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Evaluating the Efficacy of Varying Concentrations of *Metarhizium anisopliae* Against *Bactrocera cucurvitae* Under In-Vitro Conditions

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Abstract. This study aimed to evaluate the effectiveness of the entomopathogenic fungus *Metarhizium anisopliae* in controlling the cucurbit fruit fly (*Bactrocera cucurbitae*) under in-vitro conditions, addressing the need for sustainable alternatives to chemical pesticides. The research focused on developing eco-friendly pest management strategies to mitigate the economic losses caused by *B. cucurbitae* infestations in squash cultivation. A randomized complete block design (RCBD) was employed, testing five varying concentrations of *M. anisopliae* spore solution. Each treatment was replicated four times, using 20 fruit flies per replicate. The spore solutions were allocated for each treatment, and mortality rates, mummification times, and toxicity levels were recorded. Statistical analyses, including ANOVA and log-probit analysis, were used to determine lethal concentrations and times for effective control. The results demonstrated a clear dose-response relationship, with higher spore concentrations leading to significantly higher mortality rates and faster mummification times. A concentration of 6.0×10^5 Colony-Forming Unit CFU/mL was necessary for substantial population suppression, with lethal concentrations for 50% and 99% mortality calculated as 2.65×10^5 (CFU)/mL and 1.38×10^5 CFU/mL, respectively. These findings suggest that *M. anisopliae* is an effective biological control agent against *B. cucurbitae*, offering a sustainable alternative to chemical pesticides. Further research is recommended to assess its efficacy in field applications and to evaluate potential long-term ecological impacts.

Keywords: *Bactrocera cucurbitae*, Efficacy, *Metarhizium anisopliae*, Mortality rates, Pest Management.

1.0 Introduction

The cucurbit fruit fly (*Bactrocera cucurbitae*) is a highly destructive pest that primarily targets cucurbit crops, including squash, causing substantial economic losses globally (Zhang et al., 2021). Squash is particularly vulnerable to *B. cucurbitae* infestations due to its soft, fleshy fruit, which provides an ideal environment for the pest's egg-laying and larval development. Infestations lead to significant yield losses and reduced marketability, posing a major threat to farmers' livelihoods and food security, especially in regions where squash is a staple crop.

The prevalence of *B. cucurbitae* is notably high in tropical and subtropical regions, particularly in Asia, with countries such as India, Thailand, and the Philippines being heavily affected (Prabhakar et al., 2020). However, due to global trade, its distribution has expanded, making it a growing concern in parts of Europe, Africa, and the Pacific Islands, where cucurbit cultivation is also essential (Cai et al., 2023).

Historically, chemical pesticides have been the primary means of controlling *B. cucurbitae* populations. Although effective in the short term, their widespread and prolonged use has raised concerns over pesticide residues in crops, contamination of soil and water, and the development of resistant pest populations (Iqbal et al., 2022). These problems are further compounded by stricter regulations and increasing consumer demand for pesticide-free produce, driving the need for safer and more sustainable pest management alternatives (Alfenas et al., 2019).

In response to these challenges, entomopathogenic fungi (EPFs) have gained attention as biological control agents due to their natural ability to target a wide range of insect pests. *Metarhizium anisopliae*, in particular, has shown promise for its effectiveness against *B. cucurbitae* and other pests while posing minimal risks to non-target organisms (Gond et al., 2019). Moreover, unlike chemical pesticides, *M. anisopliae* is biodegradable and does not persist in the environment, making it a more sustainable option (Hussain et al., 2022). Importantly, no known cases of resistance to EPFs have been reported, further supporting their potential as long-term solutions for pest management (Mansoor et al., 2023).

Despite the growing body of research supporting *M. anisopliae*, studies evaluating its effectiveness against *B. cucurbitae* under controlled conditions remain limited. Furthermore, the optimal concentrations and application methods for maximizing its efficacy against this pest are yet to be fully explored. This study aims to fill this gap by evaluating the effectiveness of *Metarhizium anisopliae* in controlling *Bactrocera cucurbitae* under controlled in vitro conditions. The research seeks to identify optimal concentrations and conditions for its application by examining the fungus's impact on fruit fly mortality. The findings from this study will contribute to developing eco-friendly, sustainable, and precise pest management strategies, ultimately reducing dependence on chemical pesticides and addressing their associated risks.

