

PRODUCTION, VIRULENCE, AND VIABILITY OF *Beauveria bassiana* (Bals.) Vuill. CONIDIA OBTAINED BY FERMENTATION OF AMARANTH STUBBLE

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ABSTRACT

Entomopathogenic fungi biological formulations require viable concentrations of infective units (conidia) with high virulence. These are mainly produced by solid-state cultivation of agro-industrial residues with high C/N ratios as substrates, such as amaranth stubble (*Amaranthus hypochondriacus* L.), which is discarded in the fields due to its lack of postharvest utility and thus becomes a source of contamination. Therefore, its use can reduce production costs when compared to common substrates such as rice (*Oryza sativa*). The objective of this work was to compare the effect of amaranth stubble on the production, virulence, and viability of *Beauveria bassiana* (Bals.) Vuill. (Hypocreales: Cordycipitaceae) conidia produced by solid-state cultivation to those produced using rice as a substrate. The results showed that the yield of *B. bassiana* conidia produced with rice was 15 higher than that produced with amaranth stubble. However, there were no significant differences in the virulence and viability of conidia produced by both substrates. Therefore, the use of amaranth stubble is an economical alternative to produce *B. bassiana* conidia compared to common substrates like rice.

Keywords: *Amaranthus hypochondriacus* L., lethal time 50.

INTRODUCTION

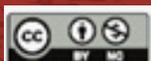
Biological control involves the application of natural enemies or their components to pest insects in order to reduce their population density to an economically acceptable level (Bueno *et al.*, 2021). Entomopathogenic fungi (EPF) are among the most widely used agents for this purpose due to their high infectivity and relatively simple production. *Beauveria bassiana* (Bals.) Vuill. (Hypocreales: Cordycipitaceae) is one of the most commonly used EPF in biological control due to its ability to infect various

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orders of insects, as well as its widespread distribution in nature (Imoulan *et al.*, 2016; Gebremariam *et al.*, 2021).

The production of hydrolytic enzymes in this fungus is regulated by induction-repression mechanisms at the substrate level, meaning that its production is directly influenced by the carbon and nitrogen sources in the culture medium, and it is also involved in its virulence (Altinok *et al.*, 2019). Virulence is not universal between host/pathogen pairs, although virulence of *B. bassiana* strains often varies among insects, indicating great plasticity (Zhang *et al.*, 2020).

Furthermore, the use of indigenous isolates in biocontrol can minimize the risk of introducing non-native strains that could threaten local ecosystems. In addition, native isolates may have greater potential for commercial production as they may be easier and more cost-effective to produce on a larger scale (Gebremariam *et al.*, 2021). Therefore, the identification and characterization of local strains of *B. bassiana* is crucial for the development of effective and sustainable biological control strategies in agriculture.

EPF can be produced through liquid or solid-state cultivation, with the latter method producing conidia with better viability and resistance to abiotic factors. Aerial conidia are grown on inexpensive substrates that ensure high yields. Agro-industrial residues are a popular choice due to their low cost and high carbon-nitrogen ratio (C/N) (Jaronski, 2023). The objective of this study was to evaluate the effect of amaranth stubble (*Amaranthus hypochondriacus* L.) on the production, virulence, and viability of *B. bassiana* conidia produced through solid-state cultivation, compared to those produced using rice (*Oryza sativa*) as a substrate.

MATERIALS AND METHODS

Microorganism

Beauveria bassiana (Bals.) Vuill. (Hypocreales: Cordycipitaceae) DS3.17 was isolated from coffee fields in the Papaloapan basin region, in the community of Rancho Grande, Valle Nacional, Oaxaca (17° 50' 39.3216" N, 96° 19' 59.4906" W). Mycosed insects were collected and transported in humid chambers to the Bioprocesses laboratory of the University of Papaloapan, Tuxtepec campus, where they were cultured on potato dextrose agar. The cultures were serially propagated until pure cultures were obtained (Gandarilla-Pacheco *et al.*, 2013). The genomic DNA of the pure strains was extracted using a commercial kit (UltraClean®), and the samples were sent to the Institute of Biotechnology of the UNAM Cuernavaca unit (IBt) for sequencing. The results were analyzed using the MEGA X bioinformatics software, and a phylogenetic tree was constructed.

