



Toxicity assay of *Tenebrio molitor* test larvae inoculated with entomopathogenic fungi singly and in consortium

Alfarisy FK^{1*}, Wagiyana W², Hoesain M², Prayoga GH², Ibtisam I¹, Yessy Y¹, Ulfindrayani IF¹, Ullah R³, Handoko H⁴, and Al Ramadhani FM⁵

¹ Graduate School, State University of Surabaya, Lidah Wetan 60213, Surabaya, Indonesia

² Department of Plant Protection, Faculty of Agriculture, University of Jember, Jember, Indonesia

³ Department of Biomedical Engineering, Chung Yuan Christian University, Taoyuan 320, ROC. Taiwan

⁴ Division of Material Sciences, Nara Institute of Science and Technology, Takayama-cho, Ikoma, Nara 630-0192, Japan

⁵ Department of Agrotechnology, Faculty of Agriculture, University of Pekalongan, Pekalongan, Indonesia

Alfarisy FK, Wagiyana W, Hoesain M, Prayoga GH, Ibtisam I, Yessy Y, Ulfindrayani IF, Ullah R, Handoko H, Al Ramadhani FM. 2026 – Toxicity assay of *Tenebrio molitor* test larvae inoculated with entomopathogenic fungi singly and in consortium. Asian Journal of Mycology 9(2), 88–103, Doi 10.5943/ajom/9/2/9

Abstract

The increasing concern over pesticide residues and their negative impact on food safety, environmental health, and human well-being has encouraged the exploration of biological alternatives such as entomopathogenic fungi for pest control. This study aimed to evaluate the toxicity of single and combined entomopathogenic species of *Beauveria bassiana* and *Metarhizium anisopliae* against mealworm larvae, *Tenebrio molitor*. Fungal isolates were cultured, prepared at specific spore densities, and applied through topical methods under controlled laboratory conditions. A randomized complete design was used with five treatments, including a negative control (aquades), a positive control (synthetic pesticide, Profenofos 2 mL L⁻¹), single application of *B. bassiana* and *M. anisopliae* and then combined application of entomopathogenic species. Larval mortality and infection symptoms were observed daily, and the Lethal Time (LT₅₀) was determined using probit analysis. The results clearly demonstrated that the single-species treatment of *B. bassiana* exhibited the highest toxicity, achieving an LT₅₀ value of 4.5 days. In contrast, *M. anisopliae* required 7.8 days, while the combined application of both fungi resulted in an LT₅₀ of 6.4 days, indicating no synergistic advantage over the single application of *B. bassiana*. Microscopic observations confirmed fungal colonization, mummification, and hyphal proliferation in infected larvae. Statistical analysis revealed significant differences among treatments ($P < 0.05$), with *B. bassiana* consistently outperforming both *M. anisopliae* and their combined application. These findings highlight that a single application of *B. bassiana* is the most effective treatment and has strong potential as a biopesticide agent. This study provides important implications for sustainable pest management. The effectiveness of entomopathogenic fungi may vary depending on the application strategy, and combined treatments do not always result in improved efficacy compared to single-species applications.

Keywords – Biological agents – Entomopathogenic fungi – Mortality – Probit – Toxicity

Introduction

Food and environmental safety are major global issues, necessitating sustainable conservation efforts. The long-term impact of synthetic pesticides has caused problems in various aspects (Curl et al. 2020, Ahmad et al. 2024). Recent findings by Panis & Lemos (2024) indicate that rural women are at an elevated risk of pesticide residue exposure, which may contribute to breast cancer epidemiology. This increased risk is attributed not only to genetic predispositions but also to occupational exposure in agriculture. Contamination can occur via diverse routes, including specific spraying techniques and equipment for applying synthetic pesticides. Pesticide exposure can be direct during agricultural spraying, indirectly through crop production, or enter the body through consumption. A total of 43 case studies has identified relationships between receptor-based *in vitro* activities and the arylhydrocarbon receptor (AhR), estrogen receptor (ER), and androgen receptor (AR) in women (Vinggaard et al. 2021).

Mitigating pesticide residue contamination requires implementing various technical strategies, including Integrated Pest Management (IPM), biological control, and sustainable agricultural practices (Bruce & Boateng 2025, Wu et al. 2025, Zhao et al. 2025). Each active ingredient in pesticide has a distinctive half-life, during which it decomposes into simpler compounds. Research has identified the top 10 active pesticide compounds categorized by type, horticultural product (fruit), and plant parts (interior, leaf, surface, and stem), and has correlated these factors with their associated health impacts (Kumar et al. 2024).

Entomopathogenic fungi (EPF) have emerged as promising candidates for development as biopesticides in biological control. These fungi produce secondary metabolites that can infect and contribute to the mortality of insect pests (Kobmoo et al. 2025, Muhammad 2025, Scovero et al. 2025). Secondary metabolites are a diverse array of low molecular weight compounds produced by microorganisms to adapt to various environmental stress. Fungi, as an important source of secondary metabolites, have several important functions including drug discovery and biopesticide development (Macheleidt et al. 2016, Wang et al. 2022). The cluster of secondary metabolites consists of nonribosomal peptides (NRPS) and polyketides (PKS). *Metarhizium anisopliae* mainly produces destruxins (cyclic hexadepsipeptides), while *Beauveria bassiana* produces beauvericin and bassianolide (Pedrini 2022). Notable species developed as biopesticides include *Aspergillus terreus*, *Beauveria bassiana*, *Metarhizium anisopliae*, *Penicillium steckii*, and *Purpureocillium lilacinum* (Bhoi et al. 2025, Renson et al. 2025). The infectious compounds produced by these fungi encompass polyketides, non-ribosomal peptides, and other secondary metabolites such as beauvericin and destruxin (Tang et al. 2020, Yin et al. 2021, Liu et al. 2022, de Vicente et al. 2025).

Beauveria bassiana species is a well-known entomopathogenic fungus that can infect various insect pests. The fungus is abundant and can survive under various soil conditions (Niu et al. 2019, Majchrowska-Safaryan & Tkaczuk 2021). Several studies have shown its effectiveness in controlling the Asian corn borer (*Ostrinia furnacalis*) (Zhan et al. 2026), *Tetranichus urticae* (Sharma 2026), *Spodoptera frugiperda* (Wakil et al. 2026), and *Liriomyza sativae* (Zhang et al. 2025b). Meanwhile, *M. anisopliae* can control various types of pests such as fly larvae on fruits (Prince et al. 2026), *Spodoptera frugiperda* (Cheng et al. 2026), and *Bactrocera oleae* (Abbasi-Mozhdehi & Zibae 2026). Both fungi produce secondary metabolite compounds that have different functions. In general, the infection mechanism of both fungi involves spores attaching to the pest's cuticle through contact and penetrating the outer layer (Mannino et al. 2019, Zhang et al. 2025a). This contact and chemical factors cause the spores to release toxins that can attack the pest's nervous system (Zhang et al. 2024).

In general, most research on pest control using entomopathogenic fungi still focuses on single-species applications (Abbasi-Mozhdehi & Zibae 2026, Sharma 2026). This approach is considered practical but does not fully optimize control efficacy. Furthermore, each type of EPF has a distinct infection mechanism, including variations in cuticle penetration, enzyme production, and synthesis of secondary metabolites that play a role in the pathogenesis process. These

differences in characteristics indicate the potential for synergistic interactions when multiple species are combined. Currently, the application of entomopathogenic fungi is commonly associated with the combinations of synthetic pesticide applications rather than fungi. Rice & Furlong (2023) reported that β -cyfluthrin and imidacloprid induced significant sublethal developmental effects in *Alphitobius diaperinus* larvae, whereas Spinosad showed no significant effect on larval development and only limited effects on movement behavior, although it still produced moderate synergistic interactions with *B. bassiana*. However, the use of EPF combinations alone has not been systematically explored, comprehensive conclusions cannot be drawn regarding their effectiveness compared to single applications. Therefore, further research is needed to evaluate the potential of EPF combinations, particularly in improving pest control efficacy and understanding the types of interactions that occur.

