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Article

Mortality of *Galleria mellonella* L. by *Beauveria bassiana* (Balsamo) Vuill (Ascomycota: Hypocreales)

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Abstract

One of the most commonly used fungi in pest control, which has a wide range of hosts, is *Beauveria bassiana*. This entomopathogen has adapted to different environments and can be found in several places, so in the present work, 60 soil samples were collected in 2020 in Acatlán de Pérez Figueroa, Oaxaca, by means of *Galleria mellonella*, used as bait for its isolation. Five isolates were obtained from this procedure, which were evaluated in larvae of *Galleria mellonella* at 24, 48, 72, 96, 120, 144, and 168 h in order to know their efficacy in mortality and mycosis. In the methodology, the DNA of the fungi was extracted and the ITS region of the 5.8S rRNA gene was amplified by PCR, the product was sequenced and the sequences were compared with others existing at the National Center for Biotechnological Information. The result was one isolate of *Beauveria pseudobassiana* and four of *Beauveria bassiana*, of which isolate 11 showed 100% mortality and mycosis at 96 h, unlike the commercial isolate and the control, which needed an additional 72 h to kill all larvae, in addition to showing 20% and 60% of mycosis, respectively. The time of 96 h was significantly different (*p*# 0.05) because during this time, most of the larvae died and mycosis occurred. Identifying entomopathogenic fungi and conducting studies of their effectiveness in larvae facilitates the start of new research experiments.

Palabras clave:

Beauveria bassiana, *Galleria mellonella* L., ADN, efficacy, isolate, time.

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Introduction

The biological effectiveness of microorganisms for pest control is of great importance when it is sought to prevent, repel, or reduce the damage they cause to crops (Castro and Martínez, 2019). This effectiveness involves the action of organisms such as entomopathogenic fungi, which were the first microorganisms to be observed as causing diseases in insects since it was possible to see their growth on the insects' bodies (Van Driesche *et al*., 2007). Such is the case of *Beauveria bassiana*, known since ancient times as white muscardine, which was one of the first entomopathogenic fungi used as a biological insect controller (Barbosa *et al*., 2017; Barbosa *et al*., 2018).

This fungus can cause disease without being ingested since it can enter through the cuticle and invade the internal cavity, attacking fatty tissues and organs, so the insect stops feeding, dies and the fungus multiplies inside it and after a period of 4 to 10 days after infection, it dies (Serna-Domínguez *et al*., 2019; Pacheco *et al*., 2020). The fungus *Beauveria bassiana* has the characteristic of presenting colonies with a velvety or powdery white appearance and as time passes, it turns yellowish (Orduño-Crúz *et al*., 2011).

The genus *Beauveria* is currently used for the control of several pest species, such as; pepper weevil, banana weevil, cotton aphid, melon aphid, whitefly, coffee berry borer and coffee bean weevil, which is why it is considered a generalist as it attacks various insects (Pacheco *et al*., 2020). Nonetheless, not all species of this genus have the same effectiveness in pest control, so it is necessary to know the biological effectiveness of isolates before using them for this purpose (Ríos *et al*., 2020).

In this regard, there are insects such as *Galleria mellonella* L. that have the advantage of surviving and reproducing easily in captivity when they are in the larval stage, making them ideal for establishing bioassays and generating valuable results in hours (Jorjao *et al*., 2018; Kavanagh and Sheehan, 2018). Thus, in the present research work, soil was sampled and fungi of the genus *Beauveria* were identified, the biological effectiveness of which was evaluated with the use of larvae of *Galleria mellonella* L.

Materials and methods

Collection of soil samples and isolation of native fungi

A total of 60 samples of soil cultivated with sugarcane were collected in Acatlán de Pérez Figueroa, Oaxaca. The sampling was carried out in three seasons in 2016, in which 300 g of each sample was collected in Ziploc bags, which were labeled and transferred in coolers to the insect pathology laboratory of the College of Postgraduates, where they were isolated. To isolate entomopathogenic fungi, the trap insect technique was used (Zimmermann, 1986), for which soil samples were placed in cups 10 cm high by 5 cm in diameter, in which five larvae of *Galleria mellonella* were placed and they were monitored until the larvae passed to the pupal stage.

