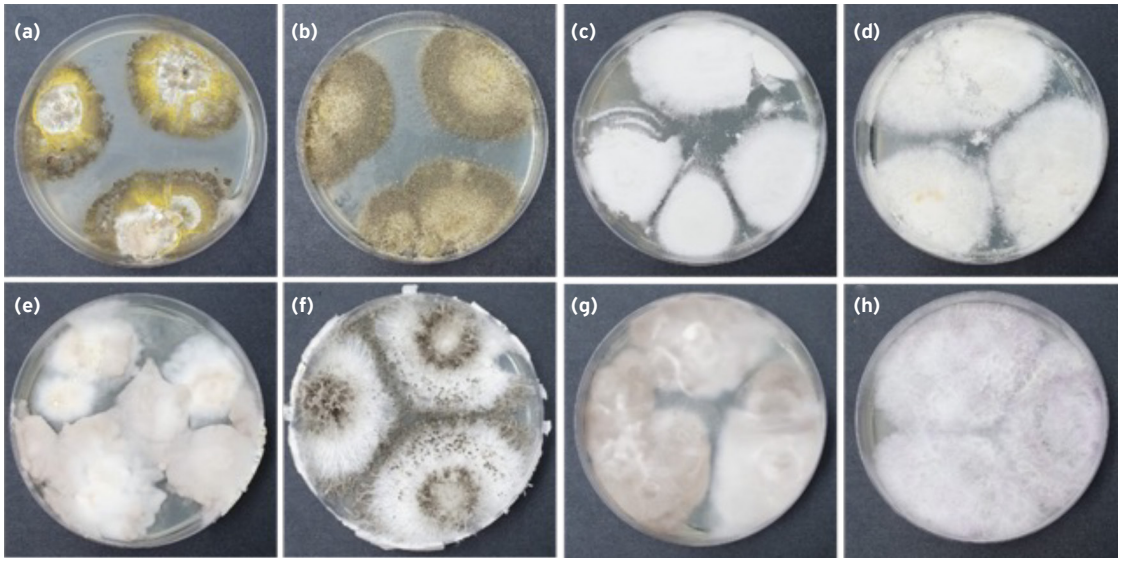

Figure 1. Colonies of entomopathogenic fungi on potato dextrose agar. (a and b) *Metarhizium anisopliae*; (c and d) *Beauveria* sp.; (e) *Cordyceps* sp.; (f) *Clonostachys* sp.; (g) *Paecilomyces* sp.; (h) *Fusarium* sp.
Source: Elaborated by the authors.

Ten adult females of the coffee berry borer were placed inside the cryogenic tubes for immersion in the suspensions of conidia of the entomopathogens. These tubes were then lightly stirred manually for 30 seconds, and then the insects together with the suspension were transferred to a 30-mL plastic pot with a lid containing four pieces of overlapping blotter filter paper cut into 2 cm^2 previously sterilized, for absorption of the conidia suspension and drying of the insects. The control treatment was done with immersion in distilled water solution + Tween 0.2%. After 1 hour, the coffee berry borers were transferred to another plastic pot (30 mL) containing a small piece of diet for feeding the insects and a piece of blotter filter paper cut to the size of 1.5 cm^2, so that the filter paper does not come into contact with the diet. Two hundred µL of sterilized distilled water was added on top of the filter paper to keep the microclimate moist inside the pots (Fig. 2a).

