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Original Article

Effect of temperature and substrate on conidia yield of entomopathogenic fungi using the JKI solid-state bioreactor system

Efeito da temperatura e do substrato na produção de conídios de fungos entomopatogênicos utilizando o sistema de biorreator em estado sólido JKI

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Abstract

The growing demand for sustainable pest control has intensified the need for large-scale biopesticide production. *Beauveria bassiana* and *Metarhizium anisopliae* are widely used in Integrated Pest Management (IPM), but their production still requires more efficient, scalable methods. This study is the first to evaluate the simultaneous cultivation of *B. bassiana* (IBCB227) and *M. anisopliae* (IBCB425) in the JKI-Solid Fermenter System (JKI-SFs) under different temperatures and novel substrate combinations, including rice-barley mixes. Solid-state fermentation was conducted at 15, 20, 25, 30, and 37 °C on parboiled rice, barley, and a rice-barley mix (4:1). Optimal conidia yields were achieved at 25 °C for *B. bassiana* and 25-30 °C for *M. anisopliae*, with no sporulation at 37 °C. The rice-barley matrix enhanced production: a 194% increase in conidia yield was observed for *B. bassiana* and 5.8% for *M. anisopliae* compared to rice alone. The JKI-SFs bioreactor enabled yields up to 6.0×10^9 conidia/g dry mass without contamination and with better handling. These findings demonstrate the potential of JKI-SFs as an efficient, scalable, and contamination-free system for biopesticide production using optimized substrate strategies.

Keywords: biological control, conidia yield, food security, solid-state fermentation, sustainable agriculture.

Resumo

A crescente demanda por controle sustentável de pragas intensificou a necessidade de produção de biopesticidas em larga escala. *Beauveria bassiana* e *Metarhizium anisopliae* são amplamente utilizadas no Manejo Integrado de Pragas (MIP), mas sua produção ainda requer métodos mais eficientes e escaláveis. Este estudo é o primeiro a avaliar o cultivo simultâneo de *B. bassiana* (IBCB227) e *M. anisopliae* (IBCB425) no Sistema JKI-Fermentador Sólido (JKI-SFs) sob diferentes temperaturas e novas combinações de substratos, incluindo misturas de arroz e cevada. A fermentação em estado sólido foi conduzida a 15, 20, 25, 30 e 37 °C em arroz parboilizado, cevada e uma mistura de arroz e cevada (4:1). Rendimentos ótimos de conídios foram alcançados a 25 °C para *B. bassiana* e 25-30 °C para *M. anisopliae*, sem esporulação a 37 °C. A matriz arroz-cevada aumentou a produção: observou-se um aumento de 194% na produção de conídios para B. bassiana e 5,8% para *M. anisopliae* em comparação com o arroz isolado. O biorreator JKI-SFs permitiu rendimentos de até $6{,}0\times10^9$ conídios/g de massa seca sem contaminação e com melhor manuseio. Esses resultados demonstram o potencial dos JKI-SFs como um sistema eficiente, escalável e livre de contaminação para a produção de biopesticidas, utilizando estratégias otimizadas de substrato.

Palavras-chave: controle biológico, rendimento de conídios, segurança alimentar, fermentação em estado sólido, agricultura sustentável.

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1. Introduction

Integrated pest management (IPM) is an ecological approach to controlling pest populations by combining multiple strategies, such as biological, mechanical, chemical, and cultural methods, to minimize the reliance on chemical pesticides. This integrated approach increases the demand for biopesticides, leading to a need for large-scale production (Muñiz-Paredes et al., 2017).

Microorganisms are multiplied in solid-state fermentation on solid and moist particles with minimal water use at normal incubation temperatures (between 20 and 37 °C), replacing submerged fermentation (SmF). Bioreactors used to multiply microorganisms are classified according to aeration and agitation strategies into types such as tray, fixed bed, rotary drum, and continuous or intermittent mixing with forced aeration (Couto and Sanromán, 2006).

The mass production of fungi for pest management aims to generate sustainable biopesticides that target a variety of agricultural pests. Fungal-based pesticides, or mycopesticides, produced from entomopathogenic fungi such as *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin and *Metarhizium anisopliae* sensu lato (Metschnikoff) Sorokin (Hypocreales, Clavicipitaceae), rely on the production of conidia, which infect and kill insects (Zimmermann, 2007a, b; Li et al., 2010). The growing demand for sustainable agricultural practices has driven the development of biopesticides as a viable and environmentally friendly alternative to conventional chemical pesticides. Among the most promising biological control agents, the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* have been widely used in Integrated Pest Management (IPM) due to their specificity against target insects, lower environmental impact, and biodegradability (Akutse et al., 2020; Bamisile et al., 2021). However, large-scale production of these biopesticides still faces challenges, such as the need to optimize conidia production in efficient, economically viable, and scalable systems.

Solid-state fermentation (SSF) has emerged as a promising alternative to submerged fermentation (SmF), allowing mass production of conidia on natural substrates with lower water and energy consumption (Muñiz-Paredes et al., 2017). However, many of the methodologies currently used for fungal multiplication remain rudimentary, limiting the yield and quality of the biopesticides obtained (Li et al., 2010). In countries like Brazil, entomopathogenic fungi are often produced in autoclavable plastic bags or trays without adequate control of key parameters such as nutrients, oxygenation, pH, temperature, and water activity, which can negatively affect production efficiency (Li et al., 2010; Moura Mascarin, 2019).

Beauveria bassiana and *Metarhizium anisopliae* were chosen for this study due to their proven efficacy as entomopathogenic fungi, widely recognized for their ability to infect and control a variety of insect pests in agricultural systems (Gebremariam et al., 2021). These fungi have a well-documented history of use in biological control programs worldwide, particularly in countries with large agricultural sectors like Brazil (Meyling and Eilenberg, 2007). Their selection is based on their broad host range, high virulence, and adaptability to diverse environmental conditions, making them ideal candidates for large-scale biopesticide production (Song et al., 2019). Research has shown that these fungi infect their hosts by adhering to the insect cuticle, germinating, and penetrating the exoskeleton, ultimately killing the host through a combination of mechanical damage and the production of insecticidal toxins (Padilla-Guerrero et al., 2011). Several field trials and laboratory studies have highlighted their potential as eco-friendly alternatives to chemical pesticides, supporting their use in IPM programs (Bharti and Ibrahim, 2020).

