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Performance and Virulence of the Entomopathogenic Fungi *Beauveria bassiana* Grown in Media Derived from Biodegradable Agricultural Wastes Enriched with Cricket Powder

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ABSTRACT

Entomopathogenic fungi (EPF) can be an eco-friendly alternative to control pests. To produce large-scale EPF requires underused economical substrates containing high carbon and nitrogen; hence the production could be cost-effective. This study investigates the effects of organic agricultural waste, i.e., rice bran, rice husks, tea dregs, and wheat bran enriched with cricket powder as culture media on the mycelial growth, sporulation, and conidia viability of *Beauveria bassiana* and the fungal pathogenicity towards *Spodoptera litura* larvae. For each type of medium, five independent cultures of *B. bassiana* are treated as replications. *B. bassiana* grown on a PDA medium is treated as a control. This study shows that rice bran enriched with cricket powder became the most suitable waste medium to support the mass production of virulent *B. bassiana*. Therefore, rice bran media enriched with cricket powder can be used as an appropriate medium for the mass production of *B. bassiana*.

INTRODUCTION

The use of chemical insecticides has become a common practice in agricultural cultivation around the world. However, using insecticides resulted in various negative impacts (Bolzonella et al., 2019; Rani et al., 2021). In this case, entomopathogenic fungi (EPF) such as *Beauveria bassiana* (Bals.) Vuill can be an alternative for controlling pests (McKinnon et al., 2017). The development of insecticides derived from EPF is minimal compared to chemical insecticides. One of the reasons is the difficulty in mass propagation of EPF and limited information on the nutritional requirement or geometry of EPF (Mishra et al., 2016).

Globally, organic waste generated annually from the industrial processing of agricultural raw materials is relatively high. In Indonesia alone, 11 million tons of rice husks and 5.5 million tons of rice bran are produced yearly (BPS, 2022). Meanwhile, the worldwide production of wheat bran every year reaches 650 million tons from wheat flour production (Prückler et al., 2014). Most of this waste is used as animal feed or burned for destruction. About 160,000 tons of tea dregs in China are discarded yearly (Cui et al., 2021). The utilization of tea dregs is still minimal, only used as a mixture for compost. To produce large-scale EPF requires underused economical substrates containing high carbon and nitrogen. Hence the production cost could be decreased. In this case, organic agricultural waste such as rice bran, rice husks, tea dregs, and wheat bran can serve as inexpensive media for EPF production. However, continuous mass production can reduce the virulence of EPF. As a consequence, the addition of insect-derived substrate such as cricket powder is of great importance to re-increase the virulence of EPF.

Entomopathogen fungi depend on the production of Cuticle Degrading Enzymes (CDEs) to penetrate the host cuticle. CDEs are induced by cuticular components in insect cuticles, where protein is the main component of insect cuticles. Sari et al. (2018) reported that cricket powder

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in the growth medium provides nutrition for the entomopathogenic fungi *Metarhizium majus* and increases its virulence on *Oryctes rhinoceros* larvae. An alternative approach would be using additives of cricket powder on organic agricultural waste such as rice bran, rice husks, tea dregs, and wheat bran as a medium that may enhance the viability dan pathogenicity of *B. bassiana*. To date, no information is available on the effect of supplements on *B. bassiana* production to enhance conidia viability dan pathogenicity of *B. bassiana* towards *Spodoptera litura.* This study aims to investigate the effects of organic agricultural waste, i.e., rice bran, rice husks, tea dregs, and wheat bran medium with cricket powder, on the mycelial growth, sporulation, and conidia viability of *B. bassiana* and the fungal pathogenicity towards *S. litura*.

MATERIALS AND METHODS

Research Location

The research was conducted at the Laboratory of Biological Control, Department of Plant Pests and Diseases, Faculty of Agriculture, Universitas Brawijaya from July to December 2022.

