Effects of Cricket and Fruit Fly Flour in Growth Media on *Beauveria bassiana* (Bals.) Vuill Pathogenicity Against *Zeugodacus cucurbitae* (Coquillet) Prepupae

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ABSTRACT

Zeugodacus cucurbitae (Diptera: Tephritidae) is a major insect pest of horticultural crops, causing significant yield losses.The efficacy of *Beauveria bassiana*, a potential biocontrol agent, can be influenced by growth media composition. This study examined the effects of cricket and fruit fly flour on *B. bassiana* growth and pathogenicity against *Z. cucurbitae*. The methods added *B. bassiana* growth media with different concentrations (0%, 0.5%, 1%, and 1.5%) of cricket and fruit fly flour and assessing its pathogenicity against *Z. cucurbitae* at a spore density of 10^6 and 10^8 spores/mL. The results showed that 1% cricket flour combined with a spore density of 10^8 spores/mL resulted in the highest mortality rate (29.33%) and the fastest infection (2 days). The lowest average mortalities were found in treatments without flour addition which were 21.3%, 19.3%, and 19%, respectively. The longest time to cause infection was observed in 7 days. Infection symptoms are marked by the emergence of white fungal mycelia covering the cuticle, while infected adults exhibit deformed, wrinkled, and smaller wings. These findings highlight the potential of growth media optimization to enhance *B. bassiana* virulence, contributing to the development of more effective and sustainable biocontrol strategies against *Z. cucurbitae*.

Keywords: Biological control; Entomopathogenic fungi; Spore density; Insect Flour; Virulence

INTRODUCTION

The fruit fly *Zeugodacus cucurbitae* (Diptera: Tephritidae) is important in horticultural crop cultivation (Lei Li et al., 2019). The host plants of *Z. cucurbitae* belong to the families Cucurbitaceae (Nair & Pal., 2020), Caricaceae, Fabaceae, Loganiaceae, Malvaceae, Myrtaceae, Pandanaceae, Passifloraceae, Rhamnaceae, Saptoaceae, Solanaceae, Agavaceae, Capparidaceae, Moraceae, Rutaceae, and Vitaceae. *Z. cucurbitae* is widely distributed in regions with temperate, tropical, and subtropical climates. The damage caused by fruit fly infestations can reach up to 100% (Sari et al., 2020).

Integrated pest management components to control *Z. cucurbitae* can be achieved using entomopathogenic fungi. According to Altinok et al. (2019), one of the commonly employed entomopathogenic fungi for pest-insect control is *Beauveria bassiana*. *B. bassiana* fungus produces toxins such as beauvericin, beauverolite, bassianolite and isorolite that act by damaging tissues, which leads to mortality, as reported by Bagariang et al. (2023). This entomopathogenic fungus is effective in sup-

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pressing the development of pest insects in the orders Hemiptera (Sani et al., 2020; Atta et al., 2020), Orthoptera (Romero-Arenas et al., 2020), and Diptera (White et al., 2021).

The decline in the quality of entomopathogenic fungal spores and virulence can occur during the in vitro subculture process (Hussien et al., 2021). The reduction in the spore quality of entomopathogenic fungi may be attributed to the diminished sources of carbon, chitin, starch, and protein in the propagation media (Islam et al., 2021). Adding carb and nitrogen sources in the growth media can aid in preserving the infectivity of entomopathogenic fungi by activating chitinase enzymes crucial for the penetration of the host cuticle. Therefore, a propagation technique that can maintain the fungus's quality and virulence is essential (Sari & Khobir, 2020).

Adding protein and chitin sources to growth media can maintain the virulence of entomopathogenic fungi and activate protease and chitinase enzymes involved in the degradation of insect cuticles (Susila et al., 2023). Chitin and protein sources are most abundant in insect integuments (Khayrova et al., 2021). Incorporating insect flour into the growth media can enhance spore density and fungal viability due to its nutrient content closely resembling the natural host (Mascarin et al., 2024). The types of insect flour used in this study originated from adult crickets and fruit flies. This research investigated the effects of adding cricket and fruit fly flour to *B. bassiana* growth media with varying concentrations and spore densities on the infection symptoms, development, mortality, and the speed of infection onset in *Z. cucurbitae* pupae.