This study aims to bridge this gap by evaluating the conidia production of *B. bassiana* (IBCB227) and *M. anisopliae* (IBCB425) at different temperatures and on various substrates using the JKI-SFs solid fermentation system, based on Prophyta® technology. The goal is to optimize cultivation conditions to maximize sporulation and enable the large-scale, sustainable, and efficient production of these fungal-based biopesticides.

This study is novel in integrating the JKI-SFs solid-state bioreactor based on Prophyta® technology with optimized substrate combinations and temperature regimes for large-scale production of *B. bassiana* and *M. anisopliae*. While prior research has explored conidial production on isolated substrates or in conventional systems, this work systematically evaluates a scalable, contamination-free bioreactor using a synergistic rice-barley matrix under controlled conditions. This approach provides new insights into process standardization and scale-up potential for industrial biopesticide production.

2. Material and Methods

2.1. Location and biological material

Beauveria bassiana (Balsamo-Crivelli) Vuillemin (IBCB227) and *M. anisopliae* sensu lato (Metschnikoff) Sorokin (Hypocreales, Clavicipitaceae) were respectively obtained from larvae of *Spodoptera frugiperda* Smith, 1979 (Lepidoptera: Noctuidae) and from soil samples of the Atlantic Forest collected in the municipalities of Casa Branca (21°46'23"S, 47°05'24"W) and Iporanga (24°35'15"S, 48°35'30"W), São Paulo state, Brazil. Isolates of these fungi were deposited in the "Odemar Cardim Abreu" Collection of Entomopathogenic Microorganisms at the Experimental Centre of the Biological Institute of Campinas, São Paulo state, Brazil. Pure conidia from these fungi were preserved, respectively, at -80 °C and -20 °C in 10% glycerol and at 6 °C in tubes with malt, soybean peptone, and agar-MPA culture medium (malt extract 30 g/L [Merck KGaA, Germany], soybean peptone 5 g/L [Merck KGaA, Germany], agar 18 g/L [Roth, Germany]) (Muñiz-Paredes et al., 2017). The initial inoculum was obtained by propagating the fungi on Petri dishes with MPA culture medium at 25 ± 1 °C in the dark. The experiments were conducted at the Laboratory of Microbiology and Bioengineering, Julius Kühn Institute of Biological Control, Darmstadt, Germany. Conidia from these fungi were measured, and their morphology analysed

three times under the same conditions, using different batches at different times.

The identification of *B. bassiana* and *M. anisopliae* s.l. was based on the morphological characteristics of colonies and conidia, following the taxonomic keys described by Driver et al. (2000) and Zimmermann (2007a, b). Although these genera are known to include cryptic species, no molecular characterization was performed in this study. Therefore, the isolates are referred to as *B. bassiana* and *M. anisopliae* sensu lato, in accordance with morphological taxonomy.

2.2. In vitro characterization of fungal growth and sporulation

Conidia of the IBCB227 and IBCB425 isolates were harvested by superficial scraping with the aid of a Drigalski spatula from colonies grown for 14 days on MPA medium, suspended in 0.1% Tween 80® [Sigma, Germany], and calibrated to a concentration of 1.0 x 10^7 conidia/mL. Next, 100 µL of the conidial suspension was distributed with the aid of a Drigalski spatula onto a freshly prepared MPA medium plate (90-15 mm) and incubated at 25 °C in a biochemical oxygen demand (B.O.D.) for 14 days in the dark. Ten mL of 0.1% Tween 80® were added per sample, and this solution subjected to vortexing. Then, 100 µL of the conidial suspension was placed onto glass slides, and a coverslip placed over the fungal suspension. The fresh slides were analysed by capturing images of the conidia using an optical microscope [Carl Zeiss Microscopy GmbH, Germany] equipped with an Olympus camera and Olympus CellP software. The experiment was conducted with five replications (Petri dishes) and repeated three times under the same conditions, using different batches of the fungus at different times.

2.3. Radial growth under temperature regimes

A total of 100 µL of conidia from the isolates IBCB227 and IBCB425 were suspended in 0.1% Tween 80® and adjusted to a concentration of 1.0 x 107 conidia/mL (Howard et al., 1993). This suspension was distributed using a Drigalski spatula onto Petri dishes containing MPA culture medium (90-15 mm) and incubated at different temperatures (15, 20, 25, 30, and 37 °C) in a B.O.D. in the dark. The radial growth of the isolates IBCB227 and IBCB425 was evaluated on circular agar discs (5 mm in diameter), obtained from a four-day pre-culture of the fungi. The radial growth was measured using a millimetre ruler from three to twenty days after inoculation.

2.4. Sporulation under temperature regimes

The conidia of the isolates IBCB227 and IBCB425 were collected using a Drigalski spatula from a 14-day pre-culture grown on MPA medium, suspended in 0.1% Tween 80® and adjusted to a concentration of 1.0 x 10^7 conidia/mL. Next, 100 µL of the conidial suspension was distributed using a Drigalski spatula onto freshly prepared MPA medium plates (90-15 mm). These plates were kept at 15, 20, 25, 30, and 37 °C in B.O.D. incubators for 14 days in the dark. Ten µL of 0.1% Tween 80® were added per sample, vortexed, and the conidia counted using a hemocytometer under an optical microscope, obtaining the total spore count per temperature.

2.5. Inoculum preparation for bioreactor assay

A fungal pre-culture was prepared in adapted Samsinakova liquid medium (Glucose 25.0 g/L [Merck KGaA, Germany], 20.0 g/L corn steep solid [Sigma, Germany], 15.0 g/L sodium chloride [Merck KGaA, Germany]). Erlenmeyer flasks with 50 mL of the culture medium were sterilized at 121 °C for 20 minutes in a Tuttnauer autoclave 5075EL steam sterilizer, Netherlands. Next, 1 mL of a suspension from 14-day-old cultures of each isolate (IBCB227 and IBCB425) was inoculated into MPA culture medium. Each Erlenmeyer flask was inoculated with 1.0 x 10^7 conidia/mL counted using a hemocytometer under an optical microscope. Next, the flasks were placed in a shaker incubator (Multitron, Infors HT, Switzerland) at 25 °C and 150 rpm for 72 hours to allow fungal growth.