2.0 Methodology

2.1 Experimental Design

This study employed five (5) treatment levels, replicated four (4) times, and arranged according to a Randomized Complete Block Design (RCBD). The blocking factor was the container from which fruit fly specimens were collected. Each experimental unit consisted of 20 fruit fly specimens. The treatment levels were defined as follows:

M1 = 0 Colony-Forming Unit CFU/mL
M2 = 1.5×10^5 CFU mL-1
M3 = 3.0×10^5 CFU mL-1
M4 = 4.5×10^5 CFU mL-1
M5 = 6.0×10^5 CFU mL-1

2.2 Procedures

Media Preparation

The fungus *Metarhizium anisopliae* was initially purified on semi-selective media (rose-bengal chloramphenicol agar) and later subcultured onto enrichment media (potato dextrose agar). The media was prepared by mixing with distilled water, cooking at 85°C for 15 minutes using a magnetic stirrer, and sterilizing at 121°C for 15 minutes in an autoclave. After sterilization and cooling to 50°C, 15 mL of the media was poured into sterile petri plates. Once the media solidified, the plates were flipped to prevent contamination.

Purification of the Fungal Culture

Metarhizium anisopliae was purified by inoculating a loopful of sorghum culture onto rose-bengal chloramphenicol agar (RBCA) plates, incubated for seven days at 27±0.5°C. Colonies with typical *M. anisopliae* characteristics were subcultured repeatedly on potato dextrose agar (PDA) until pure cultures were obtained. These pure colonies were transferred to PDA slants and stored at 4°C.

Collection of Fruit Fly Specimens

Fruit flies were collected using a pheromone trap from a 1-gallon plastic container filled with cotton soaked in the commercial methyl-eugenol solution. The trap was hung near the Pest Clinic Laboratory, College of Agriculture, MSU-General Santos. Trapped flies were collected using a fly net and placed in aerated plastic containers.

Treatment Preparation

To prepare the spore solutions, slant cultures of *M. anisopliae* were flooded with 10 mL of physiological saline, and the spores were scraped using an inoculating loop. The resulting suspension was filtered through eight layers of sterile gauze. Serial dilution was performed up to 10^{-5}, and spore concentrations were adjusted to the required levels using a physiological sodium chloride (NaCl) solution. Fifty milliliters of each treatment solution was prepared.

Treatment Application

Sterile 50 mL plastic spray bottles were used to apply the treatment solutions to the fruit fly specimens. Care was taken to avoid excessive dripping.

Processing

The study proceeded with the following stages (see Figure 1):

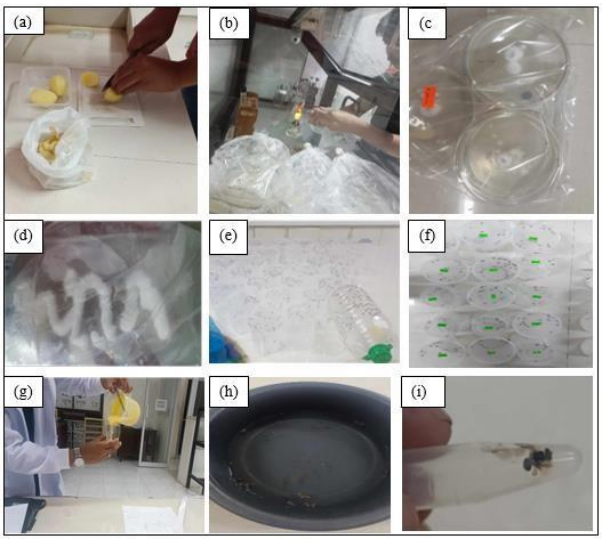

Figure 1. Using culture media and mass production of *Metarhizium anisopliae* led to the assignment and application of treatments.