Isolate Preparation

Beauveria bassiana isolate was obtained from the collection of the Department of Plant Pests and Diseases, Faculty of Agriculture, Universitas Brawijaya. The isolate was evidenced to be virulent on insect and mite pests (Afandhi et al., 2020; Puspitarini, Fernando, Widjayanti et al., 2022). The isolate was initially cultured on Potato Dextrose Agar (PDA) media.

Agricultural Waste Media Preparation

The agricultural wastes, i.e., rice bran, rice husk, tea dreg, and wheat bran, were dried at room temperature, then ground to powder, and sieved using a 0.5 mm mesh sieve to obtain uniform powder size. Rice bran and rice husk were obtained from Sumber Ngepoh Farmer Association in Malang, East Java, Indonesia; tea dreg was collected from the cafeteria of the Faculty of Agriculture, Universitas Brawijaya; and wheat bran was purchased from the local pet store in Malang. Culture media were made by soaking 60 g of each agricultural waste powder in 200 ml of boiling distilled water. The powder was then filtered using filter paper. The retained powder was then put into a heat-resistant plastic and steamed for 30 minutes. As much as 25 g of steamed powder was placed into a Petri dish. The dish was sealed using plastic wrap. For the treatment with cricket powder, 15% cricket powder was added to the agricultural waste powder and mixed well before being soaked in hot distilled water. Lastly, all plated media were sterilized using an autoclave at 121°C and 15 psi for 60 minutes. Furthermore, all agricultural waste media's carbon, nitrogen, and moisture content were analyzed.

B. bassiana Inoculation to Culture Media

The sterilized media were inoculated with *B. bassiana* using a 0.5 cm diameter cork borer. This inoculation was carried out aseptically at LAFC to avoid contaminating other microorganisms. After that, the media were incubated in a sterile place for 14 days. For each type of medium, five independent cultures of *B. bassiana* were treated as replications. *Beauveria bassiana* grown on PDA media was treated as a control.

Conidia Density Calculation

The conidia of *B. bassiana* from each culture medium were harvested by surface scraping. The obtained conidia were suspended with 10 ml of sterilized distilled water containing 0.05% Tween 80. The suspension was centrifuged at 3,000 rpm for 5 minutes to separate the conidia from the mycelium. The obtained conidia pellet was then mixed with 5 ml of distilled water, and the conidia density was calculated using a haemocytometer with the aid of a compound microscope (Puspitarini, Fernando, Sianturi, et al., 2022). Conidia density is calculated using the Eqn 1.

$$C = \frac{t \times d}{n \times 0.25} \times 10^6 \qquad \text{..1)}$$

Where: C is the conidia density; t is the total number of conidia in the observed sample box; d is the dilution factor; n is the number of sample boxes (5 large boxes x 16 small boxes); 0.25 is the correction factor for the use of a small-scale sample box on the haemocytometer (Indriyanti et al., 2017).

Conidia Viability Calculation

Conidia viability assessment was carried out by incubating the suspension for 24 hours on a glass slide. After the incubation process, the number of germinated conidia was counted. The number of germinated conidia was calculated using the Eqn 2.

$$V = \frac{G}{G+U} \times 100\% \quad \text{..2)}$$

Where: V : conidia viability (%); G is the number of germinated conidia; U is the number of conidia that did not germinate (Indriyanti et al., 2017).

Pathogenicity Assay

The third instar larvae of *Spodoptera litura* used as test insects were obtained from the Research Institute for Sweetener and Fiber Crops (Balittas), Karangploso District's laboratory Malang Regency, East Java. The larvae were immersed in each suspension for 30 seconds and placed in a plastic container. For the control group, larvae were immersed in sterilized distilled water. The larvae were fed with green mustard, which was changed every day. Each experimental unit consisted of 20 larvae.