Conidia production kinetics

Conidia production kinetics were carried out in triplicate using plastic containers (10 cm length x 10 cm width x 4 cm depth) with 25 g of either rice (*Oryza sativa*, C/N ratio

8.38) or amaranth stubble (*Amaranthus hypochondriacus* L., C/N ratio 6.54) inoculated under aseptic conditions with 32.5 mL of a conidia suspension at a concentration of 2×10^9 conidia mL⁻¹, with an initial humidity of 75 % and a water activity of 0.99 (López-Sosa *et al.*, 2017). The containers were then incubated at 25 °C for 8 days (Sev Prendo INO 650V-7, Mexico). Every 24 hours, 2 g of the fermented substrate were taken, and 20 mL of distilled water were added to each sample. The suspension was homogenized for 2 minutes with a vortex (Genie 2, Scientific Industries, New York, USA). Subsequently, aliquots of 1 mL were taken to make serial dilutions ranging from 1×10^{-1} to 1×10^{-3} , which were used to count spores with a hemacytometer (Neubauer chamber) and an optical microscope (Olympus Ch2) (Núñez-Gaona *et al.*, 2010).

Viability

At the end of the solid-state cultivation (SSC), 3 g of each substrate were resuspended in 10 mL of distilled water. The supernatant was serially diluted until a final concentration of 50 to 300 conidia in 500 µL of distilled water was reached. This volume was then inoculated into Petri dishes containing 4 % Sabouraud dextrose agar (Bioxon, Mexico). The dishes were incubated at 25 °C (Sev Prendo INO 650V-7, Mexico) and colonies were counted after 24 h (Monzón, 2001). The percentage of viability was calculated as the ratio between the number of colonies that emerged and the initial count of conidia, multiplied by 100 (Qin, 2014).

Bioassays

Tenebrio molitor (Coleoptera: Tenebrionidae) colonies were established in rectangular plastic boxes at different developmental stages. The insects were kept at room temperature and fed a mixture of oats and wheat bran (1:1 w/w). Additionally, a sterile moist cotton was placed in each box. Healthy and active *T. molitor* larvae groups of a single color and a length of 2 cm were selected for the experiment. For each experimental unit, 12 larvae were submerged for 15 seconds in a suspension containing 1×10^8 conidia mL⁻¹ or distilled water (control). Each experimental unit was then placed in a transparent plastic box with sterile oatmeal and incubated at 25 °C with a photoperiod of 12:12 h (Montesinos-Matías *et al.*, 2011). Each treatment was carried out in triplicate. The results were reported as a percentage of daily mortality.

Statistical analysis

The results were statistically analyzed using a two-way ANOVA with a significance level (α) of 0.05 using statistical software (Minitab™, USA). For the bioassays (mortality), a one-way ANOVA was performed using the Statistics and Machine Learning Toolbox (Matlab™, USA) with a significance level (α) of 0.05. The value for lethal time 50 was obtained through a manual interpolation of the results.

RESULTS AND DISCUSSION

Conidia production

Conidia production kinetics for *Beauveria bassiana* (Bals.) Vuill. (Hypocreales: Cordycipitaceae) DS3.17 using rice or amaranth stubble (*Amaranthus hypochondriacus* L.) as substrates were registered (Figure 1). The production profiles for both substrates were similar for the first 4 days of the bioprocess. However, conidia production started on the third day in both cases, with the highest concentration being obtained with rice on the fifth day (5.63×10^9 conidia g^{-1} of substrate), which then gradually decreased