Entomopathogenic fungi's effectiveness can be assessed using larvae such as *Tenebrio molitor*. *Tenebrio molitor* or mealworm is a well-established model organism in entomopathogenic research because its immune responses and infection processes are well characterized. This allows researchers to conduct controlled and reproducible bioassays without the variability and risks associated with using field pest species (Praprotnik et al. 2021). Evaluating virulence is essential for determining the fungi's infective capabilities. In vitro assessments of pathogenicity serve as critical indicators of the success of candidate entomopathogenic fungi. The common application methods include both oral and topical routes (Mantzoukas et al. 2022, Ferreira & Soares 2023). The chosen application methods can significantly influence the infection mechanism and the interaction between the fungi and the target organism. Depending on the materials utilized, applications may employ either the fungi themselves or specific secondary metabolites as the active ingredients (Wang et al. 2024, Marks et al. 2025). For example, research by Islam et al. (2023) demonstrated that topical application resulted in an 82% mortality rate in second-instar *S. litura* larvae seven days post-treatment. In contrast, using bioactive compounds derived from *B. bassiana* on *S. littoralis* second-instar larvae produced a maximum mortality rate of 61.11% (Bakr et al. 2025). These studies illustrate that entomopathogenic fungi exhibit varying levels of efficacy based on species, formulation, and target organism. Moreover, investigations have suggested that these fungi can demonstrate synergistic effects when used with other species (Batool et al. 2020, Půža & Tarasco 2023, Sharma et al. 2024). Consequently, this study aims to evaluate the toxicity of entomopathogenic fungi against *T. molitor* larvae when utilized in a consortium.

Materials & Methods

Fungal isolation and formulation

Isolates of *B. bassiana* and *M. anisopliae* were obtained from previous research conducted at the Food and Horticultural Plant Pest Disease Observation Laboratory in Tanggul District, Jember Regency, Indonesia (Wagiyana et al. 2025). Rejuvenating isolates was carried out by growing fungi on PDA (potatoes dextrose agar) media with Dionicol antibiotics (containing thiamphenicol). A single streak of the fungal isolate was carefully placed in the centre of a Petri dish using an ent needle and incubated at a controlled room temperature of 25 °C for seven days. Subsequently, the fungal biomass was transferred to a liquid medium consisting of PDB (Potato Dextrose Broth) GM403, supplemented with 10 mL of Tween 80 in a 250 mL Erlenmeyer flask. Following this, the collected fungal material was scraped and inoculated into the liquid PDB medium, which was then subjected to shaking at 80 rpm for 96 hours using an H-MSR 200 shaker. Fungal propagules produced in this liquid medium were blastospores, which were harvested by filtering culture through sterile gauze and suspended in deionized water containing 0.01% Tween 80 (Corval et al. 2021).

Spore density and characteristics microscopy

Spore density was determined by measuring 1 mL of fungal culture suspension with a micropipette (DIAB Hipette) and adding it to 9 mL of sterile distilled water in a test tube. The mixture was then vortexed for 10 minutes at 500 rpm to achieve a homogeneous solution, as

described by Sudarjat et al. (2024). A total of 1 mL was taken and then placed on a hemocytometer with a magnification of 400 times. After obtaining conidia, calculate the spore density using the following formula (Indriyanti et al. 2017):

$$S = \frac{t \times d}{n \times 0.25} \times 10^6$$

where,

- S : Conidium density / mL
- t : Total number of spores in the observed sample coax
- n : Number of sample boxes
- 0.25 : Correction factor for using a small-scale box on a haemocytometer
- d : Dilution factor
- 10^6 : Spore density standard

Bioassay test

Each petri dish contained 10 of *T. molitor* larvae, ranging from 10 to 29 mm. The dishes were lined with filter paper, and the larvae were provided with artificial cabbage cut into pieces measuring 4 cm by 4 cm. Incubation was conducted at room temperature, specifically maintained at 25 ± 2 °C. To minimize the risk of cannibalism, the feed was routinely replaced as necessary. Application was carried out with a concentration of spore density in each type of fungus, *B. bassiana* and *M. anisopliae*, as much as 1.06×10^8 conidia mL⁻¹ and 6.86×10^8 conidia mL⁻¹. Inoculation of the test larvae was carried out topically to ensure precise application. Two bioassay methods were used: oral and topical. For the oral bioassay, all artificial feeds were soaked in an entomopathogenic fungal (EPF) suspension was prepared at a specific concentration. The feed was soaked for 2 minutes, then removed and allowed to dry briefly before being fed to the larvae. For the topical bioassay, the EPF suspension was applied directly to the larvae using a fine brush to ensure uniform contact. Larvae were then reared individually in sterile containers under controlled conditions of 25 ± 2 °C and 70–75% relative humidity, with a 12-hour light: 12-hour dark photoperiod. Mortality and infection symptoms were recorded daily for 10 days.

Toxicity test

Toxicity indicators are used to determine the effectiveness of candidate entomopathogenic fungi against test larvae. This indicator used the mortality variable or larval death as the primary response. Larval mortality was observed daily as an initial screening measure to evaluate the pathogenic potential of the fungal candidates. Toxicity LT₅₀ (median lethal time) was used as an indicator of the time required to kill 50% of the test larval population due to entomopathogenic fungal infection. Application was carried out using two methods: oral application through immersion of artificial feed in fungal suspension, and topical application by directly applying the suspension on the larvae's body. Mortality observations were accompanied by observations of symptoms of acute infection in larvae. LT₅₀ values were measured by recording cumulative larval mortality at regular time intervals and analyzing the data using probit analysis or regression models to estimate the time point at which 50% of the larvae had died.

The experiments were carried out under controlled conditions at a temperature of 25 ± 2 °C and ambient laboratory humidity. A randomized complete block design (RCBD) was used to assess the virulence and toxicity of two entomopathogenic fungi, *M. anisopliae* and *B. bassiana*, applied individually and in combination against larvae of *Tenebrio molitor*. In total, five different treatments were tested (as illustrated in Fig.1), each with four biological replicates.

Two types of observations were carried out in this study. First, we assessed quantitatively the virulence and toxicity of the treatment group against *T. molitor* larvae. Here, the observation period lasted 11 days post-inoculation, during which larval mortality rates (%) was monitored and calculated the 50% lethal dose (LT₅₀). The five different treatments consisted of E0 = control (-) - aquades, E1 = control (+) - (synthetic pesticide, Profenofos 2 mL L⁻¹), E2 = 6.86×10^8 conidia mL⁻¹

of *M. anisopliae*, E3 = 1.06×10^8 conidia mL⁻¹ of *B. bassiana*, E4 = a combination of *M. anisopliae* and *B. bassiana* (6.86×10^8 conidia mL⁻¹ + 1.06×10^8 conidia mL⁻¹). Second, we qualitatively observed the symptoms and signs of fungal infection, particularly the mummification that covered the entire surface of the *T. molitor* larval body 30 days post-inoculation.

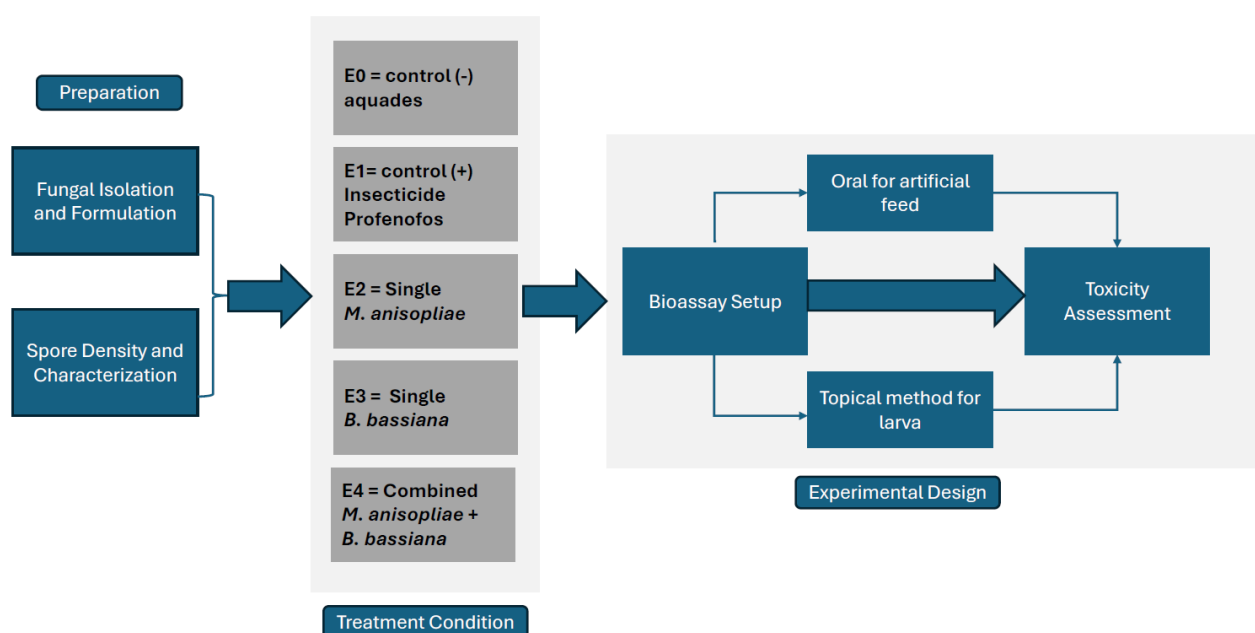


Fig. 1 – Experimental design and treatment condition.