a) Media Preparation: Culture media for growing *M. anisopliae* was mixed with agar, dextrose, and water, then sterilized to provide a nutrient-rich environment for the fungus.
b) Initial Purification: Fungal culture was transferred onto semi-selective agar plates to isolate *M. anisopliae* from other microorganisms.
c) Slant Cultures and Mass Production: The purified fungus was subcultured on fresh media to produce slants for storage and mass production.
d) Pure Cultures: After one week, pure cultures of *M. anisopliae* were obtained, ensuring the fungus's growth for experimental use.
e) Setup of Containers: Containers were prepared and sterilized to house fruit fly specimens.
f) Treatment Preparation: Spores were diluted to create different spore concentrations for treatment application.
g) Experimental Setup: Experimental units were organized according to the study's design, with random treatment assignments.
h) Surface Sterilization: Dead fruit flies were surface-sterilized to ensure accurate postmortem inspection.

i) Mummification: The study observed the mummification of melon fruit flies due to *M. anisopliae*, documenting this effect as part of the pest control mechanism.

2.3 Data Gathering Procedure

Data collected data were computed as follows:

a) The final mortality rate (%) was calculated as the proportion of dead fruit fly specimens at the end of the experiment relative to the acclimatized population, as follows:

$$\text{Final Mortality Rate (\%)} = \frac{\text{total number of dead fruit fly}}{\text{acclimitized population}} \text{x } 100 \quad \text{Eq 1}$$

b) Average time to mummification. The time (in days) until the onset of mummification was recorded for dead fruit fly specimens, given the characteristic shrinkage due to the loss of moisture and nutrients caused by mycosis.

c) Lethal concentration analysis. Lethal concentrations (LC50 and LC99) of the tubli root extract were projected by regressing the common logarithm (log base 10) of the test concentrations against the probit value of the response percentage (mortality rate). Lethal concentration analysis is expressible using the probit model:

$$P = \alpha + \beta[\log_{10}(\text{Concentration})] \quad \text{Eq 2}$$

where,

$P = 5 + \Phi^{-1}(p)$, given p = corrected mortality rate, and $\Phi^{-1}(p)$ is the probit value of the corrected mortality rate. The corrected mortality rate of the non-uniform population was computed based on Sun-Shepard's formula:

$$\text{Corrected mortality \%} = \frac{\text{Mortality \% in treated plot } \pm \text{ Change \% in control plot}}{100 \pm \text{change \% in control plot}} \quad \text{Eq 3}$$

where,

$$\text{Change \% in control plot} = \left(\frac{\text{n after treatment} - \text{n before treatment}}{\text{n before treatment}}\right) \quad \text{Eq 4}$$

Given that,

n = insect population, α = estimated value of the intercept, and β = estimated value of the slope

d) Lethal time analysis. The lethal timeframes (LT50 and LT99) of tubli root extract were projected by the same log-probit analysis as lethal concentration analysis by regressing the common logarithm (log base 10) of different time periods (in days) against the probit value of the response percentage (mortality rate per day).

e) The relative toxicity. Relative toxicity was taken as the comparison between the lethal time 50 and the treatment levels employed and computed as follows:

$$\text{Relative toxicity} = \frac{LT_{50(\text{treatment})}}{Lt_{50(\text{control})}} \text{ x } 100 \quad \text{Eq 5}$$

The fiducial limits (upper and lower) were then computed as,

$$R_{lower} = \frac{\text{Lower limit}_{\text{treatment}}}{\text{Upper limit}_{\text{control}}} \quad \text{Eq 6}$$

$$R_{upper} = \frac{\text{Upper limit}_{\text{treatment}}}{\text{Lower limit}_{\text{control}}} \quad \text{Eq 7}$$

2.4 Statistical Analysis

One-way analysis of variance (ANOVA) following a randomized complete block design was performed using STAR software for average mortality rates and time to mummify the five treatments. A comparison of means was performed at the 5% significance level using Scheffe's post hoc test to determine significant differences between treatment means.

