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Enhancing the Virulence and Conidial Quality of *Beauveria bassiana* Using Insect-Derived Chitin Sources Against *Bactrocera pupae*

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Abstract

Background: One effective method for controlling *Bactrocera carambolae* is the use of *Beauveria bassiana*. However, a common challenge is the decline in the conidial quality and virulence due to the lack of chitin and protein sources. The insect *Tenebrio mollitor* and the pupal casings of *Hermetia illucens* are rich in chitin and have the potential to enhance the growth medium. **Methods:** The study used RAKF, consisting of 3 factors and 3 replications. The first factor was the Type of Flour (T), consisting of T1: *T. mollitor* and T2: *H. illucens*. The second factor was Flour Concentration (K), which consisted of K1 (0.5%), K2 (1%), and K3 (1.5%). The third factor was Drying Temperature (S), with S1: 100°C, S2: 110°C, and S3: 120°C. **Results:** The treatments significantly affected pupal mortality, pupal phase duration, and conidial viability (ANOVA, $p < 0.05$; $n = 570$ pupae). The best treatment was T2K2S2 (1% *Hermetia illucens* flour at 110°C), resulting in 76.7% pupal mortality, a pupal duration of 9.3 days, and conidial viability of 92.2%. **Conclusions:** Insect flour enrichment, particularly *H. illucens* flour at 1% concentration and 110°C drying temperature, significantly improved the biological performance of *B. bassiana* against *B. carambolae* pupae under laboratory conditions.

Keywords: *Bactrocera carambolae*; insect flour; conidial viability; fungal virulence; pupal mortality; drying temperature

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Introduction

Bactrocera carambolae is one of the fruit fly species commonly found in Indonesia and other Southeast Asian countries. This species has a wide host range, with approximately 75 host plant species belonging to 26 plant families reported in the region (Drew & Romig, 1996). Damage caused by *B. carambolae* is characterized by ovipositor puncture marks on fruit surfaces, which lead to quality losses due to fruit rot and quantitative losses resulting from premature fruit drop caused by larval feeding activity inside the fruit.

Beauveria bassiana is a well-known entomopathogenic fungus that has been reported to control *B. carambolae* populations (Hadi et al., 2013). This fungus produces various secondary metabolites, including beauvericin, bassianin, bassianolide, beauverolides, tenellin, oosporein, and oxalic acid, which contribute to insect mortality. In addition, *B. bassiana* produces extracellular enzymes, such as chitinase, lipase, and protease, that facilitate the penetration and degradation of the insect cuticle during infection (Wang et al., 2021). The effectiveness of entomopathogenic fungi is strongly influenced by spore quality and biological performance, including conidial viability and host infection capability. However, reduced spore quality may occur when growth media lack sufficient chitin and protein sources required for fungal development (Lapinangga et al., 2022). Therefore, efforts to enhance fungal performance can be achieved by enriching growth media with insect-derived chitin and protein sources.

Tenebrio mollitor larvae are reported to contain approximately 48.1% protein and 12.8% chitin, while *Hermetia illucens* pupal casings contain 34.42% protein and up to 31% chitin (Hahn et al., 2020; Utami, 2018). Previous studies have demonstrated that incorporating insect-based materials into fungal growth media can improve conidial quality and viability. For example, the addition of *H. illucens* insect flour to *Metarhizium anisopliae* growth media increased conidial viability by up to 90.97% (Aufa & Jadmiko, 2023). Similarly, Marlina (2025) reported that the application of 1% cricket flour in M. anisopliae cultures with a conidial density of 10^8 resulted in 30% mortality of *Zeugodacus cucurbitae*. Nevertheless, processing factors such as drying temperature may influence the nutritional quality of insect-derived materials. Drying *H. illucens* at 100°C for 2 hours has been reported to yield suboptimal moisture reduction (54.1%), potentially affecting substrate quality for fungal growth (Setyaji et al., 2020). Therefore, determining appropriate drying temperatures and enrichment concentrations is essential to support optimal fungal performance.

Although several studies have reported the use of insect-derived materials to enrich growth media of entomopathogenic fungi, information regarding the combined effects of insect flour type, concentration, and drying temperature on *Beauveria bassiana* performance against *Bactrocera carambolae* pupae remains limited. Most previous studies have focused on larval stages or evaluated single enrichment factors without optimizing processing conditions. Therefore, this study provides novel insight by systematically evaluating insect flour enrichment derived from *T. mollitor* larvae and *H. illucens* pupal casings at different concentrations and drying temperatures, and by linking these factors to biological indicators of fungal performance, including pupal mortality, pupal phase duration, and conidial viability. This approach contributes to optimizing insect-based enrichment strategies to enhance the biological performance of *B. bassiana* under laboratory conditions.