Statistical Analysis

Based on the Shapiro-Wilk and Levene test, all the data obtained in this study have met the assumption of normality and homogeneity. Therefore, all data were then submitted to a one-way analysis of variance (ANOVA). In addition, the mortality data were not subjected to Abbot's formula of corrected mortality since the mean mortality in control was less than 5% (Puspitarini, Fernando, Sianturi, et al., 2022). The means were compared by Tukey's multiple comparison tests at $P < 0.05$. Additionally, average values of mortality of *S. litura* larvae from the pathogenicity assay were submitted to probit analysis for calculating LT_{50}. All analyses were performed using R statistics and used "aod" package for probit analysis.

RESULTS AND DISCUSSION

Nutritional Characterization of the Agricultural Waste Media

Table 1 shows the nutrient content of various types of agricultural waste media used to produce *B. bassiana*. The carbon content of each waste medium varies between 13-26%, with rice husk + cricket powder medium having the highest carbon content. The C/N ratio among media ranges from 8.9-19.67%, with the highest value found on rice husk medium.

Nutrients needed by fungi to grow include carbon, nitrogen, non-metallic elements such as sulfur and phosphorus, metallic elements such as Ca, Zn, K, Cu, Mn, Mg, and Fe, vitamins, water, and energy. However, the primary energy sources fungi need to grow are carbon and nitrogen (Jaronski, 2023; Litwin et al., 2020). *B. bassiana* is a heterotrophic organism that requires food as an energy source. Carbon sources are needed as a source of energy and structural fungal cells that encourage the vegetative growth of fungi because they are used in their metabolic processes. While nitrogen is needed by fungi for protein synthesis, purines, pyrimidines, chitin, and plays a role in the formation of fungal organelles, such as hyphae apical cells, for the growth of *B. bassiana* (Sadad et al., 2014). Carbon and nitrogen are essential macronutrients for forming carbohydrates, nucleic acids, and structural proteins such as the cytoskeleton and cell membranes, as well as for producing functional proteins such as enzymes (Mondal et al., 2016; Srikanth & Santhalakshmi, 2012).

Table 1. Nutritional characteristics of various types of agricultural substrates used as production media for EPF *B. bassiana*

Media	Carbon (%)	Nitrogen (%)	C/N	Moisture (%)
Rice husk	24	1.22	19.67	5.40
Tea dreg	14	1.53	9.15	4.50
Rice bran	14	1.35	10.37	6.70
Wheat bran	13	1.46	8.90	6.25
Rice husk + cricket powder	26	1.43	18.18	4.10
Tea dreg + cricket powder	21	1.69	12.43	4.51
Rice bran + cricket powder	23	1.51	15.23	5.82
Wheat bran + cricket powder	22	1.61	13.66	8.03

B. bassiana Growth on the Evaluated Agricultural Waste Media

Agricultural waste media, with the addition of cricket powder, can increase the growth of colonies of *B. bassiana*. Waste media with the addition of cricket powder shows longer colony diameters than waste media without cricket powder, with the longest colony diameters found in wheat bran enriched with cricket powder (7.43 cm) and rice bran enriched with cricket powder (6.6 cm). The shortest colony diameter was found on tea dregs (2.06 cm) (Table 2).

Table 2. Average diameter of *B. bassiana* colonies on agricultural waste media at 14 days after inoculation

Treatment	Colony diameter (cm) ± SD
Control (PDA)	2.16 ± 0.70 a
Rice husks	2.56 ± 0.55 ab
Tea dregs	2.06 ± 0.20 a
Rice bran	5.03 ± 0.45 c
Wheat bran	3.23 ± 0.25 ab
Rice husks + cricket powder	4.76 ± 0.55 c
Tea dregs + cricket powder	4.03 ± 0.81 bc
Rice bran + cricket powder	6.60 ± 1.37 d
Wheat bran + cricket powder	7.43 ± 1.46 d

Remarks: Means followed by the same letters within each column are not significantly different at $P < 0.05$ according to Tukey's test

