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Coffee Grounds as a Sustainable Alternative Substrate for Large-Scale Production of *Beauveria bassiana*

Borra de Café: Adição que Otimiza a Produção Industrial de Beauveria bassiana

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Abstract

This study aimed to evaluate the feasibility of using coffee grounds as an alternative substrate for the mass production of the entomopathogenic fungus *Beauveria bassiana*, comparing its performance with conventional solid-state fermentation systems, particularly the rice-based method commonly used in industry. The proposed approach addresses the need to reduce production costs, improve process efficiency, and valorize low-commercial-value agro-industrial residues, while maintaining the biological effectiveness of the fungus. Different substrate formulations composed of coffee grounds alone or mixed with soil or clay at proportions of 100% and 50–50% were tested, and three initial moisture levels (60%, 70%, and 80%) were evaluated. After inoculation with 10^7 conidia·g^{-1} and incubation at 26 °C for ten days, conidial production was quantified. The most promising formulation was then compared with the standard rice substrate in terms of spore yield and maintenance of fungal virulence. Bioassays were performed with *Sitophilus oryzae* and *Rhyzopertha dominica* using conidial concentrations ranging from 10^5 to 10^9 conidia·mL^{-1}. Mortality, confirmed fungal infection (conidiogenesis), and lethal time were assessed.The results showed that the coffee-grounds/soil mixture (1:1) at 70% moisture yielded the highest spore production, surpassing the conventional rice-based system. The fungal strain maintained high virulence, reaching 100% mortality in both insect species at the highest concentrations tested. LT_{50} values ranged from three to four days, depending on the target species. Coffee grounds,particularly when combined with soil—constitute an efficient, economically viable, and sustainable substrate for *B. bassiana* production, improving conidial yields while preserving fungal infective potential. This substrate represents a promising innovation for large-scale bioinsecticide production.

Keywords: Agro-Industrial Waste. Entomopathogenic Fungi. Solid-State Fermentation. Bioinsecticide. Agricultural Sustainability.

Resumo
O modelo agrícola contemporâneo enfrenta um desafio crescente: a dependência de pesticidas sintéticos. Embora eficazes a curto prazo, esses agroquímicos representam um risco significativo à saúde humana e causam graves desequilíbrios ambientais. Diante disso, o controle biológico de pragas surge como uma alternativa sustentável e de importância vital. Entre as estratégias disponíveis, a utilização de fungos entomopatogênicos—organismos que naturalmente infectam e matam insetos—destaca-se como um dos modelos mais eficientes. A crescente demanda por bioinseticidas baseados em fungos, como o *Beauveria bassiana*, impulsiona a necessidade urgente de aprimorar os sistemas de produção em escala industrial. O principal gargalo reside nos altos custos operacionais e na busca por substratos de cultivo que sejam simultaneamente efetivos e economicamente viáveis. Neste contexto, vários pesquisadores têm focado na valorização de resíduos de baixo valor comercial. O presente estudo investigou a viabilidade da utilização de um resíduo doméstico e agroindustrial amplamente disponível: a borra de café, como substrato nutritivo para a produção maciça de *Beauveria bassiana* via fermentação em estado sólido. Os achados desta pesquisa são promissores e demonstraram que formulações utilizando a borra de café não apenas são economicamente viáveis, mas também se revelaram mais eficientes na esporulação do fungo quando comparadas ao sistema de produção convencional, que tipicamente utiliza grãos de arroz. Esta descoberta estabelece a borra de café como um substrato superior para bioinseticidas, oferecendo uma solução de duplo impacto positivo: reduzindo um resíduo e otimizando a produção de agentes de controle biológico.

Palavras-chave: Resíduo Agroindustrial. Fungos Entomopatogênicos. Fermentação em Estado Sólido. Bioinseticida. Sustentabilidade Agrícola. Valorização De Resíduos. *Beauveria Bassiana*.

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1 Introduction

Agricultural biotechnology has demonstrated substantial advances, with applications include biological control as a method for pest management. This approach differs from synthetic insecticides due to the nature of its agents and the absence of persistent residues, positioning it as a component of sustainable agricultural systems (Ferreira *et al*., 2024; Van Der Vlugt, 2018).