Methods

Time and Place

This study was conducted from July to October 2024 at the Plant Health Laboratory, Faculty of Agriculture, National Development University "Veteran", East Java.

Tools and Materials

The tools used in this study were a 30x30x30 cm rearing cage, a Memmert UN 30 oven, a 1 mm sieve, a Vortex MaxiMix II Thermolyne, an Olympus microscope CX33, a sprayer, and a digital microscope camera magnifier 500X. The materials for rearing were star fruit infected with fruit flies from Tasikmadu star fruit farm, oxoid yeast, gulaku sugar, sawdust, and artificial feed (consisting of 185 g wheat bran, 180 ml distilled water, 43.2 g sugar, 10.8 g yeast, 0.3 g nipagin, and sodium benzoate). The raw materials for insect flour on the growth media of *B. bassiana* were insect flour from *T. mollitor* larvae and pupal casings of *H. illucens*, and Potato Dextrose Agar (in 250 ml PDA consisting of 250 ml sterile distilled water, 62.5 g potato, 5 g dextrose, and 5 g agar). Starfruit infected with fruit flies, yeast, sugar, sawdust, purified water, 70% alcohol, *B. bassiana* isolate, *T. mollitor* larvae, and *H. illucens* pupal casing.

Research Methods

This study employed a factorial randomized block design (RAKF) with three factors and three replications. Blocking was applied based on experimental time and the biological batch of *Bactrocera carambolae* pupae. Due to the limited availability of uniform-age pupae, treatments were conducted in three experimental batches on different days. Each batch represented one block to minimize variability associated with pupal physiological condition and handling time. Within each block, treatments were randomly assigned to experimental units under homogeneous laboratory conditions.

3 factors in the form of insect flour types (T) consisted of T1: PDA + *T. mollitor* flour and T2: PDA + *H. illucens* flour. The flour concentration factors (K) were K1: 0.5%, K2: 1%, and K3: 1.5%. The insect-drying temperature factor (S) consisted of S1 (100°C), S2 (110°C), and S3 (120°C). The control treatment was only grown on pure PDA media. This study used 19 treatment combinations and 3 replications, yielding a total of 57 experimental units. The number of pupae tested was 570 (10 pupae/treatment).

Procedure

1. *Rearing of B. carambolae*

Exploration of infected fruit was conducted by collecting as many fruits as possible, and the rearing activities were carried out according to Suputa et al. (2007) using a 30x030x30 cm cage. Imago were fed with yeast and sugar. The eggs produced were then collected and placed on star fruit and artificial feed. The manufacture of artificial feed followed the method of Yolanda & Rivaie (2014) for *B. carambolae* larvae. Furthermore, the pupae were collected in sterile sawdust and were ready for use in the treatment.

2. *Propagation of B. bassiana*

Insect flour was made by drying the insects in an oven at the treatment temperature for 3 hours, then grinding and sieving through a 1mm sieve. The flour obtained was then added to 250ml of PDA media at the following concentrations: 0.5% (1.25 g), 1% (2.5 g), and 1.5% (3.75 g). The media, with the addition of prepared flour, was used as the inoculum for B. bassiana, which was incubated for 14 days; the conidia density was then calculated at 1×10^8 conidia mL^{-1}.

Conidia viability was assessed using a microscope at 400x magnification after incubation for 24 hours. The viability calculation formula, according to Gabriel & Riyatno (1989), was:

$$V = \frac{g}{g+u} \times 100\%$$

Note:
V: conidia germination (viability)
g: number of germinated conidia
u: number of non-germinated conidia

3. *Application of B. bassiana to B. carambolae pupae*

The application used a spray technique, with 1.6 ml of suspension containing 1×10^8 conidia mL-1 applied to 1-day-old pupae. The condition of the pupae was observed daily for 14 days after treatment to record observation parameters.

Data analysis

The observation data were then analyzed to determine the effect of the treatment applied by using Analysis of Variance (ANOVA). If the results indicate a significant impact, the test continues with Duncan's Multiple Range Test (DMRT) at the 5% level of significance.

Result

Infection Symptoms

Most of the treated *B. carambolae* pupae show symptoms of infection in the form of mycelium on the cuticle or the outer part of the pupa. Normal pupae appear to have a blackish-brown cuticle with a slightly shiny color (Figure 1A). In contrast, in infected pupae, the cuticle tends to be covered with white mycelium, which is a characteristic of *B.*

bassiana mycelium (Figure 1B). The presence of mycelium on the cuticle is interpreted as the success of the fungal conidia entering the insect's body and reproducing in it. The cuticle layer of 1-day-old pupae is still very flexible, allowing fungal penetration more easily. The penetration process involves several essential enzymes, such as chitinase, lipase, and protease, which are known as insect cuticle degraders (Wang et al., 2021).

