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Initial ability of moulting of Ecdysone hormone on armyworm (*Spodoptera litura*) larvae and combination with Entomopathogenic fungi

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Abstract

Background: The tobacco armyworm *Spodoptera litura* (Lepidoptera: Noctuidae) is a notorious insect pest of crops. Tobacco armyworm (*Spodoptera litura*) is a destructive insect pest of crops that has acquired broad spectrum resistance to conventional chemical insecticides. Entomopathogenic fungi (EPF) are widely acknowledged as environmentally friendly biological control agents and have enormous potential in integrated pest management (IPM) programs. However, their use is restricted because they are slower in infecting and killing insects than chemical insecticides. The molting hormone of insects, ecdysone, controls the development of larvae, and during molting, it can cause a temporary rise in physiological vulnerability because of the loss of integrity of the cuticle and changes in immune activity.

Objective: The entomopathogenic fungi, *Metarhizium anisopliae* (*M. anisopliae*) and *Paecilomyces javanicus* (*P. javanicus*), were used in this study to determine their pathogenicity against third-instar larvae of *Spodoptera litura* (*S. litura*) when present in an exogenously supplemented fungal supplement.

Methods: Third-instar larvae were exposed to sublethal doses of 20-hydroxyecdysone, fungal suspension and 20-hydroxyecdysone and fungal suspension. Seven days of Larval mortality and

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developmental responses took place in a controlled laboratory setting. The interaction between treatments was evaluated using ANOVA.

Results: Exogenous ecdysone significantly affected molting dynamics and increased larval susceptibility to fungal infection. The highest larval mortality (92% on day 7) was obtained when the larvae were fed both ecdysone and M. anisopliae, which was significantly higher than that of the fungal treatment alone. A synergistic effect was observed with the combination of ecdysone and *P. javanicus*, but the overall mortality was slightly lower. The results obtained indicate that physiological changes during molting induced by ecdysone promote the penetration and infection efficiency of the fungus.

Conclusion: Exogenous ecdysone exposure in the pre-larval stage of *S. litura* significantly enhances its susceptibility to entomopathogenic fungi. This hormone-based approach could enhance the effectiveness of fungal biological control products and mitigate the delay in the action of EPF in IPM applications.

Keywords:

Spodoptera litura; Ecdysone; Biocatalysis; Metabolic Engineering; Entomopathogenic fungi; Paecilomyces javanicus; Metarhizium anisopliae

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Introduction

Insect pests are a significant threat to global agricultural production, resulting in considerable production and economic losses. The tobacco armyworm, *Spodoptera litura* (Lepidoptera: Noctuidae), is among these pests and a highly destructive polyphagous insect that feeds on many crops, such as rice, cotton, vegetables, and legumes. Rapid development, high reproductive capacity, and a wide host range all contribute to the significant impact of this pest on agricultural systems globally (Agui & Hiruma, 1982; Smagghe et al., 1999). Synthetic chemical insecticides are important for managing *S. litura* in many areas. However, long-term and repeated use of these chemicals has led to insecticide resistance, environmental pollution, and toxic effects on nontarget species (Batool et al., 2022). The difficulties point to the necessity of pest control approaches which are environmentally sustainable and biologically based. Entomopathogenic fungi (EPF) are pathogens used in biological control that infect insects through direct penetration of the host cuticle (Khan et al., 2021; Perveen, 2012). Species such as *M. anisopliae* and *P. javanicus* have been well studied and are known to infect and kill a wide range of insect pests (Hu et al., 2007; Fronza et al., 2017). The fungi attach to the insect cuticle, germinate, and bore into the host mechanically and enzymatically. Inside the hemocoel, the fungi multiply and produce secondary metabolites, including destruxins and other toxins, causing host death. EPF are potential substitutes for chemical insecticides because of their specificity and environmental harmlessness and play an important role in integrated pest management (IPM) programs.