2.6. Substrate selection and preparation

The "JKI-Fermenter System" (JKI-SFs) consisted of a laboratory-scale bioreactor based on solid-state fermentation, developed at the Julius Kühn Institute. Each unit was composed of a 1000 mL Schott Duran wide-mouth glass bottle (height: 215 mm; diameter: 101 mm), with two fixed connections at the bottom: one for drainage and one for aeration, and an exhaust outlet in the lid. The aeration system included sterile 0.2 µm PTFE membrane filters to ensure a contamination-free environment. The fermenters operated under forced humidified aeration (0.5 L min^{-1}), and the temperature was controlled at 25 °C inside a B.O.D. incubator. Each unit contained 100 g of Seramis® granular clay at the bottom and 200 g of solid substrate (parboiled rice, barley, soybean, or a rice-barley mix). This setup allowed uniform air distribution and moisture retention while facilitating scale-up for industrial applications (Figure 1).

2.7. Solid-state fermentation in JKI-SFs

The design of the JKI-SFs bioreactor reflects key features required for industrial fermentation: forced aeration through sterile PTFE filters (0.2 µm), uniform moisture retention via a Seramis® clay layer, and internal temperature control using an incubator system. The modular nature of the JKI-SFs, based on 1L Schott bottles with aeration and drainage ports, allows for easy replication and parallel operation, making it suitable for scalability assessments. The forced aeration system also simulates industrial air supply, enhancing oxygen diffusion across the substrate mass and reducing heat accumulation—critical factors in SSF scalability.

2.8. Matrix compositions for conidia production

The units of JKI-SFs were filled with 200, 200, 200, and 160 + 40 mg of parboiled rice, soybean, barley, or parboiled rice + barley (4:1), a ratio selected based on preliminary trials and supported by Ye et al. (2006) and Castro et al. (2015), which indicated that this proportion balances aeration (provided by barley) and nutrient content (from

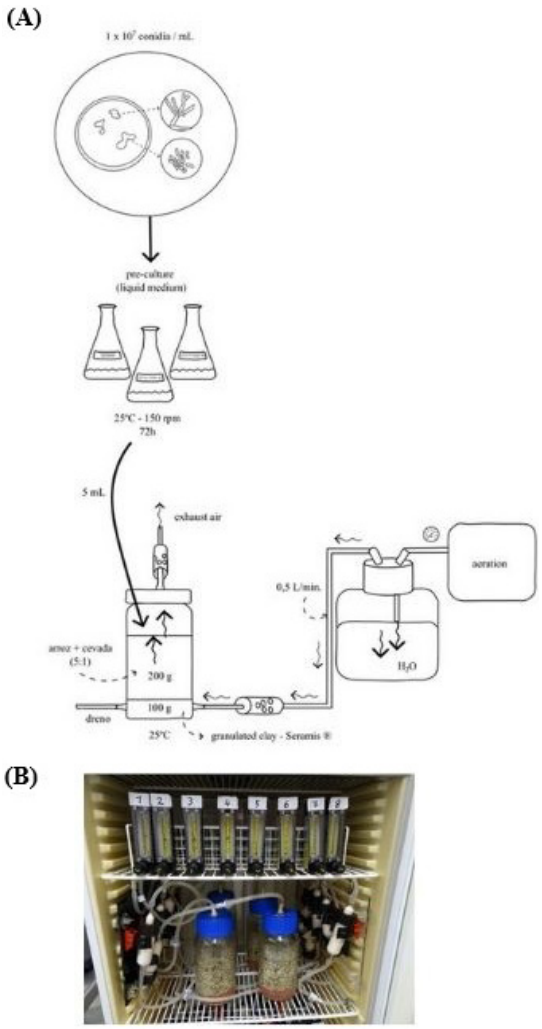


Figure 1. Methodology for solid-state fermentation of entomopathogenic fungi using the JKI-Solid Fermenter System at laboratory scale (A) and in operation (B).

rice), while also facilitating post-fermentation handling (Figure 2). One hundred grams of Seramis® granular clay were placed at the bottom of each flask, separated from the substrate matrix layer (boiled for 5 minutes in 500 mL of deionized water, except for soybean boiled for eight minutes) by a 90 mm filter paper. The JKI-SFs units were autoclaved for 20 minutes at 121 °C.

Each bioreactor was filled with sterilized deionized water, covering the substrate before inoculation. Next, 5 mL of the 72-hour pre-culture was pipetted onto the surface of the sterile water inside the bioreactor. Afterward, the container was gently shaken to suspend the biomass, the water was slowly drained through a tube, and the biomass distributed over the surface of the substrate, completing the inoculation process. The fermenters were removed from the cultivation flasks after 14 days of fermentation at 25 °C with an aeration rate of 0.5 L*min-1, and 25 g of the substrate washed with 225 mL of 0.1% Tween 80® and the conidia collected. The resulting suspension was shaken for 10 minutes on a reciprocal shaker to disperse and individualize the conidia. The conidia concentration was determined in five replicates using a hemocytometer and an optical microscope and expressed in relation to the quantity of dry matter. Moisture content of the substrates was determined by drying approximately 5 g of each sample in a forced-air oven at 105 °C until constant weight, following standard gravimetric methods

2.9. Statistical analysis

All data were analyzed using Generalized Linear Models (GLMs) to account for the non-normal distribution of biological variables. Radial growth data, being continuous and positive, were analyzed using a GLM with a gamma distribution and a log link function. Sporulation and conidia production data were analyzed using GLMs with a negative binomial distribution to handle overdispersed count data. Pairwise comparisons among treatments were performed using the Tukey-Kramer test (Westfall et al., 1999) to determine significant differences among means. Model fit was verified through residual analysis and goodness-of-fit statistics. All analyses were performed using the PROC GENMOD procedure.

3. Results

3.1. Morphology of conidia produced in solid state

The morphology of conidia of *B. bassiana* (IBCB227) and *M. anisopliae* (IBCB425), produced in solid medium, was predominantly cylindrical (Figure 3), with average lengths of 6.49 ± 0.04 µm and 3.07 ± 0.03 µm, and diameters of 2.98 ± 0.03 µm and 2.61 ± 0.02 µm, respectively

3.2. Radial growth and sporulation under different temperatures

The *B. bassiana* exhibited significantly greater radial growth at 25 °C (2.68 ± 0.06 cm), which was statistically different from all other temperatures tested (Tukey-Kramer, $p < 0.05$). For *M. anisopliae* (IBCB425), growth was significantly higher at both 25 °C and 30 °C, with no statistical difference between these two temperatures (Table 1).