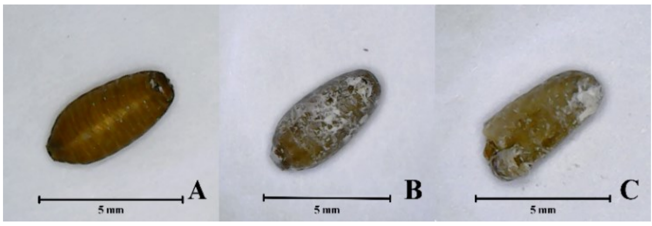

Figure 1. Pupae of *B. carambolae*; (A) Normal Pupae; (B) Pupae Infected with *B. bassiana*; (C) Puparium Infected with *B. bassiana*

Pupae that have shown symptoms of infection do not all fail to form imago. Some pupae can still develop into imago and leave behind a puparium (pupal sheath) with symptoms of infection (Figure 1C). Different levels of infection can influence this. Some pupae with low infection levels can survive and develop into adults, even with tissue damage. Another factor is that the insect's immune system, which has adapted, can increase anti-fungal regulation or activate the innate immune response (Zibaee & Malagoli, 2014).

Changes in Imago Morphology

The results of the study show abnormalities in several adults that have successfully resolved after treatment with *B. bassiana*. Several variations of wing deviation include the absence of wings (Figure 2B), puckered wings on both sides (Figure 2C), and wings that are not perfectly formed (Figure 2D). The three abnormal adults appear quite different from normal adults, who seem to have perfectly spread wings (Figure 2A). Abnormalities in the wings of adults have a significant impact on the survival rate of fruit flies since they will limit the mobility of fruit flies in finding food or finding partners to mate. In field conditions, abnormal adults will find it difficult to survive, leading to a decline in the population. This wing abnormality was also observed in a study in which infected rotten fruit was buried at a depth of 60 cm, causing the imago's wings to shrivel into various shapes (Susanto et al., 2022).

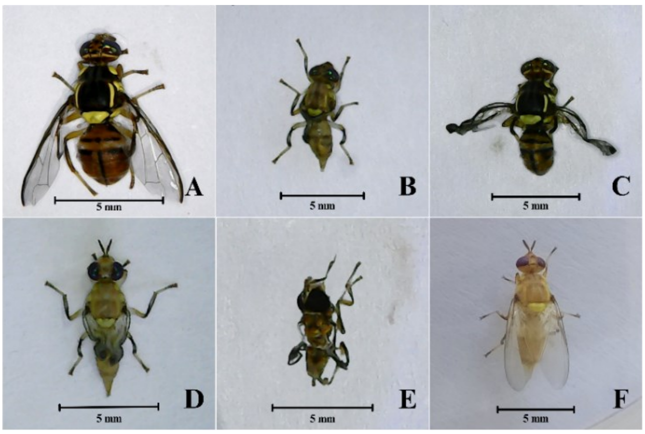

Figure 2. Morphological Changes in Imago; (A) Normal Imago; (B) Absence of Wings; (C)Puckered Wings; (D) Wings Not Perfectly Formed and Shrinked Abdomen; (E) Smaller Imago and Puckered Abdomen; (F) Imago with Faded Body Color and Lines

Other abnormalities are found in the abdomen. Normal imago has a rounded abdominal shape, with female imago equipped with an ovipositor so that the tip of the abdomen forms a little point. The abnormal imago is seen to have a shrunken abdomen with a cone-shaped appearance (Figure 2D). In addition, in Figure 2E the imago is also seen to have a puckered abdomen. This result is reinforced by the research of Mohanpuria et al. (2021), who added Escherichia coli bacteria expressing dsRNA to the artificial feed for Bactrocera dorsalis larvae; the final results showed abdominal abnormalities in the imago, appearing as an underdeveloped abdomen or an absence of a complete abdomen.