MATERIALS AND METHODS

Propagation of *Zeugodacus cucurbitae*

The mating process of *Z. cucurbitae* adults occurred within the rearing cage (30 cm x 30 cm x 30 cm). The hatching substrate consisted of 3 × 3 cm pieces of black cloth moistened with yellow pumpkin juice. *Z. cucurbitae* adults were provided with an artificial diet of sugar and yeast, which plays a role in sexual maturity and egg production (Gokulanathan et al., 2023). Adults deposited their eggs on the black cloth after being provided with the artificial diet. Eggs attached to the cloth were harvested by dipping the black cloth into 150 ml of distilled water until they detached.

The eggs poured into the artificial feed of wheat bran hatched after 24 hours and developed into larvae. Larvae reaching the final instar stage crawled onto the sawdust and transformed into pupae. The pupae within the sawdust were sifted for subsequent placement into the rearing cage to propagate the next generation.

The Diet for *Z. cucurbitae*

The artificial diet for *Z. cucurbitae* larvae combined 185 grams of wheat bran, 180 ml of distilled water, 43.2 grams of sugar, 10.8 grams of yeast, 0.3 grams of nipagin, and 0.3 grams of sodium benzoate. Wheat bran was poured into a tray measuring 20 × 15 cm. Distilled water, sugar, nipagin, and sodium benzoate were mixed and stirred until homogeneous, then allowed to stand for 5 minutes. After swelling, this solution was poured into the wheat bran tray and stirred until well distributed. The artificial feed containing eggs was placed in a 35 × 25 × 10 cm container containing 300 grams of sterilized sawdust. It was then covered with black cloth and secured with a string to prevent any interference from pests.

The Production of Cricket and Fruit Fly Flour

The production of cricket flour and fruit fly flour was carried out based on previous research by Septiani et al. (2020). Adult crickets and fruit flies were dried and sterilized simultaneously using a Memmert UN 30 oven at 100°C for 3 hours. Subsequently, crickets and fruit flies were finely ground into flour and sifted through a sieve with a mesh size of 1 mm.

The Production of Growth Media

PDA (Potato Dextrose Agar) growth media are commonly used for cultivating fungal microorganisms. The composition of the ingredients in 250 ml of PDA consists of 250 ml of distilled water, 62.5 grams of potatoes, 5 grams of dextrose, and 5 grams of agar. The potatoes were peeled, cut into small diced pieces, placed in an Erlenmeyer flask containing distilled water, and boiled until soft. The potato extract was then supplemented with dextrose and agar (Wahyu et al., 2021).

The growth media were supplemented with cricket flour and fruit fly flour at concentrations of 0.5%, 1%, and 1.5%. Determining the media composition with the addition of cricket flour and fruit fly flour utilized the dilution formula of $M_1V_1=M_2V_2$. In 250 ml of PDA, 1.25, 2.5, and 3.75 grams of flour were added for a concentration of 0.5%, 1%, and 1.5%, respectively. The solution was then boiled until homogeneous. The homogenized medium was sterilized in an autoclave at a temperature of 121°C for 25 minutes and a pressure of 1 atm.

Inoculation of *B. bassiana*

The isolates of *B. bassiana* fungi used in this study are from the collection of the Biological Control Laboratory, Plant Pest and Disease Program, Faculty of Agriculture, Universitas Gadjah Mada. These isolates were obtained initially from the Pest and Disease Observation Laboratory in Banyumas, with *Leptocorisa oratorius* as the host insect. The fungal isolates were cultured using the streaking technique within a Laminar Air Flow cabinet on all tested growth media. Subsequently, they were incubated for 14 days.