3.3. Substrate selection on a laboratory scale for industrial production in a bioreactor

The production of conidia, by *B. bassiana* (IBCB227) and *M. anisopliae* (IBCB425), was higher in the substrates parboiled rice and barley (4:1), 1.0 x 10^9 and 6.0 x 10^9 conidia/g of mass dry, respectively, in JKI-SFs and without contaminants. Furthermore, the manipulation of *B. bassiana* (IBCB227) and *M. anisopliae* (IBCB425) was better when cultivated on parboiled rice with barley than on pure rice, where they formed a compact block at the end of fermentation. The conidial yield per gram of dry mass was similar among substrates, except for *B. bassiana*, higher with soybean (Table 2).

Although the moisture content varied among substrates (e.g., 22.24% for rice and 75.68% for soybean), no evident



Figure 2. *Beauveria bassiana* (IBCB227) and *Metarhizium anisopliae* (IBCB425) produced, respectively, on parboiled rice (A, E); soybean (B, F); barley (C, G); and parboiled rice and barley (4:1) (D, H).

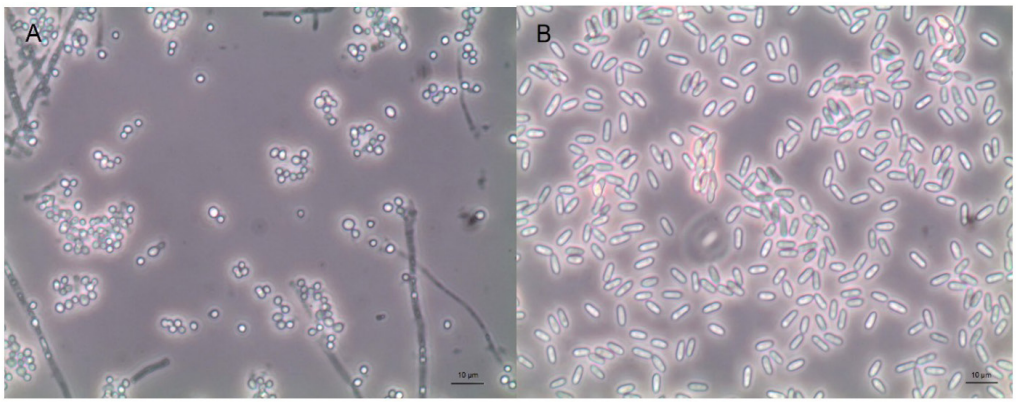


Figure 3. Conidia of *Beauveria bassiana* (IBCB227) (A) and *Metarhizium anisopliae* (IBCB425) (B) produced in solid MPA medium in the JKI-Solid Fermenter System bioreactor based on the Prophyta® technological system.

Table 1. Average radial growth (cm) of *Beauveria bassiana* (IBCB227) and *Metarhizium anisopliae* (IBCB425) cultivated on MPA medium at different temperatures (15-30 °C) over 20 days. Values are means ± standard error (n = 5), with minimum–maximum in parentheses.

Temp. (°C)	Isolate IBCB227	Isolate IBCB425
15	1.19 ± 0.04 (1.15 - 1.23) Da	0.98 ± 0.03 (0.95 - 1.01) Cb
20	1.80 ± 0.04 (1.76 - 1.84) Cb	2.27 ± 0.07 (2.20 - 2.34) Ba
25	2.68 ± 0.06 (2.62 - 2.74) Ab	3.34 ± 0.09 (3.25 - 3.43) Aa
30	2.43 ± 0.05 (2.38 - 2.48) Bb	3.27 ± 0.09 (3.18 - 3.36) Aa
37	0.00 ± 0.00 Ea	0.00 ± 0.00 Ea

Treatments at 37 °C showed no radial growth or sporulation. These null values are included to illustrate thermal limits of fungal development but were excluded from statistical comparison. Means followed by the same uppercase letter per column or lowercase letter per row do not differ by Tukey-Kramer test (Westfall et al., 1999) at a significance level of 5%. Means followed by the same lowercase letter within a row (comparison between species at the same temperature) or the same uppercase letter within a column (comparison among temperatures for the same species) do not differ significantly by the Tukey-Kramer test ($p < 0.05$).

Table 2. Production (conidia/g of dry mass) of *Beauveria bassiana* (IBCB227) and *Metarhizium anisopliae* (IBCB425) on rice, rice + barley (4:1), barley, and soybean substrates using the JKI-Solid Fermenter System, values are means ± standard error (n = 5).

Substrate	Isolate IBCB227	Isolate IBCB425
Rice	$3.67 \times 10^8 \pm 6.69 \times 10^7$ Ab	$5.57 \times 10^9 \pm 1.57 \times 10^9$ Aa
Rice + Barley (4:1)	$8.88 \times 10^8 \pm 3.45 \times 10^8$ Ab	$5.84 \times 10^9 \pm 1.39 \times 10^9$ Aa
Barley	$1.08 \times 10^9 \pm 2.29 \times 10^8$ Aa	$1.43 \times 10^9 \pm 1.28 \times 10^8$ Ba
Soybean	$2.12 \times 10^8 \pm 6.54 \times 10^7$ Bb	$9.16 \times 10^8 \pm 2.44 \times 10^8$ Ba

Means followed by the same uppercase letter within a column, or lowercase letter within a row, do not differ according to the Tukey–Kramer test (Westfall et al., 1999) at a significance level of 5%.

Table 3. Biomass production after 14 days of solid-state fermentation of *Beauveria bassiana* (IBCB227) and *Metarhizium anisopliae* (IBCB425) values are means ± standard error (n = 5) in the JKI-Solid Fermenter System.