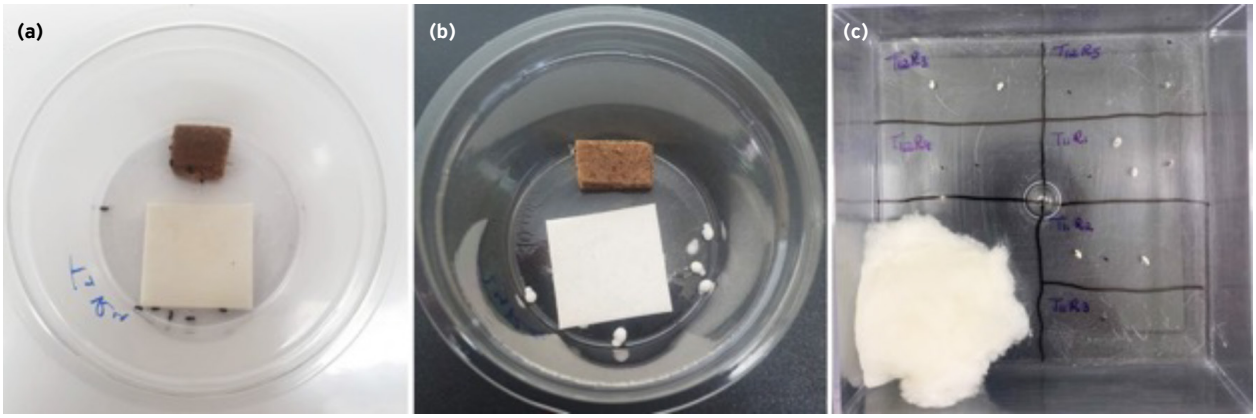

Figure 2. Bioassay stages. (a) Plastic container with the treated coffee berry borers, diet, and moistened filter paper. (b) Coffee berry borer colonized by *Beauveria bassiana* after seven days of incubation. (c) Wet chamber with dead insects after four days of incubation.
Source: Elaborated by the authors.

The pots were placed in an acclimatized room with the temperature of 25 ± 3°C and relative humidity of 80 ± 10%, regulated by the periodic activation of an air humidifier to maintain the external microclimate for seven days. Every 48 hours, another 100 µL of sterile distilled water was added to the pieces of filter paper. The moistened paper and the external humidity of the air served to provide the necessary conditions for the germination of the conidia.

After 10 days, the experiments were evaluated by separating the live and dead insects with the aid of a brush no. 00. Mortality was confirmed by observing the insects with mycelial growth and sporulation characteristic of the isolate, in addition to the morphology of conidiophores and conidia observed under an optical light microscope (Fig. 2b). Dead borers without apparent colonization were superficially disinfected in 30 seconds in NaClO1% solution and placed on paper towels to dry. Soon after, they were placed in a humid chamber in plastic Gerbox boxes containing cotton soaked in distilled water for four days at 25°C to confirm whether there was death by colonization (Fig. 2c).

The isolates of entomopathogenic fungi were divided into two groups to perform two bioassays, the first with 15 isolates and the control treatment totaling 16 treatments, and the second bioassay with 12 isolates plus the control treatment, totaling 13 treatments (Table 2). The experiments were conducted in a completely randomized design with six replications. Each plastic pot of 30 mL capacity, with 10 insects, was considered a repetition.

Outliers were identified by plotting externally studentized residuals (RStudent) versus predicted values (variable Y). From RStudent, values that were outside the -2 to 2 range were considered outliers, and their corresponding observations were removed from the database. Confirmed mortality data were transformed into arc-sen $\sqrt{x+0.5}$. The data obtained were analyzed for normality using the Jarque-Berra test; homoscedasticity by the Hartley's test; and the independence of residues by graphical analysis. Subsequently, once the assumptions were met, the data were subjected to analysis of variance using the F test ($p \leq 0.05$). With statistical significance at the 5% level, the effects of the treatments were compared using Tukey's test ($p \leq 0.05$). Statistical analysis was performed using the software Statistica 7.0 (STATSOFT, 2007).

Table 2. Treatments used in the two bioassays to evaluate the pathogenic potential and virulence of the coffee berry borer.

Treatment	Bioassay I		Bioassay II	
	Species	Isolated	Species	Isolated
T_1	Control	-	Control	-
T_2	*Beauveria bassiana*	IBCB 66	*Cordyceps fumosorosea*	IBCB 130
T_3	*Beauveria bassiana*	IBCB 170	*Cordyceps* sp.	NCTI 01
T_4	*Beauveria bassiana*	CBMA 1306	*Cordyceps* sp.	NCTC 01
T_5	*Beauveria* sp.	UFUMCB 1	*Clonostachys* sp.	NCTC 02
T_6	*Beauveria* sp.	UFUMCB 3	*Fusarium* sp.	UFUMCF 1
T_7	*Beauveria* sp.	NCTB 02	*Metarhizium anisopliae*	IBCB 348
T_8	*Beauveria* sp.	NCTB 03	*Metarhizium anisopliae*	IBCB 353
T_9	*Beauveria* sp.	NCTB 04	*Metarhizium anisopliae*	IBCB 364
T_{10}	*Beauveria* sp.	NCTB 05	*Metarhizium anisopliae*	IBCB 383
T_{11}	*Beauveria* sp.	NCTB 06	*Metarhizium anisopliae*	IBCB 391
T_{12}	*Beauveria* sp.	NCTB 07	*Metarhizium anisopliae*	IBCB 425
T_{13}	*Beauveria* sp.	NCTB 08	*Paecilomyces* sp.	NCTP 08
T_{14}	*Beauveria* sp.	NCTB 09		
T_{15}	*Beauveria* sp.	NCTB 10		
T16	*Beauveria* sp.	NCTB 11		