The main drawback of EPF is its slow action speed, which affects its effectiveness in the field and its uptake by farmers (Suarez et al., 2022). Recent studies have indicated that entomopathogenic fungi can be more effective and pathogenic when used in combination with other biological or physiological stresses. Furthermore, synergistic or additive effects of fungal pathogens and insect growth regulators have been reported for several insect pests. These interactions can be synergistic and may facilitate insect susceptibility to infection by interfering with important physiological functions or reducing host resistance. Ecdysone is a steroid hormone that is important in the regulation of insect growth and development, especially during larval moulting and metamorphosis. The 20E (active form) binds to the ecdysone receptor complex (EcR/USP) and triggers a cascade of gene expression which is linked to the shedding of the old cuticle and the formation of the new exoskeleton (Jia et al., 2022; Zhu et al., 2021). Insects experience significant physiological changes during moulting, such as weakened cuticles and changes in immune responses. This period is characterized by susceptibility, and the host may be more susceptible to pathogens. Exogenous ecdysone or ecdysone agonists have been shown to disrupt normal moulting processes and affect insect development in several lepidopteran species. Abnormal moulting, development, reproduction, and sex ratios are caused by the external application of ecdysteroids, which are present in high concentrations. Increased mortality or arrest (Zhu et al., 2024). Importantly, hormonal disturbances can also affect the immune responses and structural defense of insects, which can lead to susceptibility to microbial pathogens (Karaiyan et al., 2002).

Although there is increased interest in hormone-mediated pest control methods, little attention has been given to the potential of hormone-fungus interactions when exogenous ecdysone is used. If pest populations can be induced to moult in synchrony, in theory, a short period of physiological susceptibility may result in higher infection efficiency by the fungal pathogens. Knowledge of these interactions may offer a new approach to improving the effectiveness of biological control agents based on their mechanism of action. Thus, the present study examined the synergism between exogenously applied ecdysone and the entomopathogenic fungi *M. anisopliae* and *P. javanicus* for third-instar larvae of *S. litura*. Specifically, we aimed to determine the effect of ecdysone exposure on larval moulting and development, whether the application of ecdysone in advance of fungal infection enhances fungal pathogenicity, and whether ecdysone application in combination with fungal infection was more effective than ecdysone alone in the two fungal species. This study aims to contribute to the development of better biocontrol methods for the sustainable management of *S. litura* by exploring these interactions.

Materials and Methods

Insect Rearing

A laboratory colony of *S. litura* was established at the Plant Protection Department of the College of Agriculture, Can Tho University, Vietnam. Larvae were reared on fresh leaves of host plant species (e.g., *Ricinus communis*, soybean, and cabbage) that were changed daily. Animals were reared in a controlled environment at 26 ± 1 °C, 70 ± 5% relative humidity, and 12:12 h light: dark photoperiod. Only healthy third-instar larvae of uniform size were used in the experiments to minimize variations due to developmental differences.

Fungus isolates and preparation of spore suspensions

In this study, two entomopathogenic fungi, *M. anisopliae* and *P. javanicus*, were used. The fungal isolates were collected from the Plant Protection Department, Can Tho University. Conidia were produced on potato dextrose agar (PDA) plates at 25 °C until mature. Conidia were collected by gently scraping the culture surface and dispersing them in sterile distilled water with 0.05% Tween-80. Mycelial fragments were filtered out of the suspension using a sterile cheesecloth. The concentration of spores was estimated using a hemocytometer and adjusted to 1×10^8 conidia mL^{-1} for bioassays. A small sample of the suspension was placed on PDA plates at 25 °C for 24 h prior to the experiments to determine conidial viability, and only suspensions with germination rates >90% were used. To estimate the median lethal concentration (LC50) for each fungal species, preliminary bioassays were performed, and the results were used for combination treatments.

Ecdysone Preparation

Exogenous ecdysone treatment was performed using the molting hormone 20-hydroxyecdysone (20E). The compound was not very soluble in water; therefore, a stock solution was first prepared by dissolving the compound in a small amount of ethanol. The stock solution was diluted with distilled water to obtain the desired experimental concentrations. The final ethanol concentration in the treatment solutions was not allowed to exceed 1% to prevent solvent effects on the larval physiology. A sublethal concentration of 10 ppm was chosen for the combination experiments based on preliminary bioassays and the literature. This concentration enabled the evaluation of possible synergistic interactions with the fungal treatments but reduced direct mortality from the hormone itself.