Substrate	Isolate IBCB227	Isolate IBCB425
Rice	67.34 ± 3.09 (64.25 - 70.43) Ab	48.33 ± 1.95 (46.38 - 50.28) Cd
Rice + Barley (4:1)	63.11 ± 2.16 (60.95 - 65.27) Bb	51.57 ± 1.74 (49.83 - 53.31) Cd
Barley	66.81 ± 2.17 (64.64 - 69.98) Ab	59.69 ± 1.72 (57.97 - 61.41) Bd
Soybean	36.48 ± 1.94 (34.54 - 38.42) Cc	31.83 ± 2.92 (28.91 - 34.75) Dc

Means followed by the same uppercase letter within a column, or lowercase letter within a row, do not differ according to the Tukey–Kramer test (Westfall et al., 1999) at a significance level of 5%.

relationship with conidia production was observed based on the current dataset. Therefore, the variables were not included in further statistical comparisons. The moisture contents of rice, barley, and soybean were, respectively, 22.24%, 73.21%, and 75.68%.

In addition to conidia yield, biomass production varied among substrates and fungal isolates (Table 3). Higher biomass accumulation was observed for *B. bassiana* on barley and for *M. anisopliae* on the rice–barley mixture, whereas soybean showed the lowest biomass values.

4. Discussion

The cylindrical conidia observed for *B. bassiana* and *M. anisopliae* in solid media are consistent with previous reports, for white cultures of the first fungus with conidia, generally hyaline, with a length and diameter of 2.98 ± 0.03 µm and 6.49 ±0.04 µm and 3.07 ± 0.03 µm and 2.61 ± 0.02 µm, respectively (Benham and Miranda, 1953; Zimmermann, 2007b). Conidia of *M. anisopliae*, of colonies with green or dark green tones, were also described as cylindrical with a length and diameter, respectively, of 5 to 8 µm and 10 to 16 µm and these differences were attributed to the cultivation conditions and observation methods (Tulloch, 1976; Driver et al., 2000).

Unlike previous studies that focused on traditional cultivation methods (e.g., Petri dishes, trays, plastic bags), this research introduces and validates a solid-state fermentation strategy using the JKI-SFs bioreactor. This system, combined with a mixed substrate strategy, allowed for high conidia yield with better handling properties and minimal contamination. These features represent a technological advancement toward industrial-scale, sustainable, and efficient biopesticide manufacturing.

The radial growth of *B. bassiana* (IBCB227) and *M. anisopliae* (IBCB425) at all tested temperatures, except 37 °C, with the highest values at 25 °C and at 25 °C and 30 °C, respectively, is similar to that reported for the optimal growth and conidial viability of the latter fungus at 25 °C on substrates such as corn flour, wheat flour and yellow cornmeal, with inhibition of germination at 37 °C (Ottati-de-Lima et al., 2010). The lack of growth of the isolates IBCB227 and IBCB425 at 37 °C agrees with reports of thermos sensitivity at lower and higher temperatures (Wang and Zheng, 2012), with reduced growth rates at 15 °C and 35 °C and optimal temperature between 20 °C and 30 °C (Tefera and Pringle, 2003), faster thermal inactivation at higher temperatures (Miller et al., 2018), and higher efficiency of *B. bassiana* and *M. anisopliae* against locusts at 20 °C to 30 °C in Madagascar (Fernandes et al., 2008). The absence of sporulation at 37 °C increases the safety of the isolates IBCB227 and IBCB425 for humans (Bergman and Casadevall, 2010). The optimal sporulation of *B. bassiana* at 15 °C, 20 °C, 25 °C, and 30 °C, and of *M. anisopliae* at 20 °C, 25 °C, and 30 °C, with higher values for the former fungus except at 20 °C, aligns with better control of *Chilo partellus* (Lepidoptera: Pyralidae) by this fungus at 25 °C to 30 °C (Tefera and Pringle, 2003). However, fungal sporulation can vary with the composition of the culture medium and other environmental conditions besides temperature (Howard et al., 1993).

The absence of sporulation at 37 °C may indicate thermosensitivity in both isolates, which is consistent with their ecological origin and potential adaptation to moderate climates. This trait reinforces their suitability for application in field conditions where temperatures rarely exceed 30 °C, particularly in tropical and subtropical regions.

Furthermore, the higher sporulation of *B. bassiana* at lower temperatures may reflect its greater tolerance to cold, which has been previously reported in studies targeting pests in temperate zones (Fernandes et al., 2008).

The rice-barley mixture not only increased yield but also promoted better post-fermentation handling. This combination may have favored aeration and nutrient distribution, essential in solid fermentations. These operational characteristics are critical for industrial scale-up. Compared to single-substrate systems, the use of a rice-barley (4:1) matrix resulted in a 194% increase in conidial yield for *B. bassiana* and a 5.8% increase for *M. anisopliae* when compared to rice alone. These improvements demonstrate improvements in process conditions, especially when using the rice-barley matrix in the JKI-SFs system. Although absolute yields remain lower than those reported under highly specialized conditions (e.g., 6.2×10^{10} conidia/g in Prakash et al., 2008), our results were achieved under controlled, scalable, and reproducible conditions using an industrial-compatible bioreactor, which reflects a more practical and transferable production system.

Recent research evaluating low-cost alternative substrates including algaroba and malt for *B. bassiana* showed promising results in conidiogenesis (Paiva-Guimarães et al., 2020), which complement our findings using rice-barley mixes in a bioreactor system. Broken rice has also been reported as efficient for the production of conidia by *B. bassiana* and *M. anisopliae*, especially with 24 hours of light exposure (Bich et al., 2018). The absence of contaminants in the substrates highlights the efficacy of the JKI-SFs bioreactor as a sterile environment. However, controlling it poses challenges due to process variability, biological system complexity, and operation under sterile conditions, especially over extended periods like 21 days, validating the need to maintaining a stable and contaminant-free environment (Alford, 2006; Li et al., 2014). The superior performance of the rice-barley mix may be attributed to the combination of structural aeration provided by barley and the nutrient availability of parboiled rice, which together may optimize oxygen diffusion and metabolic activity. These factors are critical in solid-state systems, where oxygen limitations are a common constraint (Pandey et al., 2000; Gervais and Molin, 2003).