In cups with soil contaminated with *Beauveria* spores, larvae with white mycelium were observed and after five days, the fungus was isolated from these larvae in the sabouraud dextrose agar (SDA) medium (Bioxon®) and incubated at 32 °C + 2. All grown fungi were placed in 20 ml vials with SDA medium and saved for DNA amplification by a polymerase chain reaction (PCR) test.

DNA extraction and product amplification by PCR

DNA extraction by PCR was done in the laboratory of the Faculty of Agricultural and Livestock Sciences of the Meritorious Autonomous University of Puebla (BUAP, for its acronym in Spanish) in 2020. For this purpose, five isolates with *Beauveria* morphological characteristics were developed on cellophane paper adhered to the surface of the SDA culture medium for five days at 30 °C + 2. The mycelium obtained was harvested with a spatula and placed in 1.5 ml vials and then lyophilized for 24 h.

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Subsequently, this mycelium was macerated with a micro pestle and liquid nitrogen in 1.5 ml vials and the instructions of the kit (Qiagen®) manufacturer were followed to extract the DNA, which was visualized in an agarose gel and a transilluminator (UVP, Model 3UV-LMS26), each DNA sample obtained was stored in a refrigerator at 7 °C. For the amplification of the ITS region of the 5.8S rRNA gene by PCR, the primers ITS1: 5'-ATTACCGAGTTTTCAACTCCC-3' and ITS2: 5'-ACCTGATTCGAGGT CAACGTTC-3' (White *et al*., 1990) and a kit (Promega madison) were used, according to the procedure described by Rehner and Bunkley (2005).

The reaction mixture was prepared to a final volume of 50 µl and included 0.2 µM of each primer; 20 mM TrisHCl; 50 mM KCl; 2.5 mM $MgCl_2$; 0.1 mM of each deoxynucleotide (dATP, dCTP, dGTP, and dTTP); 1 U Taq DNA polymerase and 50 ng of genomic DNA. Samples were amplified by a program of 15 s at 94 °C, followed by 40 cycles at 94 °C for 15 s, 50 °C for 30 s, 72 °C for 30 s, and a final extension of 7 min at 72 °C. The amplified product was sequenced by Macrogen Laboratories in Korea. The sequences were edited with the BioEdit program version 7.1.9 (Hall, 1999) and the phylogenetic tree was generated with the Neighbor-joining method with the Mega program version 3.6 (Tamura *et al*., 2013).

Efficacy of mortality and mycosis of isolates on larvae of *Galleria mellonella*

To establish the bioassay, concentrations of $1x10^6$ spores ml^{-1} of each identified isolate were prepared, a commercial strain of *Beauveria bassiana* (PHC) and a control were also used, each of these was used to inoculate 12 larvae distributed on six circles of Sanita paper; that is, two larvae per circle of paper moistened with 300 µl of sterile distilled water. All dishes with larvae were placed at room temperature and the number of live larvae, number of dead larvae, and number of larvae with mycosis were recorded at 24, 48, 72, 96, 120, 144, 168.

Experimental design and statistical analysis

The experiment was completely randomized with seven treatments (five compared isolates plus one commercial isolate and one control), where the experimental unit was a larva and it was repeated twice over time to reduce the standard error that could be generated in the statistical analysis and obtain reliable results. With the results obtained from the experiment, a statistical analysis was performed with the SAS statistical package for Windows 9.0 in a factorial arrangement (two factors; factor= isolate and factor= time to death and mycosis). A comparison of means was carried out with the Tukey test to determine if there was a significant difference between the results of the variables (number of dead larvae and number of larvae with mycosis).

Results and discussion

A total of five isolates (6, 7B, 11, 18, 21) were obtained from 60 analyzed samples, which presented morphological characteristics that agree with the description of *Beauveria* by Humber (1996); Bustillos (2001); Rodríguez and Del Pozo (2003) in PDA medium. The mycelium had a white shade, with a soft, powder-like texture. The growth of each of the isolates was not uniform, while the shapes of the colonies were raised and thin. Under the microscope, the conidiogenous cells presented denticulated and extended apex, in repeated shapes that grew into a conidium just below the new conidium. The conidia were round in shape and adhered to the branched conidiophores.