Entomopathogenic fungi represent a class of biological agents widely used in biological control. Their cultivation and field application are considered accessible procedures, and their use has experienced widespread growth in Brazil. Among the genera employed, *Beauveria* is one of the most used, serving as the basis for the production of bioinsecticides (Andrade *et al*., 2020; Goettel, 2016).

The genus *Beauveria* (Bals.) Vuill., belonging to the order Hypocreales and the family Cordycipitaceae, comprises six species: *Beauveria alba*, *B. amorpha*, *B. bassiana*, *B. brongniartii*, *B. velata*, and *B. vermiconia* (Ghikas *et al*., 2010; Mugnai; Bridge; Evans, 1989). Among these species, *Beauveria bassiana* stands out due to its broad use. This species produces hyaline, rounded conidia, which can be uninucleate or multinucleate. Its conidiophores occur singly or in irregular groups and exhibit a structure that widens at the base and tapers toward the apex, from which the conidia emerge (Barnett, 1958).

When cultivated on Petri dishes, colonies exhibit a morphologies ranging from cottony to powdery, with a white to slightly pigmented coloration, and may produce pigments in the culture medium (Luna-Alves-Lima, 1992; Vilas Boas; Paccola-Meirelles). Furthermore, yeast-like strains isolated from insect hemolymph were described by McLeod (1954) and later termed blastospores by Ferron (1978). Currently, several strains of *Beauveria bassiana* are employed on a commercial scale for the production of bioinsecticides (Ferreira *et al*., 2024).

While bioprospecting efforts aimed at the discovery of new strains of commercial interest are consistently conducted, studies addressing the ideal substrates for the production of these biological agents remain limited.

The main objective of the present study is to evaluate the growth of *Beauveria bassiana* on an alternative substrate composed of coffee grounds. The research aims to compare the effectiveness of this formulation with conventional fermentation methods, thereby contributing to the optimization bioinsecticide production processes.

2 Material and Methods

2.1 Microorganism

The strain used in this study was *Beauveriabassiana* - Bb 01, provided by the Bioprocesses Laboratory of the Universidade Estadual do Centro Oeste do Paraná. The strain was maintained at 4 °C and cultured on Potato Dextrose Agar (PDA) prior to use in the experiment.

Beauveria bassiana was produced in Erlenmeyer flasks (250 mL) containing 50 mL of PDA, incubated at 26 °C for 10 days, under static conditions. The spore suspension was prepared by adding 40 mL of sterile distilled water, 15 g of glass beads, and 0.1% Tween 80 followed by shaking for 30 min on a magnetic stirrer. Spores were counted using a Neubauer chamber.

2.2 Substrate selection

To select the best substrate/support for spore production, the following formulations were tested: 1- coffee grounds (100%); 2 - mixture of coffee grounds and clay (50−50%); 3 - mixture of coffee grounds and soil (50−50%). The initial moisture content for each formulation was adjusted to 60%, 70%, or 80% . The experiments were performed with three replicates, [. The substrate/support was inoculated with a spore suspension (107 spores·g^{-1} of dry matter, DM) and the pH was adjusted to 6.2±0.2 with NaOH (1 M). The flasks were incubated at 26 °C for 10 days. After this period, 1 g of the substrate/support was mixed with 30 mL of distilled water, 0.01% Tween 80 , and 15 g of glass beads. After 30 min of shaking, the mixture was filtered 200 μm nylon sieve, and the spore concentration was determined using a Neubauer chamber.

2.3 Economic viability assessment of the substrate

Economic viability was assessed by comparison between the results of the best formulation obtained in the substrate selection and the cultivation of *Beauveria bassiana* on rice (Alves *et al*., 1998), a method currently used in industry.The experiment was conducted in triplicate with both cultivation systems receiving an inoculum of 107 spores·g^{-1} of dry matter, incubated at 26°C for 10 days.

2.4 Assessment of strain virulence after cultivation on different substrates

The conidia obtained from the different fermentation systems were suspended in autoclaved distilled water, and the concentration of viable spores was determined using a Neubauer chamber. For the bioassay, the following concentrations were tested in triplicate: 1.0×10^5; 1.0×10^6; 1.0×10^7; 1.0×10^8, and 1.0×10^9 conidia·mL^{-1}.