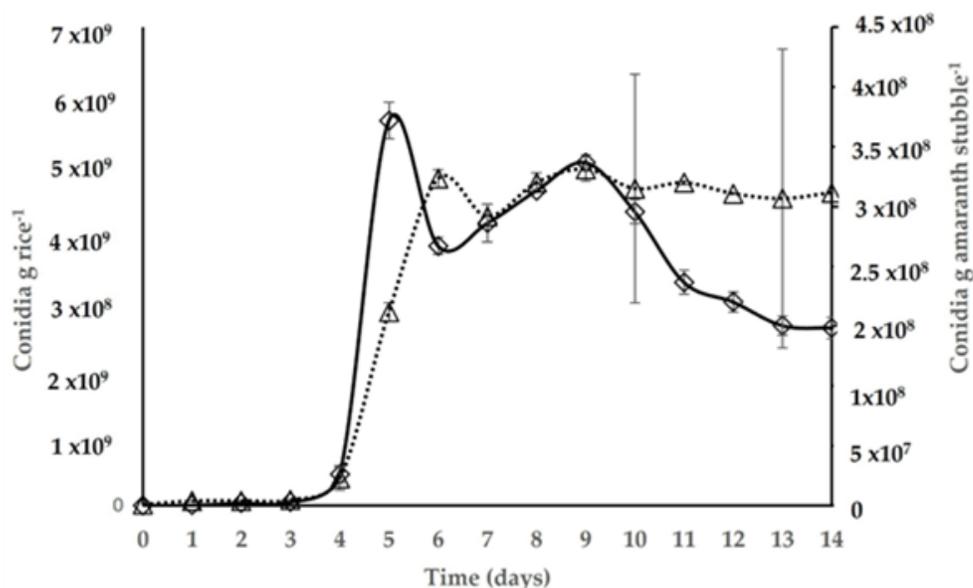


Figure 1. Production of *Beauveria bassiana* (Bals.) Vuill. (Hypocreales: Cordycipitaceae) DS3.17 conidia through solid fermentation using rice (*Oryza sativa*) (\diamond) or amaranth stubble (*Amaranthus hypochondriacus* L.) (Δ) as substrates. The different scales on the graph are used to highlight the conidia production profiles for both substrates, as there is a significant difference in magnitude between them.

until the ninth day before decreasing steadily until the end of fermentation. In contrast, for amaranth stubble, the maximum production (2.23×10^8 conidia g^{-1} of substrate) was reached on the sixth day and remained constant until the end of fermentation.

The concentration of conidia was 15 times higher when rice was used as a substrate, which was attributed to the concentration of simple carbohydrates in rice compared to complex carbohydrates (cellulose, hemicellulose, and lignin) found in amaranth stubble. These complex carbohydrates cause slow assimilation by the fungus (García-Pereyra *et al.*, 2009; Méndez-Arango *et al.*, 2019). Even though a higher concentration of conidia was obtained with rice, the viability and virulence did not show significant

differences between substrates. This is relevant because amaranth stubble is a low-cost agricultural byproduct, often an agro-industrial waste that causes pollution problems when burned.

In comparison to rice, which is a grain for human consumption, amaranth stubble is a more sustainable and cost-effective alternative. However, conidia concentrations higher than 1×10^8 conidia g^{-1} of substrate were obtained with amaranth stubble and were sufficient to evaluate virulence using bioassays (Montesinos-Matias *et al.*, 2011). In previous reports, the profile of conidia production by solid-state cultivation with *Beauveria bassiana* 885.2 was independent of the substrate used, whether it was broken rice, broken maize, or wheat bran (López-Sosa *et al.*, 2017). When comparing the conidia production of *Beauveria bassiana* 885.2 and *Beauveria bassiana* DS3.17 on broken rice, both strains had similar results; however, the use of amaranth stubble as a substrate influenced conidia production.

Viability

Conidia viability was not significantly different between rice (87.4 %) and amaranth stubble (88.9 %) (Figure 2). Montesinos-Matías *et al.* (2011) reported viability values between 68 and 82 % for conidia produced in Sabouraud maltose agar with 3 % oat and 0.5 % yeast extract, while Núñez-Gaona *et al.* (2010) reported values of 87 % using *Beauveria bassiana* 885.2 on similar media. In a study on the growth of *Beauveria bassiana* in agar/water media, Lopes *et al.* (2018) reported viability values between 98.7 and 99.2 %. However, in this study, values ranging from 87.4 to 88.9 % were obtained. Viability is a crucial parameter in the production of EPF as the efficacy of biological formulations depends on values higher than 85 % (Jaronski, 2023).

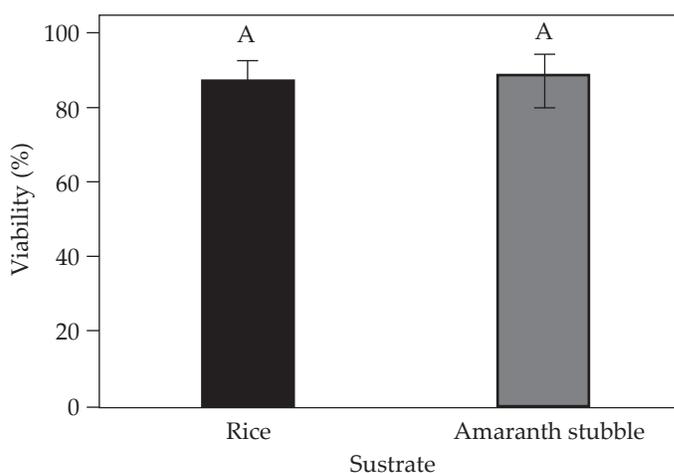


Figure 2. Viability of *Beauveria bassiana* (Bals.) Vuill. (Hypocreales: Cordycipitaceae) DS3.17 conidia produced by solid fermentation. (■): rice (*Oryza sativa*) stubble; (▒): amaranth (*Amaranthus hypochondriacus* L.) stubble. Equal letters mean that there are not significant differences ($p \leq 0.05$)

Bioassays

The percentage of *T. molitor* larvae mortality reached 100 % in 8 and 9 days when using *Beauveria bassiana* DS3.17 conidia produced with rice and amaranth stubble, respectively. The mortality profiles were similar regardless of the substrate used (Figure 3). The virulence of the fungus is one of the relevant parameters for using EPF as a biocontrol agent in agriculture. It is necessary to develop strategies to increase the virulence of infective units (Méndez *et al.*, 2010; Gandarilla-Pacheco *et al.*, 2013).