Source: Elaborated by the authors.

Multiplication of isolates

To obtain conidia of the selected entomopathogenic fungi, in a laminar flow chamber, five mycelium discs of 10-day-old isolates grown in PDA were added in Erlenmeyer containing 200 mL of liquid culture medium potato dextrose (PD) previously sterilized and cooled. Once this was done, these Erlenmeyers were placed in agitation of 180 rpm and temperature of 26°C for 48 hours. As a solid growth substrate, plastic bags containing parboiled rice (Gringo rice) were prepared. Six-kg of rice was soaked in water for 30 minutes. After the excess water was removed with a rice colander, 250 g of moist rice was fractionated into polypropylene plastic bags, 20 × 30 cm, closed with a staple, and sterilized in an autoclave for 30 minutes.

After stirring, in laminar flow, with the aid of a syringe, 20 mL of inoculum was added into five bags with rice and manually revolved for homogenization of the fungus throughout the rice. The bags were arranged on shelves at 25 ± 3°C temperature. On the third and sixth day, the rice was manually returned for mycelium rupture and gas exchange. On the eighth day, the rice was spread on trays for another three days at the same temperature. After that, to remove the conidia, the substrate was manually sieved in a sieve of 20 and 100 mesh.

Mean lethal concentration (LC_{50} and LC_{90}) and mean lethal time (LT_{50} and LT_{90})

To carry out the LC_{50} and LT_{50} experiments, the viability of the conidia of the selected isolates multiplied in rice was verified. For viability analysis, 15-uL aliquots of the 10^{-4} dilution were placed in Petri dishes containing PDA medium and incubated for 16 hours at 25°C. After this period, lactophenol blue dye was added to the conidia in the Petri dish, and under the optical microscope, about 500 germinated and non-germinated conidia were counted to determine the viability in percentage. Subsequently, to set up the experiments, serial dilutions were performed from the conidia sieved in a solution of distilled water + 0.2% Tween, previously sterilized, and the concentration of total conidia was estimated in a Neubauer chamber.

The LC bioassays were performed by immersing the insects in conidia solutions and, later, transferring them to a plastic pot with dampened filter paper and a diet piece according to the methodology used in the selection of isolates. The concentrations were: 2.5×10^9; 2.5×10^8; 2.5×10^7; 2.5×10^6; 2.5×10^5; and 2.5×10^4 viable conidia/mL for *M. anisopliae* (IBCB 353, IBCB 364, IBCB 425) and 6.0×10^9; 3.0×10^9; 3.0×10^8; 3.0×10^7; 3.0×10^6; and 3.0×10^5 viable conidia/mL for *B. bassiana* isolates (NCTB 04 e IBCB 66). In total, there were 30 treatments, with six replicates (180 plots), each plot with 15 adult coffee berry borers from mass rearing in an artificial diet. After seven days of incubation in an air-conditioned room, mortality, and wet chamber preparation were evaluated as previously described.

Lethal time (LT) bioassays were performed by immersing the insects in conidia solutions and transferred to a 30-mL plastic pot with dampened filter paper and diet piece according to the methodology used in the selection of isolates. The concentrations used were 2.5×10^9 and 2.5×10^8 viable conidia/mL for the isolates of *M. anisopliae* (IBCB 353, IBCB 364, IBCB 425); 6.0×10^9 and 3.0×10^9 conidia viable/mL for *B. bassiana* isolates (NCTB 04 and IBCB 66); and control treatment containing distilled water solution + Tween 0.2%. Both for the LC and LT assays, tests were performed to determine the doses to be used.