Experimental Design

The experiment was designed as a completely randomized design with five treatments: a control The larvae were divided into four groups: group in which larvae were treated with sterile distilled water, an ecdysone treatment group exposed to 20-hydroxyecdysone, a *M. anisopliae* (M.a) group exposed to the fungal suspension, and a *P. javanicus* (Pae) group exposed to its fungal suspension, and a combination treatment group in which larvae were first exposed to ecdysone followed by fungal application. A total of 100 third-instar larvae were used for each treatment, divided into four replicates of 25 larvae each. Each larva was reared in a separate container and treated according to the treatment group. For the combination treatments, larvae were first treated with a sublethal dose of ecdysone, followed by fungal suspensions at LC50 doses, to determine whether there was any synergistic effect between the hormonal treatment and fungal infection.

Monitoring of Mortality and Developmental Impacts

The larvae were counted daily for seven days after treatment. All deaths were reported on an hourly basis. To confirm fungal infection, dead larvae were examined for typical signs of mycosis, such as fungal growth on the cadaver surface. Developmental parameters were also measured to evaluate the physiological effects of the treatments, in addition to mortality. The parameters were molting frequency, developmental abnormalities, and timing of molting events. Selected larval samples were collected at 24 and 48 h after treatment for future molecular analysis. Samples were flash-frozen in liquid nitrogen and stored at −80°C until further processing.

Environmental Accounting as an Instrument for the Integration of the SDGs

Environmental accounting is a key aspect of the convergence of business management with the 2030 Agenda and sustainable development goals (SDGs). The adoption of environmental accounting practices results in the environmental performance of organizations, which, in turn, contributes to the SDGs. Including SDGs in administrative and sustainability reports, firms must set quantifiable objectives and indicators and specific activities that show their sustainability dedication to increasing their credibility and transparency. Within the scope of this research, it is important to note that the analysis of compliance with the following goals is considered good practice.

Statistical Analysis

Abbott's formula was used to correct the mortality data in the control group for natural mortality. Median lethal concentrations (LC50) and median lethal time (LT50) values were estimated using probit analysis. To assess the interaction effects between ecdysone and fungal treatments, the observed mortality in combination treatments was compared with the expected mortality values derived from the individual treatments. This method enabled the classification of the interaction as synergistic, additive, or antagonistic. Analysis of variance (ANOVA) was used to analyze the differences among the treatment groups. If significant differences were found, mean comparisons were made using the Least Significant Difference (LSD) test at the 0.05 level of significance. Data were analyzed with IBM SPSS Statistics version 26.0.

Results

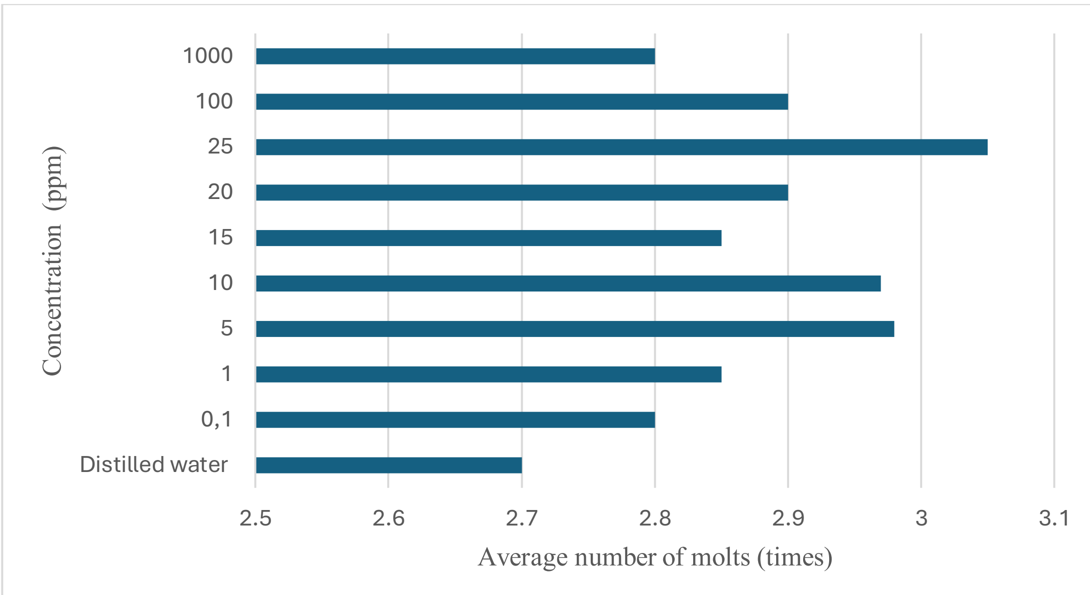
Effect of Exogenous Ecdysone on Larval Moulting