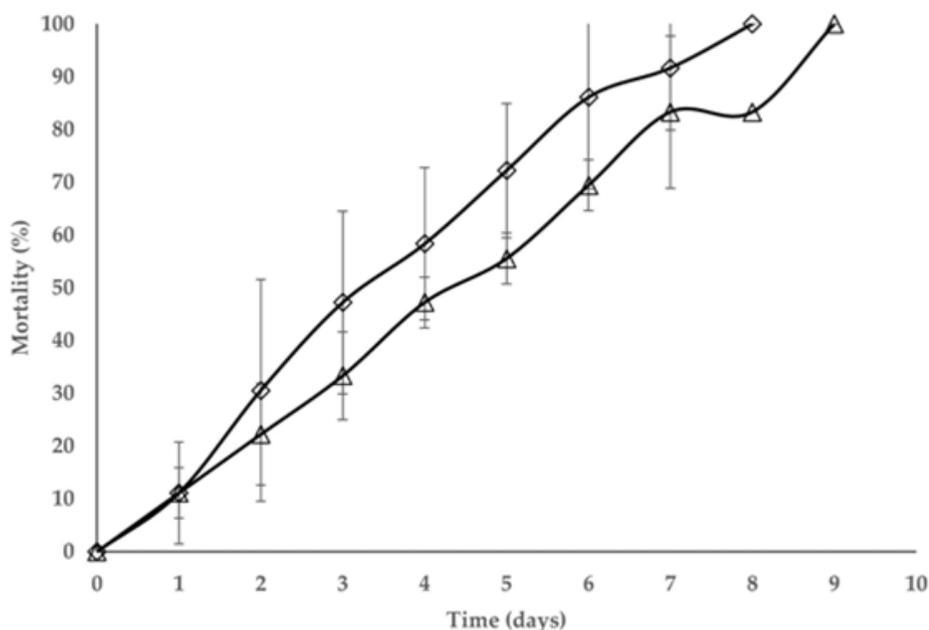


Figure 3. Mortality of *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae with *Beauveria bassiana* (Bals.) Vuill. (Hypocreales: Cordycipitaceae) DS3.17 conidia produced by solid fermentation using rice (*Oryza sativa*) (◇) or amaranth stubble (*Amaranthus hypochondriacus* L.) (△) as substrate.

Several studies have been carried out on different strains and culture media. For instance, Lopes *et al.* (2018) evaluated the effect of Sabouraud Dextrose broth at 4 % with 1 % (w/w) yeast extract and obtained 25 % mortality in 7.2 days with *Beauveria bassiana* J21. These results were attributed to the effect of keeping the conidia in a 2 % mixture of water and agar. In another study, Montesinos-Matías *et al.* (2011) used a different strain of *B. bassiana* cultured in Sabouraud maltose agar (SMA) and reported 97 % mortality on day 15. In this work, the time required to achieve total mortality was shorter than that reported in the literature (Molina and Espinal, 2000; Montesinos-Matías *et al.*, 2011). There were no significant differences ($p \leq 0.05$) between the means of the results for both substrates.

Lethal Time 50

The values for lethal time 50 (LT_{50}) obtained with *Beauveria bassiana* DS3.17 conidia produced on both substrates were four days. Our results consistent with Molina and Espinal (2000), who used a strain of *Beauveria bassiana* produced by solid fermentation with rice as a substrate to control *Hypothenemus hampei*, *Anthonomus grandis*, *Cosmopolites sordidus*, and *Plutella xylostella* in stored corn, reporting LT_{50} values between 3.84 and 5.17 days.

CONCLUSIONS

The use of amaranth stubble (*Amaranthus hypochondriacus* L.) as a substrate for solid fermentation produced a lower concentration of conidia compared to rice (*Oryza sativa*); however, the results suggest that the use of amaranth stubble is a viable alternative for *Beauveria bassiana* (Bals.) Vuill. (Hypocreales: Cordycipitaceae) DS3.17, as it still exhibited high viability and a high mortality against *T. molitor*, both of which are suitable parameters for developing biological formulations. Additionally, amaranth stubble is an agricultural residue with low added and economic value in comparison to common substrates such as rice.

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