3.0 Results and Discussion

3.1 Mortality rate

The data on mortality rate are shown in Table 1. It indicates that higher *Metarhizium anisopliae* concentrations increase mortality in *Bactrocera* fruit flies. The mortality rate grows from 58.33% at 1.5 x 10^5 CFU/mL to 96.00% at the highest concentration of 6.0 x 10^5 CFU/mL, signifying a clear dose-response relationship. These findings align with more recent research, which also demonstrates that *Metarhizium anisopliae* significantly increases mortality rates in various insect pests, including fruit flies, supporting its role as a viable biocontrol agent in integrated pest management (Onsongo et al., 2022; Prince et al., 2024; Cai et al., 2023; Mansoor et al., 2023).

Table 1. Effects of different concentrations of *Metarhizium anisopliae* on the mortality rate of fruit flies (from *Bactrocera*)

Treatment Level (CFU/mL)	Mortality Rate
M1 = 0 CFU/mL	46.33 ^{d}
M2 = 1.5 x 10^5 CFU mL^{-1}	58.33 ^{cd}
M3 = 3.0 x 10^5 CFU mL^{-1}	73.67 ^{bc}
M4 = 4.5 x 10^5 CFU mL^{-1}	85.67 ^{ab}
M5 = 6.0 x 10^5 CFU mL^{-1}	96.00 ^{a}
F test	**
P value	<0.001
%CV	7.42

3.2 Time to Mummification

Table 2 illustrates the impact of varying concentrations of *Metarhizium anisopliae* on the days it takes for *Bactrocera* fruit flies to mummify. As the concentration of *M. anisopliae* increases, the time required for mummification decreases significantly. For instance, mummification occurs at the lowest concentration (0 CFU/mL) in 22.67 days, whereas at the highest concentration (6.0 x 10^5 CFU/mL), it takes just 6.00 days. This demonstrates a clear inverse relationship between fungal concentration and time to mummification. These findings align with recent research, which has shown that higher concentrations of *M. anisopliae* accelerate the mummification process in various insect species, underscoring its effectiveness as a biocontrol agent in pest management strategies (Hussain et al., 2022; Mansoor et al., 2023; Prince et al., 2024).

Table 2. Effects of different concentrations of *Metarhizium anisopliae* on the number of days until mummification of the fruit fly (*Bactrocera*)

Treatment level (CFU/mL)	Days to Mummification
M1 = 0 CFU mL^{-1}	22.67 a
M2 = 1.5 x 10^5 CFU mL^{-1}	15.00 b
M3 = 3.0 x 10^5 CFU mL^{-1}	11.67 c
M4 = 4.5 x 10^5 CFU mL^{-1}	9.00 d
M5 = 6.0 x 10^5 CFU mL^{-1}	6.00 e
F test	**
P value	<0.001
%CV	3.01

Means with the same letter are not significantly different according to Scheffe's test at the 5% level.

3.3 Concentration Response Relationships between Metarhizium anisopliae and the Mortality Rate

Table 3 shows the relationship between different concentrations of *Metarhizium anisopliae* and the resulting mortality rates in fruit flies, providing estimates for the concentrations needed to achieve 50%, 90%, and 99% mortality. The LC50, or the concentration required to kill half of the fruit fly population, is determined to be 2.65 x 10^5 CFU/mL. To reach higher mortality levels, such as 90% and 99%, the necessary concentrations increase to 6.59 x 10^5 CFU/mL and 1.38 x 10^6 CFU/mL, respectively. These findings are consistent with recent research on using entomopathogenic fungi for pest control. For instance, Hussain et al. (2022) and Mansoor et al. (2023) reported similar dose-response trends, indicating that higher fungal concentrations are required for significant pest mortality. Additionally, Iqbal et al. (2022) demonstrated the efficacy of *M. anisopliae* in managing various insect pests, further supporting its role in targeted pest control strategies. The alignment of these results

with current research emphasizes the importance of lethal concentration estimates in the practical application of biocontrol methods.