Data analysis

The data analysis was performed utilizing OriginPro version 2025b software. A one-way ANOVA was conducted, applying a significance threshold of P-value < 0.05 or F-value > 0.001. Subsequent analyses were carried out using Tukey's, Bonferroni, and Fisher's tests. A regression analysis was performed to evaluate the relationship between each treatment and larval mortality, following the equation $y = a + bx$, where y denotes larval mortality (the dependent variable) and x represents the treatment (the independent variable).

Results

Characteristics of entomopathogenic fungi

Microscopic identifications in Fig. 2 show *B. bassiana* isolates with oval-shaped conidia. Hyphae are shaped like fine threads and are not septate. The hyaline hyphae form fungal mycelium, as observed at a magnification of 400 times (Fig. 2C). The conidiophores are irregularly branched, hyaline, and spread in a zigzag pattern. Fig. 2A shows a *B. bassiana* isolate on PDA media after 14 days of incubation. Fig. 3A shows the *M. anisopliae* isolate on PDA media after 7 days of incubation. The hyphae are septate and hyaline, forming a fungal mycelium. As the colony develops, the mycelium may appear whitish to greenish white due to conidial production. The conidiophores are upright and branched, arising from the hyphae and producing conidia. Conidia in *M. anisopliae* are elongated and cylindrical to oval in shape (Fig. 3C), and are arranged at the end of the phialides, forming greenish cylindrical structures.

Symptoms and signs of the *T. molitor* test larvae

The results of the bioassay showed that entomopathogenic fungal inoculation caused death in *T. molitor* larvae. Figure 4 explains the symptoms and signs of fungal infection. Several signs of mummification were observed covering the entire surface of the larvae. Fig. 4A shows infection by

both entomopathogenic fungi *B. bassiana* and *M. anisopliae*. Figure 4B shows the infection dominated by *B. bassiana*. Figure 3C shows infection resulting from a consortium of *B. bassiana* and *M. anisopliae*. Mummification observations were made under a Leica microscope under 100× magnification. Based on treatment with topical techniques, the process of the infection mechanism begins with the attachment of entomopathogenic fungal spores to the cuticle of the larvae, followed by germination and development on the host surface.

Figure 5 shows the mummification process on the 30th day after inoculation. After the spores successfully penetrate the cuticle, fungal colonization occurs, covering the entire surface. Figure 5A shows *B. bassiana* with fine and long fungal hyphae. Figure 5B shows that the *M. anisopliae* forms denser mycelium compared to *B. bassiana*. Figure 4 shows infection by the entomopathogenic fungal consortium. After the colonization process, each entomopathogenic fungus release bioactive compounds and other secondary metabolites that cause larval death (Litwin et al. 2020). Based on morphological observations, larval death occurs physiologically. Entomopathogenic fungal infection covers all parts of the larval body, starting from the abdomen, segments, thorax, and Kopf. In Fig. 5A, the lower thorax is dominated by *B. bassiana* fungi. In Fig. 5C, the metathorax, mesothorax, and protothorax are dominated by *M. anisopliae*. The Kopf section (Fig. 5B) vertically contains a colony of *M. anisopliae* mycelium.

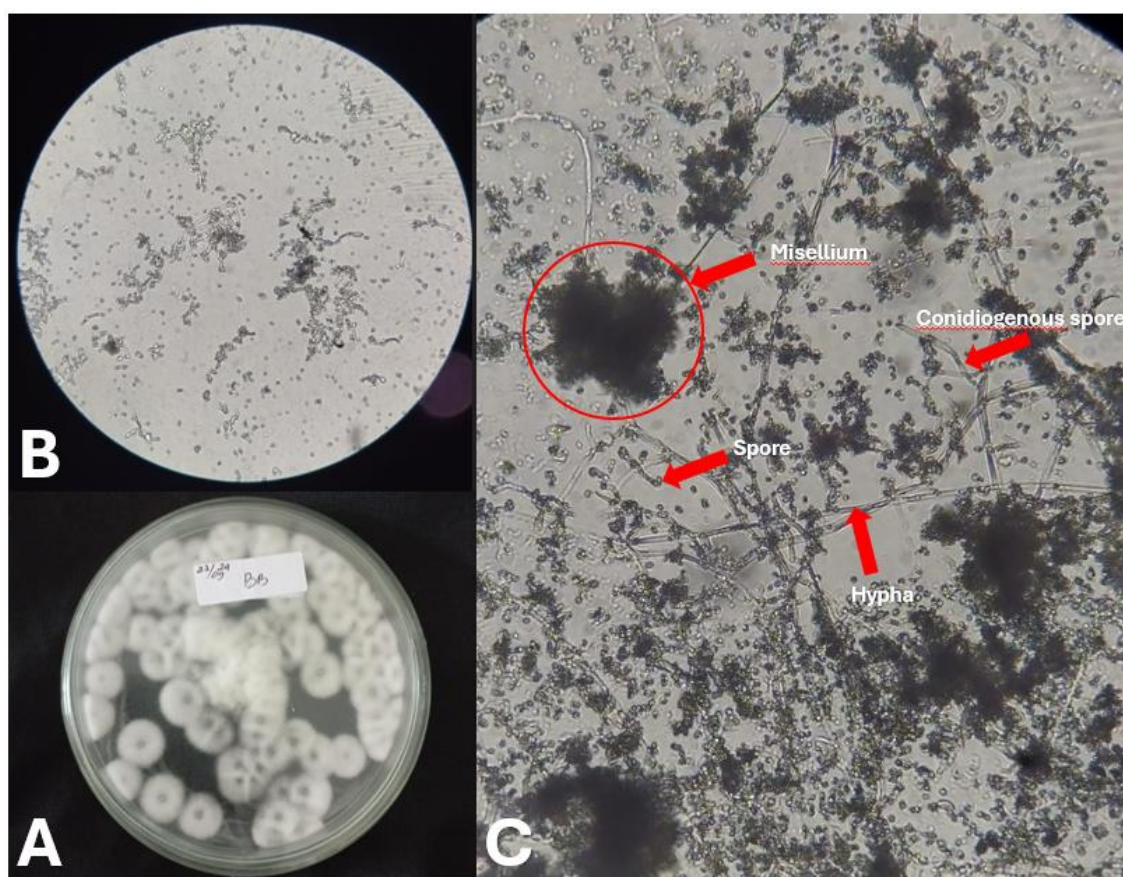


Fig. 2 – Microscopy of *B. bassiana* isolate. A *Beauveria bassiana* isolate. B Low-magnification view of fungal spores and hyphal fragments. C Microscopic structures.

Mortality of *T. molitor* larvae

Based on the treatment, larval mortality was observed for eleven days after inoculation. In the E0 treatment (negative control; Fig. 6), larval mortality tended to be stable or negligible, with no deaths recorded. However, some larvae died due to cannibalism by larger larvae. Treatments E1 (positive control; synthetic pesticide, Profenofos 2 mL L⁻¹), E2 (conidia of *M. anisopliae*), E3 (conidia of *B. bassiana*), E4 (consortium of *M. anisopliae* + *B. bassiana*) showed a progressive

increase in mortality over time. On the fourth day, treatment E3 (the single fungus *B. bassiana*) had reached >50% mortality. By day five, treatment E1 (positive control; synthetic pesticides) reached 50% larval mortality. On the eighth day, treatments E4 and E2 reached 50% larval mortality. Therefore, treatment E1 on the 11th day reached the highest mortality of 92.5%. Meanwhile, treatments E2, E3, and E4 resulted in lower mortality rates, with the maximum values of 67.5%, 77.5%, and 60%, respectively. In addition to treatment E1, some larvae remained alive and survived in all treatments.