In total, there were 11 treatments, with six replicates (66 plots), each plot with 15 adult coffee berry borers from mass rearing in an artificial diet. After two days and every 24 hours until the eighth day, a count of dead individuals was performed, and a wet chamber was prepared for confirmed mortality evaluation. Lethal time mortality data were corrected by ABBOTT's formula (1925). The experiments were conducted in a completely randomized design, and the mortality data obtained at different concentrations and times were submitted to Probit analysis, determining the LC_{50}, LC_{90}, LT_{50} and LT_{90} using the software IBM Statistical Package for the Social Sciences Statistics 28.0 (IBM CORPORATION, 2021).

RESULTS

Selection of isolates of entomopathogenic fungi to coffee berry borer

In the first bioassay with different isolates of *Beauveria* spp., it was observed that all isolates were pathogenic to coffee berry borer, with confirmed mortality above 30% (Table 3). There was a significant difference in all isolates about the control treatment, but the isolate NCTB 04 stood out, presenting mortality higher than the isolates UFUMCB 1, NCTB 06, NCTB 15, NCTB 16, NCTB 13, NCTB 07 UFUMCB 3 and NCTB 08, being the only isolate to present confirmed mortality above 85%. Bioassay II, with entomopathogenic fungi of the genera *Cordyceps, Fusarium, Metarhizium*, and *Paecilomyces* also showed significance in confirmed mortality. Isolates IBCB 364, IBCB 353, IBCB 391, IBCB 383, IBCB 348, IBCB 425, NCTC 01, NCTC 02 and NCTI 01 differed statistically from the control treatment. Isolates IBCB 364 and IBCB 353 were the only ones with more than 85% confirmed mortality.

Table 3. Effect of different isolates of entomopathogenic fungi on coffee berry borer mortality.

Bioassay I			Bioassay II		
Treatment	**Confirmed mortality (%)**		**Treatment**	**Confirmed mortality (%)**	
Control	0.0	g[1]	Control	0.0	f
NCTB 08	*30.3*	f	IBCB 130	1.0	ef
UFUMCB 3	*45.0*	e	NCTP 08	1.7	ef
NCTB 07	*58.3*	de	UFUMCF 1	1.7	ef
NCTB 13	58.4	de	NCTI 01	4.2	de
NCTB 16	60.8	cd	NCTC 02	11.7	d
NCTB 15	64.4	bcd	NCTC 01	44.2	c
NCTB 06	67.5	bcd	IBCB 425	53.8	bc
UFUMCB 1	68.2	bcd	IBCB 348	58.3	bc
IBCB 66	70.8	abcd	IBCB 383	63.3	b
CBMA 1306	74.2	abcd	IBCB 391	68.3	ab
IBCB 170	74.2	abcd	IBCB 353	86.7	a
NCTB 15.1	75.0	abcd	IBCB 364	87.5	a
NCTB 18	76.1	abc			
NCTB 05	77.5	ab			
NCTB 04	87.5	a			
CV (%)	5.5			24.3	

[1]Means followed by the same letters do not differ from each other, using the Tukey's test at 5% probability; CV: coefficient of variation.
Source: Elaborated by the authors.

Assays of concentration and lethal time of entomopathogenic fungi to the coffee berry borer

In the analysis of lethal concentrations (LC_{50} and LC_{90}) for the isolates of *M. anisopliae* IBCB 353, 364, and 425 and of *Beauveria* sp. NCTB 04, the calculated χ^2 was lower than the tabulated χ^2, indicating that the lethal effect of these entomopathogenic isolates on *H. hampei* is suitable for the Probit model, not being observed the same effect for the IBCB 66 isolate. Non-commercial ones showed higher lethality compared to their commercial peers, that is, IBCB 353 and 364 show lower CLs than IBCB 425 and NCTB 04 lower than IBCB 66, demonstrating the potential of these isolates to be inserted in the coffee berry borer management. When comparing the *M. anisopliae* isolates with each other, it was possible to state that the two non-commercial isolates showed higher lethality than the commercial isolate (IBCB 425), as their lethal concentrations were lower and, moreover, the confidence intervals did not overlap (Table 4).