Another abnormal symptom is the smaller imago than the normal imago (Figure 2E). The body length of the normal imago in Figure 2A is 7.5 mm. In comparison, in the abnormal imago in Figure 2E, the body length only reaches 4 mm. Suwarno et al. (2018) mentioned that *B. carambolae* fruit fly imago has a body length between 6 and 7 mm. Smaller imagoes are formed when B. bassiana conidia penetrate the pupa, releasing and activating toxins and enzymes that interfere with development, resulting in abnormalities in the imago's body size. Kalvnadi et al. (2018) stated that entomopathogenic fungi can interfere with growth, reduce fertility, and fitness, leading to abnormal development in insects. An imago with these symptoms can survive for only 2 days due to limitations in conducting activities. The last symptom is imago with faded body color and lines. Compared to normal imagoes, abnormal imagoes appear with a faded yellow body color with faint body lines (Figure 2F). This symptom lasts for about 3 to 4 days after the imago emerges, accompanied by behavioral characteristics that are less active than normal imago. The imago's body slowly becomes normal due to the feeding in the treatment so that the imago gets optimal nutrition.

Pupal Mortality

The results showed that insect flour enrichment significantly affected the mortality of *Bactrocera carambolae* pupae ($p < 0.05$). Pupal mortality varied among treatments, indicating differences in the biological performance of *Beauveria bassiana* under different enrichment conditions. Higher mortality was generally observed in treatments enriched with insect flour compared with the control, suggesting that improved nutrient availability supported fungal growth and infection. Previous studies have reported that adequate nutritional composition in growth media can enhance conidial quality and infection efficiency of entomopathogenic fungi, thereby increasing host mortality (Hahn et al., 2020; Wang et al., 2021).

Table 1. Percentage of Pupal Mortality

Treatment	Average Pupal Mortality (%)
Control	10.0 ± 10.00
T1K1S1	16.7 ± 15.28 a
T1K1S2	26.7 ± 5.77 ab
T1K1S3	23.3 ± 5.77 ab
T1K2S1	50.0 ± 10.00 bcd
T1K2S2	66.7 ± 15.28 cd
T1K2S3	29.3 ± 18.48 bcd
T1K3S1	33.3 ± 30.55 ab
T1K3S2	43.3 ± 25.17 abc
T1K3S3	36.7 ± 11.55 abc
T2K1S1	33.3 ± 11.55 ab
T2K1S2	36.7 ± 15.28 abc
T2K1S3	23.3 ± 5.77 ab
T2K2S1	53.3 ± 5.77 bcd
T2K2S2	76.7 ± 5.77 d
T2K2S3	53.3 ± 15.28 bcd
T2K3S1	43.3 ± 20.82 abc
T2K3S2	46.7 ± 20.82 abcd
T2K3S3	43.3 ± 15.28 abc

Note: Numbers followed by the same letter in the same column indicate no significant difference in the DMRT test at 5%.

However, the observed increase represents a relative increase in pupal mortality rather than a direct measurement of fungal virulence, as virulence parameters such as lethal concentration (LC_{50}) or lethal time (LT_{50}) were not quantified in this study. Additionally, relatively high standard deviation values in several treatments, including T1K3S1, indicate biological variability in pupal responses to fungal infection, which differences may influence pupal physiological condition and fungal–host interactions.

The best treatment for each type of flour (T1K2S2 and T2K2S2) has the same combination of concentration and drying temperature, namely 1% and 110°C. These results indicate that the combination of a concentration of 1% and a drying temperature of 110°C in both flours is the best combination to increase the ability of *B. bassiana* to control *B. carambolae* pupae. The results of this study are in line with the research conducted by Rohman et al. (2017), which found that adding 1% T. mollitor chitin to the growth medium results in the highest colony diameter growth rate in B. bassiana.

Pupal Phase Length

Based on the results, there is a significant effect of treatment on the length of the pupal phase. Treatments T2K2S1, T2K2S2, and T2K2S3 yielded the highest average pupal phase length, namely 9.3 days each (Table 2). Meanwhile, the shortest average pupal phase length is observed in T1K1S1 and T1K1S2, at 7.0 days, which is not different from the control.

Table 2. Pupal Phase Length

Treatment	Average Length of Pupal Phase (day)
Control	7.0 ± 0.00
T1K1S1	7.0 ±0.00 a
T1K1S2	7.0 ±0.00 a
T1K1S3	7.3 ±0.58 ab
T1K2S1	7.7 ±0.58 abc
T1K2S2	8.0 ±0.00 abcd
T1K2S3	7.7 ±0.58 abc
T1K3S1	7.3 ±0.58 ab
T1K3S2	7.7 ±0.58 abc
T1K3S3	7.3±0.58 ab
T2K1S1	8.3 ±0.58 bcde
T2K1S2	9.0 ±1.00 de
T2K1S3	9.0 ±1.00 de
T2K2S1	9.3 ±0.58 e
T2K2S2	9.3 ±0.58 e
T2K2S3	9.3 ±0.58 e
T2K3S1	9.0 ±1.00 de
T2K3S2	8.7 ±0.58 cde
T2K3S3	8.7 ±0.58 cde

Note: Numbers followed by the same letter in the same column indicate no significant difference in the DMRT test at 5%.