Table 3. Projected lethal concentrations of *Metarhizium anisopliae* at the 5% significance level

Lethal Concentration		Slope ± (standard error)	Chi-Square	P value*
Response Percentage	Concentration (CFU/mL)			
50	2.65×10^5			
90	6.59×10^5	3.242 ±0.577	1.677	0.4325
99	1.38×10^5			

* Chi-square test for goodness of fit: measures goodness of fit to the weighted regression line with p>.05 , indicating a good fit of the data to the line

3.4 Time Response Relationships between Metarhizium anisopliae and Mortality Rate

Table 4 shows how different concentrations of *Metarhizium anisopliae* affect the time it takes to achieve 50% mortality (LT50) in fruit flies and the associated toxicity index compared to a control group. The control treatment (M1 = 0 CFU/mL) has an LT50 of 4.676 days and serves as the baseline with a toxicity index of 100.00. As the concentration of *M. anisopliae* increases, the LT50 decreases, indicating that higher concentrations kill the fruit flies faster. For instance, at 6.0×10^5 CFU/mL (M5), the LT50 is reduced to 2.106 days, and the corresponding toxicity index rises to 222.03. This pattern demonstrates that higher concentrations of the fungus are more lethal, as reflected in their higher toxicity indices. Although the indices for some treatments overlap, indicating nonsignificant differences, the overall trend suggests that *M. anisopliae* is increasingly effective at higher concentrations. These findings align with recent research indicating that entomopathogenic fungi like *M. anisopliae* exhibit greater efficacy at elevated concentrations, reducing time to significant mortality in insect populations. Recent studies by Mansoor et al. (2023) and Hussain et al. (2022) have similarly reported that the speed of insect mortality increases with higher fungal concentrations, highlighting the effectiveness of *M. anisopliae* as a biocontrol agent in pest management strategies.

Table 4. Time response relationships between different levels of *Metarhizium anisopliae* and mortality rate, and the corresponding toxicity indices relative to those of the control

Treatment	LT50	Toxicity Index
M1 = 0 CFU mL^{-1}	4.676	100.00 b
M2 = 1.5×10^5 CFU mL^{-1}	3.400	137.53 ab
M3 = 3.0×10^5 CFU mL^{-1}	2.735	170.97 ab
M4 = 4.5×10^5 CFU mL^{-1}	2.275	205.54 a
M5 = 6.0×10^5 CFU mL^{-1}	2.106	222.03 a

Indices with the same letter indicate overlapping fiducial limits at the 5% significance level, suggesting nonsignificant differences in toxicity.

4.0 Conclusion

The study highlighted the potential of *Metarhizium anisopliae* as an effective biocontrol agent against *Bactrocera cucurbitae* under controlled laboratory conditions. Higher concentrations resulted in greater mortality and quicker mummification of fruit flies, with mortality rates increasing from 58.33% at 1.5×10^5 CFU/mL to 96.00% at 6.0×10^5 CFU/mL. The time required for mummification they decreased significantly from 22.67 days to 6.00 days as the fungal concentration increased. The study also identified an LC50 of 2.65×10^5 CFU/mL, emphasizing the viability of *M. anisopliae* as a sustainable alternative to chemical pesticides in squash cultivation. Recent research reinforces these findings, demonstrating the effectiveness of *M. anisopliae* in increasing mortality rates and decreasing developmental times of various insect pests (Hussain et al., 2022; Mansoor et al., 2023; Prabhakar et al., 2020). Future research should explore its practical application under field conditions, evaluate any potential impact on non-target species, and refine application methods. Collaborating with agricultural stakeholders will be essential to effectively integrate this biocontrol agent into broader pest management strategies for sustainable farming practices.

5.0 Contribution of Authors

The specific contributions of each author are as follows: Author A designed the study and wrote the manuscript; Author B conducted the experiments and analyzed the data; Author C contributed to data interpretation and manuscript revision. All authors have read and approved the final manuscript.

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7.0 Conflict of Interests

The authors declare no conflicts of interest or competing interests related to this research.

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