Table 4. Probit analysis and lethal concentrations (LC_{50} and LC_{90}) were predicted based on *Hypothenemus hampei* response data to the application of different entomopathogenic fungi isolates.

Isolated	N	Inclination ± SE	LC_{50} (95%CI) conidia/mL	LC_{90} (95%CI) conidia/mL	DF	χ^2	p
IBCB 353	540	0.77 ± 0.04	2.13×10^6 (1.42×10^6–3.19×10^6)	8.16×10^7 (4.61×10^7–1.66×10^8)	4	5.18	0.27
IBCB 364	540	0.57 ± 0.05	4.26×10^5 (2.21×10^5–7.54×10^5)	1.11×10^8 (5.07×10^7–3.05×10^8)	4	5.22	0.26
IBCB 425	540	0.74 ± 0.04	1.87×10^7. (1.22×10^7–2.80×10^7)	8.50×10^8 (4.86×10^8–1.69×10^9)	4	7.15	0.13
IBCB 66	540	0.67 ± 0.23	8.60×10^7 (1.46×10^7–4.77×10^8)	3.58×10^{10} (3.64×10^9–9.23×10^{12})	4	15.7	0.01^{ns}
NCTB 04	540	0.63 ± 0.09	6.80×10^6 (1.54×10^6–2.08×10^7)	1.17×10^9 (2.90×10^8–1.34×10^{10})	4	9.65	0.05

^{ns}not significant; N: number of insects; SE: standard error; LC_{50}: lethal concentration 50%; LC_{90}: lethal concentration 90%; DF: degree of freedom; 95%CI: 95% confidence interval; p: probability.
Source: Elaborated by the authors.

Regarding LC_{50}, the lowest value verified (4.6×10^5 conidia/mL) was for the isolate IBCB364, which stood out with the highest lethality, and its confidence interval did not overlap with any of the isolates tested. For LC_{90}, the lowest value observed (8.16×10^7 conidia/mL) was for the isolate IBCB 353, presenting itself as the most lethal one (Table 4). This fact is related to the isolate in question presenting the highest slope of the straight line, thus the mortality response is faster as concentrations increase. These results showed that about 5.44 and 16 times fewer conidia/mL of IBCB 364 are needed to kill 50% of coffee berry borers compared to isolates IBCB 353, 425, and NCTB 04, respectively, and to kill 90% of the borers about the isolates IBCB 425 and NCTB 04, eight and 10 times less conidia of IBCB 364 and 10 and 14 times less conidia of IBCB 353 are needed.

For mean lethal time analysis (LT_{50}), the confirmed mortality data did not fit the Probit model, since the calculated χ^2 was higher than the χ^2 tabled. Thus, it was decided for the Probit analyses of LT_{50} with mortality data corrected by ABBOTT's formula (1925) since the data were adjusted to the Probit model by the χ^2 test. The lethal action of the isolates evaluated on the females of *H. hampei* began on the third day after treatment with suspensions of conidia. According to Probit analysis, the LT50 of the isolates tested was close, ranging from 3.2 to 4.4 days. A lower LT_{50} of the coffee berry borer was observed for isolate IBCB 66 (3.2 days), followed by isolates IBCB 353 (3.5 days), IBCB 425 (3.8 days), IBCB 364 (4.3 days), and NCTB 04 (4.4 days) (Table 5).

For the LT_{90}, the variation was from 5.6 to 7.3 days. The isolate IBCB 66 had the lowest LT_{90} (5.6 days), followed by the isolates IBCB 353 (6 days), NCTB 04 (6.9 days), IBCB 425 (7.2 days), and IBCB 364 (7.3 days) (Table 5). Thus, it can be inferred that the commercial isolate IBCB 66 had a shorter lethality time and, therefore, greater virulence due to the shorter lethal times. In addition, the lethal times of the same do not overlap the others, except for the isolated IBCB 353.

Table 5. Analysis of Probit and lethal times (LT_{50} and LT_{90}) predicted based on *Hypothenemus hampei* response data to the application of different isolates of entomopathogenic fungi.