Exogenous ecdysone treatment of third-instar *S. litura* larvae had a significant effect on molting dynamics. Larvae fed ecdysone had a greater number of moults than those not fed ecdysone. The average number of moults per larva was 2.95 for the 10 ppm ecdysone application compared to approximately 2.7 moults per larva in the control group (Table 1, Figure 1). The increase is an indication that the exogenous ecdysone at moderate concentrations stimulated moulting and reduced the duration of the larval instar. However, a higher concentration (25 ppm) resulted in a different response. Larvae exposed to 25 ppm ecdysone exhibited fewer moults and developmental abnormalities, indicating that high levels of ecdysone may disrupt normal molting hormone regulation. This disruption is similar to that reported in other studies, in which high concentrations of exogenous ecdysteroids affected normal developmental processes in lepidopteran larvae. When applied alone, ecdysone treatment did not result in significant mortality, suggesting that the hormone was not directly toxic but rather exerted its effect on developmental regulation.

Table 1. Effect of exogenous ecdysone concentration on moulting frequency of third instar larvae of *S. litura*

Treatment	Ecdysone concentration (ppm)	Averga moul per larva
Control	0	2.7
Ecdysone treatment	10	2.95
High concentration treatment	25	3.05

Figure 1. The average number of molts after the 3rd larvae are exposed to Ecdysone



Mortality Induced by Entomopathogenic Fungi

In the present study, both entomopathogenic fungi tested resulted in measurable mortality in third-instar *S. litura* larvae.The cumulative larval mortality after 7 days was approximately 73% when treated with *M. anisopliae* alone at 10^8 spores mL^{-1} (Table 2). In contrast, the control group had very little mortality during the same period. Larvae infected with the fungal pathogen showed typical symptoms of mycosis, such as decreased feeding activity, decreased mobility, and progressive melanization prior to death. After death, fungal growth was observed on the cadaver of the larvae, indicating successful infection by the applied fungal isolate. The results of statistical analysis showed that the larval mortality in the fungal-treated groups was significantly higher than that in the control group (ANOVA, $p < 0.05$), suggesting that the entomopathogenic fungi had a significant pathogenic effect on *S. litura* larvae under the experimental conditions.

Table 2. Mortality of *S. litura* larvae following treatment with entomopathogenic fungi

Treatment	Spore concentration	Mortality after 7 days (%)
Control	-	0
M. anisopliae	10^8 spores mL^{-1}	73.3
P. javanicus	10^8 spores mL^{-1}	68.3