The bioassays were conducted using a completely randomized design, with three replicates for each of the five concentrations, plus a control group. Each replicate consisted of a disposable 300 mL plastic container with a transparent lid. Filter paper treated with 0.5 mL of the conidial suspension was placed at the bottom of each container; the control received 0.5 mL of distilled water. Ten adult insects of each species were placed in each container, selected from the most active and healthy individuals from the rearing colony. Each container received a code, and its position in the culture chamber was randomized. Two species of beetle (*Sitophilus oryzae* and *Rhyzopertha dominica*) were used in the bioassays. Inoculation occurred through contact with the treated surface; they remained on the treated paper for the entire period of the bioassays. Wheat grains were provided as food for the insects.

Evaluations were performed every 24 h for six consecutive days. Dead insects were transferred to new disposable plastic containers (150 mL) lined with filter paper moistened with distilled water to confirm fungal-induced mortality. The mortality data (total and confirmed) of the tested concentrations and the lethal time at the concentration of 10^9 conidia·mL^{-1} were subjected to linear and polynomial regression analyses. The conidiogenesis rate (number of insects with sporulation divided by thetotal number of dead insects) was also calculated.

3 Results and Discussion

The results demonstrate that the formulation at a 1/1 ratio (coffee grounds and soil) showed the highest spore production compared with the other formulations tested. This superior performance was observed across all evaluated moisture levels. An initial moisture content of 70\% was the most efficient for fungal spore production.

The coffee grounds and clay formulation also proved to be effective; however, spore production was lower than that of the current commercial system based on rice . Furthermore, analysis of Table 1 indicated that coffee grounds usedin their integral composition (100%) resulted in the lowest spore production for the growth of *Beauveria bassiana*.

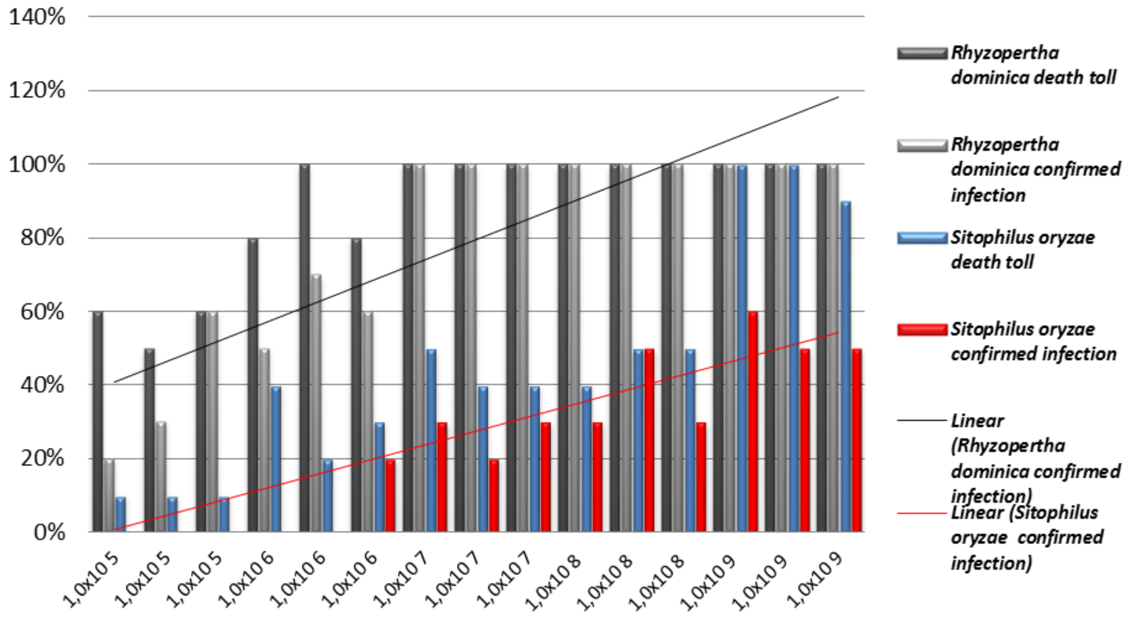
Table 1 - Conidial count on each of the substrates and at all initial moisture contents evaluated