Isolated	N	Inclination ± SE	LC_{50} (95%CI) conidia/mL	LC_{90} (95%CI) conidia/mL	DF	χ^2	p
IBCB 353	540	5.78 ± 0.53	3.5 (3.3–3.7)	6.0 (5.6–6.5)	5	8.55	0.13
IBCB 364	540	6.02 ± 0.50	4.3 (4.1–4.5)	7.3 (6.8–7.9)	5	9.44	0.09
IBCB 425	540	4.80 ± 0.48	3.8 (3.5–4.0)	7.2 (6.7–8.0)	5	8.84	0.12
IBCB 66	540	5.46 ± 0.46	3.2 (3.0–3.4)	5.6 (5.2–6.1)	5	8.09	0.15
NCTB 04	540	6.30 ± 0.48	4.4 (4.2–4.6)	6.9 (6.5–7.5)	5	9.09	0.11

N: number of insects; SE: standard error; LT_{50}: lethal time 50%; LT_{90}: lethal time 90%; DF: degree of freedom; 95%CI: confidence interval; p: probability.
Source: Elaborated by the authors.

DISCUSSION

Among the 27 isolates tested for coffee berry borer control, the ones with the best effect were the entomopathogenic fungi of the genera *Beauveria* and *Metarhizium*. All biological products registered in Brazil to control this pest are composed of fungal propagules of these genera. In total, there are five isolates of *B. bassiana* and one of *M. anisopliae* registered (AGROFIT, 2022). Therefore, research is needed to prospect new isolates that control this insect pest, which is one of the main pests of coffee growing.

Several studies have already been and continue to be carried out for the selection of entomopathogenic fungi virulent to coffee berry borer, and in all of them, the isolates that presented the highest mortality of this insect were of the genera *Beauveria* and *Metarhizium*, corroborating the data of this study. The confirmed mortality was chosen as an evaluation parameter for the selection of isolates because, in addition to confirming the cause of death of the insect, it demonstrates the virulence and capacity of the isolate to remain in the environment. According to NEVES; HIROSE (2005), in perennial crops such as coffee, the conidiogenesis of the fungus in the insect is very relevant as it guarantees its permanence in the environment.

NEVES; HIROSE (2005) selected 11 *B. bassiana* isolates with confirmed mortality above 60% at the concentration of 2.5×10^7 conidia/mL six days after inoculation. DE LA ROSA et al. (1997) selected nine isolates of *B. bassiana* with confirmed mortality above 90%. For selection, the coffee berry borers were submerged for 30 seconds in conidia suspension at the concentration of 1×10^8 conidia/mL. MORALES et al. (2019) evaluated the confirmed mortality of the coffee berry borer by the *B. bassiana* isolate, which was 23, 49, 67, and 83% at concentrations of 1×10^5, 1×10^6, 1×10^7, and 1×10^8, respectively.

In the experiments by LECUONA et al. (1986), the fungus *M. anisopliae* was shown to be pathogenic for coffee brocade *H. hampei* in the laboratory, presenting corrected maternity above 60% at the concentration above 1.5×10^8 four days of incubation.

PAVA-RIPOLL et al. (2008) immersed coffee berry borers in conidia solutions of two *M. anisopliae* isolates, Ma549 and AaIT-Ma549 (genetically modified). At concentrations of 10^1, 10^2, and 10^3 conidia/mL, the recombinant isolate significantly increased the mortality of the coffee berry borer by 32.2, 56.6, and 24.6%, respectively, after 21 days about the original treatment. After three days of treatment, the isolate AaIT-Ma549 killed 75% of the coffee berry borers, while Ma549 killed 51.3%. However, AaIT-Ma549 had lower conidiogenesis in *H. hampei* cadavers than the original isolate.

LEZCANO et al. (2015) found an average of 87% mortality of coffee berry borer infested with solutions of 10^9 conidia/mL of *Cordyceps* (= *Isaria*).

Thus, to proceed with the LC_{50} and LT_{50} mortality tests, the NCTB 04 isolates were selected from bioassay I for having the highest confirmed mortality rate, and the IBCB 66 isolate, which, in addition to being among the *Beauveria* isolates that stood out the most statistically about the control, it is the commercial isolate with the highest number of products registered to control the coffee berry borer. In bioassay II, the isolates of *M. anisopliae* IBCB 364 and IBCB 353 were selected for having the highest confirmed mortality rates (87.5 and 86.7%) and IBCB 425 for being a commercial isolate with registration for the coffee berry borer. Thus, the isolates that presented a confirmed mortality rate greater than 80% and the commercial isolates were chosen (Fig. 3).