PCR amplification of DNA of native isolates

It was possible to amplify by PCR the DNA of 5 isolates with morphological characteristics of the genus *Beauveria*. The amplified DNA was visualized between 200 and 350 bp when compared to a molecular weight marker in a manner similar to the ITS region amplification results obtained by Rehner and Buckle (2005); Zhang *et al*. (2020) to identify species of *Beauveria bassiana* extracted from different regions and study their diversity.

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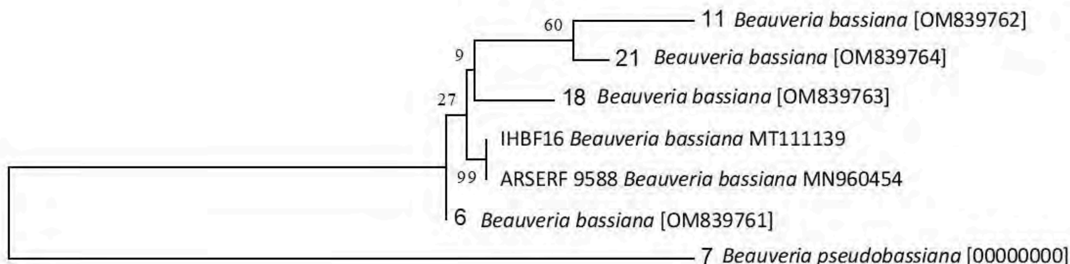
The numbers of accessions to the gene bank generated with the recording of the isolates are shown in Figure 1, which also shows the phylogenetic position of each of them within the tree, which was obtained from the ITS data of the 5.8S rRNA gene by the Neighbor-joining method (Tamura *et al*., 2013). The results of this analysis placed all isolates in the genus *Beauveria* with four isolates of *Beauveria bassiana* and one of *Beauveria pseudobassiana* (Figure 1).

Figure 1. Phylogeny of *Beauveria* isolates. Sequences from the USDA-ARS collection of access numbers: MT111139.1 and MN960454.1 of fungal cultures were used as references. Only bootstrap values above 80% are displayed.



This result showed a low number of isolates extracted, as well as low diversity, unlike the results obtained by Pérez-González *et al*. (2014); Medo *et al*. (2016); Serna-Domínguez *et al*. (2019), who found greater diversity of this genus in different sites; likewise, these authors mention the association of genetic groups of *Beauveria bassiana* with the geographical origin and types of habitats, factors that could be involved in the low diversity in this case because samples were only taken from one place.

On the other hand, according to Serna-Domínguez *et al*. (2019); Toledo *et al*. (2019), it is common to detect intraspecific diversity in this genus of fungus in addition to the dominance of some of them in agricultural soils, and although in this case diversity within the species was not analyzed, there was dominance of the species *Beauveria bassiana* in this study site.

Mortality and mycosis of isolates on larvae of *Galleria mellonella*

Mortality of *Galleria mellonella* caused by native isolates of *Beauveria*: the statistical analysis of the data showed a significant difference between the interactions of the factors, isolates x time (*p*# 0.05) and although a difference was observed in isolate 11 (100% mortality at 96 h) (Figure 2).