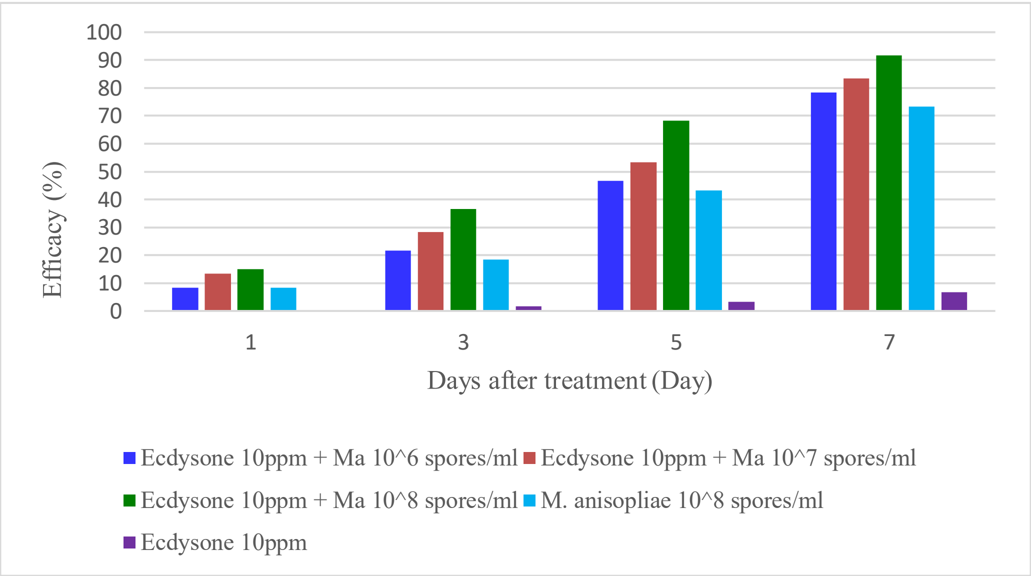
Combined Effect of Ecdysone and *M. anisopliae*

A synergistic interaction was observed between ecdysone and M. anisopliae. Larvae pre-treated with 25 ppm ecdysone and then exposed to *M. anisopliae* had significantly higher mortality than those exposed to the fungus alone (Table 3) (Figure 2). The larval mortality was approximately 92% after 7 days in the combined treatment, whereas the fungal-only treatment yielded 73% mortality. Furthermore, the combined treatment resulted in a rapid onset of mortality, with approximately 60% mortality by day 3, whereas fungal treatment alone resulted in significantly lower mortality by the same day. These results suggest that exogenous ecdysone increases the susceptibility of the host to fungal infection and decreases the median lethal time of the fungus.

Table 3. Combined effect of ecdysone and *M. anisopliae* on larval mortality

Treatment	Ecdysone concentration	Mortality Day 3 (%)	Mortality Day 7 (%)
Control	-	0	0
M. anisopliae only	-	30	73
Ecdysone only	25 ppm	<5	<5
Ecdysone + *M. anisopliae*	25 ppm	60	92

Figure 2. Efficacy of Ecdysone combined with the *M. anisopliae* fungus in *S. litura* larvae.



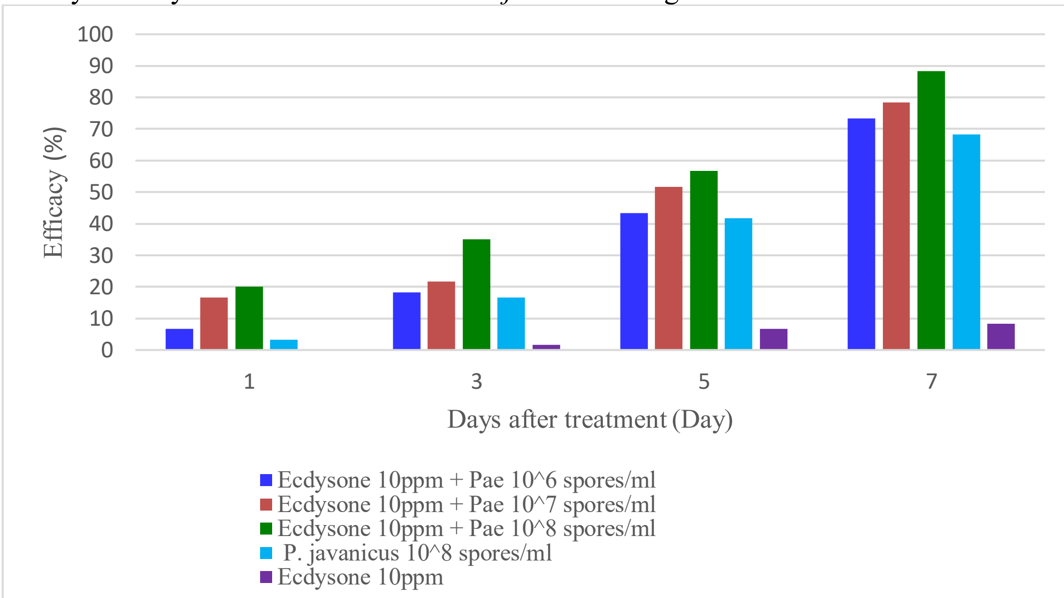
Interaction Between Ecdysone and *P. javanicus*