According to PAVA-RIPOLL et al. (2008), the evaluations of mean lethal time (LT_{50}), mean lethal concentration (LC_{50}), duration of the phases of pathogenesis, saprogenesis, and conidiogenesis of entomopathogenic fungi in insects are important parameters for evaluating the efficiency of these biocontrol agents. These variables are relevant to verify the environmental and economic implications of the application of these entomopathogens in the field.

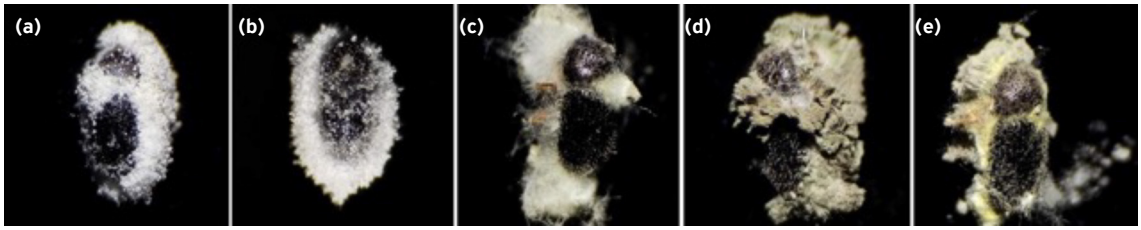

Figure 3. Coffee berry borers colonized by entomopathogenic fungi isolates. (a) IBCB 66 (*Beauveria bassiana*); (b) NCTB 04 (*Beauveria* sp.); (c) IBCB 353 (*Metarhizium anisopliae*); (d) IBCB 364 (*M. anisopliae*); (e) IBCB 425 (*M. anisopliae*).
Source: Elaborated by the authors.

Most studies of entomopathogenic fungi to control coffee berry borer under laboratory conditions found in the literature were conducted with isolates of the species *B. bassiana*, demonstrating higher natural occurrence since most studies were carried out from direct isolation of colonized coffee berry borers found in the field and potential of this species to control this insect pest.

The data obtained by NEVES; HIROSE (2005) were similar to that obtained in the present experiment. These authors selected 11 isolates of *B. bassiana* in the in-vitro control of *H. hampei*. Confirmed mortality ranged from 51.6 to 82.8% and LC50 2.5×10^6 to 6.2×10^7 conidia/mL. The isolate CG425 presented higher confirmed mortality (82.8%) and lower LC_{50} (2.5×10^6 conidia/mL). The authors suggest the use of a group or mixture of isolates in the coffee berry borer management program to increase tolerance limits and the permanence of biocontrol agents in the field, what may improve control levels.

DALVI (2008) tested four Beauveria isolates for *H. hampei* control by spraying conidia solutions on insects and LC_{50} ranged from 4.0×10^4 to 1.2×10^5 conidia/mL. FRANÇOIS et al. (2021) found LC50 of two isolates from *Beauveria* to *H. hampei*, which was 2.63×10^4 and 1.51×10^7 conidia/mL for the isolates Bb-IRAD.Nkoe and Bb-IRAD.Fbt, respectively. The LC_{90} was 8.49×10^{11} and 7.18×10^{12} conidia/mL for the isolates Bb-IRAD.Nkoe and Bb-IRAD.Fbt, respectively. MORALES et al. (2019) sprayed conidia concentrations of an isolate of *B. bassiana* on adults of *H. hampei*. The estimated LC_{50} was 2.47×10^6 conidia/mL, and LT_{50} for concentrations of 1×10^7 and 1×10^8 were four and 1.5 days, respectively. The authors also tested the control of the coffee berry borer with this same isolate in dilution 10^6 associated with the chemical insecticide Spinosad. This treatment caused 95% mortality, while separated one caused 61 (fungus) and 49% (chemical insecticide). Thus, as long as chemical and biological studies are compatible, they can be carried out to control this insect pest.