The Calculation of Spore Density

The 14-day-old isolates were harvested by adding 10 ml of distilled water (aquades) and taking them from the growth media. The resulting solution was transferred to sterile test tubes and vortexed. One milliliter of the fungal suspension was pipetted into another test tube containing 9 ml of distilled water, and it was vortexed again. This step can be repeated to achieve the desired dilution level. The fungal suspension was then placed onto a hemocytometer chamber and covered with a cover slip. Spore density calculations were carried out manually using a binocular microscope and were computed using the formula from Gabriel & Riyanto (1989).

$$K = \times \frac{n}{t \times 0{,}25} \times 10^6 \qquad (1)$$

K represents the spore density per ml of solution. T is the number of conidia in the observed square. N is the number of hemocytometers counting squares (5 large squares × 16 small squares), and 0.25 is the correction factor for using the small-scale sample squares on the hemocytometer.

The application of *B. bassiana* to *Z. cucurbitae* Prepupae

This research was arranged in a Completely Randomized Factorial Design (CRFD). The factors involved in the study include the type of insect diet, diet concentration, and spore density. There were 21 treatment combinations, each replicated 3 times, resulting in 63 experimental units, as shown in Table 1.

Table 1. Treatment Combination

Code	Treatment of spore/mL
B1V1	*B. bassiana*on pure PDA with spore density 10^8
B1V2	*B. bassiana*on pure PDA with spore density 10^7
B1V3	*B. bassiana* on pure PDA with spore density 10^6
B2V1	*B. bassiana*onPDA + Cricket Flour 0.5% spore density 10^8
B2V2	*B. bassiana*on PDA + Cricket Flour 0.5% spore density 10^7
B2V3	*B. bassiana*on PDA + Cricket Flour 0.5% spore density 10^6
B3V1	*B. bassiana* on PDA + Cricket Flour 1% spore density 10^8
B3V2	*B. bassiana* on PDA + Cricket Flour 1% spore density 10^7
B3V3	*B. bassiana* on PDA + Cricket Flour 1% spore density 10^6
B4V1	*B. bassiana* on PDA + Cricket Flour 1,5%spore density 10^8
B4V2	*B. bassiana* on PDA + Cricket Flour 1,5% spore density 10^7
B4V3	*B. bassiana* on PDA + Cricket Flour 1,5% spore density 10^6
B5V1	*B. bassiana* on PDA + Fruit Flies Flour 0,5% spore density 10^8
B5V2	*B. bassiana* on PDA + Fruit Flies Flour 0,5% spore density 10^7
B5V3	*B. bassiana* on PDA + Fruit Flies Flour 0,5% spore density 10^6
B6V1	*B. bassiana*on PDA + Fruit Flies Flour 1% spore density 10^8
B6V2	*B. bassiana*on PDA + Fruit Flies Flour 1% *spore* density 10^7
B6V3	*B. bassiana*on PDA + Fruit Flies Flour 1% spore density 10^6
B7V1	*B. bassiana*on PDA + Fruit Flies Flour 1,5% spore density 10^8
B7V2	*B. bassiana* on PDA + Fruit Flies Flour 1,5% spore density 10^7
B7V3	*B. bassiana on* PDA + Fruit Flies Flour 1,5% spore density 10^6

The fungal suspension had 10^8, 10^7, and 10^6 spore/mL spore densities. 30 prepupae of *Z. cucurbitae* per replication were placed in sterilized sawdust-filled Petri dishes. The application of *B. bassiana* fungus was carried out using a spray technique with a manual sprayer. Each treatment received 5 ml of suspension, administered only once. Observations were conducted daily for 14 days. Samples were collected for microscopic identification in the presence of *B. bassiana* symptoms.

Data Analysis

The observation results were analyzed using analysis of variance (ANOVA). Means were separated by Duncan's Multiple Range Test (DMRT) at the 5% significance level using the SPSS software.