H. illucens flour (T2) showed a higher average value than *T. mollitor* flour (T1). The T2K2S1 treatment can suppress the formation of *B. carambolae* imago for 3.3 days longer, while the best treatment in T. mollitor flour (T1K2S2) only suppresses for 1 day longer than the control. This pupal phase provides an opportunity for B. bassiana to develop longer, enabling it to cause morphological deviations or death.

The best treatments, T2K2S1, T2K2S2, and T2K2S3, which have the same value for the pupal phase length parameter, indicate that the type of *H. illucens* insect flour works better at a concentration of 1%. The right concentration is needed to achieve optimal fungal conidia growth. Under- or over-concentration can affect the fungal reproductive process. In addition, Herlinda et al. (2006) stated that accumulation of metabolites can occur when the nutrient source provided exceeds the fungus's needs, triggering it to produce enzymes that inhibit fungal reproduction.

Viability Rate

The treatments given show a significant effect on conidial viability rate. Based on Table 3, the highest average viability rate is obtained by the T2K2S2 treatment with a value of 92.2% followed by T2K2S3 with a viability of 91.5% (Table 3). These results are significantly different from the control, which achieved only a viability rate of 85.7%. The combination of treatments with *T. mollitor* results in lower viability than the combination with *H. illucens*. The best viability rate in *T. mollitor* flour is obtained with T1K2S2 at 89.3%, while the T1K1S2 and T1K3S2 treatments show values that are not significantly different, namely 88.4% each.

On the other hand, the addition of *H. illucens* flour appeared to be effective in increasing conidial viability, although it increased only 6.5% compared to the control. This increase in viability can be caused by the high chitin content in insect flour, as it is known that *H. illucens* contains relatively high chitin. It is supported by research conducted by Rohman et al. (2017), which found that the addition of chitin from *Gryllus assimilis*, *Perna viridis*, and *Scylla serrata* insects can increase the viability and virulence of *B. bassiana*.

Table 3. Viability of *B. bassiana*

Treatment	Average Conidial Viability (%)
Control	85.7 ± 1.48
T1K1S1	87.9 ± 0.90 ab
T1K1S2	88.4 ± 1.26 abc
T1K1S3	88.1 ± 1.34 ab
T1K2S1	88.2 ± 0.96 ab
T1K2S2	89.3 ± 0.48 abcd
T1K2S3	88.1 ± 0.62 ab
T1K3S1	87.4 ± 1.51 a
T1K3S2	88.4 ± 0.77 abc
T1K3S3	87.4 ± 0.90 a
T2K1S1	89.3 ± 0.52 abcd
T2K1S2	89.9 ± 1.54 bcde
T2K1S3	89.5 ± 0.80 bcde
T2K2S1	90.6 ± 0.92 def
T2K2S2	92.2 ± 1.83 f
T2K2S3	91.5 ± 0.66 ef
T2K3S1	90.3 ± 0.87 cdef
T2K3S2	91.2 ± 0.68 def
T2K3S3	90.9 ± 0.85 def

Note: Numbers followed by the same letter in the same column indicate no significant difference in the DMRT test at 5%.

Based on the data in Table 3, the combination of T1K2S2 and T2K2S2 is the best treatment for both types of flour. Moreover, this combination is found to be the best treatment for the parameters listed above. The combination of 1% concentration and 110℃ drying temperature effectively supported optimal conidia germination in both types of flour. These results are supported by research by Pramesti et al. (2014), which found that adding cricket flour at 0.5% and 1% resulted in the most significant increase in germination production and tended to produce more stable growth each week. The germination process plays an important role, since conidia viability is one of the factors that influence the fungus's virulence. The higher the conidia's viability, the higher the fungus's virulence is expected to be, and the greater the fungus's ability to interfere with pupal development, thereby increasing pupal mortality.

Conclusions

The provision of insect flour with various concentrations and drying temperatures can cause abnormalities in imago, increase the mortality rate, suppress the formation of imago for longer, and increase the viability of conidia. The best treatment is T2K2S2 (1% H. illucens at 110°C), which yields a mortality rate of up to 76.7%, a pupal period of 9.3 days, and a viability rate of 92.2%. However, these findings were obtained under controlled laboratory conditions. Further studies are required to validate the effectiveness of insect flour-enriched B. bassiana under semi-field or field conditions before practical application.

Declaration statement

The authors reported no potential conflict of interest.

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