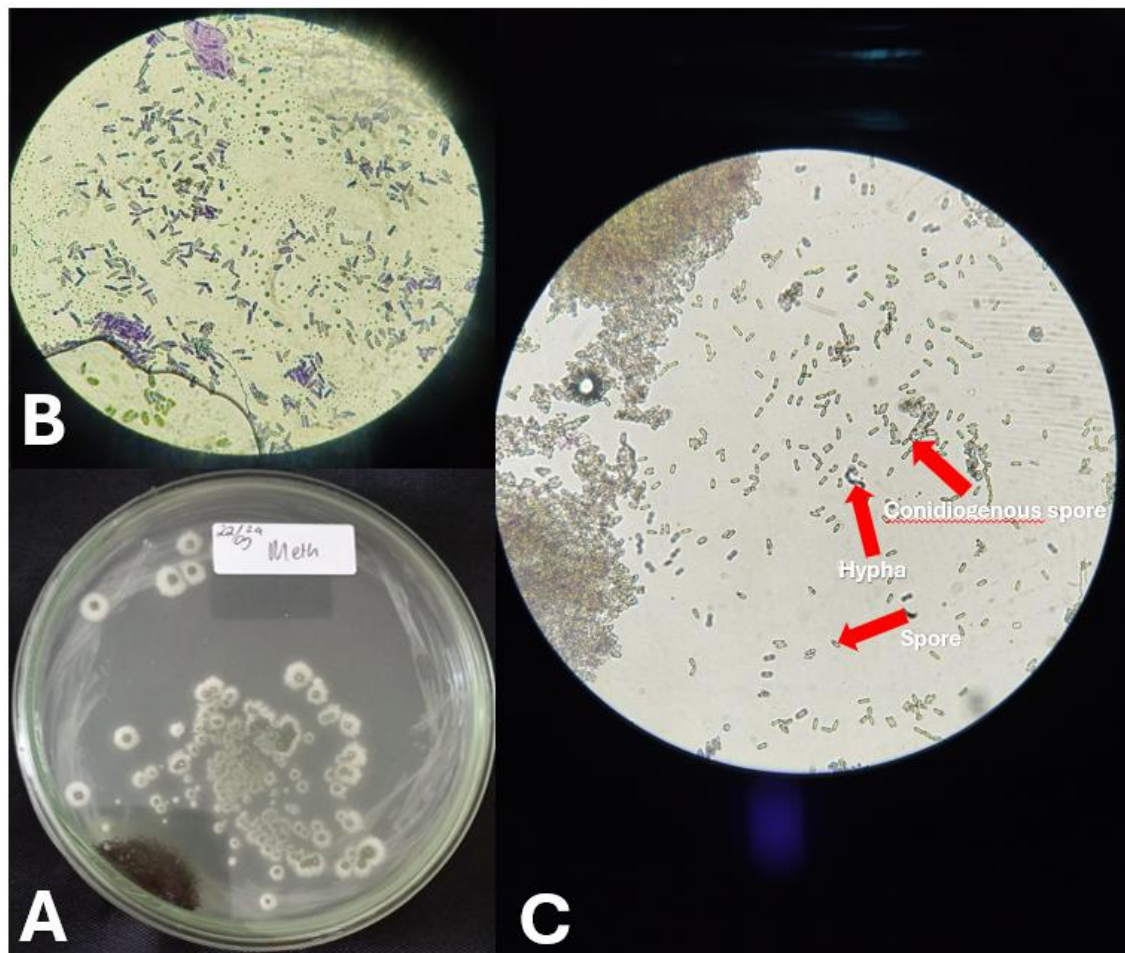


Fig. 3 – Microscopy of *M. anisopliae* isolate. A *Metarhizium anisopliae* isolate. B Low-magnification view of fungal spores and hyphal fragments. C Microscopic structures.

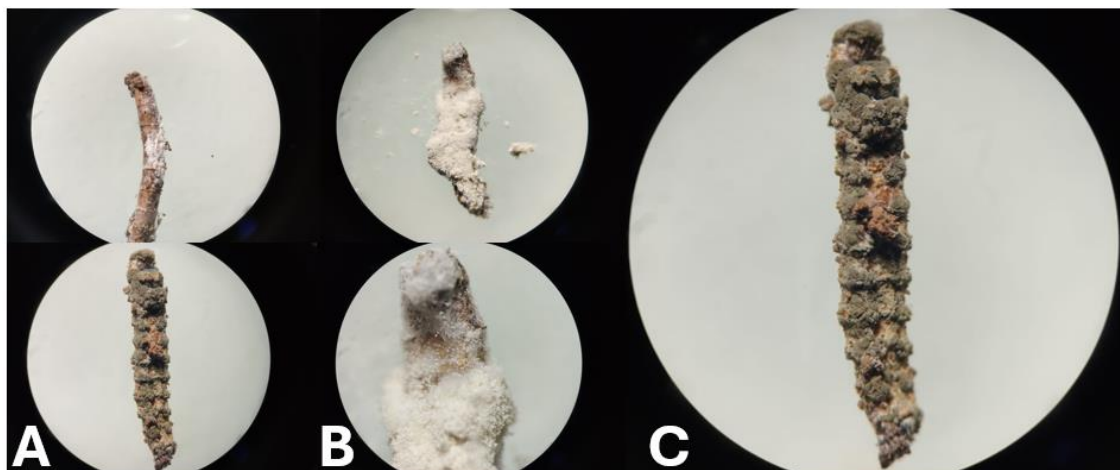


Fig. 4 – Symptoms of entomopathogenic fungal infection in *Tenebrio molitor* larvae. A Early cuticle colonization. B Mycelial growth and sporulation. C Advanced infection with fungal overgrowth and larval mummification.

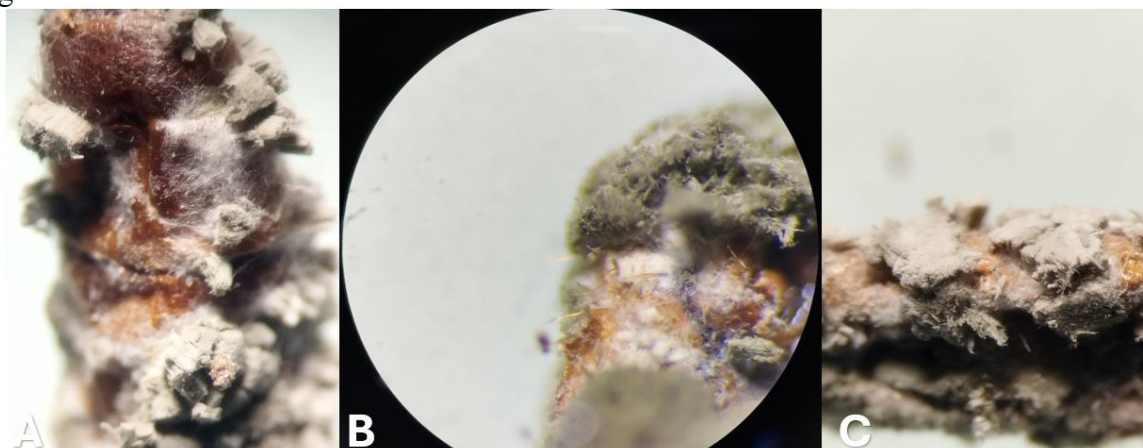


Fig. 5 – Mummification of *Tenebrio molitor* larvae induced by a consortium of entomopathogenic fungi observed under Leica microscope (400×). A Early fungal colonization with sporulation. B Microscopical view of fungal invasion on larval cuticle. C Advanced mummification with extensive fungal coverage.

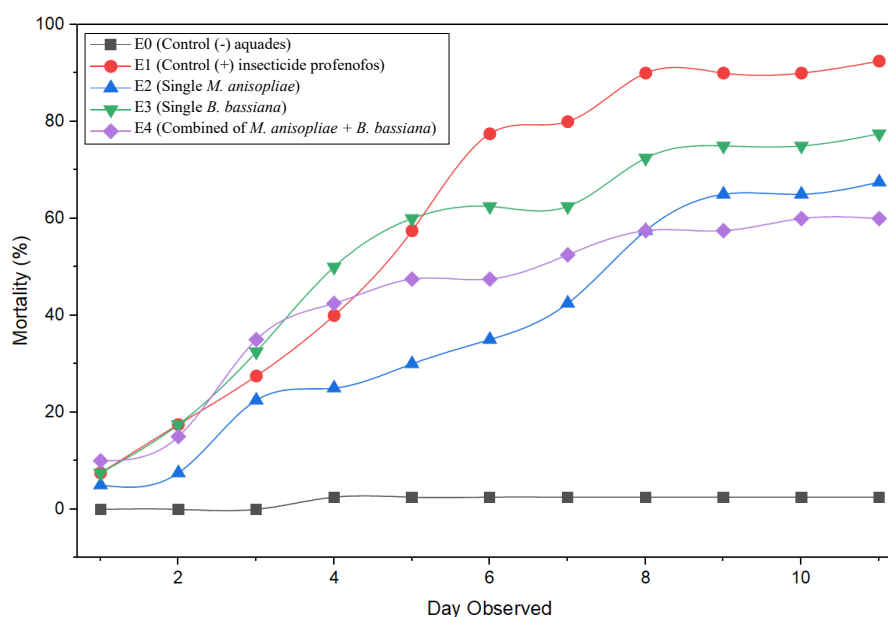


Fig.6 – Larval death rates based on time and type of treatment.