RESULTS AND DISCUSSION

Symptoms *of B. bassiana* Infection

The pre-pupal stage appeared normal, with a milky white color and a still soft larva-like texture (Figure 1A). Based on this research, *B. bassiana, when* applied to the pre-pupal stage, started to

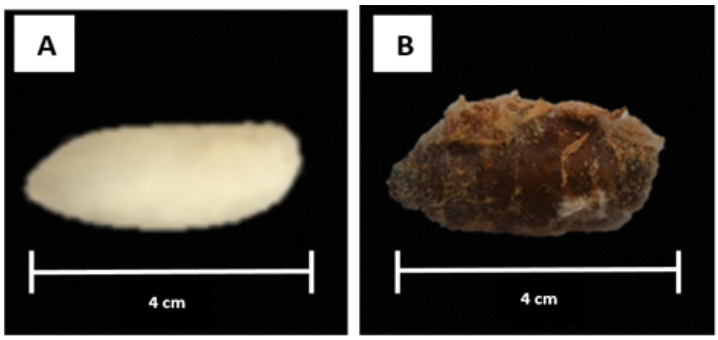

Figure 1. Healthy Prepupae of *Z. cucurbitae* (A) and Pupae Infected with *B. bassiana* (B)

exhibit symptoms during the pupal stage. Pupae infected with *B. bassiana* were characterized by the appearance of white fungal mycelium. Initially, the mycelium was only on one side of the pupal cuticle. However, as they aged, the *B. bassiana* fungus spread to cover the entire surface of the *Z. cucurbitae* pupal cuticle (Figure 1B). Microscopic observations were conducted to confirm that the entomopathogenic fungus infecting *Z. cucurbitae* was *B. bassiana*. Based on the observations, it was found that the entomopathogenic fungus *B. bassiana* exhibited characteristic morphology, which is hyaline and unicellular (Figure 2).

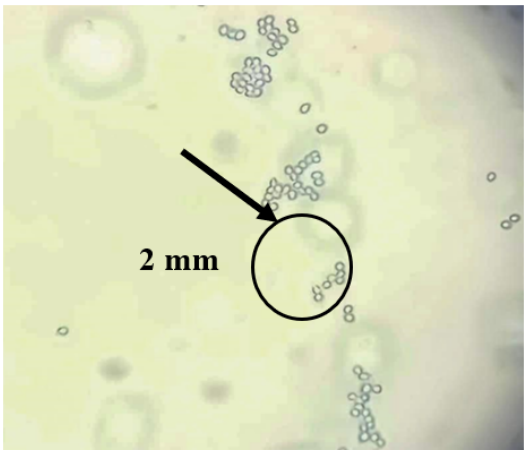

Figure 2. Conidia of *B. bassiana*

The presence of toxins within the *B. bassiana* was acknowledged for its potential to inflict tissue damage, particularly to muscles and the nervous system of *Plutella xylostella*, as stated by Ardan et al. (2020). These toxins are believed to impede the development of pupae into adults. The characteristics of *B. bassiana,* which is hyaline and unicellular, align with the statement by Sopialena et al. (2022) that *B. bassiana*, under microscopic examination, exhibits hyaline, round-shaped conidia that are unicellular.

The Growth of *Z. cucurbitae* Adults

Observations on day 14 indicated that pupae successfully developed into the adult stage. There are distinct characteristics between normal and abnormal adults. Normal adults have wings with a distinctive dark-colored costal band narrowing in the R2+3 area and widening towards the wing's apex. Another characteristic feature is a dark transverse line along the dm-cu area and a spot at the

wing's apex that does not reach the M vein and narrows along r-m (Figure 3A). In contrast, adults infected with the *B. bassiana* fungus exhibit morphological deviations, primarily in the wing area. Observable characteristics include wrinkled wings and relatively smaller size (Figure 3B). These adults experienced imperfect growth due to the entomopathogenic fungus applied during the pre-pupal stage, which did not exhibit infection symptoms initially because the infection process took longer, and symptoms only became apparent in the subsequent stage, the adult.