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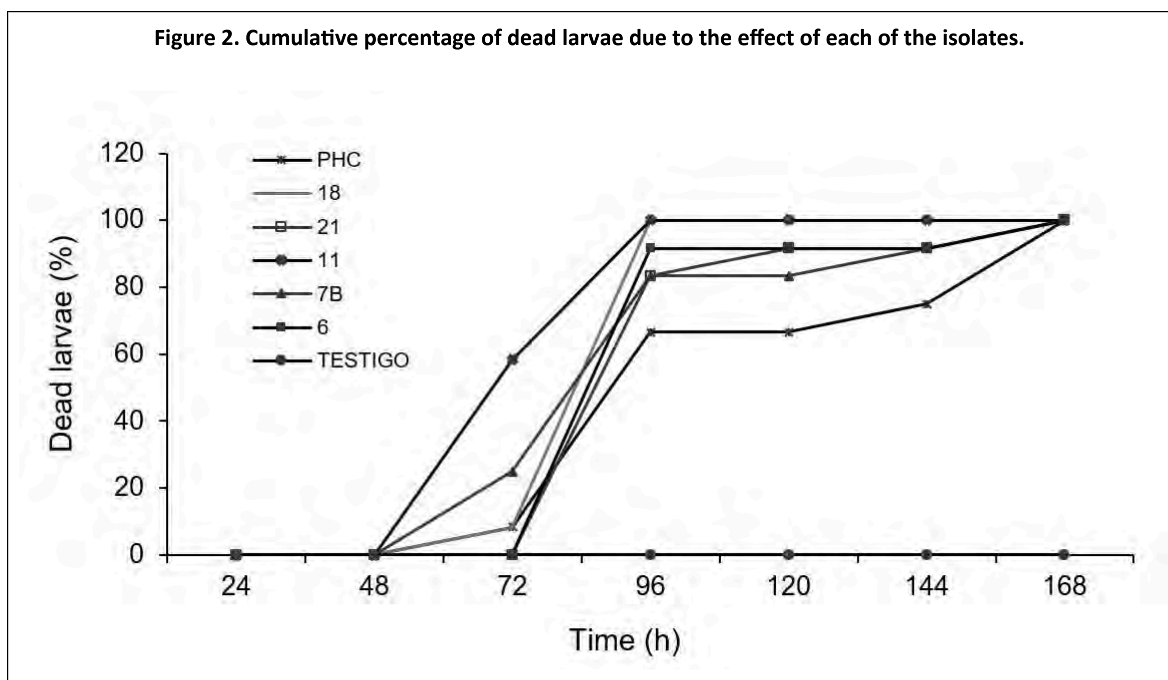
Figure 2. Cumulative percentage of dead larvae due to the effect of each of the isolates.



Statistically, no significant difference was observed between the isolates, but there was a significant difference with the control, even with the isolate identified as *Beauveria pseudobassiana* and the commercial isolate, which had 100% mortality up to 168 h. These results are consistent with those obtained by Ibrahim *et al*. (2016); Tuncsoy *et al*. (2020), who report mortality of up to 98 and 100% with $1x10^{6}$ spores ml^{-1} of *Beauveria bassiana* after 1.7 days of application. Likewise, Vertyporokh *et al*. (2019) mention that *Beauveria bassiana* kills the infected host by mechanical destruction of its tissues with the growing mycelium and by the action of secreted secondary metabolites.

On the other hand, there was a difference in the times in which the larvae died ($p\leq 0.05$), where it was observed that the largest number of larvae died at 96 h; that is, when applying spores of the isolates on larvae of *Galleria mellonella* L., their effect should be expected after 72 h and greater mortality at 96 hours (Table 1).

Table 1. Larvae killed by the effect of spores of different isolates of *B. bassiana* and *B. pseudobassiana* at differenttimes.

Species	Isolates	X̄ of the isolate factor	Time (h)	X̄ of the time factor
Beauveria bassiana	PHC	0.15476 a	24	0 c
Beauveria bassiana	18	0.15476 a	48	0 c
Beauveria bassiana	21	0.14286 a	72	0.14286 b
Beauveria bassiana	11	0.14286 a	96	0.14286 a
Beauveria pseudobassiana	7B	0.14286 a	120	0.0119 c
Beauveria bassiana	6	0.14286 a	144	0.02381 c
	Control	0 b	168	0.07143cb

Similar results were obtained by Alcazar (2007) in a study carried out with six isolates, in which mortality was at 72 h in all the isolates tested, except for one that presented greater enzyme activity at 48 h. Vertyporokh *et al*. (2019) also observed antifungal activity in the hemolymph of larvae infected with *Beauveria bassiana* at 96 h after infection.

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In addition, according to the results achieved in this study, Vertyporokh *et al*. (2019); Boston *et al*. (2020); Zhang *et al*. (2020) mention that despite the fact that low genetic diversity has been found in *Beauveria* populations, the species show differences in their virulence, which is directly related to mortality, and those organisms whose defense mechanisms or virulence seem to be more effective can survive a certain dose of spores.

Mycosis of native isolates of *Beauveria* on larvae of *Galleria mellonella*: a significant difference was observed in the interactions of mycosis and time factors (≤ 0.05). Likewise, the effect of mycosis of the isolates was also observed independently, where isolate 11, identified as *Beauveria bassiana*, was different ($p \leq 0.05$) from the control with 100% mycosis, it was even different from the commercial isolate PHC (20% mycosis) and *Beauveria pseudobassiana* (60% mycosis).