The increase in the diameter of *B. bassiana* colonies in wheat bran waste media enriched with cricket powder can be influenced by the nutritional content of cricket powder, where cricket powder contains high proteins, carbohydrates, and nucleic acids, which are sources of carbon and nitrogen that play a vital role in fungal growth. These complex molecules are the main macromolecules that make up fungal hyphae and conidia cells (Smith & Grula, 1981). High carbohydrate and protein content in the media will accelerate conidia germination so that colony formation will be faster. Chitin content in cricket powder is a source of carbon and nitrogen that supports mycelium growth and the formation of EPF conidia. Adding chitin into the media causes the media to become denser, thus triggering the fungus to grow faster.

The growth of colony diameter is also influenced by the size of the particles used as the production medium. The particle size of the waste media and cricket powder can affect the growth of *B. bassiana* fungus. In contrast, the fine particle size of the waste media and cricket powder can facilitate the absorption of nutrients by the fungus so that the fungus can grow better. The particle size on the substrate affects the speed of the fungus growing on the surface of the media. The finer the particle size of the substrate, the greater the surface area that can be degraded by the fungus (Lopez-Perez et al., 2015).

Conidia Density and Viability of *B. bassiana* on the Evaluated Agricultural Waste Media

Agricultural waste media, with the addition of cricket powder, increases the conidia production of *B. bassiana* isolates. Media added with cricket powder shows higher conidia production, with the highest conidia production in rice bran enriched with cricket powder (30.23 x 10^7 conidia/ml). The lowest conidia production is found in tea dregs, which shows conidia production of 6.15 x 10^7 conidia/ml (Table 3). While on viability, rice bran (72.75%) is observed to be the most suitable medium for conidia germination. Similarly, the media added with cricket powder showed higher viability than those without, with the highest viability in rice bran enriched with cricket powder (83.38%). The lowest viability is found in tea dregs, 51.43% and 62.09%, without and with cricket powder (Table 3).

This study shows that the increase in conidia production is related to the content of the C/N ratio and the water content of each medium. Tea dregs show the lowest conidia production because tea dregs have a low C/N ratio and water content. The moisture of the media also profoundly affects the number of conidia because EPF prefers a moist environment (Camara et al., 2022). Rice bran enriched with cricket powder had a C/N ratio of 15.23 and a moisture content of 5.82, indicating the highest conidia production. The difference in the nutrient content of carbon and nitrogen as a source of nutrients for the growth of *B. bassiana* became an essential factor in the production of EPF. The high carbon and nitrogen content in rice bran enriched with cricket powder supported the growth of *B. bassiana* isolates. Carbon and nitrogen are essential macronutrients for forming carbohydrates, nucleic acids, structural proteins such as the cytoskeleton and cell membranes, and functional proteins such as enzymes that support EPF growth. The element carbon is also known to influence the formation of lipids in the conidia of *B. bassiana*. Lipids in triglycerides are a source of energy for conidia germination and conidia formation. Carbon and nitrogen in the media determine the process of mycelium formation and conidia germination. In media with low carbon and nitrogen content, there will be fewer conidia germination, so insect mortality is also low (Safavi et al., 2007).

The increased conidia production in rice bran enriched with cricket powder also occurred due to the rich nutrient content of the growing media, which affected the growth and germination of EPF (Kim et al., 2014). Rice bran enriched with cricket powder has the most suitable nutritional content for producing *B. bassiana*. The nutritional content of rice bran, plus nutrients from cricket powder, can help *B. bassiana* to grow optimally. The addition of cricket powder chitin sources in the waste medium showed a significant increase in conidia production. The increase in conidia production in rice bran without cricket powder with rice bran enriched with cricket powder increased by 2.2 times. This indicates that conidia production increased in the media with cricket powder than in the media without. Adding chitin to the growth media can make the media more dense and trigger the EPF to form conidia in large numbers. Adding chitin in the right amount to the media directly affects the production of conidia (Palma-Guerrero et al., 2010; Włóka et al., 2022). Cultures of *B. bassiana* adding chitin as cricket powder have higher conidia density than those without chitin.