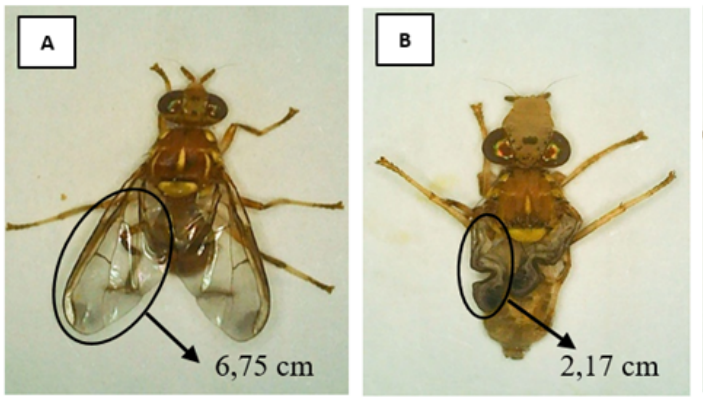

Figure 3. Morphology healthy adult of *Z. cucurbita*e (A) and adult infected with *B. bassiana* (B)

Normal adult *Z. cucurbitae* adults typically engage in activities such as flying, foraging, and reproduction, with a life cycle lasting up to 29 to 58 days in the wild (Vasudha et al., 2019; Wei et al., 2020). Conversely, abnormal adults face limitations in activities and are unable to fly, forage, or mate, resulting in a decline in *the Z. cucurbitae* population, as stated by Steck et al. (2022). Entomopathogenic fungi are known to impact insect growth and development, leading to reduced fertility, fitness, and the induction of abnormalities (Ullah et al., 2023).

The Average Mortality of *Z. cucurbitae*

Each treatment showed varying average mortality (Figure 4). The average mortality of *Z. cucurbitae* was affected by the application of *B. bassiana* with the addition of 1% cricket flour and a spore density of 10^8 was 29.33%. Adding 1 % cricket flour at a spore density of 10^7 resulted in an average mortality of 28.3 %. Treatment with adding fruit fly flour at a concentration of 1% and spore densities of 10^8 and 10^7 showed average mortalities of 27.3 % and 27 %, respectively. The treatment of adding 1.5% flour exhibited a lower average mortality. Cricket flour and fruit fly flour added at a concentration of 1.5 % with a spore density of 10^6 resulted in an average mortality of 24%. The lowest average mortalities of *Z. cucurbitae* were found in treatments without flour addition at spore densities of 10^8, 10^7 and 10^6, which were 21.3 %, 19.3 %, and 19 %, respectively (Table 3).

The research findings underscore the significant impact of adding cricket flour and fruit fly flour to *B. bassiana* growth media, resulting in significantly different average mortalities of *Z. cucurbitae* compared to treatments without flour addition. Insect flours, rich in chitin compounds, stimulate the growth of chitinase enzymes crucial for maintaining the entomopathogenic fungus's infection ability, particularly during penetration into the cuticle of test insects (Islam et al., 2021; Prayogo et al., 2019). Chemical analysis reveals that cricket flour contains 54.68 % protein and 27.49 % chitin.

Chitin and protein are abundant energy sources found in insect integuments, so their addition to the propagation medium can inhibit viability decline (Hirsch et al., 2019). Similar content is expected in fruit fly flour, influencing the entomopathogenic fungus's ability to degrade the cuticle and penetrate the insect's body.

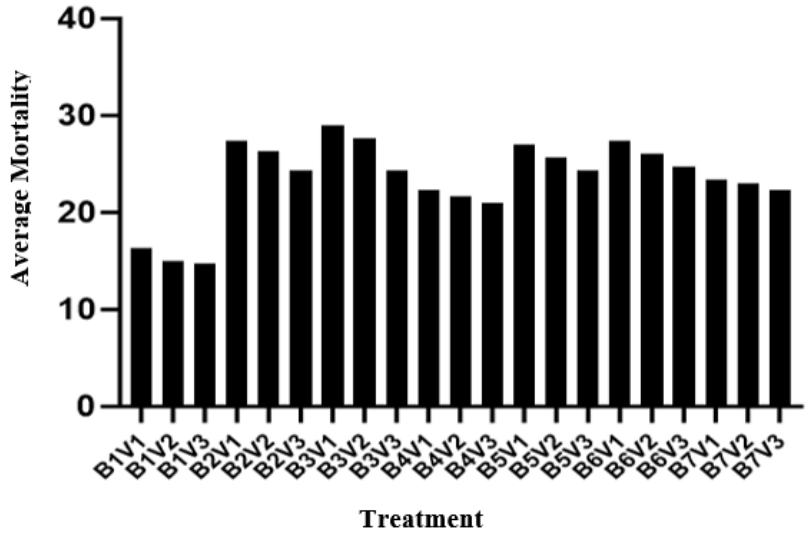

Figure 4. Mortality of *Z. cucurbitae* as affected by the application of *B. bassiana*