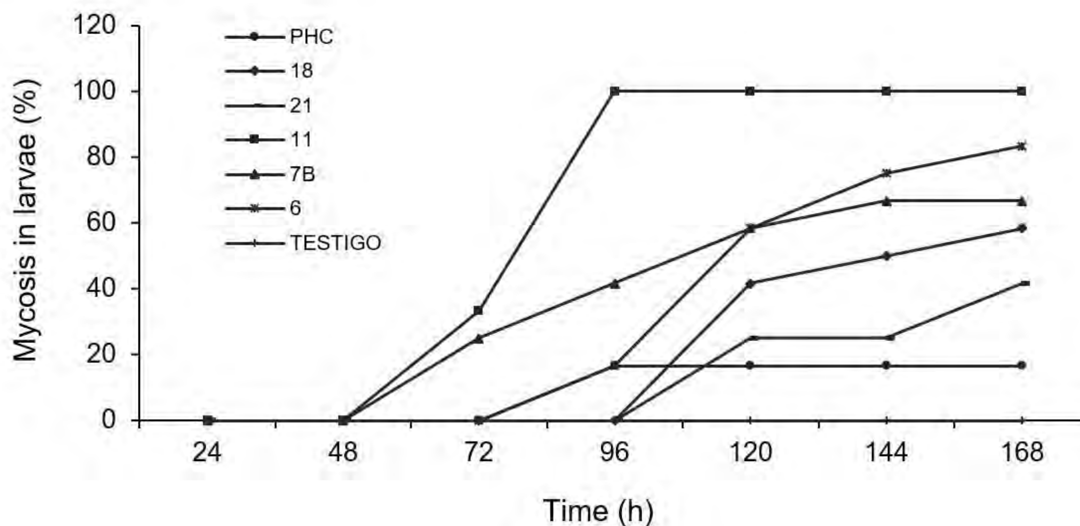
Some authors such as Hajek *et al*. (2018); Hajek *et al*. (2021) mention advantages in the mycosis capacity of fungal isolates as this represents an opportunity for spore dispersal to cause epizootics and reduce insect populations. On the other hand, there was a significant difference in the times in which larval mycosis occurred ($p \leq 0.05$) (Table 2) and the highest number of larvae with mycosis was observed at 96 h (Figure 3).

Table 2. Mycosis due to the effect of spores of different isolates of *Beauveria bassiana* and *Beauveria pseudobassiana* at different times.

Beauveria bassiana	Isolate	X̄ of the isolate factor	Time (h)	X̄ of the time factor
Beauveria bassiana	PHC	0.02381 bc	24	0 c
Beauveria bassiana	18	0.08333 bac	48	0 c
Beauveria bassiana	21	0.05952 bac	72	0.08333 bc
Beauveria bassiana	11	0.14286 a	96	0.22619 a
Beauveria pseudobassiana	7B	0.09524 bac	120	0.11905 ba
Beauveria bassiana	6	0.11905 ba	144	0.04762 bc
	Control	0 c	168	0.04762 bc

Values with different letters are significantly different.

Figure 3. Percentage of larvae with mycosis due to the effect of each of the isolates.



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The mycosis time reported by Quintero-Zapata *et al*. (2020) was 48 h in *Aedes aegypti* mosquito larvae; nevertheless, this type of larvae is smaller in size and a different species, which may influence the mycosis period, unlike *Galleria mellonella*. In this regard, Rohrlich *et al*. (2018) according to a study carried out to measure severity of the disease based on mortality and mycosis, observed a difference in mycosis that depended on the range of hosts of each species of *Beauveria*, therefore they suggest variation in the time of mycosis between different types of insects.

Conclusion

Beauveria bassiana and *Beauveria pseudobassiana* isolates may have differences in their ability to cause death in *Galleria mellonella* larvae, even between isolates of the same *Beauveria bassiana* species. In this way, by knowing these differences, we can select those isolates that have potential for the control of other larvae. Likewise, carrying out identification and mortality studies over time with native isolates is of great importance since, according to these results, it is possible to have an effective control of insects in the region, in addition to serving as a basis for the research of new experiments.

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