The ANOVA results indicated that larval mortality differed significantly among treatments ($P < 0.05$), confirming that each treatment exerted a distinct effect on mortality. As illustrated in Fig. 7, the distribution of mortality values formed clearly separated groups, reflecting biological differences among treatments. The calculated F-value (16.27) exceeded the F-value (0.01), further validating the significance of these differences. Treatment E1 (positive control, synthetic pesticide) produced the highest mean mortality at 60.90% (Standard deviation = 32.37, standard error of mean = 9.76). Among the single fungus treatments, E3 (*B. bassiana*) resulted in higher mortality than E2 (*M. anisopliae*), reaching an average of 53.86% mean mortality (Standard deviation = 24.35, standard error of mean = 7.342). However, mortality in E3 did not differ significantly from the dual-fungus consortium (E4). Figure 7 visually reinforces this pattern, where E1 clusters within the highest mortality group (“a”), E2–E4 form overlapping intermediate groups (“b” and “ab”), and E0

remains distinctly separated with minimal mortality (“c”). To further assess treatment effectiveness, additional analyses were conducted to clarify the magnitude of each treatment’s toxic effect (Table 1).

Table 1 Resume mean comparisons mortality on larvae test *T. molitor*.

	Mean (%)	Groups			t-value		q-value		t-value		Alpha (α)		
		Bonferroni	Tukey	Fisher	Bonferroni	Tukey	Bonferroni	Tukey	Bonferroni	Tukey	Bonferroni	Tukey	Fisher
Control(-) aquades	1.80	B	B	C	-0.48226	0.68201	-0.4822		0.05	0.05	0.05		
Control(+) insecticide profenofos	60.90	A	A	A	6.33225	8.95515	6.3322		0.05	0.05	0.05		
Single <i>M. anisopliae</i>	38.40	A	A	B	3.73749	5.28562	3.7374		0.05	0.05	0.05		
Single <i>B. bassiana</i>	53.86	A	A	AB	5.51975	7.80611	5.5197		0.05	0.05	0.05		
Combined of <i>M. anisopliae</i> + <i>B. bassiana</i>	44.09	A	A	AB	4.39274	6.35196E-4	4.39274		0.05	0.05	0.05		

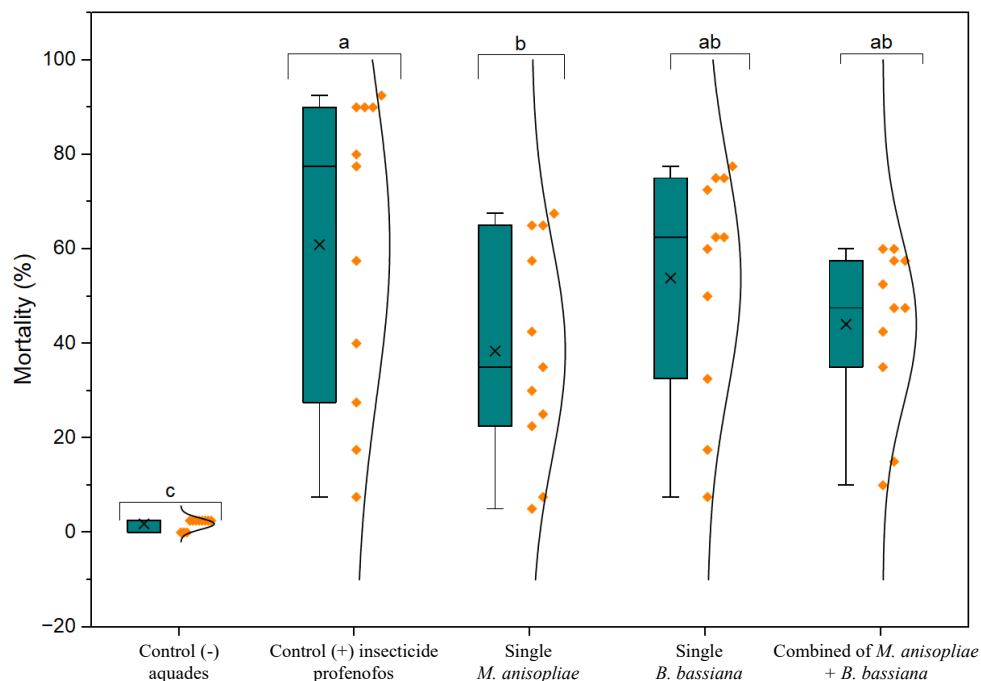


Fig. 7 – Significance of larval mortality in each treatment.

Toxicity LT₅₀ of entomopathogenic fungi

LT₅₀ toxicity describes the relationship between the time required for an active toxin ingredient to cause 50% larval mortality in a population of *T. molitor* test larvae in a petri dish. Based on probit analysis, the best larval mortality was found in treatments E1 (positive control) and E3

(Single *B. bassiana*), both took 4.5 days to achieve larval mortality above 50% of the total population. Meanwhile, E2 (Single *M. anisopliae*) required approximately 7.8 days to achieve 50% larval mortality. For treatment E4 (consortium *B. bassiana* + *M. anisopliae*), it took 6.4 days to achieve 50% larval mortality (Fig. 8).

Figure 9 presents the linear regression relationship between each treatment and larval mortality. In panel A (E0, control –), the data points appear widely scattered and do not follow the regression line, which is consistent with the low R² value of 0.55 (F = 13.5, Prob > F = 0.00512). This indicates that mortality in the control group occurred irregularly and was not driven by a consistent biological factor. In contrast, panel B (E1, synthetic pesticide) shows data points that

cluster closely around the regression line, resulting in a high R^2 of 0.91 ($F = 92.70221$, $\text{Prob} > F < 0.0001$). This pattern reflects a strong and predictable toxic effect of the synthetic pesticide, where increases in treatment exposure consistently correspond to higher larval mortality.

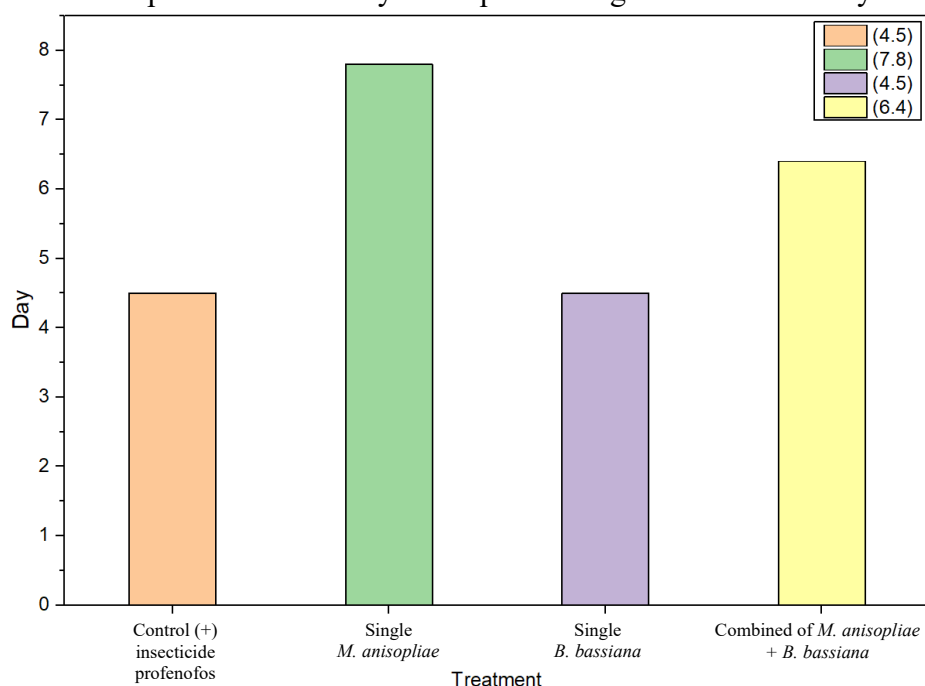


Fig. 8 – Toxicity of LT_{50} on larval mortality.