Humidity content	**Rice**	**Coffee grounds (100%)**	**Coffee grounds and clay (50-50%)**	**Coffee grounds and soil (50-50%)**	**Humidity content**
60%	3,5	1,0	2,5	3,4	60%
70%	2,5	1,2	3,0	4,0	70%
80%	2,3	0,82	2,8	2,6	80%

The values should be multiplied 1,0x109 conidia/g^{-1} and were obtained after 10 days of fermentation.
Source: research data.

Figure 1 illustrates that the tested insect species were sensitive to the *B. bassiana* strain. The spore concentration to which the insects were exposed directly influenced both the total mortality rate and the rate of fungal infection.

Figure 1 – Total and confirmed mortality for the species, subjected to different concentrations of *Beauveria bassiana*, obtained from the coffee grounds/soil (1/1) substrate

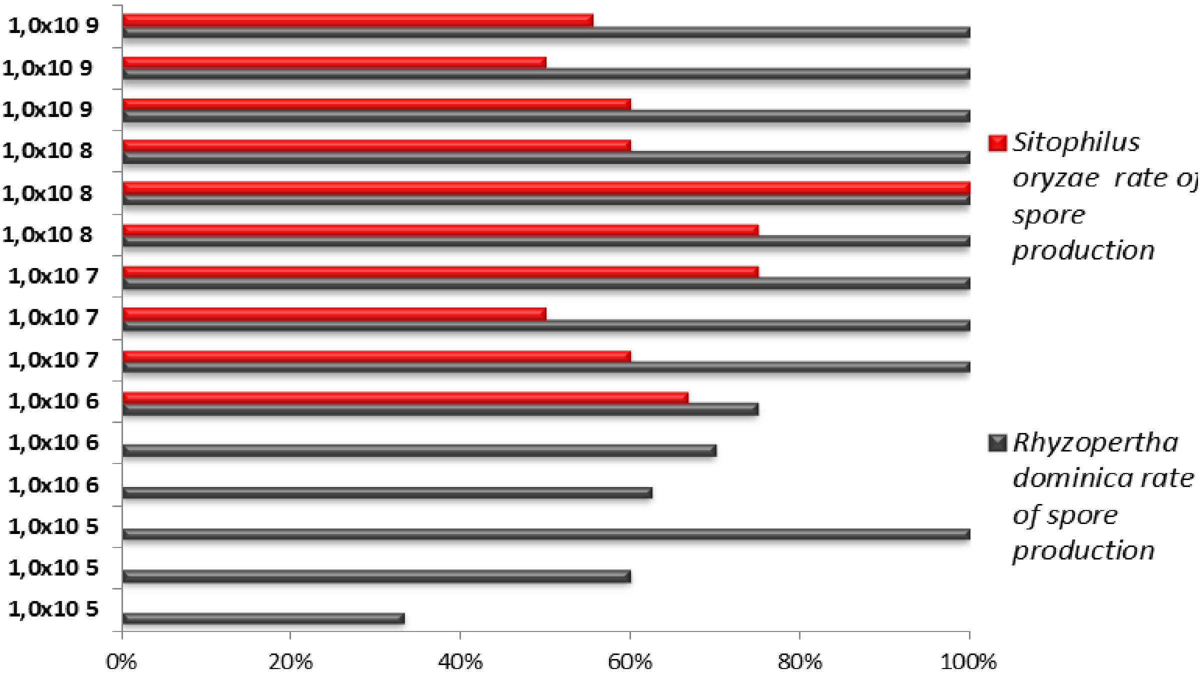


Source: research data.

It was observed that, for *Rhyzopertha dominica*, the concentration of $1.0{\times}10^7$ conidia·mL^{-1} resulted in complete mortality . In contrast , for *Sitophilus oryzae*, complete mortality was achieved only at the concentration of $1.0{\times}10^9$ conidia·mL^{-1}. Trend analyses demonstrate a positive correlation between spore concentration and insect mortality and infection rates .