Based on the results of further test analysis with viability parameters showed that rice bran (72.75%), rice bran enriched with cricket powder (83.38%), and wheat bran enriched with cricket powder (81.21%) were the same. However, those values were significantly different from other media. PDA (71.24%) and rice husk enriched with cricket powder (71.20%) showed the same viability. Rice husk (62%), wheat bran (69.36%), and tea dregs (62.09%) also produced the same viability.

The increase in viability percentage in the medium without cricket powder with the medium enriched with cricket powder is about 1.1-1.2%. When viewed from the number of conidia formed and conidia germinating, the highest chance of conidia that could infect *S. litura* larvae was in treating rice bran enriched with cricket powder. The high viability of conidia in the treatment of cricket powder-enriched waste media was caused by the nutrient content of the media used. Rich nutrition and following the needs of *B. bassiana* will increase conidia germination. Nutrient-rich growing media affect the germination of conidia of EPF (Kim et al., 2014). In addition, adding a source of chitin can increase conidia's persistence and the ability to insect infection. Conidia density and viability correlated with the virulence of EPF(Afandhi et al., 2022; Faria et al., 2015). The higher the conidia density, the higher the mortality rate of the insects tested (Puspitarini et al., 2021).

Table 3. Average conidia density and viability of *B. bassiana* on various agricultural waste media

Treatment	Conidia density (10^7 conidia/ml)	Conidia viability (%)
	$\bar{x}$ ± SD	$\bar{x}$ ± SD
Control (PDA)	12.40 ± 1.99 bc	71.24 ± 1.00 bc
Rice husks	9.40 ± 2.17 ab	62.00 ± 6.00 ab
Tea dregs	6.15 ± 1.11 a	51.43 ± 3.69 a
Rice bran	13.68 ± 2.53 bcd	72.75 ± 6.51 bcd
Wheat bran	15.30 ± 6.22 cd	69.36 ± 9.98 b
Rice husks + cricket powder	18.51 ± 2.89 d	71.20 ± 3.45 bc
Tea dregs + cricket powder	14.44 ± 2.26 bcd	62.09 ± 6.82 ab
Rice bran + cricket powder	30.23 ± 2.47 e	83.38 ± 6.14 d
Wheat bran + cricket powder	26.85 ± 3.46 e	81.21 ± 7.90 cd

Remarks: Means followed by the same letters within each column are not significantly different at $P < 0.05$ according to Tukey's test

Pathogenicity of *B. bassiana* towards *S. litura*

Various agricultural waste media used to produce *B. bassiana* showed a mortality of 76-100% on the 7th day after application (Table 4). Agricultural waste media enriched with cricket powder resulted in higher mortality than agricultural waste media without. Rice bran enriched with cricket powder and wheat bran enriched with cricket powder produced the highest mortality. Based on Table 4, the lowest percentage of mortality was in the control treatment, which was 0%. This proves that *B. bassiana* grown on waste media enriched with cricket powder can potentially kill *S. litura* larvae.

Table 4. The mean mortality of *S. litura* infected with *B. bassiana* from various agricultural waste media at 7 days after application

Treatment	Mortality (%)
	x̄ ± SD
Control (without *B. bassiana*)	0.00 ± 0.00 a
Rice husks	86.66 ± 5.77 bc
Tea dregs	76.66 ± 11.54 b
Rice bran	96.66 ± 5.77 d
Wheat bran	93.33 ± 5.77 cd
Rice husks + cricket powder	96.66 ± 5.77 d
Tea dregs + cricket powder	86.66 ± 5.77 bc
Rice bran + cricket powder	100.00 ± 0.00 d
Wheat bran + cricket powder	100.00 ± 0.00 d