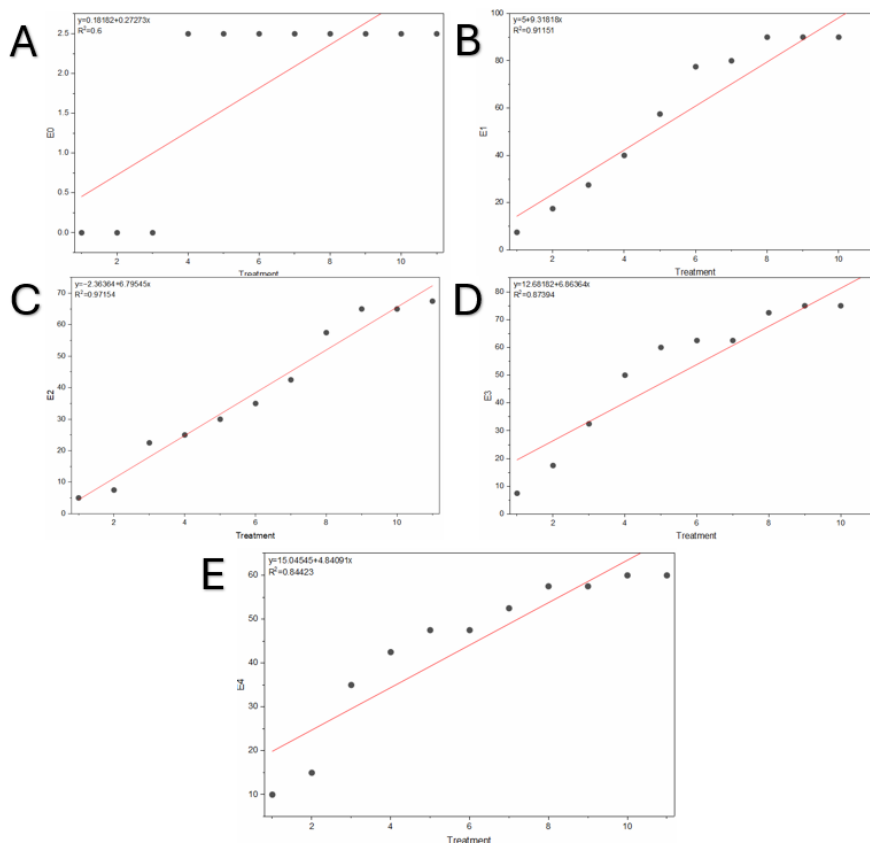


Fig. 9 – Linear regression correlation of *T. molitor* larval mortality. A E0 (negative control, aquades). B E1 (positive control, synthetic pesticide, Profenofos 2 mL L-1). C E2 (conidia of *M. anisopliae*). D E3 (conidia of *B. bassiana*). E E4 (consortium of *M. anisopliae* and *B. bassiana*).

Panel C (E2, *M. anisopliae*) exhibits the strongest linear relationship among all treatments, with an R^2 of 0.97 ($F = 307.21993$, $\text{Prob} > F < 0.0001$), and the points align tightly with the regression line, indicating very low variability and demonstrating that *M. anisopliae* induces a highly consistent mortality response across replicates. Panel D (E3, *B. bassiana*) also shows a strong linear pattern with an R^2 of 0.87 ($F = 62.39252$, $\text{Prob} > F < 0.0001$), although the data points are slightly more dispersed compared to E2, suggesting that *B. bassiana* remains effective but with moderately higher variability in larval response. Panel E (E4, fungal consortium) presents an R^2 of 0.84 ($F = 48.77804$, $\text{Prob} > F < 0.0001$), and while the relationship is still strong, the spread of data points indicates that the combined application of *M. anisopliae* and *B. bassiana* results in increased heterogeneity. Taken together, the regression patterns in Fig. 9 demonstrates that treatments E1, E2, E3, and E4 exhibit strong and biologically meaningful relationships with larval mortality, whereas E0 shows an inconsistent mortality pattern unrelated to treatment effects.

Discussion

Biological control is one component of integrated pest management (IPM) and involves the use of beneficial microorganisms such as entomopathogenic fungi. Entomopathogenic fungi are a solution for sustainable agriculture and can reduce dependence on synthetic pesticides. Species such as *B. bassiana*, *M. anisopliae*, *A. niger*, and *Lecanicillium lecani* have been widely used (Lin et al. 2021, Renson et al. 2025). Although entomopathogenic fungi can biodegrade pesticide residues and heavy metals through bioaugmentation (Dusengemungu et al. 2022, Wagiyana et al. 2025). However, their long-term use may affect soil quality and fertility (Fallah et al. 2023).

Entomopathogenic fungal species exhibit synergistic interactions and are compatible with other control agents (Iqbal et al. 2019, Batool et al. 2020). They are even synergistic with insecticide combinations, gradually affecting the mortality of *Alphitobius diaperinus* larvae (Rice & Furlong 2023). The mechanism by which entomopathogenic fungi infect larvae involves several stages, leading to larval death. First, contact between the conidia and the insect's cuticle occurs. This process involves chemical interactions that trigger enzymes to penetrate the larval skin (Baek et al. 2022). Enzymatic agents such as proteases, chitinases, and lipases play a role in penetrating and tearing the insect's cuticle (Włóka et al. 2022, Golzan et al. 2023). After successful penetration, the fungus germinates. Germination of fungal spores expands the infection's reach, leading to colonization and blastospore formation, which can cause physiological disruption to the larva's blood circulation. This disruption causes the larvae to lose blood and experience paralysis. During infection, the fungus releases secondary metabolites or primary toxins. Toxins commonly found in entomopathogenic fungi include beauvericin, destruxin, trehalase, oosporein, and tenelinolalic acid (Wang et al. 2021, Muhammad 2025). After the fungus causes internal damage, sporulation occurs, or the fungus exits the insect's body, filling the insect's cuticle layer and covering its surface. The fungus is identical to the one that infected the insect, for example, produces white or greenish thread-like hyphae. This fungus is identical to *B. bassiana* or *M. anisopliae* (Villamizar et al. 2021, Mantzoukas et al. 2022).

In this study, toxicity is an important variable for evaluating the mass production of entomopathogenic fungi as biopesticide candidates. Toxicity is the ability of a toxin possessed by an entomopathogenic fungus to infect insect pests. The higher the toxicity, the smaller the dose required for application. Therefore, this study examines the extent to which the toxin is effective in causing larval mortality. One indicator needed to test toxicity is the LT_{50} (Lethal Time). In this study, the E3 treatment (Single *B. bassiana*) was the best, namely on the 4.5th day it could cause larval mortality of 50% of the total population. The E3 treatment can be said to be equivalent to the E1 treatment (control + synthetic pesticide). The E4 treatment (consortium of *B. bassiana* and *M. anisopliae*) demonstrated substantial effectiveness, requiring 6 days to cause larval mortality of 50% of the total population. In Table 2, we summarize several toxicity capabilities of entomopathogenic fungi from various origins and main targets.

Toxicity is an early indicator that an entomopathogenic fungi has the ability to kill the target pest. The LT_{50} toxicity in this study contributes to the growing body of evidence supporting the use

of consortium of *B. bassiana* and *M. anisopliae* in pest management. Several factors that can influence the toxicity variable are the duration of contact and the condition of the larvae. In this study, larvae were fasted and fed artificial feed to increase their appetite. The entry route (mode of entry) was carried out topically to increase the potential for fungal contact with the larval cuticle.

Table 2 Summary of toxicity from entomopathogenic fungi based on LT₅₀ and test larvae.

Entomopathogenic species	Country of origin	Fungal applications	Objective test	Toxicity indicator LT ₅₀ (day)	Inoculation method	References
<i>Beauveria bassiana</i> (B1–B6).	China	Single	<i>Bactrocera dorsalis</i> larvae	20.5 ± 2.31	Immersion	Li et al. 2024
<i>Beauveria bassiana</i> Bv062	Colombia	Single	<i>Diatraea saccharalis</i> larvae	10	Topical	Lovera et al. 2020
<i>Beauveria bassiana</i>	India	Single	Nymph <i>Aphis craccivora</i>	17.69	Oral	Rajput et al. 2023
<i>Metarhizium anisopliae</i>	China	Single	Adults <i>Diaphorina citri</i>	8	Spray	Chen et al. 2025
<i>Metarhizium anisopliae</i>	Indonesia	Single	<i>Spodoptera litura</i> larvae	8	Oral	Gustianingtyas et al. 2020
<i>Metarhizium anisopliae</i>	China	Single	<i>Galleria mellonella</i> larvae	8.06	Topical	Liu et al. 2025
<i>Metarhizium anisopliae</i> + <i>Beauveria bassiana</i>	Indonesia	Consortium	<i>T. molitor</i> larvae	6.4	Topical	In this study

Conclusions

This study demonstrated that entomopathogenic fungi, both applied as single isolates or as a consortium, exhibited significant pathogenicity against *Tenebrio molitor* larvae. Among the treatments, *B. bassiana* (E3) showed the highest toxicity, achieving LT₅₀ within 4.5 days, a result comparable to the synthetic pesticide control (E1). In contrast, *M. anisopliae* (E2) required a longer period of 7.8 days, while the consortium of *B. bassiana* and *M. anisopliae* (E4) reached LT₅₀ in 6.4 days, indicating that although synergistic interactions were present, their combined efficacy did not surpass that of *B. bassiana* alone. Mortality symptoms were characterized by fungal colonization, mummification, and cuticle invasion, confirming infection as the primary cause of larval death. These findings highlight the potential of *B. bassiana*, either individually or in consortium, as a promising candidate for biopesticide development, reducing reliance on synthetic chemicals while supporting sustainable pest management strategies. Furthermore, the LT₅₀ value provides a useful screening parameter for evaluating fungal virulence *in vitro*.