Figure 2 show the conidiogenesis rate for each species, and it was found that *Rhyzopertha dominica* died primarily due to direct fungal infection, while *Sitophilus oryzae* may have died not from direct infection but from environmental factors such as stress caused by the inoculation itself. This interpretation is supported by the fact that the conidiogenesis rate remained, on average, at 60%.

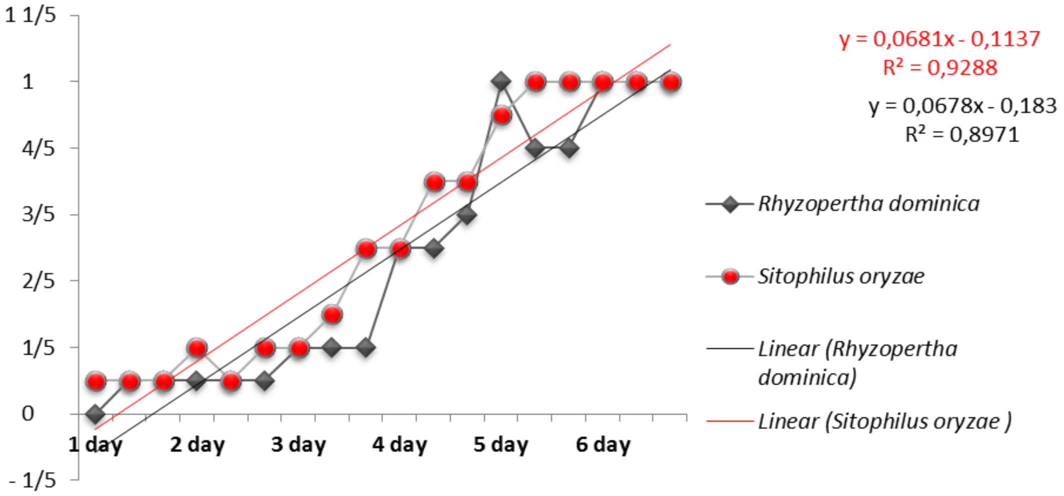
Figure 2 - Conidiogenesis rate (confirmed mortality/total mortality) in the species subjected to different concentrations of the *Beauveria bassiana* isolate, obtained from a coffee grounds/soil substrate (1/1)



Source: research data.

The lethal time curve (Figure 3) showed that *Rhyzopertha dominica* exhibited mortality within 24 h after fungal inoculation, with an LT50 (Lethal Time 50%) on the 4th day of incubation and an LT100 (Lethal Time 100%) on the fifth day. In contrast, *Sitophilus oryzae* showed the first mortality event at 48 h after inoculation, with an LT50 on the third day and an LT100 on the sixth day of incubation.

Figure 3 - Lethal time curve for the species, subjected to a concentration of 109 conidia ·mL^{-1} obtained from the coffee grounds/soil substrate (1/1)



Source: research data.

The production of spores in different agro-industrial residues has been the subject of scientific investigation , with favorable results reported for various formulations. Dalla Santa *et al*.(2005), working with potato waste and sugarcane bagasse, demonstrated that the cultivation of *B. bassiana* on these substrates results in greater growth and spore production than commercial cultivation on rice. In their review, Oliveira *et al*. (2020) argue that these results may be related to the presence of cellulosic substrates, which provides improved aeration and a larger surface area for spore production.

In this study, theresults corroborate the importance of aeration and growth surface for spore production. Pure coffee grounds (100%), due to their fine particle size, exhibited a more compact structure, whichin turn reducedaeration and the available contact surface for fungal development. Indeed, in experiments using this formulation, hyphal growth was observed only at the periphery of the culture flask, with no growth detected in the interior.

With the addition of clay an increased formation of aggregates was observed, which enlarged interparticle spaces, enhanced aeration, and allowed improved hyphal development, enabling growth to reach the interior of the culture flask.