Remarks: Means followed by the same letters within each column are not significantly different at $P < 0.05$ according to Tukey's test

Rice bran enriched with cricket powder became the most suitable waste medium to support the production of virulent *B. bassiana*. The number of fungal conidia applied influenced larval mortality. The higher the number of conidia, the more enzymes and toxins are produced, thereby increasing the mortality of the larvae tested (Afandhi et al., 2022; Puspitarini et al., 2021). The number of conidia is influenced by the nutrients contained in the medium (Iskandarov et al., 2006; Paiva-Guimarães et al., 2020). In addition to the influence of nutrients contained in rice bran, the chitin content also influences mortality in rice bran enriched with cricket powder. The death of *S. litura* larvae due to the application of EPF occurs due to the presence of compounds and enzymes that are pathogenic to their host insects.

EPF can release lipase, chitinase, amylase, protease, and phosphatase (Harith-Fadzilah et al., 2021; Vidhate et al., 2023; Wang & Wang, 2017). Protease is the most crucial cuticle-degrading enzyme, and this enzyme's activity stimulates the chitinase enzyme's emergence. Chitinase is an enzyme to degrade chitin, and lipase degrades lipoproteins, fats, and wax layers which are then used by fungi as a source of nutrients. The high chitin content in rice bran enriched with cricket powder helps promote hyphae growth and conidia germination. Chitin content in the substrate is an important source of carbon and nitrogen for EPF growth, including hyphae growth and spore germination (Palma-Guerrero et al., 2010; Włóka et al., 2022). *B. bassiana* grown on rice bran with the addition of cricket powder chitin sources can cause an increase in the chitinase enzyme. Chitinase is a specific hydrolytic enzyme that degrades chitin substrates. Adding chitin to EPF growth media can induce chitinase enzymes used in pest biocontrol. Chitinase enzyme activity generally takes place from the beginning of fungal growth. Chitinase enzyme is known to be one of the determinants of the ability of EPF infection because it plays a role in the penetration of fungi into the insect body (Fang et al., 2009; Pelizza et al., 2012).

Fig. 1 shows the larval body before and after being covered by the white hyphae of *B. bassiana*. This condition occurred due to the penetration of *B. bassiana* into the larval body. The mechanism of penetration of *B. bassiana* begins with the growth of conidia in the integument. Then the hyphae secrete enzymes such as lipolytic, proteolytic, and chitinase which cause the hydrolysis of the integument. After *B. bassiana* enters the larva's body, the fungi will release beauvericin toxin which causes damage to the insect's body tissue. The insect will die and mycelia will grow in all parts of the larva's body (Mallebrera et al., 2018). Larvae of *S. litura* infected with *B. bassiana* show characteristic symptoms such as reduced feeding intensity and limited movement, and after death, the body will be overgrown with fungal mycelia. The decrease in appetite causes the larvae to lack nutrients, so the energy generated from the metabolic process is reduced and causes the larvae to move slowly. Lack of nutrients in the larval body is also caused by *B. bassiana,* which utilizes nutrient sources in the larval body. The fungus will reproduce on the insect's body and form hyphae. When the insect has died, the fungus will continue its life cycle until its body is filled with mycelium, hardened, and mummified (Mora et al., 2018).

Lethal Time 50

The LT50 value of each type of media indicates that rice bran enriched with cricket powder is the most suitable waste medium for producing *B. bassiana* because it caused the fastest 50% mortality of *S. litura* larvae, which was 3.25 days. Meanwhile, in the tea dregs media, the 50% mortality was 5.24 days (Table 5).