LEZCANO et al. (2015) tested the virulence of a *Cordyceps* sp. and commercial isolates of *B. bassiana* and *M. anisopliae* in the control of coffee berry borer. *Cordyceps* sp. showed higher insect mortality than commercial products, demonstrating the potential of the species to control this pest. The results obtained for the *Cordyceps* sp. were LC_{50} of 1.1×10^8 and LC_{95} of 5.9×10^9 conidia/mL. Despite the potential of the species, compared with the results obtained in the present work, the isolates of *Beauveria* spp. and *M. anisopliae* evaluated need lower conidia concentrations for *H. hampei* lethality.

PAVA-RIPOLL et al. (2008) tested the pathogenicity of *M. anisopliae* isolates in *H. hampei* by immersing adults for 2 minutes at the concentration of 10^7 conidia/mL. At this concentration, the mean lethal time of the isolate AaIT-Ma549 (genetically modified) was three days, while that of Ma549 (original) was 3.7 days. Thus, the LT_{50} was reduced by 20.1% about the original isolate.

According to the work by FRANÇOIS et al. (2021), the lethal times that caused 50 and 90% mortality of coffee berry borers at the concentration of 3.0×10^8 conidia/mL of *B. bassiana* were 3.7 and 11.7 days, respectively, for the isolate Bb-IRAD. Fbt, and 2.7 and 9.1, respectively, for isolate Bb-IRAD.Nkoe. The authors observed that the tested isolates caused mortality of coffee berry borers before showing signs of conidiogenesis outside the body of the insects, as confirmed mortality was lower than total mortality, and this effect was evidenced by increasing concentration and of time.

BLANCO (2017) performed the immersion of the coffee berry borers in a solution at the concentration of 107 conidia/mL of three isolates of *B. bassiana* (13, 24, and 9205) for 3 minutes and offered coffee on parchment as food after 24 hours of infestation, to verify the pathogenicity of the isolates after four successive subcultures. The estimated LT_{50} at the first crossing was 2.4, 2.8, and 3.1 days, in the second subculture it was three, 3.8, and 3.3 days, in the third 3.9, 4.6, and 4.1 days; and 4.2, five and 4.6 days in the fourth subculture for isolates 13, 24 and 9205, respectively. This experiment demonstrated that the virulence of the isolates can be reduced according to the number of successive subcultures.

According to SILVA et al. (2003), the lethal time of microbial control agents on insect pests can be used as complementary data, since population reduction is more important than the speed of this process. Entomopathogenic fungi cause infection and colonization of the host, and this process takes time, so they may not have a rapid lethal effect.

Studies with entomopathogenic fungi of other species besides *B. bassiana* to control the coffee berry borer are important in the search for control of this insect to increase the range of products available and the number of species in the environment for natural control. According to the data obtained, concentration, and lethal time, the isolates of *M. anisopliae* IBCB 353 and 364 have the potential to be tested in the field to control the coffee berry borer.

CONCLUSIONS

The isolates NCTB 04, from *Beauveria* sp., and IBCB 353 and IBCB 364, from *M. anisopliae*, cause confirmed mortality above 85% in the coffee berry borer. The lethal concentration and dose assays in comparison with commercial isolates showed that these isolates have the potential for controlling this insect pest under laboratory conditions.

AUTHORS' CONTRIBUTIONS

Conceptualization: Andaló, V. **Data curation:** Ferreira, J. **Formal analysis:** Fuga, C. **Funding acquisition:** Soares, C. **Investigation:** Ferreira, J.; Sousa, T. **Methodology:** Ferreira, J. **Project administration:** Andaló, V.; Ferreira, J. **Resources:** Soares, C. **Software:** Soares, C. **Supervision:** Soares, C.; Andaló, V. **Validation:** Fuga, C.; Ferreira, J. **Writing – original draft:** Ferreira, J. **Writing – review & editing:** Ferreira, J.; Fuga, C.; Andaló, V.; Sousa, T.; Soares, C. **Literature search:** Ferreira, J. **Data analysis:** Fuga, C. **Critically revised the work:** Fuga, C.; Andaló, V.

AVAILABILITY OF DATA AND MATERIAL

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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CONFLICTS OF INTEREST

All authors declare that they have no conflict of interest.

ETHICAL APPROVAL

Not applicable.

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