Coffee grounds acted as a source of carbon, vitamins, and proteins for the fungus. However, in the 100% formulation, despite the greater availability of these elements, the best performance was not achieved . This outcome is attributed to substrate compaction and limitedaeration. Although the levels of tannin, caffeine, and caffeic acid, were not quantified, it is plausible that these compounds also influenced the fungal response.

The coffee grounds with soil formulation was the most suitable for the development of

Beauveria bassiana, surpassing conventional rice cultivation in spore production. Although rice hashistorically been considered the ideal substrate due to its carbon, nitrogen, and mineral content, the substrate with soil and coffee grounds exhibited a granular structure, with small aggregates , which allowed adequate aeration for fungal development.

The increase in spore production can be explained by the nutritional characteristics of the soil itself, which is not an inert material but acts as a secondary source of minerals and organic matter, providing compounds necessary for fungal development. It is also relevant to consider that, in its natural environment, *Beauveria bassiana* maintains its spores in the soil, suggesting an evolutionary adaptation of its biochemical and physiological machinery to thrive in the conditions found in this substrate.

An initial moisture content of 70% proved to be the most effective across all tested substrates. According to Mishra *et al*. (2013), initial moisture contents ranging from 35% to 80% can be used for solid-state fermentation.

The *Beauveria bassiana* isolate demonstrated good infective capacity against the tested species. Figure 1 shows that all concentrations elicited lethal responses. Complete mortality was observed starting at a concentration of 1.0×10^7 conidia/mL for *Rhyzopertha dominica* and 1.0×109 conidia/mL for *Sitophilus oryzae*.

Infection of insects occurs through direct penetration across the cuticle or via ingestion of fungal spores. In both cases, after germination, the fungus releases lytic enzymes, such as proteases (PR1 and PR2) and phospholipase B (PLB), as reported by Wan (2003). This process allows fungal conidia to penetrate the host and reach the hemolymph. Within the hemolymph, the fungus multiplies, adopting a yeast-like form in *Beauveria bassiana* and disseminating throughout the insect tissue . The variation in lethality observed demonstrates differentiated responses for the species, which was expected due to the parasite-host interaction.

The results demonstrate that there was no loss of virulence in the *Beauveria bassiana* strain cultured on the coffee grounds and soil substrate. On the contrary, high virulence was observed, accompanied by an equally high conidiogenesis rate. The virulence of *Beauveria bassiana* against the treated species is evident, as mortality reached 100% at the highest conidia concentration The high conidiogenesis and the epizootic potential presented enhance the importance of this substrate in a biological control program. High conidia production is crucial because it compensates for the low probability that most spores will survive to infect a new host, due to environmental factors such as temperature, humidity, and radiation (Charnley, 1997; Fancelli, 2013; Lohse, 2014).

A crucial factor in biological control is the speed of action of the agent. In this experiment, the lethal time 50 (LT50) at a concentration of 10^9 conidia/mL was four days for *Rhyzopertha dominica*. The conidium requires a minimum of 24 h to cross the cuticle and invade the insect's hemocel (Alves, 1998). After penetrating the cuticle , the fungus produces toxins such as Beauvericin and Beauverolides, which can cause behavioral symptoms like sluggishness and paralysis (Andrade *et al*., 2021; Rocha *et al*., 2023 ; Schrank; Vainstein, 2010). The insect then dies and, under favorable conditions, , the fungus emerges, exteriorizing its hyphal structures (Andrade *et al*., 2020).

Host death may occur indirectly, through nutrient depletion , or directly, through the release of proteases and chitinases (Santos *et al*., 2022). In many cases, death is caused by a combination of these factors. When death occurs due to direct factors, the fungus does not always develop inside the insect's body, which may be overcome by septicemia due to a reduction in its resistance capacity. In the tested concentrations, the appearance of hyphae was observed after death, although mortality occurred prior to visible hyphal development . In this regard, whether the death resulted from fungal infection or from other conditions, only the preparation of histological slides could provide evidence, which was not performed in this study."

4 Conclusion

The production of *Beauveria bassiana* on coffee grounds–based substrates is viable. The fungus maintains its virulence and exhibits high conidia production. The performance of this process is superior to that of current commercial cultivation methods, which led to the filing of a patent application (BR 10 2014 027682 3) for its use in Brazil.

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