Table 5 shows the LT50 value of each treatment of agricultural waste media. Based on Table 5, the LT50 value that is effective in influencing the mortality of *S. litura* larvae is found in the treatment of rice bran enriched with cricket powder with an LT50 value of 3.25 days, which means that within 3.25 days, it causes mortality of 50% with the regression equation y = -4.714+5.429x which indicates that every increase in the coefficient of x (time) will increase the value of the coefficient of y (probit), namely the mortality of *S. litura* by 5.429. The difference in the time of death of *S. litura* larvae is influenced by the ability to infect *B. bassiana*.

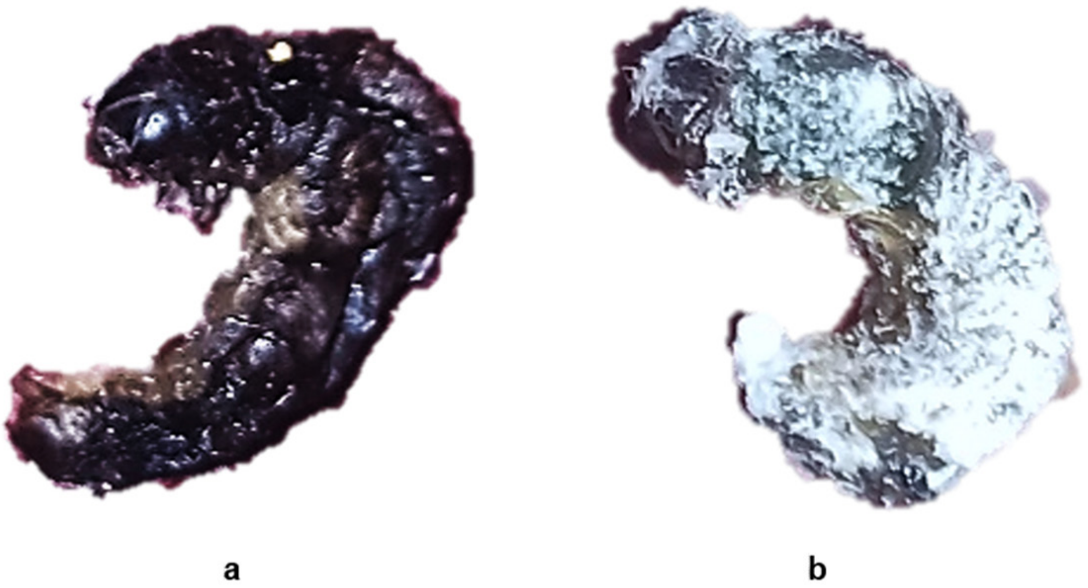

Fig. 1. Dead larvae of *S. litura* at 7 days after application of suspension of *B. bassiana* (a) Before overgrown with hyphae; (b) After overgrown with hyphae

Table 5. LT50 values of *B. bassiana* grown in various agricultural waste media against *S. litura*

Treatment	LT_{50} (days)	r		Regression	Sig.
Rice husks	4.96	0.969	0.938	y= -8.857+4.786x	<0.001
Tea dregs	5.24	0.961	0.924	y= -8.143+4.357x	<0.001
Rice bran	4.34	0.971	0.944	y= -8.714+5.393x	<0.001
Wheat bran	3.94	0.984	0.968	y= -7.143+5.321x	<0.001
Rice husks + cricket powder	4.67	0.969	0.938	y= -9.143+5.214x	<0.001
Tea dregs + cricket powder	4.99	0.965	0.931	y= -9.00+4.786x	<0.001
Rice bran + cricket powder	3.25	0.988	0.975	y= -4.714+5.429x	<0.001
Wheat bran + cricket powder	3.53	0.992	0.985	y= -5.714+5.393x	<0.001

CONCLUSION

Rice bran enriched with cricket powder was the most suitable waste medium for producing *B. bassiana*. The fungal colony diameter, conidia density, and viability were highest when *B. bassiana* was grown on rice bran medium enriched with cricket powder, leading to the fungus's higher virulence. Therefore, rice bran media enriched with cricket powder can be a sustainable medium for the mass production of *B. bassiana*.

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