Acknowledgements

We would like to thank the Ministry of Science, Technology, and Higher Education through the fundamental grant funding scheme under contract 5075/UN.25.3.1/LT/2024. All research activities were conducted at the Pest Laboratory, Department of Plant Protection, with support from the ICM (Integrated Crops Management) research group.

Conflict of Interest

None

Accessibility of data

Data will be provided if any requested.

References

- Abbasi-Mozhdehi MR, Zibae A. 2026 – A field study on a plant-based protein hydrolysate containing insecticidal protein from *Metarhizium anisopliae* Metchnikoff against *Bactrocera oleae* (Rossi) (Diptera: Tephritidae). *Journal of Natural Pesticide Research* 15, 100183.
- Ahmad MF, Ahmad FA, Alsayegh AA, Zeyauallah M, et al. 2024 – Pesticides impacts on human health and the environment with their mechanisms of action and possible countermeasures. *Heliyon* 10(7), 1–26.
- Baek S, Noh MY, Mun S, Lee SJ, et al. 2022 – Ultrastructural analysis of beetle larva cuticles during infection with the entomopathogenic fungus, *Beauveria bassiana*. *Pest Management Science* 78(8), 3356–3364.
- Bakr WH, Ghaly MF, Tantawy WG, El-Shafeiy SN. 2025 – Detection of some secondary metabolites of *Beauveria bassiana* and the potential effects on *Spodoptera littoralis*. *Chemical and Biological Technologies in Agriculture* 12(1), 1–15.
- Batool R, Umer MJ, Wang Y, He K, et al. 2020 – Synergistic effect of *Beauveria bassiana* and *Trichoderma asperellum* to induce maize (*Zea mays* L.) defense against the asian corn borer, *Ostrinia furnacalis* (Lepidoptera, Crambidae) and larval immune response. *International Journal of Molecular Sciences* 21, 1–29.
- Bhoi TK, Mahanta DK, Samreen, Samal I, et al. 2025 – Effect of endophytic entomopathogenic fungi (EPPF) in biological control of *Odontotermes obesus* (Rambur 1842) and their synergistic impact on plant physiological responses. *The Microbe* 7, 1–11.
- Bruce BB, Boateng ID. 2025 – Pineapple by-products: A critical review of their bioactive compounds as eco-friendly pesticides in pest management. *Food Chemistry: X* 28, 1–18.
- Chen D, Suo J, Yan J, Chen L, et al. 2025 – Evaluation of laboratory toxicities and field application of plant spray oil and its mixture with *Metarhizium anisopliae* against *Diaphorina citri* Kuwayama (Hemiptera: Liviidae). *Insects* 16(7), 1–11.
- Cheng Y, Li H, Yang X, Shang S, et al. 2026 – Non-target effects of *Bacillus thuringiensis* and *Metarhizium anisopliae* on egg parasitoids of *Spodoptera frugiperda*. *Biological Control* 213, 105957.
- Corval ARC, Mesquita E, Corrêa TA, Silva, et al. 2021 – UV-B tolerances of conidia, blastospores, and microsclerotia of *Metarhizium* spp. entomopathogenic fungi. *Journal of Basic Microbiology* 61(1), 15–26.
- Curl CL, Spivak M, Phinney R, Montrose L. 2020 – Synthetic pesticides and health in vulnerable populations: Agricultural workers. *Current Environmental Health Reports* 7(1), 13–29.
- de Vicente MC, de Paula LGF, de Freitas RMP, Almeida A dos A, et al. 2025 – Experimental assessment of beauvericin toxicity in tadpoles of *Dendropsophus minutus* (Anura: Hylidae). *Toxicon* 267, 108600.
- Dusengemungu L, Gwanama C, Simuchimba G, Mubemba B. 2022 – Potential of bioaugmentation of heavy metal contaminated soils in the Zambian Copperbelt using autochthonous filamentous fungi. *Frontiers in Microbiology* 13, 1–18.
- Fallah N, Pang Z, Zhou Y, Yuan Z, Lin W. 2023 – Sugarcane-peanut intercropping stimulatory effects on arbuscular mycorrhizal and entomopathogenic fungi promote soil health and crop productivity. *Field Crops Research* 303, 1–17.
- Ferreira JM, Soares FE de F. 2023 – Entomopathogenic fungi hydrolytic enzymes: A new approach to biocontrol? *Journal of Natural Pesticide Research* 3, 1–6.
- Golzan SR, Talaei-Hassanloui R, Homayoonzadeh M, Safavi SA. 2023 – Role of cuticle-degrading enzymes of *Beauveria bassiana* and *Metarhizium anisopliae* in virulence on *Plodia interpunctella* (Lepidoptera, Pyralidae) larvae. *Journal of Asia-Pacific Entomology* 26(2), 102038.

- Gustianingtyas M, Herlinda S, Suwandi S, Suparman S, et al. 2020 – Toxicity of entomopathogenic fungal culture filtrate of lowland and highland soil of South Sumatra (Indonesia) against *Spodoptera litura* larvae. *Biodiversitas* 21(5), 1839–1849.
- Indriyanti DR, Mahmuda S, Slamet M. 2017 – Effect of *Beauveria bassiana* doses on *Spodoptera litura* mortality. *International Journal of Scientific & Technology Research* 6(9), 206–210.
- Iqbal M, Shaheen FA, Mahmood R, Rafique MK, et al. 2019 – Synergistic effect of entomopathogenic fungi and bacteria against pulse beetle, *Callosobruchus chinensis*. *Pakistan Journal of Zoology* 51(5), 1685–1691.
- Islam SMN, Chowdhury MZH, Mim MF, Momtaz MB, Islam T. 2023 – Biocontrol potential of native isolates of *Beauveria bassiana* against cotton leafworm *Spodoptera litura* (Fabricius). *Scientific Reports* 13(1), 1–13.
- Kobmoo N, Mongkolsamrit S, Khonsanit A, Noisripoom W, et al. 2025 – Entomopathogenic fungi from paddy soils suppress a major insect pest and enhance rice growth under greenhouse conditions. *Biological Control* 210, 1–12.
- Kumar A, Ojha PK, Roy K. 2024 – First report on regression-based QSAR addressing pesticide dissipation half-life in plants: A step towards sustainable public health. *Science of The Total Environment* 954, 176175.
- Li XL, Zhang JJ, Li DD, Cai XY, et al. 2024 – Toxicity of *Beauveria bassiana* to *Bactrocera dorsalis* and effects on its natural predators. *Frontiers in Microbiology* 15, 1–11.
- Lin Y, Huang J, Akutse KS. 2021 – Whitefly-induced tomato volatiles enhance the virulence of *Lecanicillium lecanii*. *Journal of Invertebrate Pathology* 183, 1–7.
- Litwin A, Nowak M, Różalska S. 2020 – Entomopathogenic fungi: unconventional applications. *Reviews in Environmental Science and Biotechnology*, 19(1), 23–42.
- Liu S, Zheng J, Lin Y, Liang K, et al. 2022 – Application of in vivo solid phase microextraction in exploring dynamic metabolic alterations in living organisms under exogenous stimulation. *Advances in Sample Preparation* 2, 1–7.
- Liu T, Chen W, Tang Y, Liu F, et al. 2025 – Two novel entomopathogenic fungal species of *Lecanicillium* isolated from soil in China. *BMC Microbiology* 25(1), 1–10.
- Lovera A, Belaich M, Villamizar L, Patarroyo MA, Barrera G. 2020 – Enhanced virulence of *Beauveria bassiana* against *Diatraea saccharalis* using a soluble recombinant enzyme with endo- and exochitinase activity. *Biological Control* 144, 1–11.
- Macheleidt J, Mattern DJ, Fischer J, Netzker T, et al. 2016 – Regulation and role of fungal secondary metabolites. *Annual Review of Genetics* 50, 371–392.
- Majchrowska-Safaryan A, Tkaczuk C. 2021 – Abundance of entomopathogenic fungi in leaf litter and soil layers in forested habitats in Poland. *Insect* 12(2), 1–13.
- Mannino MC, Huarte-Bonnet C, Davyt-Colo B, Pedrini N. 2019 – Is the insect cuticle the only entry gate for fungal infection? Insights into alternative modes of action of entomopathogenic fungi. *Journal of Fungi* 5(2), 1–9.
- Mantzoukas S, Kitsiou F, Natsiopoulou D, Eliopoulos PA. 2022 – Entomopathogenic fungi: Interactions and applications. *Encyclopedia* 2(2), 646–656.
- Marks BB, Nogueira MA, Hungria M. 2025 – Microbial secondary metabolites and their use in achieving sustainable agriculture: Present achievements and future challenges. *Agronomy* 15(6), 1–35.
- Muhammad J. 2025 – Biochemical and molecular profile alterations in *Locusta migratoria migratorioides* (Orthoptera: Acrididae) induced by the entomopathogenic fungi. *Journal of Natural Pesticide Research* 14, 1–10.
- Niu X, Xie W, Zhang J, Hu Q. 2019 – Biodiversity of entomopathogenic fungi in the soils of South China. *Microorganisms* 7(9), 1–14.
- Panis C, Lemos B. 2024 – Pesticide exposure and increased breast cancer risk in women population studies. *Science of the Total Environment* 933, 1–16.
- Pedrini N. 2022 – The entomopathogenic fungus *Beauveria bassiana* shows its toxic side within insects: Expression of genes encoding secondary metabolites during pathogenesis. *Journal of*