Table 3. Average Mortality of *Z. cucurbitae* as affected by the application of *B. bassiana*

Code	Treatments	Average Mortality
B1V1	*B. bassiana*on Pure PDA and spore density 10^8	21.33±1.52^{ab}
B1V2	*B. bassiana*on Pure PDA and spore density 10^7	19.33±4.04^{a}
B1V3	*B. bassiana*on Pure PDA and spore density 10^6	19.00±4.35^{a}
B2V1	*B. bassiana*on PDA + Cricket Flour 0,5% spore density 10^8	27.33±2.51^{de}
B2V2	*B. bassiana* on PDA + Cricket Flour 0,5% spore density 10^7	26.33±2.30^{de}
B2V3	*B. bassiana* on PDA + Cricket Flour 0,5% spore density 10^6	26.67±1.15^{de}
B3V1	*B. bassiana* on PDA + Cricket Flour 1% spore density 10^8	29.33±1.15^{de}
B3V2	*B. bassiana* on PDA + Cricket Flour 1% spore density 10^7	28.33±1.52^{de}
B3V3	*B. bassiana* on PDA + Cricket Flour 1% spore density 10^6	26.67±1.15^{de}
B4V1	*B. bassiana* on PDA + Cricket Flour 1,5% spore density 10^8	25.33±0.57^{de}
B4V2	*B. bassiana* on PDA + Cricket Flour 1,5% spore density 10^7	24.67±0.57^{cd}
B4V3	*B. bassiana* on PDA + Cricket Flour 1,5% spore density 10^6	24.33±2.08^{cd}
B5V1	*B. bassiana* on PDA + Fruit fly Flour 0,5% spore density 10^8	27.33±2.08^{de}
B5V2	*B. bassiana* on PDA + Fruit fly Flour0,5% spore density 10^7	25.67±1.52^{de}
B5V3	*B. bassiana* on PDA + Fruit fly Flour0,5% spore density 10^6	25±1.00^{cd}
B6V1	*B. bassiana* on PDA + Fruit fly Flour1% spore density10^8	27.33±2.08^{de}
B6V2	*B. bassiana* on PDA + Fruit fly Flour1% spore density 10^7	27.00±2.00^{de}
B6V3	*B. bassiana* on PDA + Fruit fly Flour1% spore density 10^6	26.33±1.52^{de}
B7V1	*B. bassiana*on PDA + Fruit fly Flour1,5% spore density 10^8	25.33±0.57^{de}
B7V2	*B. bassiana*on PDA + Fruit fly Flour1,5% spore density 10^7	24.33±2.51^{cd}
B7V3	*B. bassiana*on PDA + Fruit fly Flour1,5% spore density 10^6	24±1.00^{bc}

Remarks: Means followed by the same superscript letters are not significantly different based on DMRT at 5%.

Optimal concentrations of insect flour, specifically at 0.5 % and 1 %, demonstrated the most favorable results, enhancing *B. bassiana* conidia production and maintaining a stable count. Conversely, 1.5 % insect flour addition did not significantly differ from the treatment without flour, potentially inhibiting conidia formation due to a denser growth medium, resulting in a narrower growth space for *B. bassiana* (Rosana et al., 2021). According to Saidah & Asri (2019), conidia formation was inhibited due to the accumulation of metabolites resulting from adding a chitin source with a concentration that was too high.

Spore density of the entomopathogenic fungus during application also influences *Z. cucurbitae* mortality. Higher spore density increases the likelihood of active spores germinating, penetrating the cuticle, and causing Infection (Hardiansyah et al., 2023). Spores will penetrate the cuticle and develop within the tissue, causing Infection in *Z. cucurbitae*. Sumikarsih et al. (2019) stated that the spore density, whether high or low, significantly affected the effectiveness of *B. bassiana* in the field.