A similar, albeit less pronounced, synergistic effect was observed when ecdysone was combined with *P. javanicus*. Larvae treated with both agents had higher mortality than those treated with the fungus alone (Table 4) (Figure 3). The overall mortality obtained with *P. javanicus* was lower than that obtained with *M. anisopliae*, but the combined treatment resulted in a significant increase in fungal pathogenicity compared to the single-agent treatment. The results indicate that hormonal pre-exposure may be generally beneficial for the infection of *S. litura* larvae by various entomopathogenic fungi.

Table 4. Effect of ecdysone on pathogenicity of *P. javanicus*

Treatment	Ecdysone concentration	Mortality Day 7 (%)
Control	-	0
P. javanicus only	-	68.3
Ecdysone only	25 ppm	<5
Ecdysone + *P. javanicus*	25 ppm	88.3

Figure 3. Efficacy of Ecdysone combined with the *P. javanicus* fungus in *S. litura* larvae.



Comparative Efficacy of Fungal Treatments

In the two fungal species tested, *M. anisopliae* consistently caused greater larval mortality than *P. javanicus*. The treatment with ecdysone and M. *anisopliae* had the highest mortality (92%). The results show that hormonal manipulation of insect development can greatly improve the efficacy of fungal biological control agents.

Discussion

The present study shows that ecdysone can be used as an exogenous hormone to significantly affect the developmental physiology of *S. litura* larvae. It also enhances their susceptibility to infection by entomopathogenic fungi. Exogenous 20-hydroxyecdysone was found to modulate the moulting process of third instar larvae, as evidenced by the increased number of moults. This is similar to the known function of ecdysone as a major regulator of insect moulting and metamorphosis, which regulates the timing and progression of developmental transitions in arthropods (James & Xu, 2012; Jia et al., 2016).

The higher the concentration of ecdysone, the more frequent the moulting, indicating that exogenous exposure to ecdysone can speed up developmental events in *S. litura* (Tateishi et al., 1993). It has been shown previously that the moulting cycle is associated with changes in ecdysteroid levels, which in turn control the synthesis of the cuticle and the activity of the epidermal cells [17]. Physiological stress can result from external hormone treatments that disrupt this regulatory balance and may lead to a reduction in the insect's resistance (Aviles et al., 2019; Ma et al., 2023). These alterations can lead to temporary weaknesses that make the insect more susceptible to pathogenic attack (Wu et al., 2024). *M. anisopliae* and *P. javanicus*, the two entomopathogenic fungi used in the present study, were effective in causing high larval mortality in the laboratory. It is well known that entomopathogenic fungi can infect insects by penetrating the host cuticle directly (Tong et al., 2025; Kamimura, 2012). When fungal infection was preceded by ecdysone treatment, however, a significant increase in mortality was noted. The highest mortality was obtained by the simultaneous application of ecdysone and *M. anisopliae*, which achieved approximately 92% mortality after seven days. This finding suggests that there is a strong synergy between hormonal modulation and fungal pathogenicity.

This synergistic effect could be due to the fact that hormonally induced moulting may temporarily weaken the structure of the insect cuticle. The moulting process involves the breakdown of the old cuticle and the formation of the new one, which can leave the insect vulnerable to attack during this period. Entomopathogenic fungi, including *Metarhizium*, enter insects mainly through the cuticle, and any disruption of the cuticle structure may allow the fungi to penetrate and colonize the insect (Meng et al., 2013).