Fungi 8(5), 1–9.

- Praprotnik E, Lončar J, Razingar J. 2021 – Testing virulence of different species of insect associated fungi against yellow mealworm (Coleoptera: Tenebrionidae) and their potential growth stimulation to maize. *Plants* 10(11), 1–17.
- Prince M, Cunningham JP, Sawbridge T, McKinnon AC. 2026 – Activity at the cuticle: Yeasts antagonise infection of the entomopathogenic fungus *Metarhizium anisopliae* in Queensland fruit fly larvae. *Biological Control* 216, 106006.
- Puža V, Tarasco E. 2023 – Interactions between entomopathogenic fungi and entomopathogenic nematodes. *Microorganisms* 11(1), 1–14.
- Rajput S, Suroshe SS, Yadav PR, Kumar A, Saini GK. 2023 – Bioefficacy of engineered *Beauveria bassiana* with scorpion neurotoxin, LqqlT1 against cotton mealybug, *Phenacoccus solenopsis* and cowpea aphid, *Aphis craccivora*. *PeerJ* 11, 1–22.
- Renon C, Nkundu MD, Mtei RP. 2025 – Harnessing indigenous entomopathogenic fungi for biopesticide development against *Tuta absoluta* in Tanzania. *Journal of Natural Pesticide Research* 13, 1–9.
- Rice SJ, Furlong MJ. 2023 – Synergistic interactions between three insecticides and *Beauveria bassiana* (Bals.-Criv.) Vuill. (Ascomycota: Hypocreales) in lesser mealworm, *Alphitobius diaperinus* Panzer (Coleoptera: Tenebrionidae), larvae. *Journal of Invertebrate Pathology* 200, 1–9.
- Scovero S, Moraglio ST, Tavella L. 2025 – Effects of tomato treatments with commercial formulations of entomopathogenic fungi on the pest *Tuta absoluta* and the predator *Macrolophus pygmaeus*. *Biological Control* 207, 1–8.
- Sharma A, Thakur N, Hashem A, Dawoud TM, Abd Allah EF. 2024 – Insecticidal potential of *Bacillus thuringiensis*, *Beauveria bassiana* and *Metarhizium anisopliae* individually and their synergistic effect with barazide against *Spodoptera litura*. *Heliyon* 10(17), 1–14.
- Sharma N. 2026 – Solid-state fermentation using agro-industrial residues for enhanced production of *Beauveria bassiana* s.l. JG787 and evaluation of virulence against *Tetranychus urticae*. *Bioresource Technology Reports* 33, 102588.
- Sudarjat, Widiyanti F, Salsabila Y, Mubarak S. 2024 – The effect of conidia density and application frequency of the entomopathogenic fungus *Aschersonia aleyrodis* Webber in controlling silverleaf whitefly (*Bemisia tabaci* Gennadius) on tomato (*Solanum lycopersicum* Linnaeus). *Plant Protection Science* 60(1), 80–88.
- Tang J, Zhang S, Zhang X, Chen J, et al. 2020 – Effects of pyrolysis temperature on soil-plant-microbe responses to *Solidago canadensis* L.-derived biochar in coastal saline-alkali soil. *Science of the Total Environment* 731, 1–13.
- Villamizar LF, Barrera G, Hurst M, Glare TR. 2021 – Characterization of a new strain of *Metarhizium novozealandicum* with potential to be developed as a biopesticide. *Mycology* 12(4), 261–278.
- Vinggaard AM, Bonefeld-Jørgensen EC, Jensen TK, Fernandez MF, et al. 2021 – Receptor-based in vitro activities to assess human exposure to chemical mixtures and related health impacts. *Environment International* 146, 1–18.
- Wagiyana W, Suharto S, Nurcahyanti SD, Alfarisy FK, et al. 2025 – Entomopathogenic fungal consortium of *Beauveria bassiana* and *Metarhizium anisopliae* as biodegradation from residue insecticide profenofos by in vitro studies. *Current Research in Environmental & Applied Mycology (Journal of Fungal Biology)* 15(1), 171–184.
- Wakil W, Iftikhar S, Kavallieratos NG, Gidari DLS, et al. 2026 – Laboratory evaluation of the entomopathogenic fungus *Beauveria bassiana*, bacterium *Bacillus thuringiensis*, and insecticide emamectin benzoate treatments, alone and in dual combinations against *Spodoptera frugiperda*. *Crop Protection* 200, 107446.
- Wang G, Ran H, Fan J, Keller NP, et al. 2022 – Fungal-fungal cocultivation leads to widespread secondary metabolite alteration requiring the partial loss-of-function VeA1 protein. *Science Advances* 8(17), 1–11.

- Wang H, Peng H, Li W, Cheng P, Gong M. 2021 – The toxins of *Beauveria bassiana* and the strategies to improve their virulence to insects. *Frontiers in Microbiology* 12, 1–11.
- Wang X, Han Y, Li S, Li H, et al. 2024 – Edible fungus-derived bioactive components as innovative and sustainable options in health promotion. *Food Bioscience* 59, 104215.
- Włóka E, Boguś MI, Wrońska AK, Drozdowski M, et al. 2022 – Insect cuticular compounds affect *Conidiobolus coronatus* (Entomophthorales) sporulation and the activity of enzymes involved in fungal infection. *Scientific Reports* 12(1), 1–19.
- Wu S, Jiang Q, Li L, He J, et al. 2025 – Synergistic incorporation of nano-pesticides into biological control: excellent biocompatibility with parasitic wasps (*Aphidius colemani*) can achieve efficient aphid control. *Journal of Integrative Agriculture* 25(7), 2903–2914.
- Yin F, Xiao M, Berestetskiy A, Hu Q. 2021 – The *Metarhizium anisopliae* toxin, destruxin A, interacts with the SEC23A and TEME214 proteins of *Bombyx mori*. *Journal of Fungi* 7(6), 1–7.
- Zhan Z, Batool R, Wang X, Han H, et al. 2026 – Graphene oxide enhances the dual role of *Beauveria bassiana* in biocontrol of the Asian corn borer and promotion of the maize seedling growth under field conditions. *Chemical Engineering Journal* 529, 173004.
- Zhang D, Qi H, Zhang F. 2025a – Parasitism by entomopathogenic fungi and insect host defense strategies. *Microorganisms* 13(2), 1–16.
- Zhang Q, Wang D, Liang X, Wang H, Lyu B. 2025b – Optimization and efficacy of an autoinoculation device for managing *Liriomyza sativae* with *Beauveria bassiana*. *Biological Control* 207, 105824.
- Zhang W, Chen X, Eleftherianos I, Mohamed A, et al. 2024 – Cross-talk between immunity and behavior: insights from entomopathogenic fungi and their insect hosts. *FEMS Microbiology Reviews* 48(1), 1–14.
- Zhao B, Zhang H, Mulder J, Sun X, et al. 2025 – Sustainable risk management of heavy metal contaminated agricultural soil: Current frameworks and future perspectives. *Journal of Environmental Management* 393, 127118.