Time of *B. bassiana* Infection in *Z. cucurbitae*

Based on Table 2, the shortest time required by *B. bassiana* to infect *Z. cucurbitae* was observed in treatment B3V1 (1 % cricket flour and a spore density of 10^8), which was 2 days, followed by treatment of 1% cricket flour at spore densities of 10^7 (B3V2) and 10^6 (B3V3), as well as 1% fruit fly flour at spore densities of 10^8 (B6V1) and 10^7 (B6V2), which required 3 days for infection. Meanwhile, the longest time to cause Infection was observed in treatments B1V1, B1V2, B1V3 (without flour

Table 2. Infection Time

Treatment	Infection Time (Day)
B1V1	7
B1V2	7
B1V3	7
B2V1	5
B2V2	5
B2V3	5
B3V1	2
B3V2	3
B3V3	3
B4V1	6
B4V2	6
B4V3	7
B5V1	5
B5V2	5
B5V3	5
B6V1	3
B6V2	3
B6V3	4
B7V1	6
B7V2	6
B7V3	7

addition at spore densities of 10^8, 10^7, and 10^6 spore/mL), and B7V3 (1.5 % fruit fly flour addition with a spore density of 10^6), which was 7 days.

Time is a crucial factor, as observed by Dannon et al. (2020) and Friska et al. (2023). *B. bassiana* requires time to infect *Spodoptera litura* larvae because fungal conidia need time to germinate and form hyphae before they can eventually penetrate the insect's cuticle. Hence, *Z. cucurbitae* infection symptoms appeared one day after application and entered the pupal stage. The time of infection is influenced by the germination rate, which is affected by nutrient content in the growth medium, with cricket flour and fruit fly flour contributing high levels of chitin and protein (Rehman et al., 2023). The high content of chitin and protein in the growth medium of entomopathogenic fungi can accelerate germination because they play a vital role in the formation of fungal cell structures during the germination process to infect *Z. cucurbitae.*

CONCLUSION

The effectiveness of *B. bassiana* in controlling *Z. cucurbitae* is significantly influenced by the interaction between fungal toxins, insect flours, and spore density. Adding 1% cricket flour at a spore density of 10^8 spores/mL enhance *B. bassiana* infection, accelerating mortality and reducting infection time. Infection symptoms in *Z. cucurbitae* pupae are marked by the emergence of white fungal mycelia covering the cuticle, while infected adults exhibit deformed, wrinkled, and smaller wings. The highest average mortality of *Z. cucurbitae* was found in the treatment with 1% cricket flour and a spore density of 10^8 m/L, reaching 29.3 %, with an infection time of 2 days. In contrast, the lowest average mortality was observed in the treatment without flour addition at a spore density of 10^6, which was 19%, with an infection time of 7 days. These findings highlight the potential of optimized fungal growth media in enhancing *B. bassiana* virulence, providing valuable insights for improving entomopathogenic fungus-based biocontrol strategies.

AUTHORS CONTRIBUTIONS

NR played a pivotal role in shaping the research methodology by contributing to the design and development of models, ensuring a strong foundation for the study. She also provided essential oversight and mentorship throughout the project, guiding the external team in planning and execution. Additionally, she prepared the initial draft of the publication, incorporating detailed translations to ensure clarity and precision. UUM focused on conducting experiments and collecting critical data, playing a hands-on role in the investigation process. She conceptualized the research goals and managed data-related activities, including annotation, cleaning, and preparing the data for reuse. She also ensured the accuracy and replicability of the results through validation processes while creating impactful visual presentations of the data. Her contributions extended to project administration and formal analysis, applying statistical and computational techniques to synthesize findings. RMK enhanced the publication's quality through a meticulous review and editing process, offering insightful revisions and commentary to refine the work during both pre-and post-publication stages. Together, their combined efforts ensured the project's success and impactful dissemination.

COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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