The conidial yield of *B. bassiana* (IBCB227) and *M. anisopliae* (IBCB425) confirms the potential of these isolates for large-scale production, but it was lower compared to reports under different pH, moisture, and rice types, up to 2.3×10^9 and 6.2×10^{10} conidia/g of rice, respectively (Jenkins et al., 1998; Prakash et al., 2008; Taylor et al., 2013). The lower conidial production by *B. bassiana* (IBCB227) in solid-state fermentation (SSF) with rice compared to barley can be attributed to the specificities of the substrate and cultivation conditions (Ye et al., 2006). The higher production of the isolate *M. anisopliae* (IBCB425) compared to non-optimized conditions, 1.0×10^9 conidia/g (Barra-Bucarei et al., 2016), suggests that this substrate provides a more favourable environment for this fungus. Despite these variations, the number of conidia produced by both fungi in the JKI-SFs bioreactor is enough for the development of biopesticides, especially with rice as a substrate (Pandey et al., 2000). This is important because conidia are the most practical and infectious stage of entomopathogenic fungi and are preferred for large-scale production and application (Dallastra et al., 2023). The use of bioreactors like the JKI-SFs reduces the need for labour and improves quality control compared to traditional laboratory methods such as using Petri dishes and glass flasks (Dorta et al., 1996).

These results support the potential scalability of the process by demonstrating that key production parameters such as substrate composition, temperature range, and aeration can be standardized and controlled in a laboratory-scale bioreactor that simulates industrial conditions. The JKI-SFs system enables uniform and reproducible conidia production with low contamination risk and reduced labor, which are essential requirements for large-scale manufacturing. However, the heat generated by fungal metabolic activity in solid-state fermentation must be controlled through aeration or cooling (Castro et al., 2015; Manan and Webb, 2017). Efficient aeration removes heat, but it must be controlled to reduce moisture loss from the substrate (Shojaosadati et al., 2007; Castro et al., 2015) and maintaining adequate values of this parameter in the substrate (Shojaosadati et al., 2007; Castro et al., 2015). Systems with functions similar to an industrial bioreactor, such as the JKI-SFs, with optimal conditions for gas exchange (O_2 and CO_2), moisture, nutrition, and temperature to maximize conidial production and ensure the viability of biopesticides, are essential for transferring the process to the industry.

The lack of correlation between moisture content and conidia production per gram of dry mass at the end of solid-state fermentation by the isolates IBCB227 and IBCB425 should be further evaluated, as water facilitates nutrient transfer, maintains metabolic activity, and so, fungal growth in these processes (Gervais and Molin, 2003). Interestingly, despite its significantly lower moisture content (22.24%) compared to barley and soybean (>73%), parboiled rice supported high conidia production, especially for *M. anisopliae*. However, excessive moisture levels can increase the proliferation of competing microorganisms, reduce substrate aeration and nutrient availability, and consequently, the conidial production (Prakash et al., 2008). Therefore, proper moisture management is essential to enhance the quality and quantity of conidia produced, thereby improving the development of biopesticides (Prakash et al., 2008). The production of entomopathogenic fungi in JKI-SFs bioreactors, comparable to industrial scale, allows tests to optimize this process, including the choice of substrates and control of aeration and temperature.

A significant difference in this study is the evaluation of the combination of substrates, specifically parboiled rice and barley, to optimize sporulation and conidial yield of *B. bassiana* and *M. anisopliae*. While most studies focus on conventional substrates, such as rice or corn alone, our approach explores synergies between different matrices to improve fungal production and manipulation. The mixture of parboiled rice and barley was shown not only to increase conidial productivity but also to facilitate material handling at the end of the fermentation process, making it more suitable for future industrial applications.

Although parboiled rice and barley were used in this study due to their consistency and low contamination risk, we recognize that their use in large-scale production may raise concerns regarding competition with food resources. Therefore, future applications should prioritize the use of agricultural by-products, broken rice, or lower-grade grains that are not suitable for human consumption, in order to ensure alignment with food security goals and sustainable biopesticide production (Pandey et al., 2000; Bich et al., 2018).

The implementation of the JKI-SFs bioreactor in regions with high demand for biological control can boost the production of biopesticides in an affordable manner, with greater safety and quality of the final product, benefiting small and medium-sized producers who face challenges in pest management. Large-scale production of *B. bassiana* and *M. anisopliae* conidia was effective in a sterile environment with less labor required. The ideal temperature range for the growth and sporulation of these fungi is 15 to 30 °C, with higher values of 25 °C for *B. bassiana* and 25-30 °C for *M. anisopliae*. The JKI-Fermenter system, when combined with appropriate substrate formulations and controlled cultivation conditions, offers a robust platform for the scale-up of conidia production. These findings contribute directly to the advancement of biopesticide manufacturing by demonstrating a scalable, sterile, and cost-effective alternative to conventional methods, aligning with industrial production demands and sustainable agriculture goals.

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5. Conclusion

This study demonstrated that *B. bassiana* (IBCB227) and *M. anisopliae* (IBCB425) can be effectively cultivated in a laboratory-scale solid-state fermentation system using the JKI-SFs bioreactor. Optimal conidial production occurred at 25 °C for *B. bassiana* and 25-30 °C for *M. anisopliae*, with no sporulation observed at 37 °C. Among the tested substrates, a mixture of parboiled rice and barley (4:1) resulted in higher yields and better handling properties compared to individual substrates. These findings indicate that the JKI-SFs system enables standardized, contamination-free production of entomopathogenic fungi under controlled conditions, and provide a strong basis for future greenhouse and field evaluations to confirm the biopesticide's effectiveness in real agricultural environments. While the system was tested on a laboratory scale, its structure and operation mimic key features of industrial fermentation, suggesting its potential applicability for scale-up in commercial biopesticide production.

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Data Availability Statement

All data generated or analyzed during this study are included in this published article.

References

AKUTSE, K., SUBRAMANIAN, S., MANIANIA, N., DUBOIS, T. and EKESI, S., 2020. Biopesticide research and product development in africa for sustainable agriculture and food security: experiences from the International Centre of Insect Physiology and Ecology (ICIPE). *Frontiers in Sustainable Food Systems*, vol. 4, no. 1, pp. 563016. http://doi.org/10.3389/fsufs.2020.563016.

ALFORD, J.S., 2006. Bioprocess control: advances and challenges. *Computers & Chemical Engineering*, vol. 30, no. 10-12, pp. 1464-1475. http://doi.org/10.1016/j.compchemeng.2006.05.039.

BAMISILE, B., AKUTSE, K., SIDDIQUI, J. and XU, Y., 2021. Model application of entomopathogenic fungi as alternatives to chemical pesticides: prospects, challenges, and insights for next-generation sustainable agriculture. *Frontiers in Plant Science*, vol. 12, no. 1, pp. 741804. http://doi.org/10.3389/fpls.2021.741804. PMid:34659310.