Hormonal treatments can also affect insect immune responses, in addition to structural changes in the cuticle. Several studies have demonstrated that ecdysteroid signaling can interact with immune regulatory pathways in insects, which regulate the production of antimicrobial peptides and other defense mechanisms (Sange et al., 2025; Zhang et al., 2023). Changes in these immune responses can reduce the host's resistance to fungal invasion, and thus improve the effectiveness of biological control agents.

The pathogenicity of the two fungal species tested was also found to differ in the results obtained in this study. *M. anisopliae* alone and in combination with ecdysone treatment consistently resulted in higher larval mortality than *P. javanicus* (Wang et al., 2025). This variation could be explained by differences in virulence factors, infection strategies, or host-pathogen compatibility of the fungi. *Metarhizium* spp. are known to be highly pathogenic against a large number of lepidopteran pests and are frequently employed in integrated pest management programs (Luo et al., 2020).

The increased efficacy of the combination of hormonal treatment and fungal infection indicates that the use of entomopathogenic fungi in pest management could be enhanced by combining it with hormonal treatment (Cao et al., 2016). A drawback of fungal biocontrol agents is that they are slower acting than chemical insecticides (Alfy et al., 2020). Physiological manipulation may make the host more susceptible and thus speed up the infection process and increase the mortality rate. These synergistic strategies may enhance the efficacy of microbial biocontrol agents in agricultural settings (Qu et al., 2024).

Although the results of this laboratory study are encouraging, a number of limitations should be noted. The experiments were carried out in a controlled environment, and the effectiveness of this approach in the field is yet to be determined. Fungal survival and infectivity can be affected by environmental conditions such as temperature, humidity, and UV radiation (Kumari et al., 2015). The potential of using ecdysone in combination with entomopathogenic fungi in semi-field and field conditions should be assessed for practical use in pest management in the future (Song et al., 2017).

Moreover, additional studies are needed to elucidate the molecular and physiological basis of the synergistic interaction observed. Knowledge of the effect of ecdysone on host immune responses, cuticle formation, and fungal penetration could be useful for the development of better biological control strategies (Mateos et al., 2022).

In general, the results of this study suggest that hormonal modulation of insect development can be a useful tool to increase the pathogenicity of entomopathogenic fungi against *S. litura*. Physiological manipulation combined with microbial biocontrol agents is a promising strategy for the development of environmentally friendly pest management strategies (Xuan et al., 2025; Mirani et al., 2025).

Conclusion

The results of this study show that the developmental physiology of *S. litura* larvae is greatly affected by the exogenous application of 20-hydroxyecdysone, which leads to an increase in the number of moults and temporary physiological vulnerability. The pathogenicity of the entomopathogenic fungi *M. anisopliae* and *P. javanicus* was significantly increased by pre-exposure to ecdysone, with the greatest synergistic effect observed when combined with *M. anisopliae*, giving up to 92% larval mortality. The findings indicate that the physiological changes that occur during hormonally induced moulting may make the larvae more susceptible to fungal infection, thus enhancing their mortality and the effectiveness of fungal biocontrol agents. The results suggest that hormonal modulation combined with microbial biocontrol could be a promising and environmentally friendly approach to control *S. litura* and possibly other lepidopteran pests. The underlying physiological and molecular mechanisms of this interaction should be explored in future studies, and the efficacy of this method should be tested in the field to assess its potential for use in an integrated pest management program.

Ethical and Environmental Considerations

No institutional review board was required to provide formal ethical approval for the use of invertebrate insect species (S. litura) in this study. The study followed all necessary laboratory procedures for human treatment and rearing of insect colonies, and every experimental practice aimed at reducing suffering in the test animals.

Acknowledgement

Author Contributions

All Authors equally contributed to the study and manuscript prepartion.

Conflict of Interest

The authors declared that no conflict of interest.

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