BARRA-BUCAREI, L., VERGARA, P. and CORTES, A., 2016. Conditions to optimize mass production of *Metarhizium anisopliae* (Metschn) Sorokin 1883 in different substrates. *Chilean Journal of Agricultural Research*, vol. 76, no. 4, pp. 448-454. http://doi.org/10.4067/S0718-58392016000400008.

BENHAM, R.W. and MIRANDA, J.L., 1953. The genus *Beauveria*, morphological and taxonomical studies of several species and of two strains isolated from Wharf-Piling Borers. *Mycologia*, vol. 45, no. 5, pp. 727-746. http://doi.org/10.1080/00275514.1953.12024312.

BERGMAN, A. and CASADEVALL, A., 2010. Mammalian endothermy optimally restricts fungi and metabolic costs. *mBio*, vol. 1, no. 5, e00212-10. http://doi.org/10.1128/mBio.00212-10. PMid:21060737.

BHARTI, V. and IBRAHIM, S., 2020. Biopesticides: production, formulation and application systems. *International Journal of Current Microbiology and Applied Sciences*, vol. 9, no. 10, pp. 3931-3946. http://doi.org/10.20546/ijcmas.2020.910.453.

BICH, G.A., CASTRILLO, M.L., VILLALBA, L.L. and ZAPATA, P.D., 2018. Evaluation of rice by-products, incubation time, and photoperiod for solid state mass multiplication of the biocontrol agents *Beauveria bassiana* and *Metarhizium anisopliae*. *Agronomy Research*, vol. 16, no. 5, pp. 1921-1930. http://doi.org/10.15159/AR.18.197.

CASTRO, A.M., CASTILHO, L.R. and FREIRE, D.M.G., 2015. Performance of a fixed-bed solid-state fermentation bioreactor with forced

aeration for the production of hydrolases by *Aspergillus awamori*. *Biochemical Engineering Journal*, vol. 93, pp. 303-308. http://doi.org/10.1016/j.bej.2014.10.016.

COUTO, S.R. and SANROMÁN, M.A., 2006. Application of solid-state fermentation to food industry: a review. *Journal of Food Engineering*, vol. 76, no. 3, pp. 291-302. http://doi.org/10.1016/j.jfoodeng.2005.05.022.

DALLASTRA, E.D.G., DIAS, A.C.P., MORAIS, P.B., SILVA, J.F.M., CASCIATORI, F.P. and GRAJALES, L.M., 2023. Development of a novel pilot-scale tray bioreactor for solid-state fermentation aiming at process intensification. *Chemical Engineering and Processing*, vol. 193, pp. 109526. http://doi.org/10.1016/j.cep.2023.109526.

DORTA, B., ERTOLA, R.J. and ARCAS, J., 1996. Characterization of growth and sporulation of *Metarhizium anisopliae* in solid-substrate fermentation. *Enzyme and Microbial Technology*, vol. 19, no. 6, pp. 434-439. http://doi.org/10.1016/S0141-0229(96)00017-8.

DRIVER, F., MILNER, R.J. and TRUEMAN, J.W.H., 2000. A taxonomic revision of *Metarhizium* based on a phylogenetic analysis of rDNA sequence data. *Mycological Research*, vol. 104, no. 2, pp. 134-150. http://doi.org/10.1017/S0953756299001756.

FERNANDES, E.K., RANGEL, D.E., MORAES, A.M., BITTENCOURT, V.R. and ROBERTS, D.W., 2008. Cold activity of *Beauveria* and *Metarhizium*, and thermotolerance of *Beauveria*. *Journal of Invertebrate Pathology*, vol. 98, no. 1, pp. 69-78. http://doi.org/10.1016/j.jip.2007.10.011. PMid:18096184.

GEBREMARIAM, A., CHEKOL, Y. and ASSEFA, F., 2021. Phenotypic, molecular, and virulence characterization of entomopathogenic fungi, *Beauveria bassiana* (Balsam) Vuillemin, and *Metarhizium anisopliae* (Metschn) Sorokin from soil samples of Ethiopia for the development of mycoinsecticide. *Heliyon*, vol. 7, no. 5, e07091. http://doi.org/10.1016/j.heliyon.2021.e07091. PMid:34095584.

GERVAIS, P. and MOLIN, P., 2003. The role of water in solid-state fermentation. *Biochemical Engineering Journal*, vol. 13, no. 2-3, pp. 85-101. http://doi.org/10.1016/S1369-703X(02)00122-5.

HOWARD, K.M., SMART, M.G. and BOTHAST, R.J., 1993. A novel method for identifying new conidia in submerged liquid culture. *Mycologia*, vol. 85, no. 1, pp. 127-130. http://doi.org/10.1080/00275514.1993.12026256.

JENKINS, N.E., HEVIEFO, G., LANGEWALD, J., CHERRY, A.J. and LOMER, C.J., 1998. Development of mass production technology for aerial conidia for use as mycopesticides. *Biocontrol News and Information*, vol. 19, pp. 21N-31N.

LI, S.T., LIU, Y., ZHOU, Q., LUE, R.F., SONG, L., DONG, S.W., GUO, P. and KOPJAR, B., 2014. A novel axial-stress bioreactor system combined with a substance exchanger for tissue engineering of 3D constructs. *Tissue Engineering. Part C, Methods*, vol. 20, no. 3, pp. 205-214. http://doi.org/10.1089/ten.tec.2013.0173. PMid:23822092.

LI, Z., ALVES, S.B., ROBERTS, D.W., FAN, M., DELALIBERA JUNIOR, I., TANG, J. and RANGEL, D.E.N., 2010. Biological control of insects in Brazil and China: history, current programs and reasons for their successes using entomopathogenic fungi. *Biocontrol Science and Technology*, vol. 20, no. 2, pp. 117-136. http://doi.org/10.1080/09583150903431665.

MANAN, M.A. and WEBB, C., 2017. Design aspects of solid-state fermentation as applied to microbial bioprocessing. *Journal of Applied Biotechnology and Bioengineering*, vol. 4, no. 1, pp. 511-532.

MEYLING, N. and EILENBERG, J., 2007. Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: potential for conservation biological control. *Biological Control*, vol. 43, no. 2, pp. 145-155. http://doi.org/10.1016/j.biocontrol.2007.07.007.

MILLER, M.E., SHISHKOFF, N. and CUBETA, M.A., 2018. Thermal sensitivity *of Calonectria henricotiae* and *Calonectria pseudonaviculata* conidia and microsclerotia. *Mycologia*, vol. 110, no. 3, pp. 546-558. http://doi.org/10.1080/00275514.2018.1465778. PMid:29969386.

MOURA MASCARIN, G., 2019. Current status and perspectives of fungal entomopathogens used for microbial control of arthropod pests in Brazil. *Journal of Invertebrate Pathology*, vol. 165, pp. 46-53. http://doi.org/10.1016/j.jip.2018.01.001. PMid:29339191.

MUÑIZ-PAREDES, F., MIRANDA-HERNÁNDEZ, F. and LOERA, O., 2017. Production of conidia by entomopathogenic fungi: from inoculants to final quality tests. *World Journal of Microbiology & Biotechnology*, vol. 33, no. 3, pp. 57. http://doi.org/10.1007/s11274-017-2229-2. PMid:28229333.

OTTATI-DE-LIMA, E.L., BATISTA FILHO, A., ALMEIDA, J.E.M., GASSEN, M.H., WENZEL, I.M., ALMEIDA, A.D. and ZAPELLINI, L.O., 2010. Produção semissólida de *Metarhizium anisopliae* e *Beauveria bassiana* em diferentes substratos e efeito da radiação ultravioleta e da temperatura sobre propágulos desses entomopatógenos. *Arquivos do Instituto Biológico*, vol. 77, no. 4, pp. 651-659. http://doi.org/10.1590/1808-1657v77p6512010.

PADILLA-GUERRERO, I., BARELLI, L., GONZÁLEZ-HERNÁNDEZ, G., TORRES-GUZMÁN, J. and BIDOCHKA, M., 2011. Flexible metabolism in *Metarhizium anisopliae* and *Beauveria bassiana*: role of the glyoxylate cycle during insect pathogenesis. *Microbiology*, vol. 157, no. 1, pp. 199-208. http://doi.org/10.1099/mic.0.042697-0. PMid:20929953.

PAIVA-GUIMARÃES, A.G.L., FREIRE, K.R.L., SANTOS, S.F.M., ALMEIDA, A.F. and SOUSA, A.C.B., 2020. Alternative substrates for conidiogenesis of the entomopathogenic fungus *Beauveria bassiana* (Bals) Vuillemin (Deuteromycotina: hyphomycetes). *Brazilian Journal of Biology = Revista Brasileira de Biologia*, vol. 80, no. 1, pp. 133-141. http://doi.org/10.1590/1519-6984.195711. PMid:31017241.

PANDEY, A., SOCCOL, C.R. and MITCHELL, D., 2000. New developments in solid state fermentation: bioprocesses and Products. *Process Biochemistry*, vol. 35, no. 10, pp. 1153-1169. http://doi.org/10.1016/S0032-9592(00)00152-7.

PRAKASH, G.V.S.B., PADMAJA, V. and KIRAN, R.R.S., 2008. Statistical, optimization of process variables for the large-scale production of *Metarhizium anisopliae* conidiospores in solid-state fermentation. *Bioresource Technology*, vol. 99, no. 6, pp. 1530-1537. http://doi.org/10.1016/j.biortech.2007.04.031. PMid:17590331.

SHOJAOSADATI, S.A., HAMIDI-ESFAHANI, Z., HEJAZI, P., FARAHANI, E.V. and RINZEMA, A., 2007. Evaluation of strategies for temperature and moisture control in solid state packed bed bioreactors. *Iranian Journal of Biotechnology*, vol. 5, no. 4, pp. 219-225.

SONG, M., YU, J., KIM, S., LEE, S., KIM, J., NAI, Y., SHIN, T. and KIM, J., 2019. Downstream processing of *Beauveria bassiana* and *Metarhizium anisopliae*-based fungal biopesticides against *Riptortus pedestris*: solid culture and delivery of conidia Biocontrol. *Biocontrol Science and Technology*, vol. 29, no. 6, pp. 514-532. http://doi.org/10.1080/09583157.2019.1566951.

TAYLOR, B., EDGINGTON, S., LUKE, B. and MOORE, D., 2013. Yield and germination of the entomopathogenic fungus *Beauveria bassiana* when grown on different rice preparations. *Journal of Stored Products Research*, vol. 53, pp. 23-26. http://doi.org/10.1016/j.jspr.2013.02.004.

TEFERA, T. and PRINGLE, K., 2003. Germination, Radial growth, and sporulation of *Beauveria bassiana* and *Metarhizium anisopliae* isolates and their virulence to *Chilo partellus* (Lepidoptera: Pyralidae) at different temperatures. *Biocontrol Science and*

Technology, vol. 13, no. 7, pp. 699-704. http://doi.org/10.1080/0958315031000151756.

TULLOCH, M., 1976. The genus *Metarhizium. Transactions of the British Mycological Society*, vol. 66, no. 3, pp. 407-411. http://doi.org/10.1016/S0007-1536(76)80209-4.

WANG, J. and ZHENG, C., 2012. Characterization of a newly discovered *Beauveria bassiana* isolate to *Frankliniella occidentalis* Perganda, a non-native invasive species in China. *Microbiological Research*, vol. 167, no. 2, pp. 116-120. http://doi.org/10.1016/j.micres.2011.05.002. PMid:21632224.

WESTFALL, P.H., TOBIAS, R.D. and WOLFINGER, R.D., 1999. *Multiple comparisons and 584 multiple tests using the SAS system.* Cary, NC: SAS Institute Inc.

YE, S.D., YING, S.H., CHEN, C. and FENG, M.G., 2006. New solid-state fermentation chamber for bulk production of aerial conidia of fungal biocontrol agents on rice. *Biotechnology Letters*, vol. 28, no. 11, pp. 799-804. http://doi.org/10.1007/s10529-006-9004-z. PMid:16786244.

ZIMMERMANN, G., 2007a. Review on safety of the entomopathogenic fungus *Metarhizium anisopliae. Biocontrol Science and Technology*, vol. 17, no. 9, pp. 879-920. http://doi.org/10.1080/09583150701593963.

ZIMMERMANN, G., 2007b. Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii. Biocontrol Science and Technology*, vol. 17, no. 6, pp. 553-596. http://doi.org/10.1080/09583150701309006.